รายงานวิจัยฉบับสมบูรณ์

โครงการ: อณูชีววิทยาและวิทยาภูมิคุ้มกันของเชื้อไวรัสไข้หวัดนก H5N1

โดย ศาสตราจารย์ ดร. พิไลพันธ์ พุธวัฒนะ

ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล
มหาวิทยาลัยมหิดล

12 กันยายน 2555

สัญญาเลขที่ RTA4980008

รายงานวิจัยฉบับสมบูรณ์

โครงการ: อณูชีววิทยาและวิทยาภูมิคุ้มกันของเชื้อไวรัสไข้หวัดนก H5N1

[1]พิไลพันธ์ พุธวัฒนะ, [1]ประเสริฐ เอื้อวรากุล, [1]สุดา ลุยศิริโรจนกุล,

[2]วิทวัช วิริยะรัตน์, [2]กฤษฎา ใจชื้น, [2]ปานเทพ รัตนากร,

[3]โสภณ เอี่ยมศิริถาวร, [3]คำนวณ อึ้งชูศักดิ์, [3]จักรรัฐ พิทยาวงศ์อานนท์,
[3]รุ่งเรือง กิจผาติ

[1] ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล
[2] คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล
[3] สำนักระบาดวิทยา กรมควบคุมโรค กระทรวงสาธารณสุข

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

งานวิจัยครั้งนี้ได้ตั้งเข็มที่จะศึกษาเรื่องราวเกี่ยวกับเรื่องของไข้หวัดนก แต่เนื่องจากผู้ป่วยไข้หวัดนกได้หมดไปจากประเทศไทยตั้งแต่กลางปี พ.ศ. 2549 และในปี พ.ศ. 2552 ได้มีการระบาดทั่วโลกของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ A (H1N1) ลุกลามมาถึงประเทศไทย คณะผู้วิจัยจึงได้ขยายขอบเขตของการศึกษาวิจัยครอบคลุมทั้งไวรัส H5N1 และไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ A(H1N1) ดังปรากฏอยู่ในผลงานเล่มนี้

คณะผู้วิจัยขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) เป็นอย่างสูง ที่ให้ทุนสนับสนุนโครงการวิจัยนี้ นอกจากผลงานตีพิมพ์ดังที่ปรากฏอยู่ในรายงานฉบับนี้แล้ว ทุนวิจัย สกว. ยังมีส่วนทำให้คณะผู้วิจัยได้มีส่วนช่วยเหลือหน่วยงานสาธารณสุขของประเทศ ในการเป็นทีมงานคู่ขนานช่วยวินิจฉัยผู้ป่วยไข้หวัดนกในรายที่เป็นปัญหา และสืบสวนการระบาดของโรคไข้หวัดใหญ่สายพันธุ์ใหม่ จนนำไปสู่การค้นพบผู้ป่วยสองรายแรกของประเทศ และช่วยทำการตรวจหาภูมิคุ้มกันให้แก่บุคลากรทางการแพทย์ของหลายหน่วยงาน คณะผู้วิจัยขอขอบพระคุณ US Centers for Disease Control and Prevention ที่ให้การสนับสนุนงานวิจัยไข้หวัดนกภาคสนาม; Professor Robert Webster ที่ปรึกษาการวิจัยและให้ความอนุเคราะห์ low pathogenic influenza 16 subtypes; Dr Adrian Farmer ที่ปรึกษาด้าน satellite telemetry technique; Dr. Bernard Moss, US. National Institute of Health ที่ให้ความอนุเคราะห์ plasmid สำหรับการสร้าง recombinant vaccinia virus; Southeast Asia Infectious Clinical Research Network ที่อนุเคราะห์ archival samples; ขอบคุณ บริษัท Roche, Belgium สำหรับ oseltamivir carboxylate และบริษัท GlaxoSmithKline สหราชอาณาจักร สำหรับ zanamivir ขอบพระคุณสำนักระบาดวิทยา กรมควบคุมโรค และกรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข กรมอุทยานแห่งชาติ สัตว์ป่าและพันธุ์พืช ที่ได้ร่วมโครงการวิจัยนี้ ขอบพระคุณสำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ ที่ร่วมให้การสนับสนุนค่าใช้จ่ายในการวิจัยบางโครงการ ขอขอบพระคุณผู้ร่วมวิจัยและอาสาสมัครในโครงการ ขอขอบคุณนักศึกษาบัณฑิตในความดูแลทุกคน นางสวัสดิรักษ์ ใสงาม ผู้ประสานงานโครงการวิจัย นางสาวพิษณุ ภู่รักษ์ นางสาวกรรณิการ์ นทีรมณ์ และ นางสาวรำพร กุหลาบ นักวิทยาศาสตร์ผู้ปฏิบัติงานซึ่งมีความรับผิดชอบ ทำงานหนัก และทุ่มเทเพื่อให้งานวิจัยนี้สำเร็จลงได้ ขอกราบขอบพระคุณผู้บริหาร สกว. และขอบคุณ คุณพรพิมล กิติมศักดิ์ เจ้าหน้าที่บริหารโครงการ สำนักงานกองทุนสนับสนุนการวิจัย ที่ได้ช่วยบริหารจัดการและประสานงานตลอดโครงการด้วยน้ำใจและอัชฌาสัยอันดียิ่ง สุดท้ายนี้คณะผู้วิจัยขอขอบพระคุณคณะแพทยศาสตร์ศิริราชพยาบาล และมหาวิทยาลัยมหิดล ที่ได้ให้การสนับสนุนการวิจัยในสถาบันเป็นอย่างดียิ่งตลอดมา

บทคัดย่อ

รหัสโครงการ:

ชื่อโครงการ : อณูชีววิทยาและวิทยาภูมิคุ้มกันของเชื้อไวรัสไข้หวัดนก H5N1

ชื่อนักวิจัย : พิไลพันธ์ พุธวัฒนะ

ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

E-mail Address : pilaipan.put@mahidol.ac.th

ระยะเวลาโครงการ : 3 ปี

งานวิจัยนี้ได้ทำการศึกษาคุณสมบัติทางอณูชีววิทยาระดับโมเลกุลของเชื้อไวรัสไข้หวัดนก H5N1 ที่มีความสามารถในการก่อโรครุนแรง และเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ A(H1N1) 2009 รวมทั้งศึกษาการตอบสนองทางภูมิคุ้มกันต่อการติดเชื้อไวรัสทั้งสองด้วย การศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแสดงให้เห็นว่าอนุภาคไวรัสมากกว่า 80% มีรูปร่างเป็นทรงกลม และส่วนน้อยที่เหลือ มีรูปร่างแบบสายยาว หรือรูปร่างไม่แน่นอน กลไกในการก่อโรคของไวรัส H5N1 ประการหนึ่ง คือความสามารถในการเพิ่มจำนวนและกระตุ้นให้เกิดการสร้าง cytokine ในเซลล์เยื่อบุหลอดเลือดดำของมนุษย์ได้ดี นอกจากนี้ยังได้ค้นพบกลไกใหม่อีกประการหนึ่ง ที่ทำให้ไข้หวัดนกมีความรุนแรงในการก่อโรค คือ ฮีแมกกลูตินินของเชื้อไวรัส H5N1 ช่วยให้ไวรัสสามารถทนทานต่อสารยับยั้งที่มีอยู่ในซีรั่มได้ งานวิจัยนี้ได้ทำการเฝ้าระวังและสำรวจหาไวรัสไข้หวัดนกลูกผสมที่อาจมียีนของไวรัสไข้หวัดใหญ่ของมนุษย์รวมอยู่ในอนุภาคด้วย ได้ตรวจไวรัสไข้หวัดนกทั้งหมดจำนวน 109 สายพันธุ์ ในระหว่างปี พ.ศ. 2546-2549 โดยวิธี multiplex RT-PCR แต่ก็ไม่พบว่ามีไวรัสลูกผสมเกิดขึ้น การวิเคราะห์ทาง phylogenetic แสดงให้เห็นว่าการกลับมาของการระบาดของเชื้อ H5N1 มักเกิดจากไวรัสที่รอดเหลือจากการระบาดครั้งก่อน ไม่ใช่เป็นการนำสายพันธุ์ใหม่เข้ามาจากประเทศอื่น การวิจัยยังได้พบไวรัสลูกผสม 2 เชื้อ อุบัติขึ้นในปี พ.ศ.2550 จากการรวมตัวระหว่างไวรัสไข้หวัดนก H5N1 ด้วยกันเอง ในปี พ.ศ. 2550 ได้ทำการวินิจฉัยการติดเชื้อไข้หวัดนก clade 2.3.4, genotype V ในผู้ป่วยชาวลาวซึ่งเข้ามารับการรักษาในประเทศไทยและเสียชีวิตในโรงพยาบาลที่จังหวัดหนองคาย

เทคนิค hemagglutination-inhibition (HI) assay โดยการใช้เม็ดเลือดแดงห่านร่วมกับ เทคนิค microneutralization (microNT) assay ซึ่งทำการทดสอบในเซลล์ MDCK ได้ถูกพัฒนาขึ้นเพื่อใช้ตรวจหาแอนติบอดีต่อเชื้อ H5N1 ระดับของแอนติบอดีที่เพิ่มขึ้นในซีรั่มคู่จะสามารถตรวจพบได้ที่ประมาณ 15 วัน หลังเริ่มมีอาการป่วย ทั้ง HI และ NT antibody ที่ระดับ titer ≥40 (ไม่คิดรวมปริมาตรในหลุมทดสอบ) สามารถคงอยู่ได้เป็นเวลานานถึง 5 ปี หลังจากการป่วย นอกจากนี้ยังพบว่า ผู้สูงอายุที่ได้รับวัคซีนไข้หวัดใหญ่จำนวน 5.2% มีการเพิ่มขึ้นของระดับ

แอนติบอดีที่สามารถทำปฏิกิริยาข้าม (cross-neutralizing antibody) กับเชื้อ H5N1 ได้ งานวิจัยนี้ได้ทำการสร้าง vaccinia recombinant viruses ทั้งหมด 6 แบบ ที่มีการสอดแทรกยีนต่างๆ ของไวรัส H5N1 ได้แก่ HA, NA, NP, M, และ NS รวมทั้ง vaccinia recombinant virus ที่มีการใส่เฉพาะ pSC11 plasmid vector เข้าไปเท่านั้นเพื่อใช้เป็น control จากการวิเคราะห์ด้วยวิธี Western blot พบว่าผู้รอดชีวิตจากการติดเชื้อ H5N1 ทั้ง 4 ราย มีแอนติบอดีต่อฮีแมกกลูตินิน 3 แบบ คือ HA0, HA1และ HA2 domains ในขณะที่ผู้ไม่ติดเชื้อ H5N1 จะมีแอนติบอดีต่อ HA2 domain เท่านั้น และเมื่อนำเซลล์ที่ติดเชื้อ vaccinia recombinant viruses ที่มีการแสดงออกของโปรตีนต่างๆของเชื้อไข้หวัดนกมาใช้เป็นแอนติเจนในการทดสอบหาแอนติบอดีต่อเชื้อ H5N1 ด้วยวิธี indirect immunofluorescence ได้ผลว่าซีรั่มของผู้รอดชีวิตจากการติดเชื้อ H5N1 ทั้ง 4 ราย และซีรั่มจากกลุ่มที่ไม่มีการติดเชื้อ H5N1 เกือบทั้งหมดสามารถมีแอนติบอดีต่อโปรตีนทดสอบทุกชนิด แต่กลุ่มผู้รอดชีวิตจากการติดเชื้อ H5N1 จะมีระดับของแอนติบอดีที่สูงกว่า นอกจากนี้การทดสอบด้วยวิธี ELISpot โดยใช้ overlapping peptide pools ที่ครอบคลุมโปรตีน NP และ M ทั้งสาย ยังทำให้ทราบว่าผู้รอดชีวิตจาก H5N1 มีการสร้างภูมิคุ้มกันด้านเซลล์ซึ่งสามารถคงอยู่ได้นานเท่ากับภูมิคุ้มกันแบบสารน้ำ และเมื่อทำการวิเคราะห์ต่อไปด้วยเทคนิค flow cytometry ก็พบว่า cytotoxic T cells ที่มีอยู่นั้นส่วนมากเป็นชนิด CD4+ T lymphocyte ในขณะที่พบ CD8+ T lymphocytes เป็นส่วนน้อย และ NP peptides ทำให้เกิดการกระตุ้นได้สูงกว่า M peptides

จากการใช้เทคนิคการสื่อสารผ่านสัญญาณดาวเทียม (satellite telemetry) ในการติดตามนกนางนวลที่บางปู แสดงให้เห็นว่าเส้นทางบินของนกนางนวลครอบคลุมถึง 7 ประเทศที่มีรายงานการระบาดของเชื้อไข้หวัดนก ได้แก่ ไทย กัมพูชา เวียดนาม จีน อินเดีย บังคลาเทศ และเมียนม่าร์ โดยมี 3 ประเทศเป็นถิ่นที่อยู่อาศัย ได้แก่บริเวณทะเลสาบในธิเบต และซินเจียงเป็นที่ผสมพันธุ์และวางไข่ บริเวณอ่าวไทยตอนใน และทะเลสาบกัมพูชา เป็นที่ที่นกอพยพมาอาศัยในฤดูหนาว ส่วนอีก 4 ประเทศที่เหลือเป็นจุดหยุดพักในระหว่างอพยพ เนื่องจากลักษณะพันธุกรรมของไวรัสในประเทศไทยและจีนมีความแตกต่างกัน โดยไวรัสในประเทศไทยจัดอยู่ใน clade 1 แต่ไวรัสในประเทศจีนจัดอยู่ใน clade 2 จึงไม่พบความเชื่อมโยงระหว่างเชื้อที่ก่อการระบาดในสองประเทศนี้ ในขณะที่เชื้อไข้หวัดนกที่พบในไทย กัมพูชา และเวียดนาม เป็นไวรัส clade 1 เหมือนกัน นอกจากนั้นการระบาดของโรคไข้หวัดนกครั้งแรกๆ ในประเทศทั้งสามนี้ยังเกิดขึ้นในเวลาใกล้เคียงกันแสดงว่าเชื้อไวรัสน่าจะมีที่มาจากแหล่งเดียวกัน จากข้อมูลที่ได้จากการติดตามเส้นทางการบินของนกประกอบกับผลการทดสอบเกี่ยวกับความทนทานของนกที่ฉีดเชื้อไวรัสเข้าไป ทำให้สันนิษฐานได้ว่านกอพยพอาจมีบทบาทในการแพร่กระจายของเชื้อไข้หวัดนก

หลังจากสิ้นสุดการระบาดของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 2009 ระลอกแรก พบอัตราการติดเชื้อในกลุ่มผู้บริจาคโลหิตจำนวน 7% และ ในกลุ่มบุคลากรทางการแพทย์จำนวน 12.8% การศึกษาในระดับชุมชนพบอัตราการติดเชื้อในเด็กจำนวน 58% ในขณะที่พบการติดเชื้อใน

ผู้ใหญ่เพียง 3.1% เท่านั้น นอกจากนี้งานศึกษาวิจัยนี้ยังแสดงให้เห็นว่า nasopharyngeal aspirate เป็นสิ่งส่งตรวจที่ให้ผลไวต่อการวินิจฉัยการติดเชื้อดีที่สุด รองลงมาคือ nasal swab และ throat swab ตามลำดับ การวิเคราะห์ตัวอย่างจากทางเดินหายใจที่เก็บอย่างต่อเนื่อง พบว่าปริมาณไวรัสที่ถูกปล่อยออกมาจากผู้ป่วยจะอยู่ในช่วง 2.6×10^{2} - 8.1×10^{9} copies/ml และช่วงเวลาที่ผู้ป่วยสามารถแพร่เชื้อจะอยู่ระหว่าง 1 ถึง 11 วัน (เฉลี่ย 5 วัน) การประเมินความสามารถของวัคซีนเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ H1N1 ชนิด monovalent inactivated ในการกระตุ้นภูมิคุ้มกันในผู้ติดเชื้อ HIV เปรียบเทียบกับกลุ่มผู้ไม่ติดเชื้อ พบว่าในกลุ่มผู้ติดเชื้อ HIV ที่ได้รับวัคซีน มีอัตรา seroconversion เท่ากับ 32% และ seroprotection เท่ากับ 33.3% ขณะที่ในกลุ่มผู้ไม่ติดเชื้อ HIV พบอัตรา seroconversion และ seroprotection เท่ากันคือ 35%

งานวิจัยนี้ได้นำเทคนิค 4 อย่าง ได้แก่ การหาลำดับนิวคลิโอไทด์ของยีน NA, การลดลงของจำนวน plaque (plaque reduction assay), การยับยั้งเอนไซม์นิวรามินิเดส (neuraminidase inhibition- NAI assay) และ วิธีวัดปริมาณนิวคลิโอโปรตีนที่ลดลง (ELISA-based viral nucleoprotein reduction assay) มาใช้ในการศึกษาและเฝ้าระวังการเกิดเชื้อไข้หวัดใหญ่ดื้อต่อยา โดยข้อดีของเทคนิค ELISA based viral nucleoprotein reduction assay คือ สามารถใช้ในการทดสอบกับยาหรือสารที่ออกฤทธิ์ยับยั้งต่อยีนใดของไวรัสก็ได้ ดังนั้นวิธีนี้จึงดีกว่าวิธี NAI ที่ใช้ทดสอบกับยาที่ออกฤทธิ์ต้านเฉพาะนิวรามินิเดสของไวรัสเท่านั้น นอกจากนี้วิธีนี้ใช้เวลาไม่นานและใช้สารเคมีทดสอบในปริมาณน้อยเมื่อเทียบกับวิธีมาตรฐานคือ plaque reduction assay แต่ข้อเสียของวิธี ELISA based viral nucleoprotein reduction assay คือ ไม่สามารถใช้ทดสอบกับไวรัสซึ่งมีอัตราการเพิ่มจำนวนที่ช้าผิดปกติได้

คำหลัก : ไข้หวัดนก H5N1, ไข้หวัดใหญ่สายพันธุ์ใหม่ 2009, การตอบสนองทางภูมิคุ้มกัน, ระบาดวิทยาทางซีรั่ม, นกอพยพ, ระบบสื่อสารผ่านดาวเทียม

Abstract

Project Code:

Project Title: Molecular biology and immunology of avian influenza H5N1 viruses

Investigator: Prof.Dr. Pilaipan Puthavathana, Ph.D.
Department of Microbiology, Faculty of Medicine Siriraj Hospital,
Mahidol Universisty

E-mail Address: pilaipan.put@mahidol.ac.th

Project Period: 3 years

The present study investigated the molecular biological properties of the highly pathogenic avian influenza (HPAI) H5N1 viruses and the 2009 pandemic A(H1N1) viruses (H1N1pdm virus) as well as the immunological responses against those viral infections. Electron microscopy demonstrated that more than 80% of the viral particles in a population of an influenza isolate were spherical type, while filamentous and pleomorphic forms were the minor populations. Based on M protein, morphological type of influenza viruses was unlikely under control of genetic control. A pathogenic mechanism of HPAI H5N1 viruses involved the active replication and cytokine induction in human vein endothelial cells. Additionally, a novel pathogenic mechanism that made HPAI H5N1 virus virulent involved the viral hemagglutinin mediated resistance to the serum inhibitors. Surveillance for the reassortant H5N1 virus carrying human genomic segment had been conducted in 109 HPAI H5N1 viruses collected between 2003 and 2006 by using multiplex reverse transcription-polymerase chain reaction (RT-PCR), and no reassortant was found. Our genetic analysis showed that the resurgence of the HPAI H5N1 outbreak usually occurred from the indigenous viruses surviving after subsidence of the previous outbreaks, not from the re-introducing of the new strain from the other country. Two reassortants emerged from the reassortment between two H5N1 parental viruses were discovered in 2007. An infection caused by the clade 2.3.4, genotype V virus was diagnosed in a Lao patient who came into Thailand for receiving medical care and died in the Nongkhai hospital in 2007.

Goose erythrocyte-hemagglutination inhibition (HI) assay together with microneutralization (microNT) assay in MDCK cells were used for the detection of antibody to H5N1 viruses. The increasing antibody titer in paired blood was shown

approximately 15 days after disease onset. Both HI and NT antibodies at the titer of $\geq$ 40 (regardless of total volume in the reaction) persisted for almost 5 years after the disease onset in cases that could be followed-up that far. An increase in level of cross neutralizing antibody against HPAI H5N1 could be seen in 5.2% of the old-age vaccinees who received seasonal influenza vaccine. Six recombinant vaccinia viruses carrying individual gene of HPAI H5N1 virus, i.e., HA, NA, NP, M and NS and the pSC11 plasmid vector control were constructed. Western blot analysis showed that all 3 form of HA: HA0 and its cleavage products, HA1 and HA2 domains were expressed in the HA recombinant vaccinia virus-infected TK^- cells. H5N1 survivors contained the antibodies that directed against all 3 forms of HA; while all non-H5N1 subjects contained cross reactive antibody against the HA2 domain only. The viral proteins expressed in cells infected with these constructs were used as the test antigen in the indirect immunofluorescence assay for detection of the H5N1 antibodies. The result demonstrated that sera from all 4 survivors and almost of the non-H5N1 subjects were reactive against all kinds of these viral proteins; but the antibody titers were higher in the survivors. H5N1 survivors also developed cell-mediated immunity which lasted as long as the humoral immunity as determined by ELISpot assay using overlapping peptide pools spanning the entire NP and M proteins. Flow cytometry assay demonstrated that majority of the reactive cytotoxic T cells from H5N1 survivors were CD4+T lymphocytes whereas CD8+T lymphocytes were the minor population; and NP peptides yielded stronger reactivity than M peptides.

Satellite telemetry tracking of the Brown headed gulls from the Bang Poo study site demonstrated their flyways that spanned 7 countries (Thailand, Cambodia, Vietnam, China, India, Bangladesh and Myanmar) which were affected by H5N1 AI. This gull species inhabits 3 countries; Tibet lake area and Xinjiang in west China as the breeding grounds, and the inner gulf of Thailand as well as the TonLe Sap lake area in Cambodia as the places for overwintering. The other 4 countries were the places for stopover during migration. The difference in the viral genetic clades between H5N1 viruses in Thailand (clade 1 virus) and China (clade 2 virus) suggested that there was no linkage between AI spread between the two countries. On the other hand, the HPAI H5N1 virus found in Thailand, Cambodia and Vietnam belonged to clade 1; and the firstly AI outbreaks in these countries occurred at about the same time, suggestive of the same virus origin. The data from satellite telemetry together with the result on virus

inoculation in gulls kept in captivity suggested the possible role of AI spread by migratory birds.

At the end of the first epidemic wave of the 2009 pandemic influenza the infection rates of 7% were found in blood donors and 12.8% in health care workers. The community based study reported the higher infection rates in children (58%) than adults (3.1%). Our study also showed that nasopharyngeal aspirate was the best sample which yielded the most sensitive result in the disease diagnosis, and followed in order by nasal swab and throat swab specimens. Using sequential respiratory samples, the viral load at the amount of 2.6×10^{2} - 8.1×10^{9} copies/ml were shed from the patients and duration of viral shedding lasted between 1 and 11 days (mean = 5 days). The immunogenicity of monovalent, inactivated H1N1pdm vaccine was evaluated in HIV infected individuals and the uninfected controls after single injection by HI assay. The seroconversion rate of 32% and the seroprotection rate of 33.3% were found in the HIV infected vaccinees; while the seroconversion rate as well as the seroprotection rate of 35% was found in the uninfected control group.

Our group has established 4 techniques: direct nucleotide sequencing, plaque reduction assay, neuraminidase inhibition (NAI) assay and ELISA based viral nucleoprotein (NP) reduction assay for using in the surveillance for anti-viral drug resistant influenza viruses. The advantage of ELISA based viral NP reduction assay is its ability to investigate the drug or compounds targeting any viral gene; therefore, it has an advantage over the NAI assay which can investigate only the compounds targeting NA protein. Moreover, it has an advantage over the gold standard plaque reduction assay such that it consumes less reagents and time. The pitfall of ELISA based viral NP reduction assay is its inability to test the viruses with unusually slow rate of replication.

Keywords : Highly pathogenic influenza H5N1 virus, 2009 pandemic A(H1N1) virus, satellite telemetry

สารบัญ

เนื้อเรื่องย่อ

งานวิจัยนี้ทำการศึกษาเกี่ยวกับคุณสมบัติทางชีววิทยาระดับโมเลกุลของเชื้อไวรัสไข้หวัดนก H5N1 และเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ (2009 pandemic A(H1N1) virus, H1N1pdm) รวมทั้งการตอบสนองทางภูมิคุ้มกันต่อการติดเชื้อด้วย ภาพถ่ายจากกล้องจุลทรรศน์อิเล็กตรอนของเชื้อไข้หวัดนก H5N1 ซึ่งมีความรุนแรงในการก่อโรคสูงไม่มีความแตกต่างจากไวรัสไข้หวัดนกที่มีความรุนแรงในการก่อโรคต่ำ และไม่แตกต่างจากไวรัสไข้หวัดใหญ่ของคน การย้อมสี negative stain แสดงให้เห็นรูปร่างของอนุภาคไวรัส 3 แบบ คือ ทรงกลมขนาด 80-120 nm สายยาวขนาด >300 nm และอนุภาคที่มีรูปร่างไม่แน่นอนขนาด 120-300 nm โดยอนุภาคทรงกลมมีจำนวนมากกว่า 80% ของอนุภาคทั้งหมด การศึกษาเปรียบเทียบลำดับนิวคลิโอไทด์ของ *M* gene กับข้อมูลที่เคยมีผู้รายงานไว้ก่อนชี้แนะว่าลักษณะรูปร่างของไวรัสไม่ได้ถูกกำหนดด้วยลักษณะทางพันธุกรรมซึ่งแย้งกับที่เคยมีผู้รายงานไว้ ลักษณะเด่นของโรคไข้หวัดนก H5N1 คือ ความล้มเหลวในการทำงานของอวัยวะหลายชนิดและการสร้าง cytokine มากเกินไป มีปัจจัยหลายอย่างซึ่งทำให้ไวรัสไข้หวัดนก H5N1 มีความรุนแรงในการก่อโรคในมนุษย์ ได้แก่ การที่ cleavage site ของ ฮีแมกกลูตินิน มี basic amino acids หลายตัวติดต่อกัน ซึ่งไวรัสทุกตัวที่แยกได้ในประเทศไทยมีลักษณะนี้ นักวิจัยแต่ก่อนชี้แนะว่า นี้เป็นคุณสมบัติสำคัญที่ทำให้ไวรัสไข้หวัดนกแพร่กระจายออกจากระบบทางเดินหายใจไปสู่ระบบอื่นๆได้ ซึ่งคุณสมบัตินี้พบได้น้อยมากในไวรัสไข้หวัดใหญ่ของมนุษย์ และงานวิจัยนี้ยังพบว่า กลไกในการก่อโรคของไวรัส H5N1 ประการหนึ่งคือ ความสามารถในการเพิ่มจำนวนในเซลล์เยื่อบุหลอดเลือด และมี proinflmmatory cytokine หลายชนิดถูกสร้างขึ้นด้วย ในขณะที่ไวรัสไข้หวัดใหญ่ของคนจะเพิ่มจำนวนในเซลล์เยื่อบุหลอดเลือดได้ไม่ดี นอกจากนี้ยังได้พบกลไกใหม่ที่ทำให้ไข้หวัดนกมีความรุนแรงในการก่อโรค คือเชื้อไวรัสไข้หวัดนกไม่ถูกยับยั้งโดย serum innate inhibitor ในขณะที่ไวรัสไข้หวัดใหญ่ของคนจะถูกยับยั้งได้เกือบหมดและการศึกษานี้ยังแสดงให้เห็นว่า ความทนทานนี้ขึ้นอยู่กับฮีแมกกลูตินินของเชื้อไข้หวัดนก

เชื้อไวรัสไข้หวัดนก H5N1 มีความรุนแรงมาก แต่โชคดีที่ไวรัสชนิดนี้ไม่มีการติดต่อระหว่างประชากรมนุษย์เนื่องจากเชื้อไวรัสไข้หวัดนกเกาะติดกับที่รับบนผิวเซลล์ (receptor site) ของคนได้ไม่ดี แต่เนื่องจากการผสมกันระหว่างยีนของไวรัสสองชนิดเกิดขึ้นบ่อย ดังนั้นจึงอาจเป็นไปได้ที่จะเกิดไวรัสลูกผสม (reassortant) ซึ่งได้รับคุณสมบัติความรุนแรงในการก่อโรคจากไวรัส H5N1 และความสามารถในการแพร่กระจายเชื้อในประชากรมนุษย์จากไข้หวัดใหญ่ของมนุษย์ งานวิจัยนี้จึงได้ทำการสำรวจหาไวรัสลูกผสมที่อาจปะปนอยู่กับไวรัสไข้หวัดนก ทั้งหมดจำนวน 109 สายพันธุ์ ในระหว่างปี พ.ศ. 2546-2549 โดยใช้วิธี multiplex RT-PCR ซึ่งสามารถขยายยีนทั้ง 8 ชิ้นของไวรัสไข้หวัดนกได้ แต่ก็ไม่พบว่ามีไวรัสลูกผสมเกิดขึ้น

ประเทศไทยได้ผ่านการระบาดของไวรัสไข้หวัดนก H5N1 มาแล้วหลายระลอกนับตั้งแต่ปีพ.ศ. 2547 การวิเคราะห์ทาง phylogenetic แสดงให้เห็นว่าเชื้อไวรัส H5N1 ในช่วงการระบาดแรกๆ มีลักษณะทางพันธุกรรมที่แตกต่างกันมาก แต่เมื่อมีการควบคุมการระบาดในสัตว์ปีกก็เหลือไวรัสไข้หวัดนกเพียง 2-3 lineages เท่านั้น ภายหลังจากที่การระบาดแต่ละปีสงบลง ปรากฏการณ์นี้สังเกตเห็นได้ในฤดูร้อนของปีพ.ศ. 2549-2550 การกลับมาของการระบาดมักจะเกิดขึ้นในบริเวณลุ่มแม่น้ำยม และแม่น้ำน่าน และเกิดจากไวรัสที่รอดเหลือจากการระบาดครั้งก่อนมิใช่เกิดจากการนำเชื้อสายพันธุ์ใหม่เข้ามาจากประเทศอื่น นอกจากนี้การวิจัยยังได้พบไวรัสลูกผสม 2 เชื้อ อุบัติขึ้นในปี พ.ศ. 2550 จากการรวมตัวระหว่างไวรัสไข้หวัดนก H5N1 ด้วยกันเอง ไวรัสไข้หวัดนก H5N1 ที่ก่อการระบาดในประเทศไทยตั้งแต่ปี พ.ศ. 2547 จัดอยู่ใน clade 1 genotype Z และได้ระบาดอยู่ในประเทศไทยตั้งแต่นั้นมา เป็นไวรัส clade เดียวที่อยู่ในภาคกลางและภาคเหนือของประเทศจนกระทั่งปี พ.ศ. 2551 และในปี พ.ศ. 2549 นักวิจัยกลุ่มอื่นในประเทศไทยได้พบการเข้ามาของไวรัส clade 2.3.4 ในภาคอีสานของประเทศ โครงการวิจัยนี้ได้ทำหน้าที่เป็นห้องปฏิบัติการคู่ขนานของสถาบันสุขภาพแห่งชาติ (National Institute of Health, NIH) ในการวินิจฉัยโรคไข้หวัดนกในผู้ป่วยชาวลาวที่มารับการรักษาตัวในโรงพยาบาลหนองคายในปีพ.ศ. 2550 และได้พบว่าเป็นการติดเชื้อไวรัส H5N1 clade 2.3.4 เช่นกัน ในการประเมิน selective pressure ที่เกิดกับฮีแมกกลูตินิน เกี่ยวกับ synonymous codon usage ชี้แนะว่า ไวรัส subclade 2.1 จากอินโดนีเซียมี codon volatility ต่ำสุด เมื่อเทียบกับไวรัสใน clade และ subclade อื่นๆ

จากการที่ไวรัสไข้หวัดนก H5N1 มีความจำเพาะกับ receptor ชนิด α2,3 galactose-linked sialic acid ซึ่งเป็น avian type receptor กลุ่มผู้วิจัยที่ทำการศึกษาเชื้อ H5N1 ที่ระบาดในฮ่องกงในปี พ.ศ. 2540 จึงได้แนะนำให้ใช้เม็ดเลือดแดงของม้าในการทดสอบหาแอนติบอดีต่อเชื้อ H5N1 ด้วยวิธี hemagglutination inhibition (HI) assay รวมไปถึง WHO ก็ได้มีการแนะนำให้ใช้เม็ดเลือดแดงของม้าในการทดสอบด้วยวิธีนี้เช่นกัน แต่อย่างไรก็ตาม การแนะนำนี้เกิดจากการทดสอบเปรียบเทียบโดยใช้เลือดเพียง 2 ชนิด คือ เม็ดเลือดแดงจากม้าและจากหนูตะเภาเท่านั้น สำหรับประเทศไทย เม็ดเลือดแดงม้าเป็นเลือดที่หาได้ไม่ง่ายนัก งานวิจัยนี้จึงทำการทดสอบโดยเปรียบเทียบเม็ดเลือดแดง 5 ชนิด คือ คนหมู่เลือดโอ ไก่ ห่าน หนูตะเภา และม้า จากการทดสอบ พบว่าเชื้อ H5N1 ที่ทดสอบสามารถจับกับ receptor บนผิวเม็ดเลือดแดงของห่านได้ดี และจากการวิเคราะห์ดู receptor binding site ที่อยู่บนฮีแมกกลูตินิน พบว่าเชื้อ H5N1 ที่ใช้ในการทดสอบมี recptor binding site ที่ไม่ต่างจากเชื้อ H5N1 ที่ก่อการระบาดในฮ่องกงเมื่อปี พ.ศ. 2540 เม็ดเลือดแดงห่านให้ความไวสูงในการทดสอบ hemagglutination assay ซึ่งใช้ในขั้นตอนของการแยกเชื้อไวรัส และการทดสอบหาแอนติบอดีด้วยวิธี HI assay พบว่าผลการทดสอบที่ใช้เม็ดเลือดแดงห่านกับผลการทดสอบที่ใช้เม็ดเลือดแดงม้าให้ผลไม่ต่างกัน ในขณะนี้เม็ดเลือดแดงห่านจึงเป็นที่ยอมรับสำหรับการทำ HI assay และถูกนำไปใช้ในห้องปฏิบัติการหลายแห่งของประเทศ และเม็ดเลือดแดงชนิดนี้ถูกนำไปใช้ในการทดสอบเพื่อศึกษาเชิงระบาด

วิทยาทางซีรั่มต่อไปด้วย นอกจากเชื้อ HPAI H5N1 แล้ว คณะผู้วิจัยยังพบว่าเม็ดเลือดแดงห่านมีความไวสูงในการทดสอบเชื้อ LPAI ทั้ง 16 subtypes รวมทั้งเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ด้วย

งานวิจัยนี้ได้ทำการศึกษาจลนศาสตร์และความคงอยู่ของระดับแอนติบอดีที่ตอบสนองต่อการติดเชื้อ HPAI H5N1 ในซีรั่มของผู้ป่วยที่เสียชีวิตจากการติดเชื้อ H5N1 จำนวน 4 ราย และจากผู้รอดชีวิต จำนวน 4 ราย โดยใช้วิธี microNT, HI และ Western blot ผลการศึกษาพบว่า HI antibody ถูกตรวจพบได้เร็วกว่า NT antibody โดยส่วนใหญ่แล้วการเพิ่มขึ้นของระดับแอนติบอดีในซีรั่มคู่จะตรวจพบที่ประมาณ 15 วัน หลังเริ่มมีอาการป่วย กลุ่มตัวอย่างผู้รอดชีวิตจากการติดเชื้อ H5N1 จะถูกเก็บตัวอย่างเลือดทุกๆ 6 เดือน เป็นเวลานาน 3 ปีหลังจากเข้าร่วมโครงการ ผลการวิจัยพบว่าทั้ง HI และ NT antibody ที่ระดับ titer ≥40 (ไม่คิดรวมปริมาตรในหลุมทดสอบ) สามารถคงอยู่ได้เป็นเวลานานประมาณ 5 ปีหลังจากการป่วย ดังนั้นค่า titer 40 สามารถใช้เป็นค่า cut-off ในการวินิจฉัยเพื่อจำแนกระหว่างผู้ติดเชื้อและไม่ติดเชื้อ H5N1 และสามารถใช้ประโยชน์ในการติดตามเฝ้าระวังกลุ่มเสี่ยงที่อาจติดเชื้อแต่ไม่แสดงอาการได้ เช่น กลุ่มผู้เลี้ยงสัตว์ปีก กลุ่มผู้ทำหน้าที่ทำลายสัตว์ป่วย และชาวชนบท เป็นต้น นอกจากนี้ยังพบว่าประมาณ 6.7-10.3% ของกลุ่มผู้สูงอายุมี cross-neutralizing antibody ต่อเชื้อ H5N1 ที่ titer 10 และพบ seroconversion 5.2% ในกลุ่มผู้สูงอายุที่ได้รับวัคซีนไข้หวัดใหญ่ งานวิจัยนี้ได้ทำการสร้าง vaccinia recombinant viruses ทั้งหมด 6 ชนิด โดยมีการสอดแทรกยีนต่างๆ ของไวรัส HPAI H5N1 ได้แก่ HA, NA, NP, M หรือ NS ลงในยีโนมของ vaccinia virus และชนิดที่ 6 คือ vaccinia recombinant virus ที่มีการใส่เฉพาะ pSC11 plasmid vector เข้าไปเท่านั้นเพื่อใช้เป็น control หลังจากสร้าง vaccinia recombinant viruses ขึ้นมาแล้วก็นำไปเลี้ยงในเซลล์ TK^- เพื่อให้มีการแสดงออกของโปรตีนจากไวรัสซึ่งสามารถตรวจสอบการแสดงออกของโปรตีนนั้นๆ ได้ด้วยวิธี immunofluorescence และ Western blot ในการศึกษา vaccinia recombinant virus ที่มีการแสดงออกของ H5N1 HA protein ซึ่งเป็น immunodominant epitope ที่สำคัญในการกระตุ้นให้เกิด protective antibody พบว่าเซลล์ที่ติดเชื้อมีการแสดงออกของ HA ทั้ง 3 รูปแบบ คือ HA0, HA1 และ HA2 ผู้รอดชีวิตจากการติดเชื้อ H5N1 จะมีแอนติบอดีต่อ HA ทั้ง 3 รูปแบบนั้น ในขณะที่ผู้ไม่ติดเชื้อ H5N1 จะมีเพียงแอนติบอดีที่ทำปฏิกิริยาข้าม (cross-reactive antibody) กับส่วน HA2 เท่านั้น การกระตุ้นภูมิคุ้มกันในหนู BALB/c ด้วย HA recombinant vaccinia virus สามารถกระตุ้นให้เกิดทั้ง HI และ NT antibody ต่อเชื้อ H5N1 ได้ ซึ่งได้ทำการทดสอบกับทั้ง reverse genetic virus ที่มีการแสดงออกของ H5 HA protein และ parental HPAI H5N1 virus แอนติบอดีที่ถูกสร้างขึ้นจากการกระตุ้นด้วยโปรตีนฮีแมกกลูตินินจะเป็น protective antibody สามารถป้องกันการติดเชื้อได้ แต่ทว่าโปรตีนส่วนอื่นๆ ของเชื้อไวรัสก็สามารถกระตุ้นให้เกิดแอนติบอดีที่มีหน้าที่ต่างๆกัน เช่น opsonization, antibody dependent cell cytotoxicity หรือขัดขวางการทำงานของโปรตีนของไวรัส งานวิจัยนี้ได้ใช้เซลล์ TK^- ที่ติดเชื้อ recombinant vaccinia virus และมีการแสดงออกของโปรตีน HA, NA, NP, M หรือ NS มาใช้เป็นแอนติเจนเพื่อตรวจหาแอนติบอดีโดยวิธี indirect

immunofluorescence ผลการทดสอบแสดงให้เห็นว่า ซีรั่มของผู้รอดชีวิตจากการติดเชื้อ H5N1 ทั้ง 4 ราย และซีรั่มเกือบทั้งหมดจากผู้ที่ไม่มีการติดเชื้อ H5N1 มีแอนติบอดีต่อโปรตีนต่างๆ ที่ใช้ในการทดสอบ แต่ผู้รอดชีวิตจากการติดเชื้อ H5N1 จะมีระดับของแอนติบอดีที่สูงกว่า

ในการทดสอบด้วยวิธี ELISpot assay โดยใช้ overlapping peptide pools ที่ครอบคลุมโปรตีน NP และ M ทั้งสาย พบว่าผู้รอดชีวิตจากการติดเชื้อ H5N1 มีการสร้างภูมิคุ้มกันด้านเซลล์ซึ่งสามารถคงอยู่ได้ในช่วงระยะเวลาใกล้เคียงกับภูมิคุ้มกันแบบสารน้ำ และผลจากการวิเคราะห์ด้วยเทคนิค flow cytometry โดยใช้ immunophenotype marker (CD4+ หรือ CD8+) ร่วมกับ γ-interferon, TNF-α, และ T cell-related cytotoxic marker CD107a พบว่า cytotoxic T cells ที่ถูกกระตุ้นนั้นส่วนใหญ่เป็นชนิด CD4+ T lymphocyte ในขณะที่พบ CD8+ T lymphocytes เป็นส่วนน้อย และ NP peptides มีความสามารถในการกระตุ้นได้สูงกว่า M peptides การศึกษานี้ยังแสดงให้เห็นว่า ผู้ที่ไม่เคยได้รับเชื้อ H5N1 มีการแสดงออกของ cross-reactive cellular immunity ต่อ NP peptides ได้เช่นกัน

มหาวิทยาลัยมหิดลได้รับความร่วมมือจากกรมอุทยานแห่งชาติ สัตว์ป่า และพันธุ์พืช ในการศึกษาวิจัยถึงบทบาทของนกอพยพในการแพร่กระจายของเชื้อไข้หวัดนก โดยใช้ transmitter ติดไว้กับตัวนกในการส่งข้อมูลทางไกลผ่านระบบดาวเทียม (satellite telemetry) ทำให้สามารถทราบถึงเส้นทางการบิน และถิ่นที่อยู่ของนกได้ การศึกษาครั้งนี้ใช้เครื่องส่งสัญญาณจำนวน 20 เครื่องติดกับตัวนก 4 ชนิด ได้แก่ นกนางนวลธรรมดา (Brown headed gulls), นกปากห่าง (Open-billed storks), นกยางโทนใหญ่ (Great egrets) และ นกเป็ดแดง (Lesser whistling ducks) ซึ่งการศึกษานี้ไม่สามารถติดตามเส้นทางบินของนกเป็ดแดงได้ เนื่องจากสัญญาณจากเครื่องส่งที่ติดกับตัวนกมีการขาดหายไปหลังจากเริ่มการติดตามเพียงไม่กี่สัปดาห์ โดยในขณะนี้การศึกษาถิ่นที่อยู่ของนกปากห่าง และนกยางโทนใหญ่ยังคงดำเนินต่อไป สำหรับนกนางนวลทั้ง 8 ตัวที่ได้ทำการติดตามนั้น มีเพียง 5 ตัว ที่สามารถทำการบันทึกเส้นทางอพยพระหว่างประเทศไทยและจีนได้อย่างสมบูรณ์ 1-2 รอบ เส้นทางบินของนกนางนวลครอบคลุม 7 ประเทศที่มีรายงานการระบาดของเชื้อไข้หวัดนก ได้แก่ ไทย, กัมพูชา, เวียดนาม, จีน, อินเดีย, บังกลาเทศ และพม่า แต่มีเพียงประเทศไทย กัมพูชา และจีนเท่านั้นที่เป็นถิ่นที่อยู่ของนกนางนวล ขณะที่ประเทศอื่นเป็นเพียงที่หยุดพักระหว่างการอพยพเท่านั้น ดังนั้นด้วยข้อมูลของระยะทางและช่วงเวลาของการอพยพ ประกอบกับข้อมูลจากการศึกษาทดลองเกี่ยวกับระยะเวลาที่นกนางนวลที่ถูกฉีดเชื้อ H5N1 จะขับเชื้อออกจากร่างกายจนกว่าจะตายหรือหายจากโรค ชี้ให้เห็นว่ามีความเป็นไปได้ที่นกนางนวลที่ติดเชื้อจะสามารถแพร่เชื้อไวรัสไปตามเส้นทางบินอพยพในเดือนมีนาคม ปีพ.ศ. 2552 พบการอุบัติขึ้นของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ที่ประเทศเม็กซิโก โดยผู้ป่วย 2 รายแรกของไทยนั้นเดินทางกลับมาจากประเทศเม็กซิโกเช่นกัน ทีมวิจัยได้ให้ความร่วมมือกับสถาบันสุขภาพแห่งชาติ (Thai NIH) ในการวินิจฉัยและรายงานผู้ติดเชื้อทั้งสองราย เมื่อวันที่ 4 พฤษภาคม พ.ศ. 2552 และสามารถแยกเชื้อจากผู้ป่วยสองรายนี้

ได้ และให้ชื่อว่า A/Nonthaburi/102/2009 (H1N1) และ A/Thailand/104/2009 (H1N1) ทีมวิจัยได้ทำการศึกษาลำดับพันธุกรรมของเชื้อทั้งสองนี้และได้เผยแพร่ไว้ใน GenBank database เชื้อไวรัส A/Thailand/104 นั้นสามารถเพิ่มจำนวนได้ง่ายกว่า ดังนั้นเชื้อนี้จึงถูกนำไปใช้เป็นแอนติเจนในการทดสอบหาภูมิคุ้มกันตอบสนองต่อการติดเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ การทดสอบซีรั่มที่ได้มีการเก็บไว้ก่อนปีพ.ศ. 2552 พบว่าแอนติบอดีที่มีอยู่ในซีรั่มก่อนการระบาดนั้นสามารถเกิด cross-neutralize ต่อเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ได้ โดยมีระดับ titer ที่ต่างกันไป แสดงให้เห็นว่าในกลุ่มประชากรไทยนั้นมีภูมิคุ้มกันบางส่วนต่อเชื้อชนิดนี้อยู่แล้ว และยังสามารถอธิบายถึงเหตุผลที่ว่าทำไมเชื้อชนิดนี้จึงก่อให้เกิดความรุนแรงของโรคไม่มากนัก ในการทำ HI assay โดยใช้เม็ดเลือดแดงของห่าน ร่วมกับการใช้ microNT assay ในการศึกษาซีรั่มคู่ของผู้ป่วย ทำให้ได้ค่า cut-off antibody titer ที่สามารถจำแนกระหว่างผู้ติดเชื้อกับผู้ที่ไม่ติดเชื้อ กล่าวคือ ผู้ติดเชื้อซึ่งเป็นผู้ใหญ่จะมี HI titer สูงกว่าหรือเท่ากับ 40 และเด็กที่ติดเชื้อจะมี HI titer สูงกว่าหรือเท่ากับ 20 ค่า cut-off titer นี้ได้ถูกนำไปใช้ในการศึกษาขนาดของการระบาดระลอกแรกในกลุ่มประชากรไทย โดยพบอัตราการติดเชื้อ 7% ในกลุ่มผู้บริจาคโลหิต และ 12.8% ในกลุ่มบุคลากรทางการแพทย์ ซึ่งอัตราการติดเชื้อระหว่างสองกลุ่มนี้ไม่มีความแตกต่างกันในเชิงสถิติ และจากการตอบแบบสอบถามของเจ้าหน้าที่พยาบาลเกี่ยวกับวิธีการและอุปกรณ์ป้องกันตนเองจากการติดเชื้อ (เช่น การล้างมือ การใส่หน้ากากอนามัย และการสวมถุงมือ) พบว่าการไม่สวมหน้ากากอนามัยระหว่างสัมผัสกับบุคลากรทางการแพทย์ด้วยกันที่ติดเชื้อ เป็นปัจจัยเสี่ยงสำคัญที่ทำให้เจ้าหน้าที่ติดเชื้อได้ อย่างไรก็ตามการศึกษานี้แสดงให้เห็นว่าประชากรส่วนใหญ่ยังไม่มีภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ และผลการวิจัยนี้คาดการณ์ว่าจะมีการระบาดระลอกถัดไปเกิดขึ้นภายหลังการระบาดในระลอกแรก ซึ่งต่อมาก็ได้พบว่ามีการระบาดเกิดขึ้นแล้วถึง 4 ระลอก และเชื้อ H1N1 ตามฤดูกาลที่เป็นเชื้อที่มีอยู่เดิมได้ถูกแทนที่โดยเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ตั้งแต่ปี พ.ศ. 2552 เป็นต้นมา จากการศึกษาในระดับชุมชนร่วมกับสำนักระบาดวิทยาพบว่าในพื้นที่ชนบท (จังหวัดเชียงใหม่ และนครศรีธรรมราช) ภายหลังจากการระบาดระลอกแรกพบว่ามีอัตราการติดเชื้อในเด็กสูงถึง 58% และพบในผู้ใหญ่เพียง 3.1% งานวิจัยนี้ได้มีการศึกษาระดับแอนติบอดีในกลุ่มผู้ติดเชื้อ HIV เปรียบเทียบกับกลุ่มผู้ไม่ติดเชื้อ ที่ได้รับวัคซีนไข้หวัดใหญ่ ชนิด monovalent inactivated pandemic influenza vaccine โดยใช้วิธี HI assay ผลการศึกษาพบว่า ในกลุ่มผู้ติดเชื้อ HIV ที่ได้รับวัคซีน มีอัตรา seroconversion เท่ากับ 32% และ seroprotection เท่ากับ 33.3% ขณะที่ในกลุ่มผู้ไม่ติดเชื้อ HIV ที่ได้รับวัคซีนพบอัตรา seroconversion และ seroprotection เท่ากันคือ 35%

ในการวินิจฉัยการติดเชื้อไข้หวัดใหญ่โดยการตรวจหายีโนมของเชื้อด้วยวิธี real time RT-PCR สามารถใช้สิ่งส่งตรวจจากระบบทางเดินหายใจของผู้ป่วยได้หลายชนิด เช่น nasopharyngeal aspirate (NPA), nasal swab (NS) และ throat swab (TS) งานวิจัยนี้ได้ทำการทดสอบเก็บสิ่ง

ส่งตรวจเหล่านี้จากผู้ป่วยคนเดียวกันก่อนการให้ยาต้านไวรัส ผลการทดสอบพบว่า NPA เป็นสิ่งส่งตรวจที่สามารถตรวจพบยีโนมของเชื้อได้มากที่สุด รองลงมาคือ NS และ TS ตามลำดับ

เมื่อมีการระบาดเกิดขึ้นในชุมชนปิด เช่น ในค่ายทหาร พบว่าการคัดแยกผู้ป่วยออกมานั้นเป็นวิธีการหนึ่งที่สามารถจะยับยั้งการแพร่กระจายของเชื้อได้ แต่จะต้องพิจารณาถึงระยะเวลาที่จะต้องแยกผู้ป่วยออกมา ทีมวิจัยได้ให้ความร่วมมือกับสำนักระบาดวิทยาเพื่อทำการศึกษาปริมาณไวรัสและช่วงเวลาที่ผู้ป่วยจะแพร่เชื้อ โดยใช้ real time RT-PCR ในการวิเคราะห์ตัวอย่างตรวจที่เก็บอย่างต่อเนื่องจากทางเดินหายใจของผู้ติดเชื้อ พบว่าปริมาณไวรัส 2.6×10^{2} - 8.1×10^{9} copies/ml จะถูกปล่อยออกมาจากผู้ป่วย และช่วงเวลาที่ผู้ป่วยจะสามารถแพร่เชื้อจะอยู่ระหว่าง 1 ถึง 11 วัน (เฉลี่ย 5 วัน)

ปัจจุบันยาในกลุ่มที่ออกฤทธิ์ยับยั้งเอนไซม์นิวรามินิเดสของไวรัสที่ใช้กันทั่วไปมีอยู่ 2 ชนิด คือ oseltamivir และ zanamivir งานวิจัยนี้ได้นำวิธีการ 4 อย่าง ได้แก่ การหาลำดับนิวคลิโอไทด์ของยีน NA, การลดลงของจำนวน plaque (plaque reduction assay), การยับยั้งเอนไซม์นิวรามินิเดส (neuraminidase inhibition- NAI assay) และ วิธีวัดปริมาณนิวคลิโอโปรตีนที่ลดลง (ELISA-based viral nucleoprotein reduction assay) มาใช้ในการศึกษาและเฝ้าระวังการเกิดเชื้อไข้หวัดใหญ่ดื้อต่อยา โดยก่อนหน้านี้ทีมวิจัยได้พบว่า 90-100% ของเชื้อไข้หวัดใหญ่ตามฤดูกาล H1N1 ที่ศึกษาในช่วงปี พ.ศ.2551 และ พ.ศ. 2552 ดื้อต่อยา oseltamivir ซึ่งเป็นผลจากการที่ histidine ที่ตำแหน่ง 274 ในโปรตีนนิวรามินิเดสเกิด mutation เปลี่ยนเป็น tyrosine แต่นับว่าโชคดีที่ไวรัสสายพันธุ์นี้ได้ลดน้อยและหายไปหลังจากปี พ.ศ. 2553 คณะผู้วิจัยได้ใช้วิธี ELISA-based viral nucleoprotein reduction assay ในการทดสอบคัดกรองหาสารเคมี หรือสารสกัดจากสมุนไพรที่มีฤทธิ์ต้านเชื้อไวรัสให้กับสถาบันหลายแห่ง ข้อดีของวิธีนี้คือ สามารถใช้ในการทดสอบกับยาหรือสารที่ออกฤทธิ์ยับยั้งต่อยีนใดของไวรัสก็ได้ ดังนั้นวิธีนี้จึงดีกว่าวิธี NAI ที่ใช้ทดสอบกับยาที่ออกฤทธิ์ต้านเฉพาะนิวรามินิเดสของไวรัสได้เพียงอย่างเดียว นอกจากนั้นวิธีนี้ยังใช้เวลาไม่นานและใช้สารเคมีทดสอบในปริมาณน้อยเมื่อเทียบกับวิธีมาตรฐานคือ plaque reduction assay แต่ข้อเสียของวิธี ELISA based viral nucleoprotein reduction assay คือไม่สามารถใช้ทดสอบกับไวรัสซึ่งมีอัตราการเพิ่มจำนวนที่ช้าผิดปกติได้

Executive Summary

The present study investigated the molecular biological properties of the highly pathogenic avian influenza (HPAI) H5N1 viruses and the 2009 pandemic A (H1N1) virus (H1N1pdm virus) together with the immunological responses against these two viral infections. Electron micrographs of HPAI H5N1 viruses were not different from those of the low pathogenic avian influenza (LPAI) viruses and human influenza viruses. With negative staining, 3 morphological forms of the virus particles, i.e., spherical form at size of 80-120 nm, filamentous form at size of >300 nm and the pleomorphous particles at size of 120-300 nm were visualized under a transmission electron microscope. However, the spherical form took account of greater than 80% of the entire viral population. Based on an alignment of the M genomic sequences, our study also suggested that viral morphology was not under control of the viral genetics as had been previously reported. The hallmarks of H5N1 avian influenza are multi-organ failure and cytokine storm. There are several factors that contribute to the highly virulent property of this virus subtype. Presence of multiple basic amino acids at the hemagglutinin (HA) cleavage site is present in the entire virus isolates ever reported in Thailand. Previous investigators suggested that this is an important property that facilitated the virus spreading beyond the respiratory tract to the other distal end organs, the property that is extremely rare for human viruses. Moreover, we have found that a pathogenic mechanism of HPAI H5N1 viruses involved the active replication in human endothelial cells, while human viruses poorly replicated. Production of certain pro-inflammatory cytokines/ chemokines was induced in the infected human vein endothelial cells. Additionally, we discovered a new pathogenic mechanism that made HPAI H5N1 viruses virulent, that was the ability to resist the serum inhibitors. Using guinea pig sera as the model, we demonstrated that the serum innate inhibitors could not block the infectivity of HPAI H5N1 viruses; while the infectivity of human viruses was almost completely inhibited. We also showed that this resistance was mediated through the H5 *HA* gene. Collectively, our findings add up the information on what made the HPAI H5N1 viruses become virulent.

HPAI H5N1 viruses are highly virulent; fortunately, the viruses are rarely transmitted among human population according to the difference in receptor site binding preference between human and avian viruses. As gene reassortment is common among influenza

viruses, an emergence of a reassortant virus which might gain the virulence property from the HPAI H5N1 virus and the ability to transmit among human population from the human viruses is possible. Surveillance for the reassortant virus had been conducted by our group when the occurrence of HPAI outbreaks was at its peak. A total of 109 HPAI H5N1 viruses collected between 2003 and 2006 were analyzed by multiplex reverse transcription-polymerase chain reaction (RT-PCR) using 8 primer pairs specific for each viral genomic segment. No reassortant had been found.

Thailand had suffered from many epidemic waves of H5N1 avian influenza outbreaks since 2004. Our phylogenetic analysis showed that the H5N1 virus isolates at the firstly outbreaks were genetically diverse. By various strategic control plan of avian influenza (AI) in poultry, our genetic analysis showed that only few virus lineages did exist after subsiding of the outbreaks each year as shown by the bottle neck effect in summer of 2006 and 2007. Resurgence of the outbreak usually occurred in the areas around the Yom-Nan river basin from the indigenous viruses surviving the control after previous outbreak, not the re-introducing of the virus from other countries. Additionally, we had discovered 2 reassortants emerged in 2007 from the reassortment of two H5N1 parental viruses. HPAI H5N1 viruses causing the outbreak in Thailand since 2004 belonged to clade 1, genotypeZ and had been circulating throughout Thailand since then. The clade 1 virus was the only clade remained in Central and Northern Thailand upto 2008. On the other hand, the clade 2.3.4 was reported by the other group of Thai investigators in Northeast of the country in 2006. As the parallel laboratory of the National Institute of Health, we had diagnosed an infection caused by the clade 2.3.4, genotype V virus in a Lao patient who came into Thailand for receiving medical care and died in the Nongkhai hospital in 2007. An estimation for the selective pressure on HA proteins regarding synonymous codon usage suggested that the subclade 2.1 virus from Indonesia had lowest codon volatility as compared among different clades and subclades of HPAI H5N1 viruses.

Regarding the binding preference to α2, 3 galactose linked-sialic acid (α2, 3 gal-linked SA) avian type receptor, the other group of investigators studied the HPAI H5N1 viruses from the 1997 outbreak in Hong Kong and suggested the usage of horse erythrocytes in hemagglutination-inhibition (HI) assay for detection of the viral specific antibody. This suggestion has lead to an issue of a WHO recommendation on using horse erythrocytes in HI assay. It is to be notified that this recommendation is relied on the comparison on 2 erythrocyte species (horse and guinea pig) only. Nevertheless, it is not convenient to

obtain the horse erythrocytes in Thailand. We then compared 5 erythrocyte species from human O blood group, chicken, goose, guinea pig and horse, and found that the current HPAI H5N1 viruses preferentially bound the goose erythrocytes, even though the analysis of receptor binding sites on *HA* genes of our study viruses and those of the 1997 Hong Kong viruses are not different. Goose erythrocytes yielded the most sensitive result in hemagglutination assay for recognition of the virus isolates; and the HI antibody titers obtained as using horse or goose erythrocytes were comparable. At present, goose erythrocytes are accepted for HI assay in general laboratories in Thailand; and this erythrocyte species had been employed in our subsequent seroepidemiological studies. Additionally, we also found that goose erythrocyte is also the most sensitive to detect all 16 subtypes of low pathogenic avian influenza (LPAI) viruses and the H1N1pdm virus as compared among the 5 erythrocyte species mentioned above.

Kinetics and longevity of antibody response to the HPAI H5N1 viral infection was explored in 4 fatal cases and 4 survivors by microNT, HI and Western blot assays. HI antibody was detected slightly earlier than NT antibody. In general, the increasing antibody titer in paired blood was shown approximately 15 days after disease onset. The H5N1 survivors were bled at an interval of 6 months during 3 years of the follow-up study. We found that both HI and NT antibodies at the titer of $\geq$ 40 (regardless of total volume in the reaction) persisted for almost 5 years after the disease onset in cases that we could follow-up that far. As a result, the titer of 40 has been established as the cut-off titer for differentiating the H5N1 cases from the non-H5N1 cases; and then applied in the surveillance for asymptomatic H5N1 infection in populations at risk such as poultry farmers, poultry cullers and villagers. We also found that cross neutralizing antibody against HPAI H5N1 at the titer of 10 were found in 6.7-10.3% of the elderly; and seroconversion could be seen in 5.2% of the old-age vaccinees who received seasonal influenza vaccine. We had constructed 6 recombinant vaccinia viruses carrying individual gene of HPAI H5N1 virus, i.e., HA, NA, NP, M, NS and the 6th construct carries only the pSC11 plasmid vector. These constructs infected TK^{-} cells and expressed the viral protein which could be detected by immunoflorescence and Western blot assays. The recombinant H5 HA was extensively characterized as it is the most important immunodominant epitope that induces the protective immunity. Western blot analysis showed that all 3 forms of HA were expressed in the infected cells: HA0 and its cleavage products, HA1 and HA2 domains. H5N1 survivors contained the

antibodies that directed against all 3 forms of HA; while all non-H5N1 subjects contained cross reactive antibody against the HA2 domain only. BALB/c mice immunized with the recombinant HA developed both HI and NT antibody against the reverse genetic virus carrying H5 HA gene and also with the wild type HPAI H5N1 parental virus. NT antibody mostly directs against HA protein and functions as the protective antibody. However, the other viral proteins might induce the antibody that serve other roles such as opsonization, antibody dependent cell cytotoxicity and antagonize with the action of certain viral protein. The infected TK^- cells expressing HA, NA, NP, M, or NS protein together with the pSC11 carrying cells were used as the antigen in the indirect immunofluorescence assay for detection of the antibodies in H5N1 survivors and non-H5N1 subjects including the patients infected with the 2009 pandemic virus, H3N2 virus and healthy controls. The result demonstrated that sera from all 4 survivors and almost of the non-H5N1 subjects were reactive against all kinds of these viral proteins; but the antibody titers were higher in the survivors.

H5N1 survivors also developed cell-mediated immunity which lasted as long as the humoral immunity as determined by ELISpot assay using overlapping peptide pools spanning the entire NP and M proteins. Flow cytometry assay using immunophenotype markers (CD4+ or CD8+) together with γ-interferon, TNF-α and T cell-related cytotoxic marker CD107a demonstrated that majority of the reactive cytotoxic T cells from H5N1 survivors were CD4+T lymphocytes whereas CD8+T lymphocytes were the minor population; and NP peptides yielded stronger reactivity than M peptides. The study also demonstrated that the non-H5N1 subjects also contained the cross reactive cellular immunity against the NP peptide pools, the only peptides used in this group of subjects.

Mahidol University in collaboration with the Department of National Parks, Wildlife and Plant Conservation had conducted the satellite telemetry study to explore the role of migratory birds in AI spread. An analysis in adjunct with GPS program, the birds' flyways and their habitats could be positioned. A total of 20 transmitter platforms were tagged in 4 bird species: Brown headed gulls (*Larus brunnicephalus*), Open-billed storks (*Anastomus oscitans*), Gret egrets (*Egretta alba*) and Lesser whistling ducks (*Dendrocygna javanica*). We did not success to monitor the flyway of Lessler whistling ducks because the transmitter signals were lost within few weeks after tracking. At present, the study on the habitats of the Open-billed storks and Gret egrets are ongoing. Of 8 gulls tracked, only 5 gulls could complete 1-2 migratory cycles between Thailand and China. The flyways of gulls spanned 7 countries that were affected by

avian influenza: Thailand, Cambodia, Vietnam, China, India, Bangladesh and Myanmar. Based on duration of staying for longer than one month, only Thailand, Cambodia and China were considered to be the gulls' habitats; while the other countries were the places of stopover during migration. With the distance and duration of migration together with the experiment on duration of virus shedding till death or survival in the gulls inoculated with H5N1 virus, our study suggested that it is possible for the infected gulls to spread the virus along their migratory flyways.

The emergence of the 2009 pandemic influenza began in Mexico in March 2009. The first 2 cases of Thailand were also imported from Mexico. As the companion laboratory of the National Institute of Health, Thailand, these 2 cases were diagnosed and reported on May 4, 2009. The causative viruses were isolated and named A/Nonthaburi/102/2009 (H1N1) and A/Thailand/104/2009(H1N1). Their entire genomes were sequenced and deposited in the GenBank database. A/Thailand/104 is easier to be propagated; therefore, it has been used by our group as the test antigen in many serological studies. The investigation in archival serum samples collected before 2009 showed the presence of pre-existing antibody that could cross neutralize the H1N1pdm virus to various extents of titers. This finding implied that the Thai population had been partially protected; and explained why the disease caused by the pandemic virus was not severe. Goose erythrocyte HI assay together with microNT assay were employed to investigate paired blood samples from the patients. The result led to the establishment of the cut-off antibody titer that can differentiate between the cases and non-cases, i.e., the HI titer of $\geq$40 for adults and $\geq$20 for children. These cut-off titers were applied to investigate the magnitude of the pandemic in Thai people at the end of the first epidemic wave. The infection rates of 7% were found in blood donors and 12.8% in health care workers which were statistically non-significant different. Questionnaires regarding self-reported adherence of nurses to the infection control practices (hand hygiene, masks and gloves) revealed that the exposure to health care professionals with influenza-like illness without wearing a mask was the only identified risk factor for the infection. Eventually, our study showed that most of the Thai people were lack of immunity. So, we predicted that subsequent epidemic waves would occur following the first one. Our estimation has been confirmed by the occurrence of at least 4 subsequent outbreaks thereafter. The H1N1pdm virus has completely replaced the original seasonal H1N1 virus since 2009. The community based study conducted in collaboration with the Bureau of Epidemiology (BoE) after the first epidemic wave in

rural areas (Chiang Mai and Nakhon Si Thammarat) showed that the infection rates were much higher in children (58%) than adults (3.1%). HI assay had been used to evaluate the antibody response in HIV infected vaccinees and the uninfected controls who received single injection of the monovalent inactivated pandemic influenza vaccine. The seroconversion rate of 32% and the seroprotection of 33.3% were found in the HIV infected vaccinees; while the seroconversion rate as well as the seroprotection rate of 35% were found in the uninfected control group.

Various kinds of respiratory specimens: nasopharyngeal aspirates (NPA), nasal swabs (NS) and throat swabs (TS) can be used for the viral genome detection by real time RT-PCR to diagnose the influenza disease. Our study investigated those specimens collected from the same infected individual prior to drug treatment and showed that NPA was the best sample that yielded the most sensitive result in the disease diagnosis, and followed in order by NS and TS specimens. During an outbreak in a closed community such as a military camp, the isolation of the infected case is one approach to stop the virus spread; but how long is the isolation period needed to be determined. BoE in collaboration with our group had investigated the viral loads and duration of viral shedding from the infected cases by real time RT-PCR using sequential respiratory samples. We found that the viral load at the amount of 2.6×10^{2} - 8.1×10^{9} copies/ml were shed from the patients and duration of viral shedding lasted between 1 and 11 days (mean = 5 days).

At present, 2 kinds of neuraminidase inhibitors: oseltamivir and zanamivir have been licensed for general use. Our group has established 4 techniques: direct nucleotide sequencing, plaque reduction assay, neuraminidase inhibition (NAI) assay employing 2'2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) and ELISA based viral nucleoprotein (NP) reduction assay to conduct the surveillance for anti-viral drug resistant influenza viruses. We previously showed that 90-100% of the seasonal influenza A(H1N1) viruses investigated in 2008 and 2009 were oseltamivir resistant according to H274Y substitution in *NA* gene. Fortunately, this virus has been extinct after 2010. We have successfully applied the newly developed ELISA based viral NP reduction assay to investigate the anti-viral activity of the compounds or plant extracts prepared by other institutes. The advantage of this technique is its ability to investigate the drug or compounds targeting any viral gene, while NAI assay can be used for the drugs that target the NA protein only. ELISA based viral NP reduction assay also has

an advantage over the gold standard plaque reduction assay such that it consumes less reagents and time.

บทนำ

คุณสมบัติทั่วไปของเชื้อไวรัสไข้หวัดใหญ่

ไวรัสไข้หวัดใหญ่ (influenza virus) จัดอยู่ใน Family *Orthomyxoviridae* แบ่งออกเป็น 3 immunological types คือ A B และ C โดย type A มีการติดเชื้อในคนและสัตว์หลายชนิด เช่น สัตว์ปีก หมู ม้า เสือ เป็นต้น type B มีการติดเชื้อก่อโรคในคนเท่านั้น สำหรับ type C มีรายงานการติดเชื้อในคนโดยไม่แสดงอาการ ไวรัสไข้หวัดใหญ่เป็นไวรัสที่มียีโนมเป็น RNA ซึ่งมีลักษณะเป็นท่อนจำนวน 7-8 ท่อน แต่ละท่อนล้อมรอบด้วยโปรตีน capsid เกิดเป็นแกนกลาง หรือ ribonucleoprotein ซึ่งอยู่ภายในเยื่อหุ้ม (envelope) เดียวกัน บน envelope มี glycoprotein spikes 2 ชนิด ได้แก่ hemagglutinin (HA) และ neuraminidase (NA) อนุภาคของไวรัสไข้หวัดใหญ่มีรูปร่างได้หลายแบบ คือ กลม เป็นสายยาว หรือมีรูปร่างไม่แน่นอน ไวรัสไข้หวัดใหญ่เป็นไวรัสที่มีการกลายพันธุ์สูงมาก ทั้งแบบ point mutation หรือ reassortment ซึ่งเป็นการแลกเปลี่ยน geneomic segment ของไวรัสไข้หวัดใหญ่ type A สองสายพันธุ์ ที่มีการติดเชื้อภายในเซลล์เดียวกัน ทำให้มีไวรัสลูกผสมสายพันธุ์ใหม่ หรือ subtype ใหม่เกิดขึ้นอยู่เรื่อยๆ

ไวรัสไข้หวัดใหญ่ type A สามารถแบ่งตามความแตกต่างของ H และ N ออกเป็น 17 H และ 9 N subtypes ทุก H และ N subtypes พบได้ในนกน้ำ จึงเชื่อว่านกน้ำเป็นรังโรค (reservoir host) ของไข้หวัดใหญ่ type A

ระบาดวิทยาของเชื้อไวรัสไข้หวัดนก H5N1 ในประเทศไทย

เชื้อไข้หวัดนก (avian influenza virus) ที่มีรายงานการระบาดมาสู่คนมีอยู่หลาย subtype ที่สำคัญได้แก่ H5N1, H9N2, H7N1 และ H7N3 viruses แต่เชื้อ H5N1 เป็น subtype ที่ก่อโรครุนแรงมากที่สุด การแพร่กระจายมาสู่คน มีรายงานครั้งแรกที่ฮ่องกง ในปี 2540 ประเทศไทยรายงานการเกิดขึ้นของโรคไข้หวัดนก H5N1 virus เป็นครั้งแรกและรายงานผู้ป่วยรายแรกของประเทศ เมื่อวันที่ 23 มกราคม 2547 ผู้ป่วยรายสุดท้ายของประเทศเกิดขึ้นในเดือนกรกฎาคม 2549 รวมมีผู้ป่วยทั้งสิ้น 25 ราย ถึงแก่ชีวิต 17 ราย คิดเป็นอัตราตาย 68% ส่วนการระบาดในสัตว์ปีกมีรายงานครั้งสุดท้ายในประเทศในปี 2551 ในปัจจุบัน โรคไข้หวัดนก H5N1 ก็ยังไม่หมดไป ในปี 2555 ยังพบการระบาดมีผู้ติดเชื้อและผู้เสียชีวิตใน 6 ประเทศทั่วโลก คือ จีน เวียดนาม กัมพูชา อินโดนีเซีย บังคลาเทศ และ อียิปต์

เชื้อ H5N1 virus ของฮ่องกง และ H5N1 virus ในปัจจุบันเป็นไวรัสลูกผสม (reassortant) เกิดจาก avain viruses หลาย serotype ผสมกัน โดยมียีน HA เป็นบรรพบุรุษร่วม แม้ทางฮ่องกงได้ทำลายสัตว์ปีกไปจนหมดสิ้นในการระบาดครั้งแรก แต่บรรพบุรุษของไวรัสซึ่งอยู่ทางตอนใต้ของจีนยังคงอยู่ การศึกษาทางวิวัฒนาการของไวรัส ชี้แนะว่าไวรัสเหล่านั้นได้มีการ reassort กันอีกหลายครั้ง ได้ไวรัสลูกผสมที่มีคุณสมบัติทางชีววิทยาเปลี่ยนไปโดยลำดับ และจนกระทั่งเกิดเป็นไวรัส H5N1 ในปัจจุบันซึ่งมีความรุนแรงมากขึ้น การระบาดเป็นวงกว้าง

ขึ้น อัตราตายในผู้ป่วย 60-70% ทั้งนกบกและนกน้ำตายจากการติดเชื้อ ในขณะที่ไวรัสของฮ่องกง ปี พ.ศ. 2540 มีอัตราตายในผู้ป่วยเพียง 33% และมีเพียงนกบกเท่านั้นที่ติดเชื้อแล้วตาย แต่นกน้ำจะไม่ตาย

ในขณะที่มีไวรัสไข้หวัดนกเกิดขึ้นในประเทศไทยนั้น มีคนติดเชื้อไม่มากนัก และการติดต่อจากคนสู่คนไม่ได้เกิดขึ้นง่าย เนื่องจาก receptor บนผิวเซลล์ สำหรับเชื้อไข้หวัดนกนั้นเป็น SAα2,3Gal ในขณะที่ receptor บนผิวเซลล์สำหรับเชื้อไข้หวัดใหญ่เป็น SAα2,6Gal เนื่องจากข้อกำจัดในด้านความจำเพาะต่อ receptor ทำให้เชื้อไข้หวัดนก ยังไม่สามารถติดเชื้อกับเซลล์ของคนได้ดีนัก แต่ถ้าเชื้อยังมีการระบาดอยู่โดยควบคุมไม่ได้ อาจเป็นไปได้ว่า เชื้อมีโอกาสมากขึ้นในการปรับตัวให้เข้ากับเซลล์มนุษย์ หรือเกิด reassortment กับ human virus เกิดเป็นไวรัสลูกผสมที่มีทั้งความรุนแรง ติดเชื้อและเพิ่มจำนวนในเซลล์คนได้ดี

เชื้อไวรัสไข้หวัดนกในประเทศไทยมาจากไหน

การระบาดของโรคไข้หวัดนกในโลกอาจมีสาเหตุเกิดจากนกอพยพ การค้าตามชายแดน และการค้าข้ามประเทศระยะไกล ในประเทศไทยการระบาดครั้งแรกๆ เกิดขึ้นในภาคกลางของประเทศแถบจังหวัดนครสวรรค์ ทำให้มีข้อสันนิษฐานว่า นกอพยพน่าจะเป็นตัวการนำเชื้อมาสู่ประเทศไทย เนื่องจากจังหวัดนครสวรรค์มีบึงบอระเพ็ดเป็นแหล่งน้ำขนาดใหญ่ซึ่งเป็นที่ชุมนุมของนกอพยพชนิดต่างๆ เป็นจำนวนมาก จึงอาจเป็นไปได้ว่านกอพยพเหล่านี้อาจนำพาเอาเชื้อไข้หวัดนกจากประเทศอื่นมาสู่ประเทศไทย

พยาธิวิทยาของการติดเชื้อไข้หวัดใหญ่/ไข้หวัดนก

ลักษณะพยาธิสภาพที่เด่นที่สุดของเชื้อไวรัสไข้หวัดนกที่ต่างจากไวรัสไข้หวัดใหญ่ (human influenza viruses) ทั่วไป คือ การแพร่กระจายของเชื้อไวรัสออกนอกระบบทางเดินหายใจ และภาวะการสร้าง proinflamatory cytokines มากเกินไป (cytokine storm) การติดเชื้อ human influenza จะจำกัดตัวอยู่ในทางเดินหายใจเท่านั้น และมักจะเป็นทางเดินหายใจส่วนต้น แต่เชื้อไข้หวัดนกชอบติดเชื้อในทางเดินหายใจส่วนล่าง คือ ปอดใน pneumocyte type 2 และยังสามารถแพร่กระจายไปสู่อวัยวะอื่นโดยทางกระแสเลือด เป็น free virus particle viremia หรือ cell associated viremia การตรวจพบ anti-sense RNA ของไวรัสในลำไส้ ทำให้คาดว่าไวรัสน่าจะเพิ่มจำนวนได้ในลำไส้ ผู้วิจัยได้พยายามที่จะแยกเชื้อไวรัสจากอวัยวะต่างๆ ของ autopsy แต่ไม่ประสบผลสำเร็จ อย่างไรก็ตามการตรวจทางพยาธิวิทยา ได้พบพยาธิสภาพที่เกี่ยวเนื่องจากการติดเชื้อในหลายอวัยวะ และภาวะเลือดออกในอวัยวะต่างๆ จึงเชื่อว่าไข้หวัดนกน่าจะก่อการติดเชื้อได้ในหลายอวัยวะและในเซลล์หลายชนิด แต่ก็ยังไม่ทราบว่าเป็นเซลล์ใด มีเพียงรายงานใน macrophage เท่านั้น เซลล์เหล่านั้นอาจยอมให้ไวรัสเพิ่มจำนวนได้ หรือมีเพียงการติดเชื้อ แต่ไม่มีการเพิ่มจำนวนก็เป็นได้ การตรวจหาการเพิ่มจำนวนของไวรัสในเซลล์ ซึ่งไม่ใช่ respiratory epithelial cells อาจทำให้ทราบถึงความสามารถของเชื้อในการแพร่กระจายออก

นอก respiratory system ซึ่งถือเป็นกลไกสำคัญอย่างหนึ่งที่ทำให้การติดเชื้อไข้หวัดใหญ่มีความรุนแรงเพิ่มมากขึ้น ในสัตว์ปีกได้พบว่า endothelial cell สามารถติดเชื้อไวรัสไข้หวัดนกได้ดี

ระบาดวิทยาของเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 pandemic influenza A (H1N1) ในประเทศไทย

โรคไข้หวัดใหญ่สายพันธุ์ใหม่เดิมเคยเรียกว่า swine-origin influenza A (H1N1) หรือเรียกชื่อใหม่ในปัจจุบันว่า 2009 pandemic influenza A (H1N1) เริ่มพบการระบาดครั้งแรกในประเทศเม็กซิโก เมื่อเดือนมีนาคม พ.ศ. 2552 และองค์การอนามัยโลก (World Health Organization, WHO) ได้ประกาศการระบาดอย่างเป็นทางการในวันที่ 24 เมษายน พ.ศ. 2552 การติดเชื้อมีการแพร่กระจายออกไปยังสหรัฐอเมริกา และอีกหลายประเทศในหลายทวีปทั่วโลก องค์การอนามัยโลกจึงประกาศภาวะการระบาดทั่วโลก (pandemic phase 6) ในวันที่ 11 มิถุนายน พ.ศ. 2552 และประกาศการเข้าสู่ภาวะการระบาดตามฤดูกาล (post-pandemic phase) ในวันที่ 10 สิงหาคม พ.ศ. 2553 ประเทศไทยรายงานผู้ป่วยรายแรกเมื่อ วันที่ 4 พฤษภาคม 2552 การระบาดระลอกแรกในประเทศไทยเริ่มขึ้นในปลายเดือนพฤษภาคม 2552 พบผู้ป่วยสูงสุดในเดือนกรกฎาคม และสิ้นสุดลงในเดือนพฤศจิกายน 2552 ตามมาด้วยการระบาดเล็กๆ เป็นระลอกที่ 2 ระหว่างเดือนธันวาคม 2552 ถึงเดือนเมษายน 2553 โดยผู้ป่วยสูงสุดในเดือนกุมภาพันธ์ 2553 และการระบาดระลอกที่ 3 ซึ่งเกิดขึ้นในเดือนมิถุนายน 2553 พบผู้ป่วยสูงสุดในเดือนสิงหาคม และสิ้นสุดลงในเดือนตุลาคม 2553 ซึ่งเป็นการระบาดขนาดใหญ่มีผู้ติดเชื้อจำนวนมาก และระลอกที่ 4 เกิดขึ้นในเดือนกันยายน 2554 ในปี พ.ศ. 2555 ยังคงมีการระบาดเล็กๆ และพบผู้ป่วยอยู่เป็นระยะ กลายเป็นเชื้อประจำ ในขณะที่ไข้หวัดใหญ่ A(H1N1) สายพันธุ์เดิมได้สูญหายไปจากโลก ความรุนแรงจากการติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ไม่ได้สูงไปกว่าไข้หวัดใหญ่ทั่วไป อาการของโรคก็คล้ายกับไข้หวัดใหญ่ตามฤดูกาลแต่เดิม และมีอัตราการเสียชีวิตไม่ต่างกันคือน้อยกว่า 1% แต่การแพร่กระจายโรคจะเกิดรวดเร็วกว่ามากเนื่องจากคนส่วนใหญ่ยังไม่มีภูมิคุ้มกัน จึงทำให้มีจำนวนผู้ติดเชื้อมากมาย และโดยเฉพาะเมื่อโรคเกิดขึ้นในผู้ที่มี underlying conditions อาการมักจะรุนแรงจนถึงแก่ชีวิต

ไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่มียีนทั้ง 8 ซึ่งเกิดขึ้นจากการผสมผสานของยีนจากไวรัสสายพันธุ์ของคน สุกรและนก (quadruple reassortment) ดังนี้

1) ยีน hemagglutinin (HA), nucleoprotein (NP) และ non-structural (NS) มาจากสุกรในอเมริกาเหนือ (North America swine influenza virus lineage)

2) ยีน neuraminidase (NA) และ matrix (M) มาจากสุกรในยุโรป (Eurasian swine influenza virus lineage)

3) ยีน polymerase basic protein 2 (PB2) และ polymerase acidic protein (PA) มาจากนกในอเมริกาเหนือ (North America avian influenza virus lineage)

4) ยีน polymerase basic protein 1 (PB1) มาจากไวรัสไข้หวัดใหญ่ของคน (human seasonal H3N2 virus)

เมื่อการระบาดแพร่กระจายไปทั่วโลก นักวิจัยจากประเทศต่างๆ ได้นำวิธีการทางระบาดวิทยา และ วิธีการทาง serology ซึ่งมีความแม่นยำ มาใช้เพื่อประเมินสถานการณ์และแนวโน้มว่าจะเกิดการระบาดครั้งต่อๆไปอีกหรือไม่ จากการประเมินอัตราการติดเชื้อหรือภาวะภูมิคุ้มกันโรคของประชากรภายหลังการระบาดแต่ละครั้ง งานวิจัยนี้จึงใช้เทคนิคทาง serology คือ hemagglutination inhibition assay และ microneutralization assay มาใช้ในการประเมินอัตราการติดเชื้อและตรวจหาภาวะภูมิคุ้มกัน และวินิจฉัยการเกิด asymptomatic infection ในประชากรทั่วไป ได้พบว่าวิธี microNT มีปฏิกิริยาข้ามค่อนข้างมากไม่เหมาะกับการนำมาใช้ในการประเมินอัตราการติดเชื้อ ในขณะที่สามารถใช้ HI assay ในการประเมินได้ดีกว่า นอกจากนี้คณะผู้วิจัยได้ทำการศึกษาเกี่ยวกับลักษณะทาง genotype และ phenotype ของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 A(H1N1) เปรียบเทียบกับเชื้อไข้หวัดนก H5N1 และเชื้อไข้หวัดใหญ่ตามฤดูกาล seasonal H1N1 และ H3N2 ควบคู่กันไปด้วย โดยตีพิมพ์ข้อมูลทางพันธุกรรมเหล่านี้ใน GenBank database

ภาวะภูมิคุ้มกันต่อเชื้อไข้หวัดใหญ่

ภูมิคุ้มกันโรคไวรัสทั่วไป รวมทั้งไวรัสไข้หวัดใหญ่ เกิดจากการทำงานร่วมกัน ทั้ง humoral immunity (ได้แก่ แอนติบอดีและสารน้ำ) และ cell-mediated immunity (CMI) โดย neutralizing (NT) antibody จะจับกับตำแหน่งที่ไวรัสใช้ในการเกาะติดกับเซลล์ (attachment site) ทำให้ไวรัสเข้าเซลล์ไม่ได้ หรือเข้าสู่เซลล์ได้แต่จะไม่เกิด uncoating และ replication การติดเชื้อจึงไม่เกิดขึ้น นอกจากนี้ mucosal antibody ที่อยู่ในเมือกบุทางเดินหายใจยังดักจับไวรัสไว้ ทำให้การแพร่กระจายของเชื้อไวรัสถูกยับยั้ง ส่วน CMI นั้นจะทำงานต่อเมื่อเซลล์มีการติดเชื้อไปแล้ว โดย cytotoxic T lymphocytes (CTL) จะ recognize antigenic epitopes ของไวรัสร่วมกับ HLA class I antigen ของเซลล์ แล้วทำลายเซลล์ติดเชื้อนั้น

ความรุนแรงของ H5N1 virus ทำให้องค์กรต่างๆ ตื่นตัวในการพัฒนาวัคซีนเพื่อป้องกัน H5N1 virus ความพยายามทั้งหมดมุ่งไปที่การผลิตวัคซีนเชื้อตาย ซึ่งเป็นที่ยอมรับกันโดยทั่วไปว่าวัคซีนเชื้อตายนั้นกระตุ้นการสร้างแอนติบอดี และจะมีความจำเพาะต่อ antigen ค่อนข้างแคบ และจะต้องเปลี่ยน HA gene ในไวรัสที่ใช้เตรียมวัคซีนไปเรื่อยๆ ทีมผู้วิจัยได้ใช้แนวทางของการพัฒนาวัคซีนเอดส์มาเป็นสมมุติฐานของหัวข้อวิจัย คือ เชื้อเอชไอวี คล้ายกับเชื้อไข้หวัดใหญ่ในแง่ของการมี หลาย serotype การพัฒนาวัคซีนเอดส์มุ่งไปที่การผลิต live recombinant vector vaccine โดยมียีนสำคัญคือ gag gene (group specific gene ซึ่งเป็นรหัสสำหรับสร้าง capsid protein) ซึ่งเป็น conserve gene สามารถกระตุ้นให้เกิด cytotoxic T lymphocyte activity ที่มีผลในการทำลายเซลล์ติดเชื้อเอชไอวีที่ต่าง subtype กันได้ งานวิจัยเกี่ยวกับภูมิคุ้มกันของเชื้อไข้หวัดใหญ่มีอยู่ค่อนข้างจำกัด แต่ก็มีนักวิจัยหลายกลุ่มเชื่อว่าการกระตุ้นให้เกิดภูมิคุ้มกันข้าม subtype หรือข้ามสายพันธุ์ มีความเป็นไปได้ ในการใช้ low pathogenic AI virus หรือ live virus เป็น immunogen งานวิจัยจึงเป็นการนำเสนอแนวทางใหม่ในการพัฒนาวัคซีนป้องกันไข้หวัดนก

อัตราการเสียชีวิตของผู้ป่วยไข้หวัดนกในประเทศไทย 25 ราย ถึงแก่กรรม 17 ราย หรือคิดเป็น 68% ซึ่งถือว่ามีความรุนแรงมาก นอกจากนี้คณะผู้วิจัยยังได้พบ neutralizing antibody ต่อเชื้อไข้หวัดนกในระดับต่ำๆ ในซีรั่มที่ได้จากการเจาะเลือดผู้สัมผัสกับสัตว์ที่เป็นโรค ได้แก่ เกษตรกรผู้เลี้ยง และผู้ทำหน้าที่ทำลายสัตว์ รวมทั้ง ญาติผู้ดูแลผู้ป่วย แต่ก็ไม่ทราบว่าแอนติบอดีนี้เกิดขึ้นจากปฏิกิริยาข้ามกับไวรัส subtype อื่น หรือจากการติดเชื้อไวรัสไข้หวัดนกเอง การศึกษาระดับแอนติบอดีในผู้ป่วยและผู้รอดชีวิต และระยะเวลาที่อาจตรวจพบแอนติบอดีจะเป็นประโยชน์ในการศึกษาทางระบาดวิทยาต่อไป

มีผู้ศึกษาในกลุ่มตัวอย่างจากผู้บริจาคเลือด โดยใช้วิธี CTL assay และ ELISpot พบการตอบสนองต่อไวรัสไข้หวัดใหญ่ในหลาย epitopes ได้แก่ PB1, PB2, NP, NA, HA, M1, NS1, และ M2 ซึ่ง epitope ที่น่าจะ common ในผู้เคยติดเชื้อ คือ NP และยังได้พบ CTL activity ที่ข้าม subtype กันได้ด้วย

ในขณะที่ NP gene จะมีความ conserve ภายในไวรัสไข้หวัดใหญ่ type เดียวกันยีน HA จะเป็นยีนที่มี diversity สูง การใช้วิธี ELISpot เพื่อศึกษา T cell epitopes (ทั้ง $CD4^+$ และ $CD8^+$ T cells) ของผู้ติดเชื้อไข้หวัดนก โดยใช้ overlapping peptides ของ NP ของ H5N1 virus ในการทดสอบในผู้รอดชีวิตจากโรคไข้หวัดนกและโรคไข้หวัดใหญ่ ก็จะทำให้ทราบได้ว่า epitopes ใด เป็น common epitopes และ epitopes ใด เป็น NP specific epitopes ประโยชน์ที่จะได้จากการศึกษา คือ ทำให้ทราบ immunogenicity ของ regions ต่างๆ ของ NP molecules นอกจากนี้ยังสามารถนำ peptides ที่มี immunogenic epitopes ไปศึกษา T cell และ B cell functions ต่อไปได้ ซึ่งจะนำไปสู่ความเข้าใจในเรื่องของภูมิคุ้มกันในโรคไข้หวัดนก และเป็นแนวทางในการพัฒนาวัคซีนต่อไป

การวินิจฉัยผู้ติดเชื้อไวรัสไข้หวัดใหญ่/ไข้หวัดนก

การวินิจฉัยผู้ติดเชื้อไข้หวัดนก/ไข้หวัดใหญ่ สามารถทำได้หลายวิธี ได้แก่

1. *การตรวจหายีโนม*ด้วยวิธี conventional reverse transcription-polymerase chain reaction (RT-PCR) และ real time RT-PCR ซึ่งสามารถให้การวินิจฉัยได้ถึงระดับของเชื้อต้นเหตุ วิธีการตรวจเป็นวิธีมาตรฐานที่ใช้กันทั่วไปตามข้อแนะนำขององค์การอนามัยโลกและศูนย์ควบคุมและป้องกันโรค สหรัฐอเมริกา ซึ่งจะต้องลงนามในข้อตกลง Material transfer agreement

การติดตามการขับเชื้อไวรัสออกจากระบบทางเดินหายใจของผู้ป่วย ทั้งในเชิงปริมาณและเชิงคุณภาพ มักใช้การตรวจหายีโนมแทนการแยกเชื้อ แม้ว่ายีโนมที่ตรวจพบนั้นจะไม่ได้มาจากเชื้อไวรัสที่มีชีวิตก็ตาม แต่ก็เป็นวิธีการที่ทั่วโลกนิยมใช้

2. *การแยกเชื้อไวรัส*ในเซลล์เพาะเลี้ยงและในไข่ไก่ฟัก การตรวจกรองว่าแยกเชื้อได้จะนำน้ำเซลล์เพาะเลี้ยงหรือ allantoic fluid ของไข่มาผสมกับเม็ดเลือดแดงของสัตว์บางชนิด ก็

จะเห็นปฏิกิริยาการเกาะกลุ่มของเม็ดเลือดแดง (hemagglutination) ถ้าเม็ดเลือดแดงมาจากสัตว์ที่เหมาะสมก็จะมีความไวในการตรวจจับไวรัสที่มีจำนวนน้อยได้ วิธีการแยกเชื้อก็จะมีความไวมากขึ้น เชื้อไวรัสที่แยกได้นี้จะถูกนำไปใช้ในการทดสอบยาและพัฒนาวัคซีน เชื้อจากทั่วโลกจะถูกส่งไปยังห้องปฏิบัติการอ้างอิงเพื่อนำไปพิจารณาเป็นองค์ประกอบของวัคซีนที่ใช้ในปีต่างๆ

ตัวอย่างตรวจที่ดีที่สุดสำหรับการตรวจหายีโนมและการแยกเชื้อ คือ nasopharyngeal aspirate ซึ่งเป็นตัวอย่างตรวจที่ต้องอาศัยความชำนาญของผู้เก็บ และไม่เหมาะสำหรับการออกภาคสนาม ดังนั้นตัวอย่างตรวจที่ถูกส่งมายังห้องปฏิบัติการจึงได้แก่ nasal swab, nasopharyngeal swab และ throat swab เป็นส่วนใหญ่ ยังไม่มีข้อสรุปว่า swab ชนิดใดที่มีปริมาณไวรัสมากกว่ากัน ซึ่งจะมีผลต่อความไวของวิธีทดสอบด้วย

3. *การตรวจหาแอนติเจน* อาจทำโดยการตรวจหาแอนติเจนใน epithelial cells ที่ติดเชื้อซึ่งหลุดลอกปนออกมากับ respiratory secretion ต่างๆ โดยใช้แอนติบอดีจำเพาะในการทดสอบด้วยวิธีอิมมูนเรืองแสง (immunofluorescent assay) วิธีการนี้ต้องการผู้อ่านสไลด์ที่มีประสบการณ์สูง มิฉะนั้นอาจจะเกิดผลลบปลอมหรือผลบวกปลอมได้ง่าย ในปัจจุบันมีวิธีทดสอบรวดเร็ว (rapid test) เช่นวิธี immunochromatography ทดสอบหาแอนติเจนของไวรัสไข้หวัดใหญ่ไทป์ A และ B ซึ่งได้ผลภายในเวลาไม่กี่นาที แต่ความไวในการทดสอบต่ำเพียง 50-60% เท่านั้น

4. *การตรวจหาแอนติบอดี* เพื่อการวินิจฉัยโรคไข้หวัดใหญ่ นิยมทำด้วยการตรวจหาแอนติบอดีต่อเชื้อไวรัสในซีรั่มคู่ (paired blood samples) ซึ่งเจาะห่างกันประมาณ 2 สัปดาห์ ถ้าเป็นโรคจริงระดับแอนติบอดีในเลือดครั้งที่สอง (convalescent blood) จะสูงกว่าเลือดครั้งแรก (acute blood) อย่างน้อย 4 เท่า วิธีการที่นิยมใช้ ได้แก่วิธี hemagglutination inhibition assay อย่างไรก็ดีในการวินิจฉัยผู้ป่วยไข้หวัดนก องค์การอนามัยโลกแนะนำให้ใช้วิธี microneutralization (microNT) assay และ Western blot assay เป็นวิธีตรวจยืนยันร่วมด้วย โดยในผู้ป่วยไข้หวัดนกจะต้องพบ neutralizing (NT) antibody titer มากกว่าหรือเท่ากับ 80 (หรือ titer 40 ถ้าไม่คิดปริมาตรใน reaction well) ถ้าทราบว่าแอนติบอในระดับนี้สามารถอยู่ได้นานก็จะเป็นประโยชน์ในการสำรวจหาผู้ที่ติดเชื้อไข้หวัดนกที่มีอาการอย่างอ่อนหรือไม่มีอาการได้ ซึ่งจะทำให้ทราบถึงความชุกของผู้ติดเชื้อไข้หวัดนอในประชากรกลุ่มเสี่ยง เช่น เกษตรกรผู้เลี้ยงสัตว์ปีกและผู้ทำงานในโรงฆ่าสัตว์ปีก เป็นต้น

วิธี microNT ใช้สำหรับตรวจวัด NT antibody ซึ่งเป็น protective antibody ในการคุ้มกันโรค แต่วิธีนี้ทำได้ไม่ง่ายนัก และผู้ปฏิบัติงานมีความเสี่ยงต่อการติดเชื้อ เนื่องจากต้องใช้เซลล์เพาะเลี้ยง และใช้ไวรัสที่มีชีวิต นอกจากนี้ถ้าเป็นไวรัสไข้หวัดนก ยังต้องปฏิบัติงานในห้องชีวนิรภัยระดับ BSL 3 หรือ BSL 2 enhanced ด้วย ส่วนวิธี HI ทำได้ง่ายกว่า และใช้เชื้อที่ตายแล้ว หรือใช้เชื้อที่ยังมีชีวิตเป็นแอนติเจนทดสอบก็ได้ เพียงแต่ต้องเลือกใช้ fresh erythrocyte species ที่เหมาะสม และ HI antibody ไม่สามารถบ่งถึงการมี protective antibody ได้โดยตรง แต่เนื่องจากผู้ที่มี HI antibody ก็มักจะมี NT antibody รวมอยู่ด้วย องค์การอนามัยโลกจึงอนุโลมให้ใช้วิธี HI ในการประเมินประสิทธิภาพของ seasonal vaccine แต่ละ lot ก่อนนำออกจำหน่ายในท้องตลาด วัคซีนที่ใช้ได้จะต้องกระตุ้นให้เกิดการสร้าง HI antibody ที่ titer 40

เป็นอย่างน้อย และค่านี้น่าจะสามารถนำมาใช้ในการบ่งชี้ถึงภาวะการมีภูมิคุ้มกันต่อเชื้อไข้หวัดใหญ่ได้

โดยทั่วไปแล้วเชื้อไข้หวัดใหญ่สามารถ agglutinate เม็ดเลือดแดงจากหลาย species เช่น คนหมู่เลือดโอ หนูตะเภา ไก่ ห่าน ม้า และ ไก่งวง แต่งานวิจัยที่ผ่านมาชี้แนะว่า เม็ดเลือดแดงจากม้าน่าจะมีความไวมากที่สุดในการทำ HI assay สำหรับเชื้อไข้หวัดนก H5N1 แต่เนื่องจากเม็ดเลือดแดงม้าหาได้ยากในประเทศไทยและหลายประเทศในเอเชียอาคเนย์ และยังไม่มีผู้ใดทดสอบว่าเม็ดเลือดแดงม้ายังคงคุณสมบัติในการเป็น detector ที่ดีที่สุดสำหรับเชื้อ H5N1 ในปัจจุบัน นอกจากนี้เชื้อที่มีการระบาดขึ้นใหม่ก็จำเป็นต้องมีการเลือกชนิดของเม็ดเลือดแดงที่เหมาะสมสำหรับใช้ในการทดสอบ hemagglutination เพื่อตรวจหาเชื้อไวรัส และตรวจหาแอนติบอดีโดยวิธี HI ดังนั้นชนิดของเม็ดเลือดแดงที่เหมาะสมจึงมีความสำคัญต่อการวินิจฉัยและการทดสอบระดับภูมิคุ้มกันต่อการติดเชื้อไข้หวัดใหญ่

การดื้อยาของเชื้อไวรัสไข้หวัดใหญ่

ยาที่ใช้ในการรักษาไข้หวัดใหญ่ในมนุษย์ แต่เดิมมานั้นได้แก่ amatadine rimantadine ซึ่งออกฤทธิ์เป็น M2 ion channel blocker ยานี้ออกฤทธิ์ยับยั้งได้เฉพาะไวรัสไข้หวัดใหญ่ type A เท่านั้น ต่อมาได้พบว่าเชื้อไวรัสไข้หวัดใหญ่ของมนุษย์เริ่มดื้อต่อยานี้ การเปลี่ยนแปลง amino acid เพียง 1 ใน 5 ตำแหน่ง ซึ่งอยู่ใน transmembrane portion (residues 26 , 27, 30, 31 และ 34) จะทำให้ไวรัสไข้หวัดใหญ่ดื้อต่อยาตัวนี้อย่างสมบูรณ์ จากการศึกษาโดยคณะผู้วิจัยได้พบว่า เชื้อไวรัสไข้หวัดนก clade 1 ดื้อต่อยานี้ ในขณะที่ไวรัส clade 2 ยังไม่ดื้อต่อยานี้ สำหรับเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 A(H1N1) ก็ดื้อต่อยานี้ด้วย amino acid substitution ที่ตำแหน่งนี้เช่นกัน

ปัจจุบันยาที่นิยมใช้ในการรักษาโรคไข้หวัดใหญ่รวมถึงไข้หวัดนกด้วยคือ neuraminidase inhibitor (NI) ยานี้ออกฤทธิ์ยับยั้งไวรัสไข้หวัดใหญ่ทั้ง A และ B ในขั้นตอนปลดปล่อยไวรัสรุ่นลูกออกจากเซลล์ติดเชื้อ ยาที่เป็น NI ได้แก่ zanamivir ซึ่งใช้พ่นทางปาก (oral inhalation) และยา oseltamivir ซึ่งให้โดยการกิน ดังนั้น oseltamivir จึงมีความสะดวกในการใช้งานมากกว่า NI และถูกนำไปใช้ทั้งเพื่อป้องกัน (prophylaxis) และการรักษา (treatment) แต่อาจไม่ปลอดภัยสำหรับการนำไปใช้ในทารกอายุน้อยกว่า 1 ปี การเกิด mutation ใน NA protein ที่ amino acid substitution H274Y เป็นตำแหน่งสำคัญที่ทำให้ทั้งไวรัสไข้หวัดนกและไวรัสไข้หวัดใหญ่ดื้อต่อยา oseltamivir ในขณะที่ยัง sensitive ต่อ zanamivir และนอกจากนี้ยังพบว่า mutation ในตำแหน่งอื่นๆ ก็อาจทำให้ไวรัสดื้อต่อยาทั้งสองชนิดนี้ได้ อย่างไรก็ดี mutation ที่ receptor binding site ของ HA molecule มีผลร่วมต่อการดื้อ NI ด้วย ในปี 2008-2009 เชื้อไวรัส seasonal influenza A H1N1 ดั้งเดิมจากทั่วโลกรวมทั้งประเทศไทยดื้อต่อยา oseltamivir

ในประเทศไทยได้มีการนำยา oseltamivir เข้ามาใช้ในการรักษาผู้ป่วยไข้หวัดนก นอกจากนี้ยังมีการกินยาเพื่อป้องกันในนักวิจัยบางกลุ่มด้วย การเฝ้าระวังการดื้อยาของเชื้อ

วัตถุประสงค์

ประเทศไทยมีการรายงานผู้ป่วยไข้หวัดนก H5N1 รายสุดท้ายของประเทศเมื่อเดือนกรกฎาคม 2549 แต่ต่อมาในช่วงเดือนพฤษภาคม 2552 ได้มีการระบาดของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 A(H1N1) เกิดขึ้น คณะผู้วิจัยจึงได้ทำการศึกษาวิจัยเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ร่วมด้วย เพื่อเป็นประโยชน์ในการป้องกันและควบคุมการระบาดของโรคเป็นการเร่งด่วนในขณะนั้น ดังนั้นโครงการวิจัยจึงมีวัตถุประสงค์ ดังนี้

1. เพื่อเฝ้าระวังไวรัสไข้หวัดนกลูกผสมตัวใหม่ ที่อาจมีความรุนแรงในการก่อโรคและแพร่กระจายระหว่างคนได้ดี

2. ศึกษาลักษณะทางพันธุกรรมของเชื้อไวรัสไข้หวัดนกที่พบในประเทศไทยในช่วงการระบาดระลอกต่างๆ เพื่อทราบว่าเชื้อต้นเหตุนั้นเป็นเชื้อที่ถูกนำเข้ามาใหม่ หรือเป็นเชื้อที่รอดเหลือจากการระบาดครั้งก่อน

3. ศึกษาลักษณะทั่วไปทางชีววิทยาของไวรัสไข้หวัดนก ได้แก่ ความสามารถในการจับกับเม็ดเลือดแดง species ต่างๆ ลักษณะรูปร่างเมื่อส่องดูด้วยกล้องจุลทรรศน์อิเล็กตรอน

4. เพื่อศึกษาความสามารถของเชื้อไวรัสไข้หวัดนกในการติดเชื้อและเพิ่มจำนวนในเซลล์ที่ไม่ใช่ respiratory epithelial cells โดยเฉพาะเซลล์เยื่อบุหลอดเลือดดำ ซึ่งจะทำให้เข้าใจถึงสาเหตุที่ทำให้เชื้อไวรัสไข้หวัดนกสามารถก่อพยาธิสภาพนอกระบบทางเดินหายใจได้ รวมทั้งความสามรถในการหลั่ง cytokines/mediators ในเซลล์เยื่อบุหลอดเลือดดำ โดยศึกษาเปรียบเทียบกับไข้หวัดใหญ่ของคน

5. ศึกษา innate immunity ที่มีอยู่ในซีรั่ม (serum inhibitors) ที่มีฤทธิ์ยับยั้งความสามารถในการติดเชื้อไข้หวัดนก โดยเปรียบเทียบกับเชื้อไข้หวัดใหญ่ของมนุษย์สายพันธุ์ต่างๆ

6. ศึกษาการตอบสนองทางด้าน humoral immunity ต่อเชื้อไข้หวัดนก

6.1 ศึกษา kinetics การสร้างแอนติบอดีในผู้ติดเชื้อไข้หวัดนกและติดตามการคงอยู่ของระดับแอนติบอดีโดยการเจาะเลือดเป็นระยะทุก 6 เดือน เป็นเวลา 3 ปี เพื่อใช้เป็นค่า cut-off ในการสำรวจหาประชากรกลุ่มเสี่ยงที่อาจติดเชื้อไข้หวัดนกโดยไม่แสดงอาการ

6.2 ศึกษา cross-neutralizing antibody ต่อเชื้อไข้หวัดนกที่เกิดขึ้นในกลุ่มผู้รับวัคซีน seasonal influenza

6.3 ศึกษาแอนติบอดีต่อโปรตีนแต่ละชนิดของไวรัสไข้หวัดนก ซึ่งสามารถทำได้โดยการสร้าง vaccinia recombinant viruses ซึ่งมีการสอดแทรกยีน HA NA NP M หรือ NS ของไวรัสไข้หวัดนก รวมทั้งหมด 5 constructs และใช้ recombinant viruses เหล่านี้ในการ infect TK cell เพื่อเตรียมแอนติเจนสำหรับทดสอบหาแอนติบอดีโดยวิธีอิมมูนเรืองแสง

7. ศึกษา cellular immunity ต่อ epitopes ของ NP protein ของเชื้อไข้หวัดนกในผู้รอดชีวิตโดยการวัด function ของ cytotoxic T lymphocytes ด้วยวิธี ELISpot และเนื่องจาก NP เป็น conserved gene จึงควรพบได้ในกลุ่มคนที่เป็น non-H5N1 exposure ด้วย เป็นการสร้างแนวความคิดใหม่ในการพัฒนาวัคซีนที่กระตุ้นให้เกิดภูมิคุ้มกันข้าม subtype หรือ cross immunity

8. เพื่อศึกษาความสัมพันธ์ระหว่างเส้นทางของนกอพยพในประเทศไทย กับพื้นที่ระบาดของโรค โดยใช้เทคนิคการสื่อสารผ่านดาวเทียม satellite telemetry

9. เพื่อศึกษาการตอบสนองทางภูมิคุ้มกันต่อเชื้อไข้หวัดใหญ่/ไข้หวัดนก รวมทั้งหาค่า cut-off ในการประเมินอัตราการติดเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ A/H1N1 2009 ภายหลังจากการระบาดในระลอกแรกเพื่อประเมินความเสี่ยงที่จะเกิดการระบาดของโรคในระลอกถัดไป

10. ศึกษา viral load และ viral shedding จากสิ่งส่งตรวจชนิดต่างๆ จากผู้ที่ติดเชื้อ seasonal influenza virus A/H1N1, A/H3N2 และ A/H1N1 2009 เพื่อวิเคราะห์หาสิ่งส่งตรวจที่เหมาะสมในการวินิจฉัยการติดเชื้อและเป็นประโยชน์ในการควบคุมการแพร่กระจายของโรค

11. เพื่อเฝ้าระวังเชื้อไข้หวัดใหญ่/ไข้หวัดนก และ ไข้หวัดใหญ่สายพันธุ์ใหม่ A/H1N1 2009 ดื้อต่อยา neuraminidase inhibitors: oseltamivir และ zanamivir

วิธีการทดลอง

1. จริยธรรมการวิจัย

การดำเนินงานวิจัยในมนุษย์ได้ผ่านการอนุมัติ จากคณะกรรมการจริยธรรมวิจัยในคน (Ethic committee) คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล และจากกระทรวงสาธารณสุข

การวิจัยในสัตว์ได้รับอนุมัติการทำวิจัยจากคณะกรรมการกำกับดูแลการเลี้ยงและการใช้สัตว์ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล และได้รับการรับรองจาก Standards for Humane Care and Use of Laboratory Animals of the Office of Laboratory Animal Welfare (OLAW No. A5731-01), U.S. และได้รับอนุญาตและความร่วมมือจากเจ้าหน้าที่กรมอุทยานแห่งชาติ สัตว์ป่า และพันธุ์พืช

2. เทคนิควิธีการที่ใช้ในการศึกษาวิจัย

2.1 การแยกเชื้อและเพาะเลี้ยงเพิ่มจำนวนไวรัส ในไข่ไก่ฟักและเซลล์เพาะเลี้ยง

2.2 ทดสอบหาปริมาณไวรัสโดย TCID50 virus titration, plaque assay

2.3 การวินิจฉัยการติดเชื้อไวรัสไข้หวัดใหญ่ โดยใช้เทคนิค RT-PCR, realtime RT-PCR, immunofluorescence และ serology diagnosis โดยวิธี microNT และ HI assays

2.4 การศึกษาลักษณะทางพันธุกรรมของไวรัสโดยการหาลำดับนิวคลิโอไทด์ และการทำ phylogenetic analysis

2.5 ศึกษา viral load และ viral shedding จาก clinical samples ชนิดต่างๆ โดยใช้เทคนิค quantitative real time RT-PCR ในการตรวจหา *M* gene ของเชื้อไวรัส

2.6 การศึกษาการตอบสนองทางภูมิคุ้มกันของเชื้อไวรัสไข้หวัดใหญ่/ไข้หวัดนกด้วยวิธี microNT และ HI

2.7 ศึกษาสารในซีรั่ม ซึ่งมีผลต่อการยับยั้งการติดเชื้อและเพิ่มจำนวนของเชื้อไวรัสไข้หวัดนกและไข้หวัดใหญ่สายพันธุ์ต่างๆ ด้วยวิธี infectivity reduction assay

2.8 ศึกษาภูมิคุ้มกันชนิด cross-subtype cellular immunity ระหว่างเชื้อไข้หวัดใหญ่และไข้หวัดนก (H5N1) ด้วยวิธี ELISpot assay และ Flow cytometry

2.9 การตรวจวัดระดับ cytokine/chemokine ที่หลั่งจาก mononuclear cell และเซลล์บุหลอดเลือดโดยวิธี ELISA

2.10 ศึกษาความสัมพันธ์ระหว่างเส้นทางของนกอพยพในประเทศไทยกับพื้นที่ระบาดของโรคโดยใช้เทคนิคการสื่อสารผ่านดาวเทียม (satellite telemetry)

2.11 การทดสอบหาความไวของไวรัสต่อยาต้านไวรัส neuraminidase inhibitor

2.11.1 วิธี genotypic-based assay โดยวิเคราะห์ลำดับนิวคลิโอไทด์ของยีน *NA* ของไวรัส เพื่อหาตำแหน่ง mutation ที่ทำให้ไวรัสดื้อต่อยาต้านไวรัส วิธีนี้ทำได้รวดเร็วแต่มีข้อเสีย คือ สามารถใช้กับการเกิด mutation ที่เคยมีการศึกษาไว้ก่อนแล้ว แต่ไม่สามารถใช้ในการตรวจหาเชื้อดื้อยาที่เกิดจากการ mutation ในตำแหน่งที่เกิดขึ้นใหม่ได้

2.11.2 วิธี phenotypic-based assay โดย MUNANA-based neuraminidase inhibition assay (NAI), ELISA-based viral nucleoprotein (NP) reduction assay และ plaque reduction assay

ในการศึกษานี้จะทดสอบความไวของเชื้อต่อยาที่มีฤทธิ์ยับยั้ง neuraminidase ของไวรัส 2 ชนิด คือ oseltamivir และ zanamivir ซึ่ง oseltamivir carboxylate จากบริษัท Roche, Belgium ได้รับการสนับสนุนจาก Southeast Asia Infectious Clinical Research Network และ zanamivir จาก บริษัท GlaxoSmithKline, UK ได้รับการสนับสนุนจาก US. Centre for Disease Control and Prevention ในปัจจุบันยังไม่มีเทคนิคมาตรฐานเพื่อใช้ในการทดสอบความไวต่อยาต้านไวรัสด้วยวิธีที่พิจารณาจากการติดเชื้อในเซลล์เพาะเลี้ยง (cell culture-based infectivity) ที่ยอมรับใช้ทั่วไปในห้องปฏิบัติการต่างๆ โครงการวิจัยนี้จึงได้พัฒนาเทคนิค ELISA-based viral nucleoprotein reduction assay ขึ้นเป็นครั้งแรกเพื่อใช้เป็น alternative test โดยเปรียบเทียบกับ plaque reduction assay และ neuraminidase inhibition assay (NAI)

MUNANA-based NAI assay เป็นการทดสอบการทำงานของเอนไซม์ neuraminidase ของเชื้อไวรัสโดยตรง ซึ่งใช้ 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) เป็น substrate

Plaque reduction assay เป็นเทคนิคหลักที่ใช้กันทั่วโลก แต่ยังไม่มีใครกำหนดวิธีมาตรฐานออกมาว่า ควรใช้ไวรัสปริมาณเท่าใด นอกจากนี้วิธีนี้ใช้เวลานาน และมี variation สูง นอกจากนี้ plaque ของไวรัสยังมีขนาดไม่เท่ากัน คณะผู้วิจัยจึงกำหนดว่า หากศึกษาไวรัส H5N1 ซึ่งมีขนาดใหญ่จะกำหนดไว้ที่ 50 plaque forming unit (PFU) ส่วน H1N1/ H3N2 จะกำหนดไว้ที่ 50-100 PFU แต่ทั้งนี้วิธีนี้ก็มีข้อเสีย คือ ไวรัสบางสายพันธุ์มีลักษณะการเกิด plaque ที่มีขนาดเล็กและไม่ชัดเจน จึงทำให้ไม่สามารถทดสอบการดื้อยาด้วยวิธีนี้ได้

ELISA-based NP reduction assay เป็นเทคนิคที่พัฒนาและดัดแปลงมาจากวิธี microneutralization assay ที่ใช้ในการตรวจหาแอนติบอดีต่อเชื้อไวรัส โดยใช้การตรวจหาปริมาณการสร้าง nucleoprotein ของไวรัสภายในเซลล์ที่มีการติดเชื้อโดยวิธี ELISA สามารถทดสอบกับไวรัสได้คราวละมากๆ และใช้ยาปริมาณน้อย และเมื่อนำมาผลไปศึกษาความสัมพันธ์กับเทคนิค plaque reduction assay พบว่ามีความสัมพันธ์กัน นอกจากนี้เทคนิคนี้ได้ถูกนำไปใช้ตรวจคัดกรองหาสารสกัดสมุนไพรซึ่งมีฤทธิ์ต้านเชื้อไข้หวัดใหญ่และไข้หวัดนก

ผลการศึกษาวิจัย

1. Electron micrographs of highly and low pathogenic avian influenza viruses

Background: The outbreak of highly pathogenic avian influenza (HPAI) H5N1 virus was first reported in Thailand in 2004. Up to present, electron micrographs demonstrating the morphology of HPAI H5N1 virus particle are quite limited. *Objective:* To demonstrate the morphology of free particles of human influenza viruses, HPAI H5N1 viruses and low pathogenic avian influenza (LPAI) viruses as well as the H5N1 structural components in the infected cells. In addition, the amino acid substitutions affecting the virus morphology was also investigated. *Methods:* Electron micrographs of the negative stained virus particles and the positive stained thin sections of the HPAI H5N1 virus infected cells were visualized under a transmission electron microscope (TEM). The M1 and M2 amino acid sequences were retrieved from GenBank. *Results:* Morphologically, the free influenza virus particles appeared in 3 forms: spherical, regular and irregular rods, and long filamentous particles, were demonstrated. However, spherical form was the most predominant morphological type and took account of more than 80% of the virus populations examined. In addition, the step of viral entry and exit including incomplete particles in the infected MDCK cells were found. *Conclusion:* Of all virus isolates studied, we demonstrated that the spherical particles were the major population observed regardless of virus subtypes, host of origin, virus virulence, passage history and amino acid substitutions in M1and M2 proteins.

2. A novel pathogenic mechanism of highly pathogenic avian influenza H5N1 viruses involves hemagglutinin mediated resistance to serum innate inhibitors

In this study, the effect of innate serum inhibitors on influenza virus infection was addressed. Seasonal influenza A(H1N1) and A(H3N2), 2009 pandemic A(H1N1) (H1N1pdm) and highly pathogenic avian influenza (HPAI) A(H5N1) viruses were tested with guinea pig sera negative for antibodies against all of these viruses as evaluated by hemagglutination-inhibition and microneutralization assays. In the presence of serum inhibitors, the infection by each virus was inhibited differently as measured by the amount of viral nucleoprotein produced in Madin-Darby canine kidney cells. The serum

inhibitors inhibited seasonal influenza A (H3N2) virus the most, while the effect was less in seasonal influenza A(H1N1) and H1N1pdm viruses. The suppression by serum inhibitors could be reduced by heat inactivation or treatment with receptor destroying enzyme. In contrast, all H5N1 strains tested were resistant to serum inhibitors. To determine which structure (hemagglutinin (HA) and/or neuraminidase (NA)) on the virus particles that provided the resistance, reverse genetics (rg) was applied to construct chimeric recombinant viruses from A/Puerto Rico/8/1934(H1N1) (PR8) plasmid vectors. rgPR8-H5 HA and rgPR8-H5 HANA were resistant to serum inhibitors while rgPR8-H5 NA and PR8 A(H1N1) parental viruses were sensitive, suggesting that HA of HPAI H5N1 viruses bestowed viral resistance to serum inhibition. These results suggested that the ability to resist serum inhibition might enable the viremic H5N1 viruses to disseminate to distal end organs. The present study also analyzed for correlation between susceptibility to serum inhibitors and number of glycosylation sites present on the globular heads of HA and NA. H3N2 viruses, the subtype with highest susceptibility to serum inhibitors, harbored the highest number of glycosylation sites on the HA globular head. However, this positive correlation cannot be drawn for the other influenza subtypes.

3. Surveillance for reassortant virus by multiplex reverse transcription-PCR specific for eight genomic segments of avian influenza A H5N1 viruses

Avian influenza H5N1 virus is a global threat. An emergence of a reassortant virus with a pandemic potential is a major concern. Here we describe a multiplex reverse transcription-PCR assay that is specific for the eight genomic segments of the currently circulating H5N1 viruses to facilitate surveillance for a virus resulting from reassortment between human influenza virus and the H5N1 virus.

4. Indigenous sources of 2007–2008 H5N1 avian influenza outbreaks in Thailand

Outbreaks of H5N1 avian influenza show strong seasonality. It is not clear where the source of virus originates from in each new outbreak season. This study sought to understand the nature of viral resurgence in recent outbreak seasons in Thailand, where the epidemic is relatively well controlled. In such a situation, indigenous viruses surviving the inter-outbreak season would have to pass through a bottleneck. In order to look for evidence of the bottleneck effect, viral genome sequences from recent outbreaks in the country were analysed. H5N1 avian influenza

viruses were isolated from six outbreaks in the rainy season and winter of 2007 through to early 2008. Most of the outbreaks were in the Yom–Nan River basin in the southern part of the northern region of the country. Sequences of these viral isolates were identified as clade 1, genotype Z, similar to viruses from previous years in the central region of the country. The sequences clustered into two groups, one of which was closely related to viruses isolated from the same area in July 2006. These analyses indicated that there was a strong bottleneck effect on the virus population and that only a few lineages remained in the area. In addition, evidence of reassortment among these viruses was found. These indicated re-emergence of viruses from a small pool of indigenous sources that had been silently perpetuated over the dry summer months. Therefore, an approach to eradicate H5N1 avian influenza from the area by eliminating these local reservoirs may be feasible and should be seriously considered.

5. Avian influenza virus (H5N1) in human, Laos

The first avian influenza (H5N1) outbreak in poultry in Laos occurred in 2003 and subsided in March 2004 after massive killing of poultry to contain the disease. Extensive surveillance from July 2005 through January 2006 did not detect any influenza virus subtypes in chicken, ducks, quails, and pigs in live bird markets in the Vientiane, Champasak, and Savannakhet Provinces. Avian influenza virus (H5N1) was reintroduced into Laos in February 2006 but showed a lower incidence. Viruses isolated in this country in 2004 belonged to genotype Z, clade 1, and 2006 isolates belonged to clade 2.3.4

6. Codon volatility of hemagglutinin genes of H5N1 avian influenza viruses from different clades

Codon volatility is a method recently developed to estimate selective pressures on proteins on the basis of their synonymous codon usage. Volatility of a codon was defined as the fraction of single nucleotide substitutions that would be nonsynonymous. Higher volatility may indicate that the gene has been under more positive selection in the recent past. We analyzed volatility of hemagglutinin genes of H5N1 viruses in the recent outbreaks and observed differences in the volatility among viruses of different clades. The codon volatility of subclade 2.1 viruses from Indonesia was the lowest among all H5N1 clades and subclades. Time series analyses since the beginning of the epidemic in 2004 showed that codon volatility of subclade 2.1 has gradually decreased, while those of other major clades have been increasing. This may reflect differences in the recent evolution of these viruses.

7. Erythrocyte binding preference of avian influenza H5N1 viruses

Five erythrocyte species (horse, goose, chicken, guinea pig, and human) were used to agglutinate avian influenza H5N1 viruses by hemagglutination assay and to detect specific antibody by hemagglutination inhibition test. We found that goose erythrocytes confer a greater advantage over other erythrocyte species in both assays.

8. Erythrocyte binding preference of 16 subtypes of low pathogenic avian influenza and 2009 pandemic influenza A (H1N1) viruses

All 16 subtypes of avian influenza viruses of low pathogenicity (LPAIV) as well as their hemagglutinin (H) antigens, and four 2009 pandemic influenza A (H1N1) virus isolates were assayed for hemagglutinating activity against 5 erythrocyte species: goose, guinea pig, human group O, chicken and horse. Of all viruses and antigens assayed, the highest hemagglutination (HA) titers were obtained with goose and guinea pig erythrocytes. Hemagglutinating activity of replicating LPAIV and LPAIV antigens decreased, in order, with chicken and human group O; meanwhile, horse erythrocytes yielded lowest or no HA titer. Moreover, the 2009 pandemic viruses did not agglutinate both horse and chicken erythrocytes. Our study concluded that goose and guinea pig erythrocytes are the best in HA assay for all subtypes of influenza viruses.

9. Kinetics and longevity of antibody response to influenza A H5N1 virus infection in humans

Anti-H5N1 antibody was determined by microneutralization, hemagglutination inhibition, and Western blotting assays in serial blood samples collected from eight Thai patients, including four fatal cases and four survivors. The antibody was detected as early as 5 days and, typically, with an increase in titer in paired blood at about 15 days after disease onset. The anti-H5 antibody response was long-lasting, for almost 5 years in cases which can be followed that far. In addition, cross-neutralizing activity to related clade 1 viruses was observed.

10. Induction of cross-neutralizing antibody against H5N1 virus after vaccination with seasonal influenza vaccine in COPD patients

Archival serum samples from elderly individuals with underlying chronic obstructive pulmonary disease (COPD) who were enrolled in a double-blind case-control study of seasonal influenza vaccine efficacy were assayed for cross-neutralizing antibody formation to avian influenza A (H5N1) virus. Of 118 serum samples, 58 were collected from influenza vaccinees (mean age 68.5 y), and 60 from placebo controls (mean age 68.4 y) who received vitamin B injections. Blood samples were collected before and at 1mo after seasonal influenza vaccination from all subjects; in addition, for a longitudinal follow-up period of 1 y paired-blood samples were collected again from subjects who developed acute respiratory illness. Hemagglutination inhibition assay for antibodies to influenza A (H1N1), influenza A (H3N2), and influenza B viruses was carried out to determine the serological response to vaccination, and to diagnose influenza viral infection, while microneutralization assays were performed to detect cross-reactive antibody to H5N1 virus. Pre-existing cross-reactive H5N1 antibody at reciprocal titer 10 was found in 6 (10.3%) vaccinees and 4 (6.7%) placebo controls. There was no change in H5N1 antibody titer in these subjects after vaccination. On the other hand, 3 (5.2%) vaccinees developed seroconversion to H5N1 virus at 1mo after vaccination, even though they had no pre-existing H5N1 antibody in their first blood samples. No cross-neutralizing antibody to H5N1 virus was detected in the placebo controls or in the 22 influenza patients, suggesting that influenza vaccination, but not influenza virus infection, induces cross-neutralizing antibody against avian influenza H5N1 virus.

11. Biological properties of H5 hemagglutinin expressed by vaccinia virus vector and its immunological reactivity with human sera

A recombinant vaccinia virus harboring the full length hemagglutinin (*HA*) gene derived from a highly pathogenic avian influenza A/Thailand/1(KAN-1)/2004 (H5N1) virus (rVac-H5 HA virus) was constructed. The immunogenicity of the expressed HA protein was characterized using goat antiserum, mouse monoclonal antibody and human sera. Expressed HA protein localized both in the cytoplasm and on the cytoplasmic membrane of the thymidine kinase negative cells infected with the rVac-H5 HA virus, as determined by immunofluorescence assay. Moreover, Western blot analysis demonstrated that the rVac-H5 HA protein was post-translationally processed by proteolytic cleavage of the HA0 precursor into HA1 and HA2 domains; and all of these HA forms were immunogenic in BALB/c mice. The molecular weight (MW) of each

HA domain was the same as the wild-type H5 HA produced in Madin-Darby canine kidney cells infected with the H5N1 virus, but was higher than that expressed by a baculovirus-insect cell system. Sera from all H5N1 survivors reacted to HA0, HA1 and HA2 domains; whereas sera from H5N1-uninfected subjects reacted to the rVac-H5 HA2 domain only, but not to HA0 or HA1, indicating that some cross-subtypic immuniy exists in the general population. There was a lot-to-lot variation of the recombinant HA produced in the baculovirus-insect cell system that might affect the detection rate of antibody directed against certain HA domain.

12. The difference in IL-1β, MIP-1α, IL-8 and IL-18 production between the infection of PMA activated U937 cells with recombinant vaccinia viruses inserted 2004 H5N1 influenza HA genes and NS genes

Summary *Background*: The severity of avian influenza H5N1 disease is correlated with the ability of the virus to induce an over production of pro-inflammatory cytokines from innate immune cells. However, the role of each virus gene is unknown. To elaborate the function of each virus gene, the recombinant vaccinia virus inserted HA and NS gene from the 2004 H5N1 virus were used in the study. *Methods*: U937 cells and PMA activated U937 cells were infected with recombinant vaccinia virus inserted with HA or NS gene. The expressions of HA and NS proteins in cells were detected on immunofluorescence stained slides using a confocal microscope. The cytokine productions in the cell supernatant were quantitated by ELISA. *Results*: The recombinant vaccinia virus inserted with HA genes induces the production of IL-1β, MIP-1α, IL-8 and IL-18 cytokines from PMA activated U937 cells significantly more than cells infected with wild type vaccinia, whereas the recombinant vaccinia virus inserted with NS genes it was similar to that with the wild type vaccinia virus. However, there was no synergistic nor antagonistic effect of HA genes and NS genes in relation to cytokines production. *Conclusion*: Only the HA gene from the 2004 H5N1 virus induces IL-1β, MIP-1α, IL-8 and IL-18 cytokine productions from activated U937 cells. The same HA gene effect may or may not be the same in respiratory epithelial cells and this needs to be explored.

13. Satellite tracking on the flyways of Brown-headed gulls and their potential role in the spread of highly pathogenic avian influenza H5N1 virus

Brown-headed gulls (*Larus brunnicephalus*), winter visitors of Thailand, were tracked by satellite telemetry during 2008-2011 for investigating their roles in the highly pathogenic avian influenza (HPAI) H5N1 virus spread. Eight gulls negative for influenza virus infection were marked with solar-powered satellite platform transmitters, their movements were monitored by the Argos satellite tracking system, and locations were mapped. Five gulls completed their migratory cycles, which spanned 7 countries (China, Bangladesh, India, Myanmar, Thailand, Cambodia, and Vietnam) affected by the HPAI H5N1 virus. Gulls migrated from their breeding grounds in China to stay overwinter in Thailand and Cambodia; while Bangladesh, India, Myanmar, and Vietnam were the places of stopovers during migration. Gulls traveled an average distance of about 2400 km between Thailand and China and spent 1-2 weeks on migration. Although AI surveillance among gulls was conducted at the study site, no AI virus was isolated and no H5N1 viral genome or specific antibody was detected in the 75 gulls tested, but 6.6% of blood samples were positive for pan-influenza A antibody. No AI outbreaks were reported in areas along flyways of gulls in Thailand during the study period. Distance and duration of migration, tolerability of the captive gulls to survive the HPAI H5N1 virus challenge and days at viral shedding after the virus challenging suggested that the Brown-headed gull could be a potential species for AI spread, especially among neighboring countries. This is the only study that demonstrated flyways which links between China and Southeast Asia, the epicenter of H5N1 AI outbreak.

14. Serological response to the 2009 pandemic influenza A (H1N1) virus for disease diagnosis and estimating the infection rate in Thai population

Background: Individuals infected with the 2009 pandemic virus A(H1N1) developed serological response which can be measured by hemagglutination-inhibition (HI) and microneutralization (microNT) assays. Methodology/Principal Findings: MicroNT and HI assays for specific antibody to the 2009 pandemic virus were conducted in serum samples collected at the end of the first epidemic wave from various groups of Thai people: laboratory confirmed cases, blood donors and health care workers (HCW) in Bangkok and neighboring province, general population in the North and the South, as well as archival sera collected at pre- and post-vaccination from vaccinees who received influenza vaccine of the 2006 season. This study demonstrated that goose erythrocytes yielded comparable HI antibody titer as compared to turkey erythrocytes. In contrast to the standard protocol, our investigation found out the necessity to eliminate nonspecific

inhibitor present in the test sera by receptor destroying enzyme (RDE) prior to performing microNT assay. The investigation in pre-pandemic serum samples showed that HI antibody was more specific to the 2009 pandemic virus than NT antibody. Based on data from pre-pandemic sera together with those from the laboratory confirmed cases, HI antibody titers $40 for adults and $20 for children could be used as the cut-off level to differentiate between the individuals with or without past infection by the 2009 pandemic virus. Conclusions/Significance: Based on the cut-off criteria, the infection rates of 7 and 12.8% were estimated in blood donors and HCW, respectively after the first wave of the 2009 influenza pandemic. Among general population, the infection rate of 58.6% was found in children versus 3.1% in adults.

15. Seroprevalence of 2009 H1N1 virus infection and self-reported infection control practices among healthcare professionals following the first outbreak in Bangkok, Thailand

A serologic study with simultaneous self-administered questionnaire regarding infection control (IC) practices and other risks of influenza A (H1N1) pdm09 (2009 H1N1) infection was performed approximately 1 month after the first outbreak among frontline health care professionals (HCPs). Of 256 HCPs, 33 (13%) were infected. Self-reported adherence to IC practices in >90% of exposure events was 82.1%, 73.8%, and 53.5% for use of hand hygiene, masks, and gloves, respectively. Visiting crowded public places during the outbreak was associated with acquiring infection (OR 3.1, *P*=0.019). Amongst nurses, exposure to HCPs with influenza-like illness during the outbreak without wearing a mask was the only identified risk factor for infection (OR = 2.3, *P*=0.039).

16. Monitoring the influenza pandemic of 2009 in Thailand by a community-based survey

As an international traveling hub of South-East Asia, Thailand was one of the countries hardest and earliest hit by the influenza A (H1N1) 2009 pandemic. In order to understand the epidemic spread in the country, we conducted community-based surveys in metropolitan, urban, and rural areas using questionnaire interviews. We also determined sero-positive rates from randomly selected samples within the surveyed population. Recalled incidences of fever and acute respiratory symptoms in the survey correlated well with systematic reports of 2009 pandemic influenza cases from hospitals

in the same areas, giving a ratio of total cases extrapolated from the surveyed data for persons who sought medical attention reported in the hospital-based surveillance system at 275:1. Conducting a large scale survey of the influenza outbreak is time consuming and also can be difficult to complete in a short time. Therefore, we used the survey for monitoring the outbreak of respiratory disease in the early pandemic phase. The seroprevalence rate was 8 to 10%, with higher rate for younger age groups, and suggests that sufficient herd immunity may have been reached in Thailand, especially in urban areas, while others may still be vulnerable to the second wave of the pandemic.

17. Immune response to 2009 H1N1 vaccine in HIV-infected adults in Northern Thailand

Background: In late 2009, the Thai Ministry of Public Health provided two million doses of the monovalent pandemic influenza H1N1 2009 vaccine (Panenza (®) Sanofi Pasteur), which was the only vaccine formulation available in Thailand, to persons at risk of more severe manifestations of the disease including HIV infection. Several studies have shown poorer immune responses to the 2009 H1N1 vaccines in HIV-infected individuals. There are limited data in this population in resource-limited countries. Results: At day 28 post-vaccination, seroconversion was found in 32.0% (95%CI 24.5 - 40.2) of the HIV-infected group and 35.0% (95%CI 15.4- 59.2) of the healthy controls (p = 0.79). Seroprotection rate was observed in 33.3% (95%CI 25.8-41.6) and 35.0% (95%CI 15.4-59.2) of the HIV-infected group and the control group, respectively (p = 0.88). Among HIV-infected participants, the strongest factor associated with vaccine response was age 42 y or younger (p = 0.05). Methods: We evaluated the immunogenicity of a single, 15µg/0.5ml dose of a monovalent, non-adjuvanted 2009 H1N1 vaccine in 150 HIV-infected Thai adults and 20 healthy controls. Immunogenicity was measured by hemagglutination inhibition assay (HI) at baseline and 28 d after vaccination. Seroconversion was defined as 1) pre-vaccination HI titer < 1:10 and post-vaccination HI titer $\geq$ 1:40, or 2) pre-vaccination HI titer $\geq$ 1:10 and a minimum of 4-fold rise in post-vaccination HI titer. Seroprotection was defined as a post-vaccination HI titer of $\geq$ 1:40. Conclusions: A low seroconversion rate to the 2009 H1N1 vaccine in both study groups, corresponding with data from trials in the region, may suggest that the vaccine used in our study is not very immunogenic. Further studies on different vaccines, dosing, adjuvants, or schedule strategies may be needed to achieve effective immunization in HIV-infected population.

18. Influenza A viral loads in respiratory samples collected from patients infected with pandemic H1N1, seasonal H1N1 and H3N2 viruses

Background: Nasopharyngeal aspirate (NPA), nasal swab (NS), and throat swab (TS) are common specimens used for diagnosis of respiratory virus infections based on the detection of viral genomes, viral antigens and viral isolation. However, there is no documented data regarding the type of specimen that yields the best result of viral detection. In this study, quantitative real time RT-PCR specific for *M* gene was used to determine influenza A viral loads present in NS, NPA and TS samples collected from patients infected with the 2009 pandemic H1N1, seasonal H1N1 and H3N2 viruses. Various copy numbers of RNA transcripts derived from recombinant plasmids containing complete *M* gene insert of each virus strain were assayed by RT-PCR. A standard curve for viral RNA quantification was constructed by plotting each Ct value against the log quantity of each standard RNA copy number. Results: Copy numbers of *M* gene were obtained through the extrapolation of Ct values of the test samples against the corresponding standard curve. Among a total of 29 patients with severe influenza enrolled in this study (12 cases of the 2009 pandemic influenza, 5 cases of seasonal H1N1 and 12 cases of seasonal H3N2 virus), NPA was found to contain significantly highest amount of viral loads and followed in order by NS and TS specimen. Viral loads among patients infected with those viruses were comparable regarding type of specimen analyzed. Conclusion: Based on *M* gene copy numbers, we conclude that NPA is the best specimen for detection of influenza A viruses, and followed in order by NS and TS.

19. Infection rate, duration of viral shedding and viral load in an outbreak of novel influenza A (H1N1) 2009 infections among military conscripts in a training center, Thailand, June 2009 (อัตราการติดเชื้อ ระยะเวลาของการขับเชื้อไวรัส และปริมาณเชื้อไวรัส ขณะมีการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ 200 ในกลุ่มทหารเกณฑ์ ศูนย์ฝึกทหารใหม่แห่งหนึ่ง ประเทศไทย เดือนมิถุนายน พ.ศ. 2552)

การระบาดของไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในทหารเกณฑ์ชายไทยอายุเฉลี่ย 21 ปี มีอัตราป่วยสูงถึงร้อยละ 29.3 ระยะเวลาการขับเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในทหารเกณฑ์มีระยะเวลานานเฉลี่ย 5 วัน หลังเริ่มแสดงอาการ แต่สามารถตรวจพบได้ตั้งแต่วันแรกที่เริ่มป่วยจนถึง11 วัน ซึ่งนับว่าใกล้เคียงกันมาก ซึ่งน่าจะอธิบายได้จากการที่เป็นประชากรอายุใกล้เคียงกันและเป็นการติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ในระยะต้นของการ

ระบาดใหญ่ ที่ยังมีสารพันธุกรรมใกล้เคียงกัน การเก็บตัวอย่างสารคัดหลั่งจากคอหอยหลังโพรงจมูก (Nasopharyngeal swab) มีโอกาสได้ผลบวกสูง โดยการศึกษานี้พบ ปริมาณเชื้อไวรัสจำนวน 2.6×10^{2} copies/ml ถึง 8.1×10^{9} copies/ml เฉลี่ย 2.4×10^{4} copies/ml ปริมาณเชื้อไวรัสที่พบในผู้ป่วยแต่ละราย ส่วนใหญ่จะมีปริมาณมาก สุดในวันที่ 2 ถึงวันที่ 5 หลังจากเริ่มมีอาการป่วย จึงเป็นระยะเวลาที่ต้องมีมาตรการป้องกันการแพร่เชื้อที่เข้มงวด และต้องป้องกันต่อเนื่องตลอดระยะเวลาที่มีการระบาดเพราะผู้ป่วยบางรายอาจจะขับเชื้อได้นานกว่า 10 วันอย่างไรก็ตาม ไม่พบว่ามีความสัมพันธ์ระว่างปริมาณเชื้อไวรัสกับระยะเวลาของการขับเชื้อไวรัสจากทางเดินหายใจในผู้ป่วยกลุ่มนี้ นอกจากนี้ พบว่าระยะเวลาการขับเชื้อไวรัสในผู้ป่วยที่มีน้ำหนักเกินและเสียชีวิตเท่ากับ 8 วัน และในผู้ป่วยที่กำลังป่วยเป็นวัณโรคปอดเท่ากับ 4 วันเฉลี่ยดังนั้นการเก็บตัวอย่างวิธีนี้จึงมีเหมาะสมหากทำโดยบุคลากรที่มีประสบการณ์และใช้เทคนิคที่ถูกต้องเพราะเป็นการเก็บตัวอย่างจากจุดที่พบเชื้อไวรัสไข้หวัดใหญ่อยู่ในปริมาณมากปริมาณไวรัสของผู้ป่วยที่ตรวจพบในการศึกษาครั้งนี้มี

20. ศึกษาภูมิคุ้มกันข้ามระดับเซลล์ (cellular immune response) และ ระดับสารน้ำ (humoral immune response) ระหว่างเชื้อไข้หวัดใหญ่ และไข้หวัดนก (H5N1)

20.1 ศึกษาภูมิคุ้มกันข้ามในระดับเซลล์ (cross-subtype cellular immunity)

การศึกษาวิทยาภูมิคุ้มกันข้ามระดับเซลล์ระหว่างเชื้อไข้หวัดใหญ่และไข้หวัดนกสายพันธุ์ H5N1 virus เป็นการศึกษาการตอบสนองของระบบอิมมูนในระดับ T cell ที่เกิดขึ้นต่อ peptide epitopes ที่มาจากเชื้อไข้หวัดนก การศึกษานี้ได้อาศัยเทคนิค *ex vivo* IFN-γ enzyme-linked immunospot (ELISpot) assay ในการตรวจหา cross-reactive T cells ซึ่งถูกสร้างขึ้นจากการที่ประชากรเคยมีประวัติฉีดวัคซีน หรือเคยได้รับเชื้อไข้หวัดใหญ่ตามฤดูกาลมาก่อน โดยคาดว่า memory T cells ที่จำเพาะกับโปรตีนส่วนที่ conserve เช่น neucleoprotein (NP) ของเชื้อไข้หวัดใหญ่สายพันธุ์ H1N1 หรือ H3N2 จะสามารถจดจำและทำปฏิกิริยากับ synthetic overlapping peptide epitopes ที่มาจากเชื้อไข้หวัดนกสายพันธุ์ H5N1 และเพื่อจะจำแนกได้ว่า epitope ใดที่ conserve สามารถทำให้เกิดปฏิกิริยาข้ามระหว่างเชื้อไข้หวัดใหญ่และไข้หวัดนกซึ่งจะทำการทดสอบโดยใช้เทคนิค ELISpot assay

จากผลการทดลองด้วยวิธี ELISpot assay โดยใช้ NP overlapping peptides จะทำให้ทราบถึง conserve และ H5N1 specific epitopes แต่ก็ยังไม่ทราบว่า epitope ชนิดนี้ถูกจดจำด้วย T cell ชนิดใด ดังนั้นจึงต้องทำการเลี้ยง peptide-specific T cell line ขึ้นมาเพื่อทำการหา T cell dependency โดยเทคนิค Flow cytometry

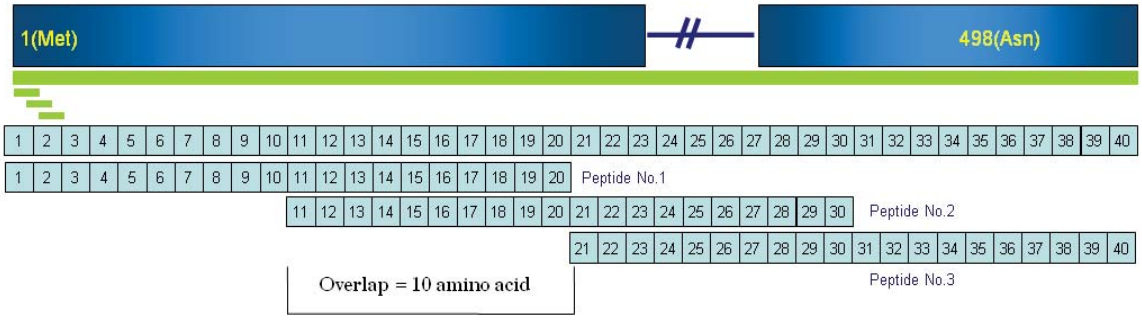
นอกจากนี้ได้ทำการสร้าง recombinant vaccinia viruses 2 ชนิด ที่มี NP และ HA protein insert ในครั้งแรกมีวัตถุประสงค์เพื่อนำไปใช้ใน CTL assay เพื่อศึกษาภูมิคุ้มกันแบบข้าม subtypes แต่มีข้อจำกัดของการทดลองคือ ได้เลือดที่เจาะได้จากผู้ป่วยปริมาณน้อย (ตามข้อกำหนดของคณะกรรมการจริยธรรมของกระทรวงสาธารณสุข) ซึ่งส่งผลให้ได้เซลล์ PBMC

ปริมาณน้อยตามไปด้วย จึงได้เปลี่ยนมาศึกษาด้วยเทคนิค ELISpot assay แทน โดยเลือกศึกษาในกลุ่มคนที่รอดชีวิต (H5N1 survivors) กลุ่มคนปกติทั้งที่เคยและไม่เคยได้รับวัคซีนและกลุ่มคนเป็นไข้หวัดใหญ่ คณะผู้วิจัยได้เลือกศึกษาเฉพาะ NP protein ซึ่งจัดเป็นโปรตีนที่ทำให้เกิด cross-reactive T cell immunity ได้มากที่สุด โดยได้ดำเนินการสังเคราะห์ peptide จำนวน 49 เส้น เพื่อหา common epitope ในกลุ่ม H5N1 survivors และ non-H5N1 subjects ทั้งสิ้น 39 ราย

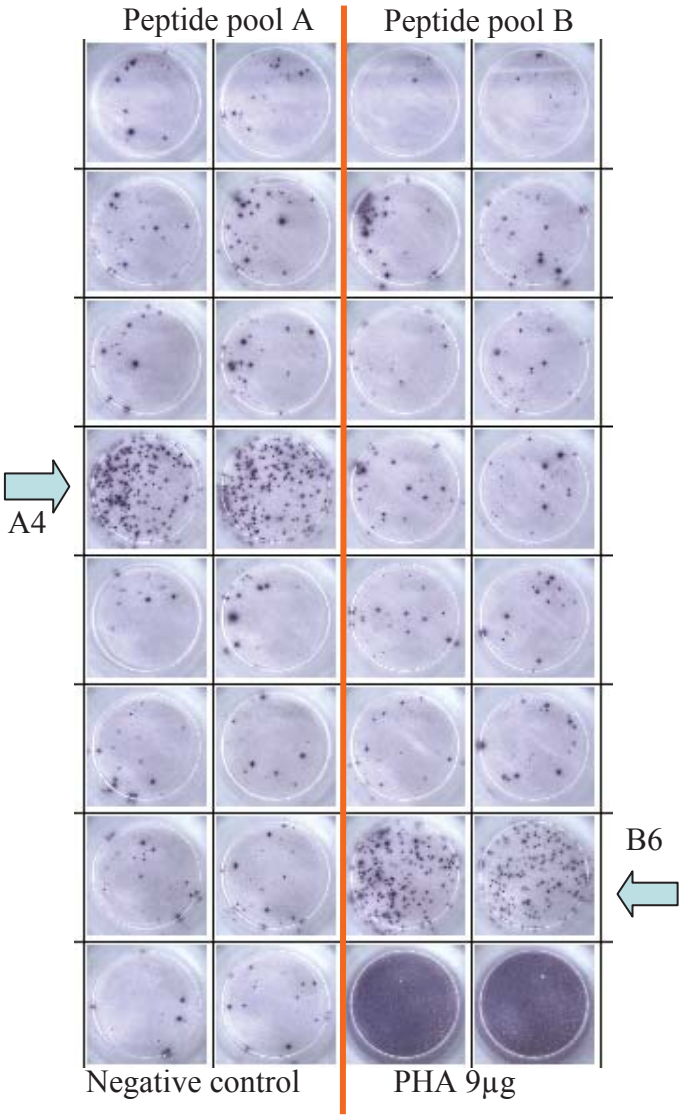
จากการทดลองได้ศึกษาส่วนของ NP protein ของเชื้อ H5N1 ซึ่งมีความอนุรักษ์ (conserve) มากกว่า 90% กับ NP protein ของเชื้อไข้หวัดใหญ่ โดยมี amino acid sequence ดังรูปที่ 1 และได้นำมาสังเคราะห์ overlapping peptides จำนวน 49 เส้น ซึ่งมีความยาวเส้นละ 20 amino acids โดยมี overlapping จำนวน 10 amino acids (รูปที่ 2) แล้วใช้เป็นตัวคัดกรองว่ามี peptide เส้นใดบ้าง ที่ T cells สามารถจดจำได้โดยวัด IFN-γ producing T cell ด้วยวิธี ELISpot assay ดังรูปที่ 3 และทำการยืนยันด้วยการทำ individual peptide ด้วย จากผลการทดลองในกลุ่ม H5N1 survivors จำนวน 4 คนพบว่า มี 2 ใน 4 คนสามารถจดจำ immunodominant NP epitopes ได้ ดังรูปที่ 4 ทั้งนี้จากการทำ serial blood samples ที่เก็บหลังจากผู้ป่วยเริ่มมีอาการพบว่า ยังสามารถตรวจพบ peptide-specific memory T cells ได้หลังจากการติดเชื้อเป็นเวลาอย่างน้อย 4 ปี โดยให้ magnitude (spot forming unit, SFU) ของการตอบสนองในระดับที่สูงพอๆ กันใน serial blood samples สำหรับในกลุ่ม non-H5N1 subjects พบว่า 16 จาก 35 คน มี memory T cells ที่สามารถจดจำ NP peptide ได้ 1-2 peptides/subject และมี magnitude ของการตอบสนองที่ต่ำกว่าเมื่อเทียบกับในกลุ่ม H5N1 survivors ดังรูปที่ 5 การทดลองนี้ยังพบว่ามี NP peptide เพียง 2 เส้นเท่านั้นที่อาจจะเป็น H5N1-specific T cell epitope ซึ่งสามารถตรวจพบได้เฉพาะใน H5N1 survivors เท่านั้น และมี 5 เส้นที่จัดว่าเป็น common epitopes ที่สามารถตรวจพบได้ในกลุ่ม non-H5N1 subjects ซึ่งมีอยู่หนึ่ง NP peptide ที่พบได้ทั้งสองกลุ่ม (งานวิจัยในส่วนนี้อยู่ระหว่างการเตรียมต้นฉบับการตีพิมพ์ผลงาน)

```
>gi|54610028|gb|AAV35112.1| nucleocapsid protein [Influenza A
virus (A/Thailand/1(KAN-1)/2004(H5N1))]
MASQGTKRSYEQMETGGERQNATEIRASVGRMVSGIGRFYIQMCTELKLSDYEGRLIQNSITIERMVLSA
FDERRNRYLEEHPSAGKDPKKTGGPIYRRRDGKWVRELILYDKEEIRRIWRQANNGEDATAGLTHLMIWH
SNLNDATYQRTRALVRTGMDPRMCSLMQGSTLPRRSGAAGAAVKGVGTMVMELIRMIKRGINDRNFWRGE
NGRRTRIAYERMCNILKGKFQTAAQRAMMDQVRESRNPGNAEIEDLIFLARSALILRGSVAHKSCLPACV
YGLAVASGYDFEREGYSLVGIDPFRLLQNSQVFSLIRPNENPAHKSQLVWMACHSAAFEDLRVSSFIRGT
RVVPRGQLSTRGVQIASNENMEAMDSNTLELRSRYWAIRTRSGGNTNQQRASAGQISVQPTFSVQRNLPF
ERATIMAAFTGNTEGRTSDMRTEIIRMMESARPEDVSFQGRGVFELSDEKATNPIVPSFDMNNEGSYFFG
DNAEEYDN
```

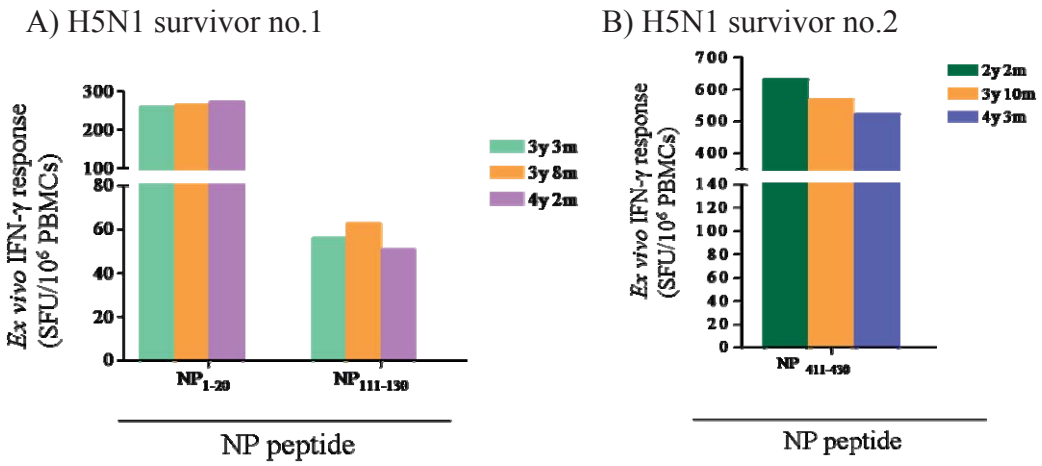
รูปที่ 1 Amino acid sequence ของ NP protein ของเชื้อ A/Thailand/1(KAN-1)/2004 (H5N1)



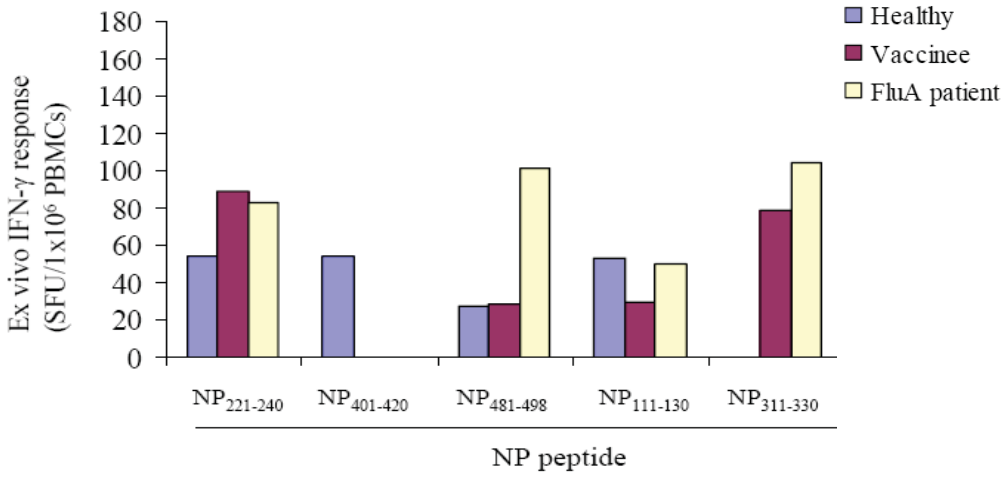
รูปที่ 2 Diagram ของการสร้าง overlapping peptide ขนาด 20 กรดอะมิโน แต่ละเส้นซ้อนทับกัน 10 กรดอะมิโน



รูปที่ 3 ตัวอย่างผลจากการทำ ELISpot assay ของบุคคลที่รอดชีวิตจากการติดเชื้อ H5N1



รูปที่ 4 การตอบสนองต่อ overlapping NP peptides ของกลุ่มตัวอย่างที่เป็น H5N1 survivors พบว่า NP peptides จากทั้งหมด 49 เส้น มีเพียง 1-2 เส้นเท่านั้นที่สามารถถูกจดจำได้โดย peptide-specific memory T cells จาก blood samples ช่วงเวลาต่างๆ หลังจาก disease onset



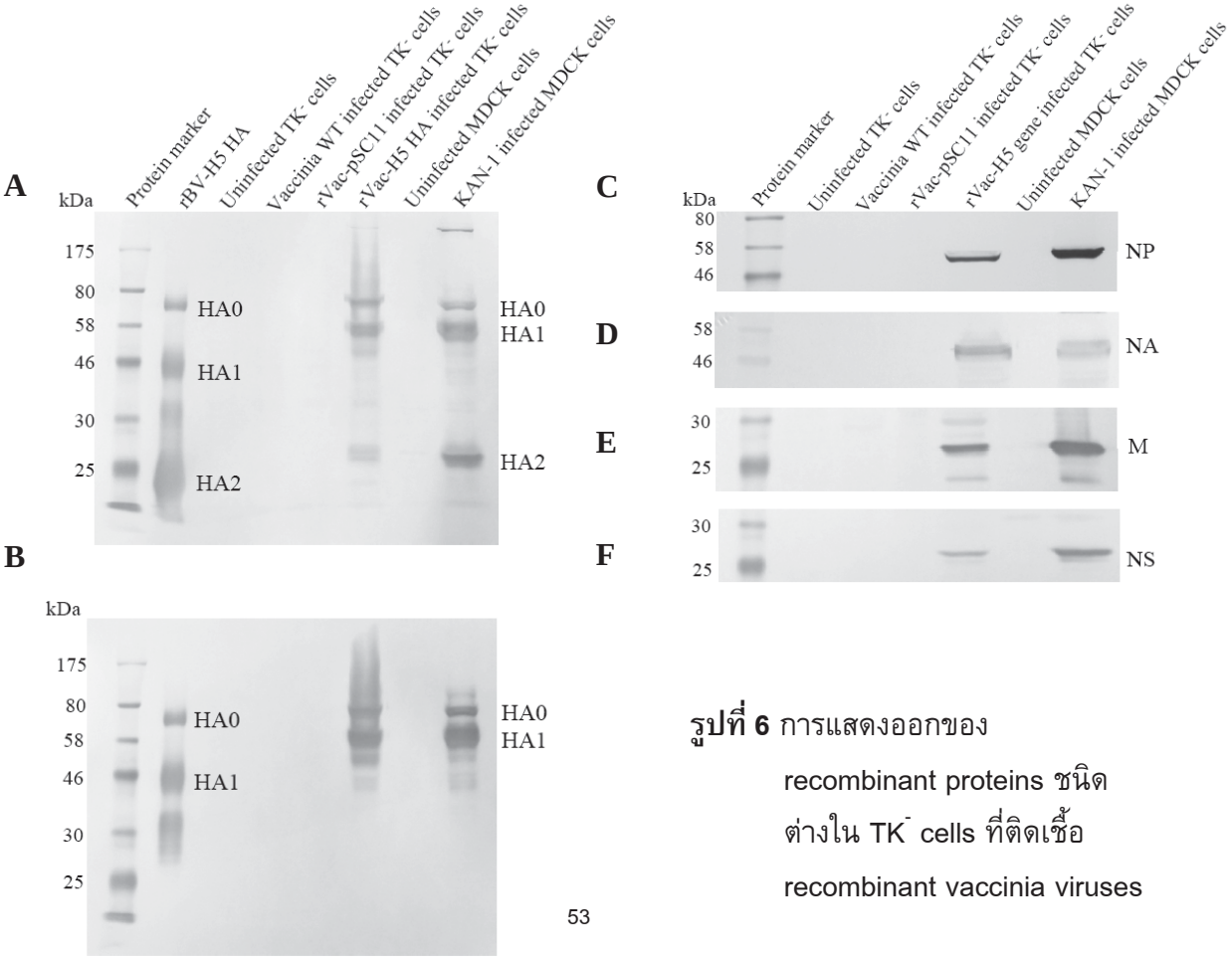
รูปที่ 5 การตอบสนองต่อ overlapping NP peptides ของกลุ่มตัวอย่างที่เป็น non-H5N1 survivors ทั้งสิ้น 35 คน พบว่ามีเพียง 16 คนที่สามารถจดจำ NP peptides 5 เส้นจากทั้งหมด 49 เส้น

20.2 ศึกษาภูมิคุ้มกันข้ามในระดับสารน้ำ (cross-subtype humoral immunity)

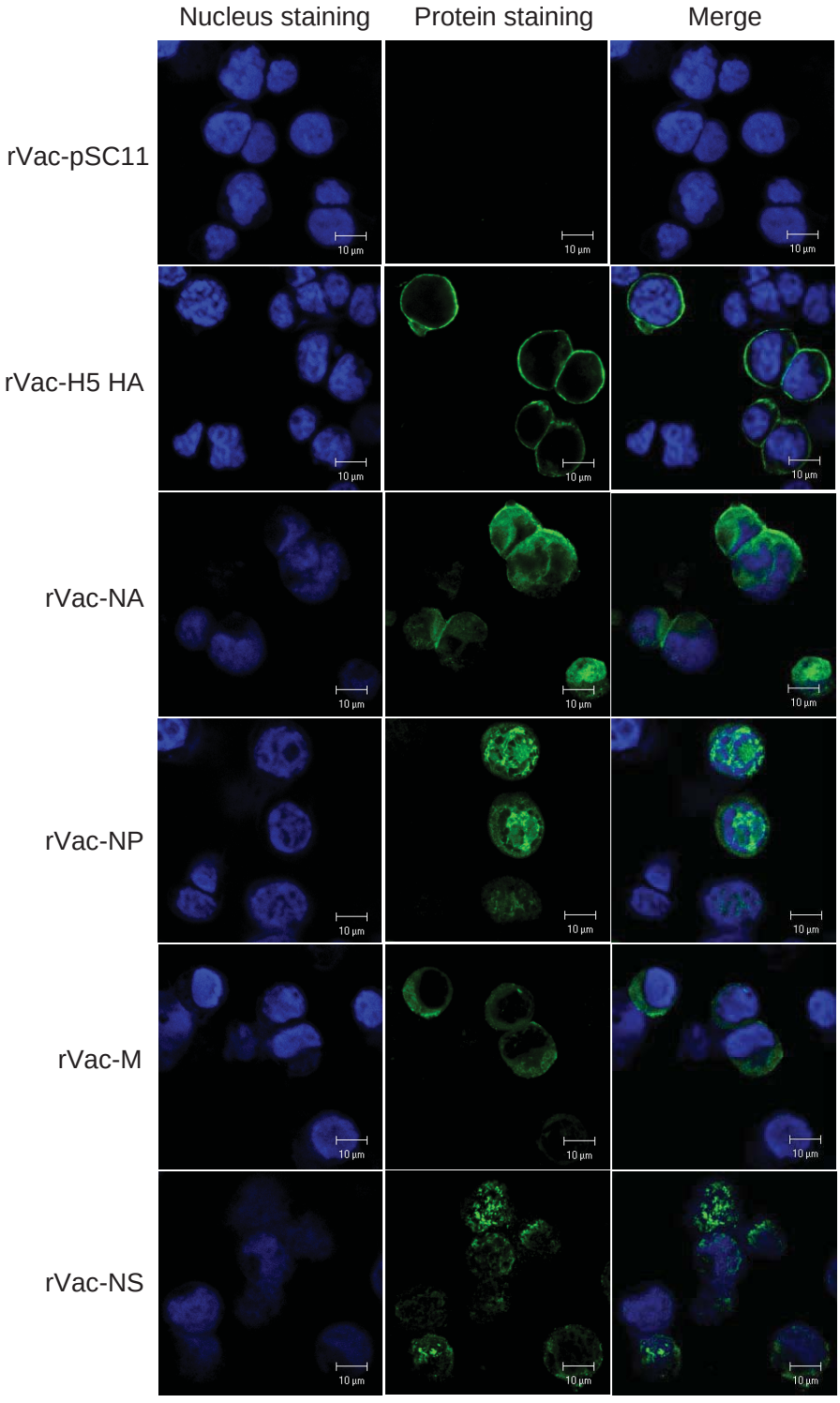
โดยทำการศึกษาภูมิคุ้มกันข้ามระหว่างเชื้อไข้หวัดใหญ่และไข้หวัดนก (H5N1) ของประชากรกลุ่มตัวอย่างที่รอดชีวิตจากการติดเชื้อ H5N1 และในกลุ่มตัวอย่างที่ไม่เคยติดเชื้อ H5N1 มาก่อนต่อโปรตีนต่างๆ ของไวรัสไข้หวัดนกที่เตรียมจาก TK^- cells ที่ติดเชื้อ recombinant vaccinia virus ที่มี insert gene: *M, HA, NP, NS* หรือ *NA* ของเชื้อไวรัส H5N1 โดยใช้เทคนิค immunofluorescence

ผลการทดลองพบว่า สามารถสร้าง recombinant H5 HA, NA, NP, M and NS proteins ใน TK^- cells ที่ติดเชื้อ recombinant vaccinia virus ที่มียีนของเชื้อไข้เหวัดนกชนิดต่างๆ อยู่ภายในยีโนม ดังรูปที่ 6 การศึกษาตำแหน่งของการแสดงออกของ recombinant proteins ชนิดต่างๆ ใน TK^- cells ที่ติดเชื้อ พบว่า HA และ NA พบได้ที่ cytoplasm และ cell membrane ของเซลล์ NP และ NS พบได้ที่ nucleus และ cytoplasm ส่วน M พบได้ใน cytoplasm ดังรูปที่ 7

จากการศึกษาแอนติบอดีต่อโปรตีนส่วนต่างๆ ในกลุ่มที่รอดชีวิตจากการติดเชื้อ H5N1 และกลุ่มที่ไม่เคยติดเชื้อ H5N1 มาก่อน พบว่า ทั้งสองกลุ่มมีแอนติบอดีต่อโปรตีนส่วนต่างๆ โดยในกลุ่มที่รอดชีวิตจะมีระดับแอนติบอดีต่อ HA และ NA ที่สูงกว่า ส่วนแอนติบอดีต่อ NP, M และ NS ทั้งสองกลุ่มมีระดับที่ใกล้เคียงกัน นอกจากนี้ยังสามารถตรวจพบแอนติบอดีต่อโปรตีนเหล่านี้ได้นานถึง 4 ปี จากการติดตามในผู้ที่รอดชีวิต ดังตารางที่ 1 (งานวิจัยในส่วนนี้อยู่ระหว่างการเตรียมต้นฉบับการตีพิมพ์ผลงาน)



รูปที่ 6 การแสดงออกของ recombinant proteins ชนิดต่างใน TK^- cells ที่ติดเชื้อ recombinant vaccinia viruses



รูปที่ 7 ตำแหน่งของการแสดงออกของแต่ละ recombinant proteins ใน TK^- cells ที่ติดเชื้อ recombinant vaccinia virus

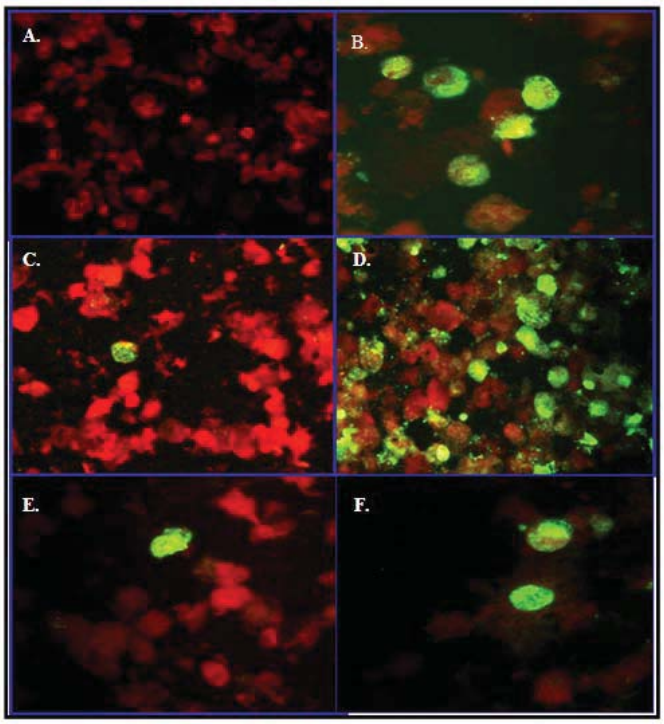
ตารางที่ 1 ระดับและการคงอยู่ของแอนติบอดีต่อโปรตีนส่วนต่าง ๆ ในกลุ่มที่รอดชีวิต

H5N1 survivor	Age (years)	Blood samples	Time at specimen collection after disease onset	NT Ab titer to KAN-1	HI Ab titer to KAN-1	IFA antibody titer to				
						HA	NA	NP	M	NS
No.1	32	1	1y 6m	160	80	640	320	80	160	160
		2	2y 6m	160	80	640	320	80	80	160
		3	3y 8m	80	80	640	160	160	80	160
No.2	29	1	2y 2m	160	80	320	160	80	40	40
		2	3y 2m	160	80	160	320	80	40	160
		3	4y 3m	80	80	320	160	80	40	80
No.3	7	1	20d	640	160	320	320	320	80	160
		2	11m	80	80	320	160	160	80	160
		3	2y 2m	80	40	160	80	160	80	160
		4	3y 1m	40	40	160	80	80	40	160
No.4	2	1	2y 3m	80	80	80	80	40	40	40
		2	3y 11m	80	80	80	160	40	40	80
		3	4y 11m	40	40	80	160	40	40	40

21. ตรวจหาการเพิ่มจำนวนของ H5N1 virus ในเซลล์ซึ่งไม่ใช่ respiratory epithelial cells

งานวิจัยในส่วนนี้ได้ทำการศึกษา kinetics การเพิ่มจำนวนและการสร้าง cytokine ของไวรัสไข้หวัดใหญ่ H1N1 และ H3N2 ในเซลล์เยื่อบุเส้นเลือดดำจากสายสะดือทารก (Human Umbilical Vein Endothelial Cells; HUVEC) เพื่อศึกษากลไกการแพร่กระจายออกนอก respiratory system เปรียบเทียบกับเชื้อไข้หวัดนก H5N1 และเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ A/H1N1 2009 โดยการตรวจหา viral nucleoprotein ในเซลล์ที่ติดเชื้อด้วยวิธี immunofluorescence assay และตรวจวัดปริมาณของไวรัสรุ่นลูกที่ถูกปล่อยออกมาในน้ำเลี้ยงเซลล์ด้วยวิธี TCID50 ในเซลล์ MDCK รวมทั้งตรวจวัดปริมาณ cytokine/chemokine (TNF-α, IP-10, IL-1β, และ IL-8) โดยวิธี ELISA

การศึกษานี้แสดงให้เห็นว่าเซลล์เยื่อบุเส้นเลือดดำจากสายสะดือทารกมีความสามารถในการติดเชื้อไวรัสไข้หวัดใหญ่แต่ละสับไทป์ และแต่ละสายพันธุ์ ซึ่งตรวจพบได้ด้วยวิธี immunofluorescence assay ดังแสดงในรูปที่ 8



รูปที่ 8 การตรวจหาแอนติเจนของเชื้อไวรัสในเซลล์เยื่อบุเส้นเลือดดำที่ถูก inoculate ด้วยเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ต่างๆ เป็นเวลา 5 วัน (A) Uninfected cells, (B) KAN-1 (H5N1), (C) KK-494 (H5N1), (D) NBL-1(H5N1) (feces), (E) Fujian(H3N2)- like virus, (F) New Caledonia (H1N1) -like virus

การศึกษา kinetic การเพิ่มจำนวนของไวรัสทำโดยการใช้ไวรัสในปริมาณต่ำ (0.005 TCID50/cell) พบว่าไวรัส H5N1 สายพันธุ์ KAN-1, Nong Khai 1 และ NBL 1 (feces) เพิ่มจำนวนในเซลล์เยื่อบุเส้นเลือดดำอย่างมีประสิทธิภาพ และสร้างไวรัสในปริมาณที่สูงกว่า H5N1 สายพันธุ์อื่น และมากกว่าไวรัสไข้หวัดใหญ่สับไทป์อื่นด้วย แสดงให้เห็นว่าประสิทธิภาพของการเพิ่มจำนวนของไวรัสไข้หวัดใหญ่ในเซลล์เยื่อบุเส้นเลือดดำมีความแตกต่างกัน ขึ้นอยู่กับ subtype และ สายพันธุ์ของไวรัส และเมื่อใช้ไวรัสในปริมาณมาก (1TCID50/cell) ประสิทธิภาพในการเพิ่มจำนวนของไวรัสไข้หวัดใหญ่ตามฤดูกาล H1N1 และ H3N2 จะสูงขึ้น และการศึกษาไวรัส A/H1N1 2009 จำนวน 3 สายพันธุ์ พบว่าไวรัสทุกตัวสามารถก่อการติดเชื้อ และเพิ่มจำนวนได้ในเซลล์เยื่อบุเส้นเลือดดำจากสายสะดือทารก ผลการศึกษา kinetic การเพิ่มจำนวนของไวรัสแสดงในตารางที่ 2-4

ตารางที่ 2 Kinetics ของการเพิ่มจำนวนไวรัสในเซลล์เยื่อบุหลอดเลือดดำที่ติดเชื้อไวรัสในปริมาณน้อย (0.005 TCID50/cell)

Viruses	Experiment	Titer of virus progenies at log 10 of TCID50 on			
	No.	Day1	Day3	Day5	Day7
A/Thailand/1(KAN-1)/2004 (H5N1)	1	4.33	5.88	5.5	4.21
	2	3.74	5	3.88	3
A/Thailand/2(SP-33)/2004 (H5N1)	1	0	0	2	0
	2	2.66	1.37	1.17	0
A/Thailand/3(SP-83)/2004 (H5N1)	1	0	0	0	0
	2	0	1.86	0	0
A/Thailand/5(KK-494)/2004 (H5N1)	1	0	0	0	0
	2	1.23	0	0	0
A/Thailand/676(NYK)/2005(H5N1)	1	3.16	2	1.83	1.67
	2	1.74	1.86	0.3	0
A/Thailand/NBL-1/2006 (H5N1)	1	2.74	1.74	1.78	0
(trachea)	2	2.58	1.88	1.58	1.88
A/Thailand/NBL-1/2006 (H5N1)	1	2	1.83	2	0
(lung)	2	2.25	0	0	0
A/Thailand/NBL-1/2006 (H5N1)	1	4.5	4.58	3.83	2.91
(feces)	2	3.88	5.12	4.08	3.25
A/Laos/Nong Khai 1/2007(H5N1)	1	3.2	6.2	5.2	4.2
	2	2.88	5.83	4.28	3.66
A/Fujian/411/2002(H3N2)-like virus	1	1.83	3.62	2.62	1.25
(Siriraj 03/04)	2	2.16	5.66	4.83	5
A/Sydney/05/97(H3N2)-like virus	1	2.83	2.25	1.32	0
(Siriraj-03/98)	2	1.08	1.75	0	0
A/Moscow/10/99(H3N2)-like virus	1	0.25	0.25	0	0
(Siriraj 06/02)					
A/New Caledonia/20/99	1	0	0	0	0
(H1N1)-like virus: Siriraj-07/00	2	0	0	0	0
A/ New Caledonia/20/99	1	0	0	1.66	0
(H1N1)-like virus : Siriraj 10/06	2	0	1.51	0	0
A/ New Caledonia/20/99	1	0	0	0	0

(H1N1)-like virus : Siriraj 02/06

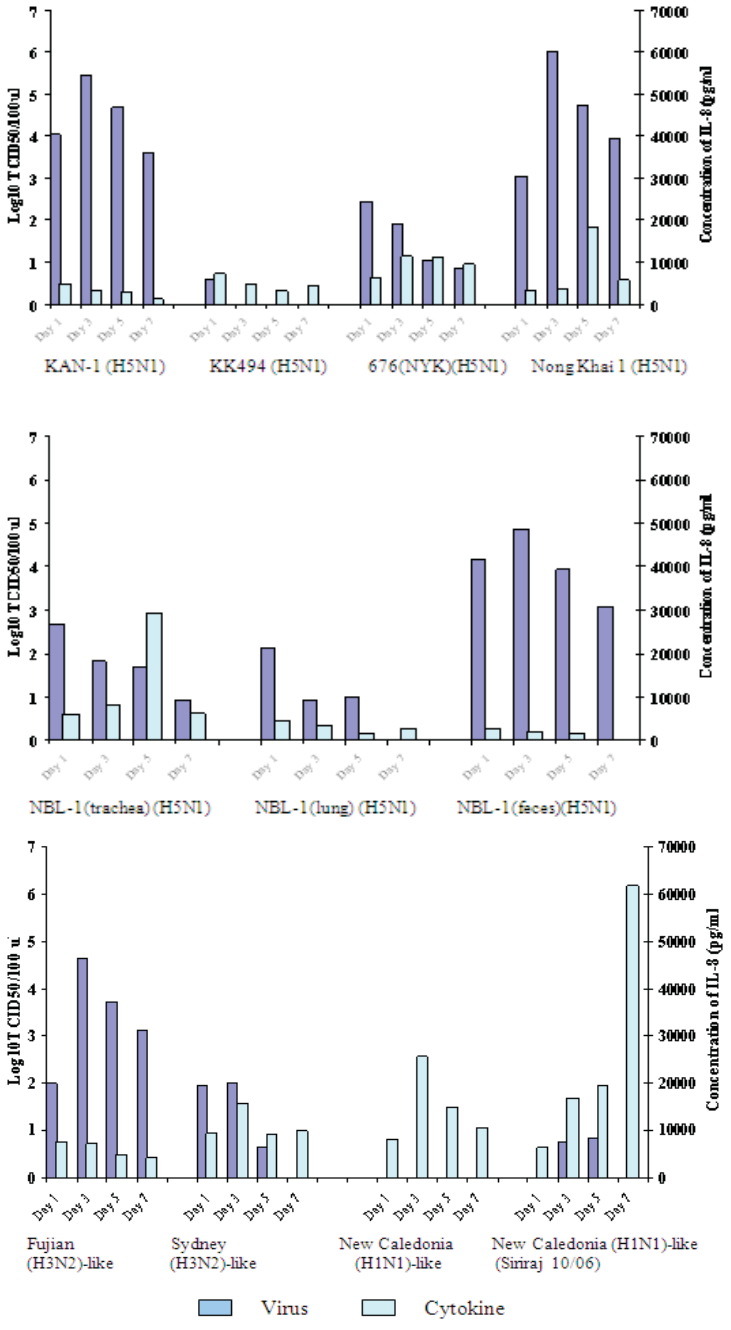
ตารางที่ 3 Kinetics ของการเพิ่มจำนวนไวรัส A/H1N1 A/H3N2 และ A/H5N1 ในเซลล์เยื่อบุหลอดเลือดดำที่ติดเชื้อไวรัสในปริมาณสูง (1 TCID50/cell)

Virus	Titer of virus progenies at log10 of TCID50 on		
	12 hpi	24 hpi	36 hpi
A/Thailand/1(KAN-1)/2004 (H5N1)	3.61	2.33	2.91
A/Thailand/676(NYK)/2005 (H5N1)	2.73	3.37	3.5
A/Laos/Nong Khai 1/2007 (H5N1)	2.75	3.83	3.74
A/Fujian/411/2002 (H3N2)-like virus	2.75	2.5	2.33
A/New Caledonia/20/99 (H1N1)-like virus (Siriraj 07/00)	2.67	3.25	2.71

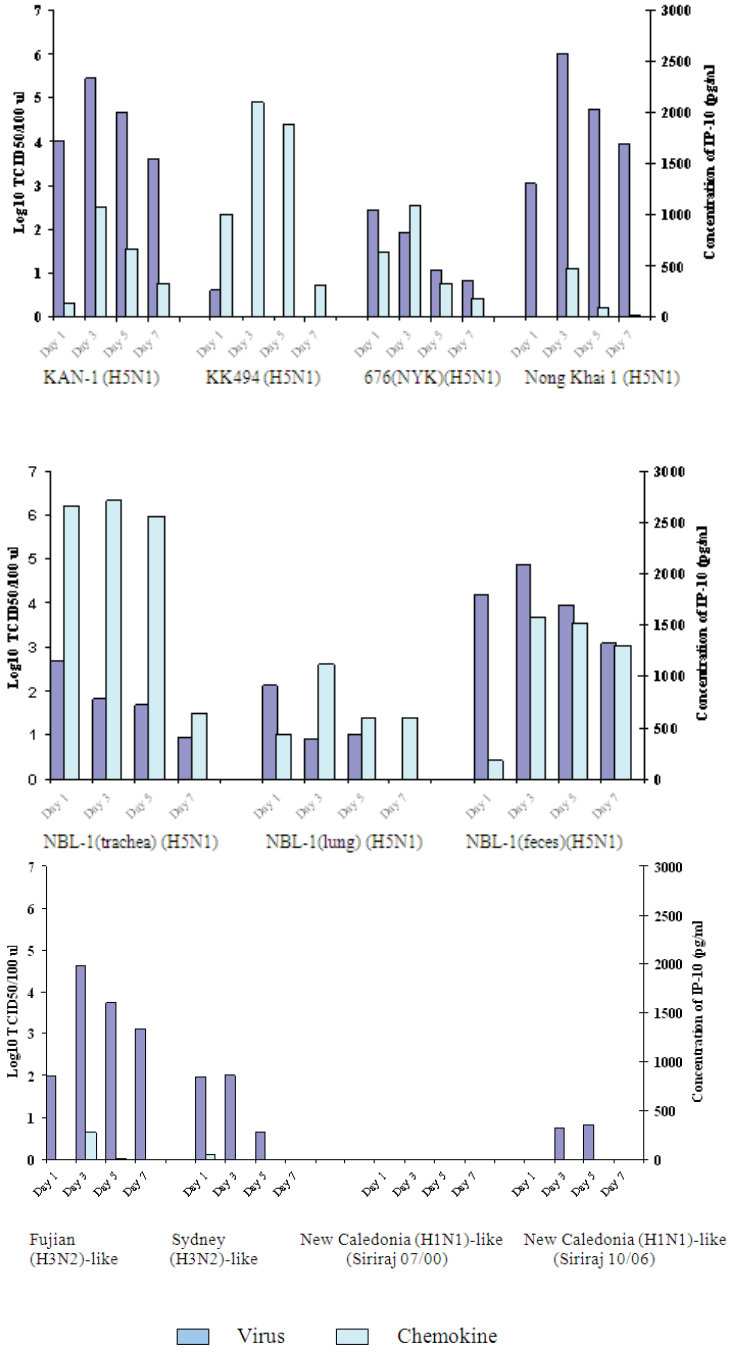
ตารางที่ 4 Kinetics ของการเพิ่มจำนวนไวรัส A/H1N1 2009 ในเซลล์เยื่อบุหลอดเลือดดำที่ติดเชื้อไวรัสในปริมาณสูง (1 TCID50/cell)

Virus	Experiment	Log10 of TCID50 titer		
	No.	12 hpi	24 hpi	36 hpi
A/Nonthaburi/102/09 (H1N1pdm)	1	0	0	0
	2	1.11	1.13	0.25
A/Thailand/104/09 (H1N1pdm)	1	3.38	2.83	3.16
	2	3.08	2.11	2.81
A/Bangkok/ICRC-1/09 (H1N1pdm)	1	3.25	3.11	2.37
	2	4	3.61	3.33

การตรวจหา cytokine/chemokine (รูปที่ 9 และ 10) ทั้งโดยใช้ไวรัสปริมาณต่ำและไวรัสปริมาณสูง ให้ผลอย่างเดียวกัน คือ ไวรัส H5N1 และ A/H1N1 2009 เหนี่ยวนำการสร้างได้ทั้ง IL-8 และ IP10 ในขณะที่ไวรัสตามฤดูกาล H1N1 และ H3N2 สามารถเหนี่ยวนำการสร้าง IL-8 ได้เพียงอย่างเดียว การศึกษานี้แสดงให้เห็นว่าไวรัสที่มีประสิทธิภาพสูงในการเพิ่มจำนวน จะมีความสามารถต่ำในการกระตุ้นการสร้าง cytokine หรือตรงข้ามกัน



รูปที่ 9 ความสัมพันธ์ในเชิงผกผันระหว่าง virus progeny titers และ ระดับของ IL-8 ซึ่งทดสอบจากน้ำเลี้ยงเซลล์ที่ติดเชื้อไวรัสในปริมาณต่ำ (0.005 TCID50/cell) ที่ช่วงเวลาต่างๆ กัน



รูปที่ 10 ความสัมพันธ์ในเชิงผกผันระหว่าง virus progeny titers และ ระดับของ IP10 ซึ่งทดสอบจากน้ำเลี้ยงเซลล์ที่ติดเชื้อไวรัสในปริมาณต่ำ (0.005 TCID50/cell) ที่ช่วงเวลาต่างๆ กัน

การศึกษานี้นำไปสู่การเสนอกลไกใหม่ในการก่อโรครุนแรง โดยเฉพาะในเชื้อไข้หวัดนก H5N1 การที่ไวรัสสามารถติดเชื้อและเพิ่มจำนวนในเซลล์เยื่อบุเส้นเลือดดำอาจเป็นกลไกหนึ่งที่สามารถอธิบายการแพร่กระจายของเชื้อไวรัสออกไปนอกระบบทางเดินหายใจ และในรายที่ผู้ป่วยถึงแก่ชีวิต การตายอาจเป็นผลจากการที่เซลล์ถูกทำลาย เนื่องจากติดเชื้อไวรัสที่มีประสิทธิภาพสูงในการเพิ่มจำนวนหรือ การตายอาจเป็นผลจากการสร้าง cytokine มากเกินไปในรายที่ติดเชื้อไวรัสที่มีประสิทธิภาพต่ำในการเพิ่มจำนวน อย่างไรก็ตามเชื้อไข้หวัดนกอาจอาศัยกลไกหลายอย่างที่ทำให้เชื้อมีความรุนแรงในการก่อโรค (งานวิจัยในส่วนนี้อยู่ระหว่างการเตรียมต้นฉบับการตีพิมพ์ผลงาน)

22. การศึกษาและเฝ้าระวังการเกิดเชื้อดื้อยา

22.1 การใช้เทคนิค genotypic based assay โดยการวิเคราะห์ลำดับนิวคลีโอไทด์ของ NA gene เพื่อหาตำแหน่ง mutation ที่ทำให้ไวรัสดื้อยา การศึกษาวิจัยนี้ ยังไม่พบตำแหน่ง mutation ใหม่ ที่ทำให้เชื้อดื้อต่อ NA inhibitors

22.2 การใช้เทคนิค phenotypic based assays

งานวิจัยนี้ใช้ 3 เทคนิค (MUNANA-based NAI assay, plaque reduction assay, และ ELISA-based NP reduction assay) ในการทดสอบความไวต่อ oseltamivir และ zanamivir ของเชื้อไวรัสไข้หวัดใหญ่/ไข้หวัดนก จำนวน 20 isolates การทดสอบทั้ง 3 วิธี ส่วนใหญ่ให้ผลไปในทิศทางเดียวกัน ดังแสดงในตารางที่ 5 อย่างไรก็ตาม ไวรัสไข้หวัดนก H5N1 ที่แยกได้จากสัตว์จำนวน 3 isolates พบค่า IC50 ที่ได้จาก NAI assay และ NP reduction assay ไม่สัมพันธ์กัน คือ NAI assay พบผลไวต่อยา แต่ขณะที่ NP reduction assay พบผลดื้อต่อยาทั้ง oseltamivir และ zanamivir เมื่อพิจารณาจาก amino acid sequence ของ *NA* gene ไม่พบการ mutation ในตำแหน่ง H274Y substitution หรือตำแหน่งอื่นๆ ที่เคยมีรายงานว่าทำให้ดื้อต่อยา ซึ่งอาจเป็นเพราะการดื้อยาอาจเกิดจาก mutation ที่ตำแหน่งอื่นหรือยีนอื่นที่เกี่ยวข้องที่ยังไม่ทราบก็เป็นได้ แต่เนื่องจากเชื้อ H5N1 ทั้ง 3 isolates นี้มีลักษณะการเกิด plaque ที่มีขนาดเล็กและไม่ชัดเจน (รูปที่ 11) ทำให้ไม่สามารถทดสอบด้วยวิธี plaque reduction assay ได้ จึงอาจเป็นไปได้ว่าไวรัสมีการทำงานของ neuraminidase ที่บกพร่องไปทำให้มีปริมาณ NP สะสมอยู่ในเซลล์ที่ติดเชื้อปริมาณสูงซึ่งทำให้ดูเหมือนไวรัสเหล่านี้ดื้อต่อยา ในกรณีนี้ ได้มีการเก็บน้ำเลี้ยงเซลล์ของไวรัสหลังจากการ treat ยา ไปทดสอบหาปริมาไวรัส พบว่าเมื่อ treat ด้วยยาพบปริมาณเชื้อไวรัสในน้ำเลี้ยงเซลล์ ในปริมาณน้อย แสดงว่าแท้จริงแล้วเชื้อนั้นยังคงมีความไวต่อยา ดังนั้นหากใช้วิธี ELISA-based NP reduction assay ในการคัดกรองหาเชื้อดื้อยา หากพบผลการดื้อยาด้วยวิธีนี้อาจต้องมีการทำการทดสอบเพิ่มเติมคือ ตรวจหาปริมาณไวรัสในน้ำเลี้ยงเซลล์หากพบว่าไม่สามารถตรวจพบไวรัสในน้ำเลี้ยงเซลล์หรือตรวจพบในปริมาณน้อย นั่นแสดงว่าเชื้อนั้นยังคงมีความไวต่อยา (งานวิจัยในส่วนนี้อยู่ระหว่างการเตรียมต้นฉบับการตีพิมพ์ผลงาน)

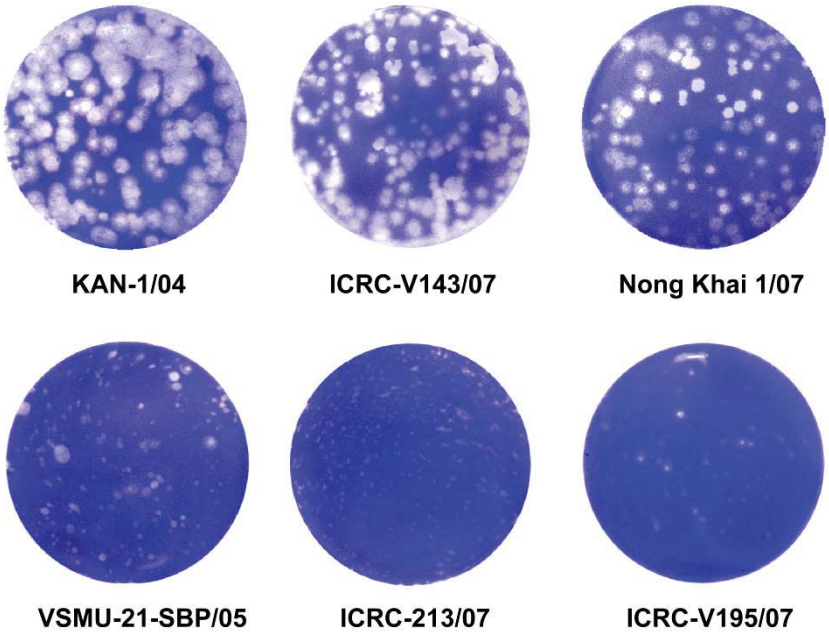
ตารางที่ 5 ค่า IC50 ที่ได้จากการทดสอบความไวต่อยาด้วยวิธีต่างๆ

	IC50[a] of oseltamivir carboxylate			IC50[a] of zanamivir		
Viruses	**NAI (nM)**	**Plaque reduction (μM)**	**NP reduction (μM)[b]**	**NAI (nM)**	**Plaque reduction (μM)**	**NP reduction (μM)[b]**
Reference strains						
Mississippi/01 (NA-274H)	1.799	0.27	0.04	1.27	0.50	0.11
Mississippi/01 (NA-H274Y)	477.76	43.64	108.23	1.33	0.52	0.331
Seasonal strains						
ICRC_SEA-3043/07	875.25	104.44	781.49	3.89	0.39	1.659
ICRC_SEA-3026/07	1.21	0.012	0.833	0.64	0.045	2.205
ICRC_SEA-3613/07	0.85	0.055	0.166	1.82	0.50	1.331
H1N1pdm						
Thailand/104/09	1.02	0.39	1.94	0.60	1.52	4.671
ICRC_CBI_20/09	1.01	0.16	0.41	0.67	1.35	0.71
H5N1 viruses						
KAN-1/04	0.15	0.181	0.447	1.82	4.42	7.92
SP-83/04	0.19	0.003	0.008	1.02	0.017	0.231
KK-494/04	0.26	0.031	0.223	2.21	0.885	0.186
676(NYK)/05	0.17	0.035	0.012	3.12	0.479	0.932
NBL-1/06 (Lung)	0.86	0.006	0.004	2.04	0.009	0.053
NBL-1/06 (Feces)	0.11	0.001	0.013	4.24	0.06	0.145
Nong Khai-1/07	8.05	1.339	5.309	1.13	1.104	3.163
VSMU-11-SPB/04	0.13	0.238	0.4395	0.40	0.042	0.099
AI-1216A/04	0.14	0.003	3.266	0.16	0.038	1.982
VSMU-21-SPB/05	0.18	NA	**13.25**	0.24	NA	**358.3**
ICRC-V143/07	1.88	0.002	0.007	7.68	0.013	0.14
ICRC-V195/07	0.53	NA	**27.63**	4.9	NA	**239.7**
ICRC-V213/07	0.28	NA	**431.3**	4.84	NA	**271.1**

[a] IC50 values are means from at least two independent experiments in duplicate wells (for NAI assay) or triplicate wells (for plaque reduction and NP reduction assays).

[b] IC50 values of ELISA-based viral NP reduction assay were determined by using the virus inoculum dose at 25 TCID50.

NA, Not applicable because the plaque formation is small and not well defined.



รูปที่ 11 ลักษณะการเกิด plaque ของเชื้อไวรัสไข้หวัดนก H5N1 ซึ่งทดสอบใน 6-well plate พบว่าไวรัส H5N1 3 isolates คือ VSMU-21-SPB/05, ICRC-213/07, และ ICRC-V195/07 มีขนาด plaque ที่เล็กและไม่ชัดเจน

บทวิจารณ์

จุดมุ่งหมายเริ่มแรกของงานวิจัยนี้คือการศึกษาเกี่ยวกับเรื่องของเชื้อไข้หวัดนก H5N1 แต่เนื่องจากผู้ป่วยไข้หวัดนกได้หมดไปจากประเทศไทยตั้งแต่กลางปี พ.ศ. 2549 และในปี พ.ศ. 2552 ได้มีการระบาดทั่วโลกของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ A(H1N1) ลุกลามมาถึงประเทศไทย คณะผู้วิจัยจึงได้ขยายขอบเขตของการศึกษาวิจัยให้ครอบคลุมทั้งไวรัสไข้หวัดนก H5N1 ไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ A(H1N1) และมีการศึกษาเปรียบเทียบกับไข้หวัดใหญ่ตามฤดูกาลด้วย

โครงการวิจัยนี้มีวัตถุประสงค์ที่หลากหลาย ทั้งด้านลักษณะทางอณูชีววิทยาในระดับโมเลกุลของตัวเชื้อ กลไกการก่อโรค ปัจจัยการแพร่กระจายของเชื้อ การตรวจวินิจฉัยการติดเชื้อ การตอบสนองทางภูมิคุ้มกันต่อเชื้อ และรวมถึงการทดสอบการดื้อต่อยาต้านไวรัส นอกจากนี้ยังมีการศึกษาเพื่อพัฒนาเทคนิคต่างๆ ที่มีประโยชน์ ซึ่งสามารถทำได้เสร็จสิ้นสมบูรณ์ และเทคนิคที่พัฒนาขึ้นมาเหล่านี้สามารถนำไปใช้ให้เป็นประโยชน์ได้จริง ซึ่งทีมวิจัยประสบความสำเร็จในการวินิจฉัยและแยกเชื้อไวรัสไข้หวัดนก H5N1 รายแรกของประเทศ และจากบทบาทในการเป็นห้องปฏิบัติการคู่ขนานกับกรมวิทยาศาสตร์การแพทย์ ทำให้ทีมวิจัยได้มีโอกาสให้การวินิจฉัยไข้หวัดนกรายที่สำคัญของประเทศในเวลาต่อๆ มา รวมถึงได้ทำการวินิจฉัยและแยกเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่รายแรกของประเทศด้วย

การศึกษาวิจัยในแต่ละด้านมีความละเอียดและค่อนข้างซับซ้อน และในกรณีของการระบาดของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ข้อมูลเกี่ยวกับลักษณะและคุณสมบัติของตัวเชื้อยังมีอยู่น้อย รวมทั้งต้องมีการพัฒนาเทคนิคที่ใช้ในการตรวจวินิจฉัยให้มีความเหมาะสมกับคุณลักษณะที่มีความจำเพาะเจาะจงกับตัวเชื้อมากขึ้น จึงต้องใช้ระยะเวลานานในการศึกษาวิจัย ประกอบกับการเก็บตัวอย่างตรวจจากผู้ป่วยจำนวนมากทำได้ยากและต้องใช้เวลานานในการเก็บรวบรวมตัวอย่างเพื่อให้ได้ข้อมูลที่มีความน่าเชื่อถือ ในงานวิจัยบางส่วนอาจต้องอาศัยเวลาในการติดตามเก็บตัวอย่างในระยะยาว เช่น การติดตามเจาะเลือดจากผู้ป่วยที่รอดชีวิตจากการติดเชื้อ H5N1 ที่ใช้ระยะเวลาติดตามนานหลายปี รวมทั้งการติดตามเส้นทางการบินของนกอพยพเป็นต้น ผลการศึกษาจากโครงการวิจัยนี้ได้ถูกนำไปใช้ในสถานการณ์จริง เพื่อประโยชน์ทางการสาธารณสุขของประเทศในการวินิจฉัยโรค การควบคุมโรค และการวางแผนการป้องกันโรค และเป็นการสร้างองค์ความรู้ใหม่ทั้งความรู้พื้นฐานและความรู้ประยุกต์ และสร้างบุคลากรนักวิจัย และนักศึกษา ที่มีศักยภาพสูง เพื่อรองรับการพัฒนางานวิจัยทางการแพทย์ในอนาคต

หนังสืออ้างอิง

1. Brown DM, Roman E, Swain SL. CD4 T cell responses to influenza infection. Semin Immunol 2004;16:171-7.

2. Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol 2004 59:1-15.

3. Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. Immunol Rev 1997;159:105-17.

4. Epstein SL, Lo CY, Misplon JA, Bennink JR. Mechanism of protective immunity against influenza virus infection in mice without antibodies. J Immunol 1998;160:322-7.

5. Gubareva LV. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res 2004;103:199-203.

6. Ito T, Suzuki Y, Mitnaul L, Vines A, Kida H, Kawaoka Y. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology 1997;227:493-9.

7. Jameson J, Cruz J, Ennis FA. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. J Virol 1998;72:8682-9.

8. Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 2004;364:759-65.

9. Lee LYH, Ha DLA, Simmons C, de Jong MD, Chau NVV, Schumacher R, et al. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. J Clin Invest 2008;118: 3478-90.

10.Lu BL, Webster RG, Hinshaw VS. Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. Infect Immun 1982;38:530-5.

11.McSharry JJ, McDonough AC, Olson BA, Drusano GL. Phenotypic drug susceptibility assay for influenza virus neuraminidase inhibitors. Clin Diagn Lab Immunol 2004;11:21-8.

12.Medeiros R, Escriou N, Naffakh N, Manuguerra JC, van der Werf S. Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology 2001;289:74-85.

13.O'Neill E, Krauss SL, Riberdy JM, Webster RG, Woodland DL. Heterologous protection against lethal A/HongKong/156/97(H5N1) influenza virus infection in C57BL/6 mice. J Gen Virol 2000;81:2689-96.

14.Roti M, Yang J, Berger D, Huston L, James EA and Kwok WW. Healthy human subjects have $CD4^+$ T cells directed against H5N1 influenza virus. J Immuol 2008;180:1758-68.

15.Smirnov YA, Gitelman AK, Govorkova EA, Lipatov AS, Kaverin NV. Influenza H5 virus escape mutants: immune protection and antibody production in mice. Virus Res 2004;99:205-8.

16.Smith BJ, McKimm-Breshkin JL, McDonald M, Fernley RT, Varghese JN, Colman PM. Structural studies of the resistance of influenza virus neuramindase to inhibitors. J Med Chem 2002;45:2207-12.

17.Stephenson I, Wood JM, Nicholson KG, Charlett A, Zambon MC. Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/Singapore/97 vaccine. Virus Res 2004;103:91-5.

18.Stephenson I, Bugarini R, Nicholson KG, Podda A, Wood JM, Zambon MC, et al. Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. J Infect Dis 2005;191:1210-5.

19.Topham DJ, Tripp RA, Doherty PC. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J Immunol 1997; 159: 5197-2000.

20.Uiprasertkul M, Puthavathana P, Sangsiriwut K, Pooruk P, Srisook K, Peiris M, et al. Influenza A H5N1 replication sites in humans. Emerg Infect Dis 2005; 11: 1036-41.

21.Chan M. World now at the start of 2009 influenza pandemic. 2009. Available: http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html. Accessed 26 June 2009.

22.Neumann G, Noda T, Kawaoka Y (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459: 931-939.

23.World Health Organization. H1N1 in post-pandemic period. 2010. Available: http://www.who.int/mediacentre/news/statements/2010/h1n1_vpc_20100810/en/index.html. Accessed 7 Oct 2010.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานที่มีการ Acknowledgements สกว. มีจำนวน 23 เรื่อง

    1.1 ผลงานที่ได้รับการตีพิมพ์เผยแพร่เรียบร้อยแล้ว มี 15 เรื่อง

    1.2 ผลงานที่ได้รับการตอบรับให้ตีพิมพ์แล้ว (in gallery proof) มี 1 เรื่อง

    1.3 ผลงานที่มี submit ฉบับ revised version manuscript ไปแล้ว 2 เรื่อง

    1.4 ผลงานที่อยู่ระหว่างการเตรียมฉบับ revised version และตอบข้อซักถามจาก reviewers มี 1 เรื่อง

    1.5 ผลงานที่อยู่ระหว่างการเขียน manuscript มี 4 เรื่อง

2. การนำเสนอผลงานในที่ประชุมวิชาการในรูปแบบโปสเตอร์ จำนวน 19 เรื่อง
3. การนำเสนอผลงานในที่ประชุมวิชาการแบบปากเปล่า จำนวน 1 เรื่อง
4. การผลิตนักศึกษาและนักวิจัยรุ่นใหม่

    4.1 นักศึกษาปริญญาโทจำนวน 4 คน

    4.2 นักศึกษาปริญญาเอกจำนวน 2 คน

    4.3 นักวิจัยหลังปริญญาเอกจำนวน 1 คน

    4.4 นักวิจัยหลังปริญญาโทจำนวน 2 คน

5. ผลงานที่เป็นผู้วิจัยร่วมในโครงการอื่นๆ มีจำนวน 28 เรื่อง

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)

    ผลงานที่ได้รับการตีพิมพ์เผยแพร่เรียบร้อยแล้ว มี 14 เรื่อง คือ

    1. Chotirosniramit N, Sugandhavesa P, Aurpibul L, Thetket S, Kosashunhanan N, Supindham T, Wongkulab P, Kaewpoowat Q, Chaiklang K, Kaewthip O, Sroysuwan P, Wongthanee A, Lerdsamran H, **Puthavathana P**, Suparatpinyo K. Immune response to 2009 H1N1 vaccine in HIV-infected adults in Northern Thailand. Hum Vaccin Immunother. 2012 Dec 1;8(12). [Epub ahead of print]

    2. Panaampon J, Ngaosuwankul N, Suptawiwat O, Noisumdaeng P, Sangsiriwut K, Siridechadilok B, Lerdsamran H, Auewarakul P, Pooruk P, **Puthavathana P**. A novel pathogenic mechanism of highly pathogenic avian influenza H5N1 viruses involves hemagglutinin mediated resistance to serum innate inhibitors. PLoS ONE 2012;7(5):e36318. (เป็น corresponding author) Impact factor 4.4

3. Vongsakul M, Kasisith J, Noisumdaeng P, **Puthavathana P**. The difference in IL-1beta , MIP-1alpha, IL-8 and IL-18 production between the infection of PMA activated U937 cells with recombinant vaccinia viruses inserted 2004 H5N1 influenza HA genes and NS genes. Asian Pac J Allergy Immunol 2011;29:349-56. Impact factor 0.649

4. Pittayawonganon C, Chootrakool H, Iamsirithaworn S, **Puthavathana P**, Chaleysub S, Auewarakul P, Kongyu S, Ungchusak K, Akarasewi P. Monitoring the influenza pandemic of 2009 in Thailand by a community-based survey. Journal of Public Health and Epidemiology 2011;3:187-93.

5. Lerdsamran H, Pittayawonganon C, Pooruk P, Mungaomklang A, Iamsirithaworn S, Thongcharoen P, Kositanont U, Auewarakul P, Chokephaibulkit K, Oota S, Pongkankham W, Silaporn P, Komolsiri S, Noisumdaeng P, Chotpitayasunondh T, Sangsajja C, Wiriyarat W, Louisirirotchanakul S, **Puthavathana P**. Serological response to the 2009 pandemic influenza A (H1N1) virus for disease diagnosis and estimating the infection rate in Thai population. PLoS One 2011;6:e16164. (เป็น corresponding author) Impact factor 4.351

6. Wiriyarat W, Lerdsamran H, Pooruk P, Webster RG, Louisirirotchanakul S, Ratanakorn P, Chaichoune K, Nateerom K, **Puthavathana P**. Erythrocyte binding preference of 16 subtypes of low pathogenic avian influenza and 2009 pandemic influenza A (H1N1) viruses. Vet Microbiol 2010;146:346-9. (เป็น corresponding author) Impact factor 2.37

7. Kositanont U, Wongsurakiat P, Pooruk P, Maranetra N, **Puthavathana P**. Induction of cross-neutralizing antibody against H5N1 virus after vaccination with seasonal influenza vaccine in COPD patients. Viral Immunol 2010; 23:329-334. (เป็น corresponding author) Impact factor 1.949

8. Ngaosuwankul N, Noisumdaeng P, Komolsiri P, Pooruk P, Chokephaibulkit K, Chotpitayasunondh T, Sangsajja C, Chuchottaworn C, Farrar J, **Puthavathana P**. Influenza A viral loads in respiratory samples collected from patients infected with pandemic H1N1, seasonal H1N1 and H3N2 viruses. Virol J 2010; 7:75 (เป็น corresponding author) Impact factors 1.882

9. Kitphati R, Pooruk P, Lerdsamran H, Poosuwan S, Louisirirotchanakul S, Auewarakul P, Chokphaibulkit K, Noisumdaeng P, Sawanpanyalert P, **Puthavathana P**. Kinetics and longevity of antibody response to influenza A H5N1 virus infection in

humans. Clin Vaccine Immunol 2009;16:978-81. (เป็น corresponding author) Impact factor 2.237

10. Auewarakul P, Chatsurachai S, Kongchanagul A, Kanrai P, Upala S, Suriyaphol P, **Puthavathana P**. Codon volatility of hemagglutinin genes of H5N1 avian influenza viruses from different clades. Virus Genes 2009;38:404-7. Impact factor 1.376

11. Chaichoune K, Wiriyarat W, Thitithanyanont A, Phonarknguen R, Sariya L, Suwanpakdee S, Noimor T, Chatsurachai S, Suriyaphol P, Ungchusak K, Ratanakorn P, Webster RG, Thompson M, Auewarakul P, **Puthavathana P**. Indigenous sources of 2007-2008 H5N1 avian influenza outbreaks in Thailand. J Gen Virol 2009;90:216-22. (เป็น corresponding author) Impact factor 3.092

12. **Puthavathana P**, Sangsiriwut K, Korkusol A, Pooruk P, Auewarakul P, Pittayawanganon C, Sutdan D, Kitphati R, Sawanpanyalert P, Phommasack B, Bounlu K, Ungchusak K. Avian influenza virus (H5N1) in human, Laos. Emerg Infect Dis 2009;15:127-9. (เป็น corresponding author) Impact factor 6.449

13. Louisirirotchanakul S, Lerdsumran H, Wiriyarat W, Sangsiriwut K, Chaichoune K, Pooruk P, Songserm T, Kitphati R, Sawanpanyalert P, Komoltri Ch, Auewarakul P, **Puthavathana P**. Erythrocyte binding preference of Avian influenza H5N1 viruses. J Clin Microbiol 2007;45:2284-6. (เป็น corresponding author) Impact factor 3.7

14. Auewarakul P, Sangsiriwut K, Chaichoune K, Thitithanyanont A, Wiriyarat W, Songserm T, Ponak-nguen R, Prasertsopon J, Pooruk P, Sawanpanyalert P, Ratanakorn P, **Puthavathana P**. Surveillance for reassortant virus by multiplex reverse transcription-PCR specific for eight genomic segments of avian influenza A H5N1 viruses. J Clin Microbiol 2007;45:1637-9. (เป็น corresponding author) Impact factor 3.7

ผลงานที่ได้รับการตอบรับให้ตีพิมพ์แล้ว (in gallery proof) มี 1 เรื่อง คือ

1. Chokephaibulkit K, Asanasaen S, Apisarnthanarak A, Rongrungruang Y, Kachintorn K, Tuntiwattanapibul Y, Judaeng T, **Puthavathana P**. Seroprevalence of 2009 H1N1 virus infection and self-reported infection control practices among healthcare professionals following the first outbreak in Bangkok, Thailand. (Accepted for submission in Influenza and Other Respiratory Viruses, Aug 10th,2012)

ผลงานที่มี submit ฉบับ revised version manuscript ไปแล้ว 2 เรื่อง คือ

1. Pirom Noisumdaeng, Phisanu Pooruk, Alita Kongchanagul, Susan Assanasen, Rungrueng Kitphati, Prasert Auewarakul, **Pilaipan Puthavathana**. Biological properties of H5 hemagglutinin expressed by vaccinia virus vector and its immunological

reactivity with human sera. (เป็น corresponding author) (Revised version in submission to Viral Immunology)

2. Suda Louisirirotchanakul, Pornparn Rojanasang, Kleophant Thakerngpol, Naree Choosrichom, Kridsda Chaichoune, Phisanu Pooruk[a], Aphinya Namsai[a], Robert Webster, **Pilaipan Puthavathana**. Electron micrographs of highly and low pathogenic avian influenza viruses. (เป็น corresponding author) (Revised version in submission to Asian Biomedicine)

ผลงานที่อยู่ระหว่างการเตรียมฉบับ revised version และตอบข้อซักถามจาก reviewers มี 1 เรื่อง คือ

1. Parntep Ratanakorn, Anuwat Wiratsudakul, Witthawat Wiriyarat, Krairat Eiamampai, Adrian H Farmer, Robert G Webster, Kridsada Chaichoune Sarin Suwanpakdee, Duangrat Pothieng, Pilaipan Puthavathana. Satellite tracking on the flyways of Brown-headed gulls and their potential role in the spread of highly pathogenic avian influenza H5N1 virus. (Submitted to PLoS ONE with under preparing the revised version)

ผลงานที่อยู่ระหว่างการเขียน manuscript มี 4 เรื่อง คือ

1. A pathogenic mechanism of HPAI H5N1 viruses involve active replication and cytokine induction in endothelial cells (ผลงานจากวิทยานิพนธ์นักศึกษาปริญญาโท)

2. Screening for drug resistant influenza A viruses: a comparative study among 3 different phenotypic based assay systems and nucleotide sequencing technique (ผลงานจากวิทยานิพนธ์นักศึกษาปริญญาเอก)

3. Immunological reactivity of specific and cross reactive antibodies against highly pathogenic avian influenza H5N1 viral proteins expresses by vaccinia virus vector (ผลงานจากวิทยานิพนธ์นักศึกษาปริญญาเอก)

4. Homosubtypic and heterosubtypic T cells mediated immune response against nucleoprotein and matrix proteins derived from highly pathogenic avian influenza A (H5N1) virus (ผลงานจากวิทยานิพนธ์นักศึกษาปริญญาเอก)

1.2 ผลงานที่เป็นผู้วิจัยร่วม มี 28 เรื่อง คือ

1. Udompornwattana S, Srajai K, Suwan P, Tangsathapornpong A, Wittawatmongkol O, Phongsamart W, Vanprapar N, Nuntarukchaikul M, Taeprasert P, Sricharoenchai S, Tanchaweng S, **Phutwattana P**, Taylor WR, Maleesatharn A, Chokephaibulkit K. The clinical features, risk of prolonged hospitalization and household

infections of hospitalized children for pandemic 2009 influenza A (H1N1) virus infection in Thailand. J Med Assoc Thai. 2012 Mar;95(3):403-11.

2. Namsai A, Louisirirotchanakul S, Wongchinda N, Siripanyaphinyo U, Virulhakul P, **Puthavathana P**, Myint KS, Gannarong M, Ittapong R. Surveillance of hepatitis A and E viruses contamination in shellfish in Thailand. Lett Appl Microbiol. 2011;53:608-13.

3. Kongchanagul A, Suptawiwat O, Boonarkart C, Kitphati R, **Puthavathana P**, Uiprasertkul M, Auewarakul P. Decreased expression of surfactant protein D mRNA in human lungs in fatal cases of H5N1 avian influenza. J Med Virol 2011;83:1410-7. Impact factor 2.47

4. Pittayawonganon C, Chootrakool H, Iamsirithaworn S, **Puthavathana P**, Chaleysub S, Auewarakul P, Kongyu S, Ungchusak K, Akarasewi P. Monitoring the influenza pandemic of 2009 in Thailand by a community-based survey. Journal of Public Health and Epidemiology 2011;3:187-93.

5. สิณีนาฏ อุทา, เกรียงศักดิ์ ไชยวงค์, สร้อยสอางค์ พิกุลสด, พิษณุ ภู่รักษ์, หทัยรัตน์ เลิศสำราญ, กรรณิการ์ นทีรมย์, **พิไลพันธ์ พุธวัฒนะ**, สุดา ลุยศิริโรจนกุล. แอนติบอดีต่อเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ (H1N1) 2009 ในผู้บริจาคโลหิตช่วงปีระบาดปี พ.ศ. 2552. วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต 2553;20:273-7.

6. Piwpankaew Y, Monteerarat Y, Suptawiwat O**, Puthavathana P**, Uipresertkul M, Auewarakul P. Distribution of viral RNA, sialic acid receptor, and pathology in H5N1 avian influenza patients. APMIS 2010;118:895-902. Impact factor 1.745

7. Apisarnthanarak A, Uyeki TM, **Puthavathana P**, Kitphati R, Mundy LM. Reduction of seasonal influenza transmission among healthcare workers in an intensive care unit: a 4-year intervention study in Thailand. Infect Control Hosp Epidemiol 2010;31:996-1003. Impact factor 2.768

8. Suptawiwat O, Tantilipikorn P, Boonarkart C, Lumyongsatien J, Uiprasertkul M, **Puthavathana P**, Auewarakul P. Enhanced susceptibility of nasal polyp tissues to avian and human influenza viruses. PLoS One 2010;5:e12973. Impact factor 4.351

9. Monteerarat Y, Sakabe S, Ngamurulert S, Srichatraphimuk S, Jiamtom W, Chaichuen K, Thitithanyanont A, Permpikul P, Songserm T, **Puthavathana P**, Nidom CA, Mai LQ, Iwatsuki-Horimoto K, Kawaoka Y, Auewarakul P. Induction of TNF-alpha in human macrophages by avian and human influenza viruses. Arch Virol 2010;155:1273-9. Impact factor 2.020

10. Wertheim HF, **Puthavathana P**, Nghiem NM, van Doorn HR, Nguyen TV, Pham HV, Subekti D, Harun S, Malik S, Robinson J, Rahman M, Taylor W, Lindegardh N, Wignall S, Farrar JJ, de Jong MD. Laboratory capacity building in Asia for infectious disease research: experiences from the South East Asia Infectious Disease Clinical Research Network (SEAICRN). PLoS Med 2010;7:e1000231. Impact factor 12.185

11. Apisarnthanarak A, **Puthavathana P**, Mundy LM. Detection of micro-neutralization antibody titer to avian influenza in an endemic avian influenza region. Clin Microbiol Infect 2009:16:1354-7. Impact factor 3.554

12. Dejpichai R, Laosiritaworn Y, **Phuthavathana P**, Uyeki TM, O'Reilly M, Yampikulsakul N, Phurahong S, Poorak P, Prasertsopon J, Kularb R, Nateerom K, Sawanpanyalert N, Jiraphongsa C. Seroprevalence of antibodies to avian influenza virus A (H5N1) among residents of villages with human cases, Thailand, 2005. Emerg Infect Dis 2009;15:756-60. Impact factor 6.449

13. Kongchanagul A, Suptawiwat O, Kanrai P, Uiprasertkul M, **Puthavathana P**, Auewarakul P. Positive selection at the receptor-binding site of haemagglutinin H5 in viral sequences derived from human tissues. J Gen Virol 2008;89:1805-10. Impact factor 3.092

14. Apisarnthanarak A, **Puthavathana P**, Kitphati R, Auewarakul P, Mundy LM. Outbreaks of influenza A among nonvaccinated healthcare workers: implications for resource-limited settings. Infect Control Hosp Epidemiol 2008;29:777-80. Impact factor 2.834

15. Leelarasamee A, Jongwutiwes U, Tantipong H, **Puthavathana P**, Siritantikorn S. Fulminating influenza pneumonia in the elderly: a case demonstration. J Med Assoc Thai 2008;91:924-30.

16. Suptawiwat O, Kongchanagul A, Chan-It W, Thitithanyanont A, Wiriyarat W, Chaichuen K, Songserm T, Suzuki Y, **Puthavathana P**, Auewarakul P. A simple screening assay for receptor switching of avian influenza viruses. J Clin Virol 2008;42:186-9. Impact factor 3.32

17. Kitphati R, Apisarnthanarak A, Chittaganpitch M, Tawatsupha P, Auwanit W, **Puthavathana P**, Auewarakul P, Uiprasertkul M, Mundy LM, Sawanpanyalert P. A nationally coordinated laboratory system for human avian influenza A (H5N1) in Thailand: program design, analysis, and evaluation. Clin Infect Dis 2008;46:1394-400. Impact factor 8.266

18. Boonnak K, Slike BM, Burgess TH, Mason RM, Wu SJ, Sun P, Porter K, Rudiman IF, Yuwono D, **Puthavathana P**, Marovich MA. Role of dendritic cells in antibody-dependent enhancement of dengue virus infection. J Virol 2008;82:3939-51. Impact factor 5.308

19. Hinjoy S, **Puthavathana P**, Laosiritaworn Y, Limpakarnjanarat K, Pooruk P, Chuxnum T, Simmerman JM, Ungchusak K. Low frequency of infection with avian influenza virus (H5N1) among poultry farmers, Thailand, 2004. Emerg Infect Dis 2008;14:499-501. Impact factor 6.449

20. Thitithanyanont A, Engering A, Ekchariyawat P, Wiboon-ut S, Limsalakpetch A, Yongvanitchit K, Kum-Arb U, Kanchongkittiphon W, Utaisincharoen P, Sirisinha S, **Puthavathana P**, Fukuda MM, Pichyangkul S. High susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-alpha and TLR ligands. J Immunol 2007;179:5220-7. Impact factor 6.068

21. Mazur I, Wurzer WJ, Ehrhardt C, Pleschka S, **Puthavathana P**, Silberzahn T, Wolff T, Planz O, Ludwig S. Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-kappaB-inhibiting activity. Cell Microbiol 2007;9:1683-94. Impact factor 5.293

22. Hourfar MK, Themann A, Eickmann M, **Puthavathana P**, Laue T, Seifried E, Schmidt M. Blood screening for influenza. Emerg Infect Dis 2007;13:1081-3. Impact factor 5.775

23. Uiprasertkul M, Kitphati R, **Puthavathana P**, Kriwong R, Kongchanagul A, Ungchusak K, Angkasekwinai S, Chokephaibulkit K, Srisook K, Vanprapar N, Auewarakul P. Apoptosis and pathogenesis of avian influenza A (H5N1) virus in humans. Emerg Infect Dis 2007;13:708-12. Impact factor 5.775

24. Apisarnthanarak A, **Puthavathana P**, Mundy LM. Risk factors and outcomes of influenza A (H3N2) pneumonia in an area where avian influenza (H5N1) is endemic. Infect Control Hosp Epidemiol 2007;28:479-82. Impact factor 2.989

25. Chan KH, Lam SY, **Puthavathana P**, Nguyen TD, Long HT, Pang CM, Chan KM, Cheung CY, Seto WH, Peiris JS. Comparative analytical sensitivities of six rapid influenza A antigen detection test kits for detection of influenza A subtypes H1N1, H3N2 and H5N1. J Clin Virol 2007;38:169-71. Impact factor 3.468

26. Perera D, Yusof MA, Podin Y, Ooi MH, Thao NT, Wong KK, Zaki A, Chua KB, Malik YA, Tu PV, Tien NT, **Puthavathana P**, McMinn PC, Cardosa MJ. Molecular phylogeny of modern coxsackievirus A16. Arch Virol 2007;152:1201-8. Impact factor 1.839

27. Auewarakul P, Suptawiwat O, Kongchanagul A, Sangma C, Suzuki Y, Ungchusak K, Louisirirotchanakul S, Lerdsamran H, Pooruk P, Thitithanyanont A, Pittayawonganon C, Guo CT, Hiramatsu H, Jampangern W, Chunsutthiwat S,

Puthavathana P. An avian influenza H5N1 virus that binds to a human-type receptor. J Virol 2007;81:9950-5. Impact factor 5.332

28. Apisarnthanarak A, **Puthavathana P**, Kitphati R, Thavatsupha P, Chittaganpitch M, Auewarakul P, Mundy LM. Avian influenza H5N1 screening of intensive care unit patients with community-acquired pneumonia. Emerg Infect Dis 2006;12:1766-9. Impact factor 5.094

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เป็นการเสริมสร้างศักยภาพให้กับประเทศในการเฝ้าระวังการอุบัติขึ้นของเชื้อใหม่ที่อาจก่อโรครุนแรงกว่าเดิม และเป็นการคงศักยภาพและความพร้อมในการวินิจฉัยโรคไข้หวัดนกให้แก่ประเทศซึ่งเป็นงานที่ทีมวิจัยได้ทำสืบเนื่องมานับตั้งแต่การระบาดครั้งแรก
- เป็นการสร้างองค์ความรู้และความเข้าใจเกี่ยวกับพยาธิกำเนิดของโรคไข้หวัดนก ซึ่งยังขาดข้อมูลทางด้านนี้อยู่อีกมาก
- เป็นการสร้างแนวความคิดใหม่ในการพัฒนาวัคซีนไข้หวัดนก
- เป็นการสร้างบุคลากรรุ่นใหม่ให้มีความรู้เรื่องไข้หวัดนก/ไข้หวัดใหญ่ เพื่อรองรับภาวะวิกฤตของประเทศ
- มีการพัฒนาเทคนิคทาง serology เพื่อใช้ในการวินิจฉัยการติดเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ A/H1N1 2009 และเป็นประโยชน์ต่อห้องปฏิบัติการอื่นๆ ในประเทศไทยเพื่อใช้เป็นแนวทางในการวินิจฉัยโรค
- เกิดผลงานตีพิมพ์ในวารสารที่มีชื่อเสียง เป็นการเผยแพร่ชื่อเสียงและเกียรติคุณของนักวิจัยไทย

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

3.1 ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ

3.1.1 อดิสรณ์ วรรธนะศักดิ์, จักรรัฐ พิทยาวงศ์อานนท์, วารินทร์ ปงกันคำ, รชต ปัญญารัตนศิลป์, วราพร วรรณา, **พิไลพันธ์ พุธวัฒนะ**, ณฐมน เงาสุวรรณกุล, พิษณุ ภู่รักษ์, หทัยรัตน์ เลิศสำราญ, สมคิด คงอยู่, โสภณ เอี่ยมศิริถาวร. อัตราการติดเชื้อ ระยะเวลาของการขับเชื้อไวรัส และปริมาณเชื้อไวรัส ขณะมีการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในกลุ่มทหารเกณฑ์ ศูนย์ฝึกทหารใหม่แห่งหนึ่ง ประเทศไทย เดือนมิถุนายน พ.ศ. 2552. รายงานการเฝ้าระวังทางระบาดวิทยาประจำสัปดาห์ 2010;41:209-13.

3.1.2 บทความฟื้นวิชา:

พิไลพันธ์ พุธวัฒนะ. การศึกษาไวรัสไข้หวัดใหญ่ทางห้องปฏิบัติการ. ประชุมวิชาการประจำปี ครั้งที่ 19 สมาคมไวรัสวิทยา (ประเทศไทย) วันที่ 20 พฤศจิกายน 2552: กรุงเทพฯ. 2552:18-30.

3.2 การเสนอผลงานในที่ประชุมวิชาการในรูปแบบโปสเตอร์

3.2.1 ประชุมและนำเสนอผลงานวิจัยในงานประชุมวิชาการนานาชาติ Keystone Symposia Conference เรื่อง Respiratory Viruses of Animals Causing Disease in humans ณ โรงแรม Swissotel The Stamford/Biopolis ประเทศสิงคโปร์ วันที่ 10-15 ธันวาคม 2549

3.2.1.1 Lerdsamran H, Louisirirotchanakul S, Wiriyarat W, Sangsiriwut K, Pooruk P, Kijphati R, Chaichoune K, Sawanpanyalert P, pittayawonganon C, Ungchusak K, Auewarakul P, **Puthavathana P.** Heamagglutinin activity of avian influenza H5N1 viruses.

3.2.1.2 **Puthavathana P**, pooruk P, Sangsiriwut K, Nateerom K, Prasertsopon J, Chaichoune K, Wiriyarat W, Korkusol A, Auewarakul P, Chokephaibulkit K, Ungchusak K, Sawanpanyalert P, Ratanakorn P. Oseltamivir sensitivity of H5N1 influenza isolates, Thailand.

3.2.2 ประชุมและนำเสนอผลงานวิจัย SEA Influenza Clinical Research Network 3rd Annual Conference ที่ บาหลี ประเทศอินโดนีเซีย วันที่ 21-23 พฤษภาคม 2551

Pilaipan Puthavathana, Kulkanya Chokephaibulkit, Chariya Saenasjja, Charoen Chuchottaworn, Phisanu Pooruk, Pisut Komolsiri, Achareeya Korkusol, Suda Louisirirotchanakul, Tawee Chotpityaasunondh, and SEAICRN staffs. Respiratory viruses associated with severe influenza like illness.

3.2.3 ประชุมและนำเสนอผลงานวิจัยในงานประชุม 8th Asia Pacific Congress of Medical Virology ที่ฮ่องกง วันที่ 26-28 กุมภาพันธ์ 2552

Pisut Komolsiri, Tawee Chotpirayasunondh, Kulkanya Chokephaibulkit, Chariya Sangsajja, Charoen Chuchottaworn, Achareeya Korkusol, Rajita Kanyaboon, Phisanu Pooruk, Nirun Vanprapar and **Pilaipan Puthavathana**. Respiratory virus associated with severe pneumonia

3.2.4 ประชุมและนำเสนอผลงานวิจัยในงานประชุม The 2nd Thailand Human Influenza Research Meeting ณ โรงแรมเชอราตัน แกรนด์ สุขุมวิท วันที่ 21-22 ตุลาคม 2552

3.2.4.1 Pirom Noisumdaeng, Thaneeya Duangchinda, Juthathip Mongkolsapaya, Susan Assanasen, Suda Louisirirotchanakul, Prasert Auewarakul, Phisanu Pooruk, Hatairat Lerdsamran and **Pilaipan Puthavathana**. Human influenza

virus-specific T cells mediated cross reactive immune response to nucleoprotein (NP) derived from avian influenza H5N1 virus.

3.2.4.2 Pornparn Rojanasang, Suda Louisirirotchanakul, Kleophant Thakergpol, Naree Choosrichom, Witthawat Wiriyarat, Kridsada Chaichoune, Robert Webster and **Pilaipan Puthavathana**. Electron micrographs of H5N1 viruses and its replication in MDCK cells.

3.2.4.3 Hatairat Lerdsamran, Phisanu Pooruk, Achareeya Korkusol, Pirom Noisumdaeng, Pisut Komolsiri, Kantima Sangsiriwut, Kannika Nateerom, Charoen Chuchottaworn, Chariya Sangsajja, Tawee Chotpitayasunondh, Kulkanya Chokephaibulkit and **Pilaipan Puthavathana**. Surveillance for sensitivity of influenza A and B viruses to neuraminidase inhibitors.

3.2.4.4 Phisanu Pooruk, Hatairat Lerdsamran, Kannika Nateerom, Roongnapa Bunrueng, Pisut Komolsiri, Chakrarat Pittayawonganon, Warin Pongkankham, Sopon Iamsiritaworn, Kulkanya Chokephaibulkit, Tawee Chotpitayasunondh, Chariya Sangsajja, Charoen Chuchottaworn and **Pilaipan Puthavathana**. Hemagglutination-inhibition and microneutralization assays for antibodies to pandemic influenza A (H1N1) 2009 virus.

3.2.4.5 Piyaporn (Nathamon) Ngaosuwankul, Pisut Komolsiri, Pirom Noisumdaeng, Kulkanya Chokephaibulkit, Tawee Chotpitayasunondh, Chariya Sangsajja, Charoen Chuchottaworn and **Pilaipan Puthavathana**. Viral load of pandemic influenza A (H1N1) 2009 virus in various types of respiratory samples.

3.2.4.6 Weena Paungpin, Kanaporn Poltep, Phirom Phompiram, Sarin Suwanpaksee, Tanasak Changbanjong, Chaiyaporn Jakapirom, Poonyapat Sedwisai, Taniyanuch Chamsai, Nam-aoy Toawan, Jarin Chatsiriwech, Parut Suksai, Kridsda Chaichoune, Witthawat Wiriyarat, Arunee Thititanyanon, Kumnuan Ungchusak, Prasert Auewarakul, Parntep Ratanakorn and **Pilaipan Puthavathana**. Seroprevalence to HPAI H5N1 virus in healthy backyards chickens living in repeated outbreak areas of Thailand.

3.2.4.7 Witthawat Wiriyarat, Weena Paungpin, Kanaporn Poltep, Phirom Phompiram, Kridsda Chaichoune, Rassameepen Phonarknguen, Nam-aoy Taowan, Sarin Suwanpakdee, Plern Yongyuttawichai, Anuwat Wiratsudakul, Arunee Thititanyanon2, Kumnuan Ungchusak, Prasert Auewarakul, Parntep Ratanakorn and **Pilaipan Puthavathana**. Seroprevalence of HPAI H5N1 virus in domestic dogs and cats living in repeated outbreak areas in Thailand

3.2.4.8 Anuwat Wiratsudakul, Witthawat Wiriyarat, Kridsada Chaichoune, Sarin Suwanpakdee, Krairat Eiamampai, Adrian Farmer, Tanasak Changbanjong, Kecha Cheewajorn, Plearn Yongyuthtawichai, Juthathip Jangjaras, Poonyapat Sedwisai, Tatiyanuch Chamsai, Nam-aoy Taowan, Ladawan Sariya, **Pilaipan Puthavathana** and Parntep Ratanakorn. Satellite telemetry reveals migratory routes of Brown-headed gulls (*Larus brunnicephalus*) captured in Thailand.

3.2.5 ประชุมและนำเสนอผลงานในการประชุมวิชาการประจำปี ครั้งที่ 19 สมาคมไวรัสวิทยา (ประเทศไทย) ณ โรงแรมรอยัลริเวอร์ วันที่ 20 พฤศจิกายน 2552

Worawat Dangsagul, Pattaraporn Cheuypratoom, Wanrangkana Aonsoung, Sontana Siritantikorn and **Pilaipan Puthavathana**. Neuraminidase inhibitor resistant seasonal influenza H1N1 viruses in Thailand during 2006 to 2009.

3.2.6 ประชุมและนำเสนอผลงานวิจัยในรูปแบบโปสเตอร์ ในงานประชุมวิชาการด้านวัคซีนไข้หวัดใหญ่และการพัฒนาวัคซีน Options for the Control of Influenza VII Conference จัดโดย The University of Hong Kong ณ Hong Kong Exhibition and Convention Center วันที่ 3-7 กันยายน 2553 ซึ่งนำเสนอผลงานโดยนักศึกษาปริญญาเอก นางสาวหทัยรัตน์ เลิศสำราญ และได้รับรางวัล International Research Scholarship ในการสนับสนุนค่าใช้จ่ายในการเดินทางและค่าเบี้ยเลี้ยง

Hatairat Lerdsamran, Phisanu Pooruk, Kantima Sangsiriwut, Pirom Noisumdaeng, Kannika Nateerom, Kridsada Chaichoune, Witthawat Wiriyarat, Kulkanya Chokephaibulkit, Chariya Sangsajja, Tawee Chotpitayasunondh, Pathom Sawanpanyalert, **Pilaipan Puthavathana**. Susceptibility of influenza A viruses to neuraminidase inhibitors as determined by phenotypic and genotypic-based assays.

3.2.7 ประชุมและนำเสนอผลงานวิจัยในรูปแบบโปสเตอร์ ในงานประชุมวิชาการประจำปีครั้งที่ 20 จัดโดยสมาคมไวรัสวิทยา (ประเทศไทย) ณ โรงแรมจอมเทียนปาล์มบีช รีสอร์ทแอนโฮเต็ล พัทยา วันที่ 25-26 พฤศจิกายน 2553

3.2.7.1 Jutatip Panaampon, Phisanu Pooruk, Pirom Noisamdaeng, Rungnapa Bunruang, **Pilaipan Puthavatthana**. Normal guinea pig serum mediated inhibitory effects on influenza virus replication. นำเสนอโดยนักศึกษาปริญญาโท นางสาวจุฑาทิพย์ พนอำพล และชนะการประกวดโปสเตอร์ได้รับรางวัลจากสมาคมไวรัสวิทยา (ประเทศไทย)

3.2.7.2 Jarunee Prasertsopon, Prawit Akarasereenont, Kitirat Techatrisak, Phisanu Pooruk, Nathamon Ngaosuwankul, Athiwat Thaworn, **Pilaipan Puthavathana**. The 2009 pandemic influenza A (H1N1) virus infection and induction of

cytokines and chemokines in human umbilical vein endothelial cells. นำเสนอโดยนักศึกษาปริญญาโท นางสาวจารุณี ประเสริฐโสภณ

3.2.8 ประชุมและนำเสนอผลงานวิจัยในรูปแบบโปสเตอร์ ในงานประชุมวิชาการ Keystone Symposiaเรื่อง Pathogenesis of Influenza: Virus-Host Interactions จัดโดย The University of Hong Kong ณ Sheraton Hong Kong Hotel & Towers, Kowloon, Hong Kong วันที่ 23-28 พฤษภาคม 2554

3.2.8.1 Jarunee Prasertsopon, Prawit Akarasereenont, Kitirat Techatrisak, Phisanu Pooruk, Nathamon Ngaosuwankul, Athiwat Thaworn, **Pilaipan Puthavathana**. Influenza A virus infection and induction of cytokines and chemokines in human umbilical vein endothelial cells.

3.2.8.2 Jutatip Panaampon, Phisanu Pooruk, Pirom Noisamdaeng, Rungnapa Bunruang, **Pilaipan Puthavatthana**. Normal guinea pig serum mediates inhibitory effects on influenza virus replication.

3.2.8.3 Nathamon Ngaosuwankul, Sopon Iamsirithaworn, Anek Mungaomklang, Rungnapa Bunruang, Phisanu Pooruk, Kulkanya Chokephaibulkit, Prasert Auewarakul and **Pilaipan Puthavathana**. Serological response to the 2009 influenza A (H1N1) virus strains derived from different epidemic waves in Thailand.

3.3 การเสนอผลงานในที่ประชุมวิชาการแบบปากเปล่า ในงานประชุมวิชาการนานาชาติ เรื่อง “Thailand Conference on Emerging Infectious and Neglected Diseases (EID 2010) ณ โรงแรมอมารีออร์คิด พัทยา จังหวัดชลบุรี วันที่ 3-4 มิถุนายน 2553

Pirom Noisumdaeng, Alita Kongchanakul, Jarunee Prasertsopon, Rungnapa Boonreung, Susan Assanasen, Phisanu Pooruk, Hatairat Lerdsamran, Rumporn Kularb, Kannika Nateerom, Prasert Auewarakul and **Pilaipan Puthavathana**. Cross reactive antibodies in non-H5N1 virus infected subjects against avian influenza H5N1 viral proteins expressed in recombinant vaccinia viruses.

3.4 หนังสือ

3.4.1 **Puthavathana P**, Auewarakul P, Buranathai C, Aungtragoolsuk N, Kitphati R, Chotpithayasunondh T. Avian influenza in Thailand. In: Sunil K. Lal (ed). Emerging viral diseases of Southease Asia. Basel: Karger. 2007: 23-34. (Issues in infectious diseases, vol 4. Zeichhardt H, Mahy B., eds)

3.4.2 **พิไลพันธ์ พุธวัฒนะ**. ไวรัสวิทยาของเชื้อไข้หวัดใหญ่. ใน: วันเพ็ญ ชัยคำภา, บรรณาธิการ. งานวิจัยพื้นฐาน ฐานรากของการพัฒนาประเทศตามปรัชญาเศรษฐกิจพอเพียง

เล่มที่ 1 การวิจัยพื้นฐานเพื่อพัฒนาสุขภาพ. สำนักงานกองทุนสนับสนุนการวิจัย (สกว.): กรุงเทพฯ. 2550:182-101.

3.4.3 **พิไลพันธ์ พุธวัฒนะ**. โรคติดเชื้ออุบัติใหม่. ใน: วันเพ็ญ ชัยคำภา, บรรณาธิการ. งานวิจัยพื้นฐาน ฐานรากของการพัฒนาประเทศตามปรัชญาเศรษฐกิจพอเพียง เล่มที่ 1 การวิจัยพื้นฐานเพื่อพัฒนาสุขภาพ. สำนักงานกองทุนสนับสนุนการวิจัย (สกว.): กรุงเทพฯ. 2550: 118-39.

3.4.4 อยู่ระหว่างการจัดทำหนังสือ “ตำราไวรัสวิทยา” ซึ่ง ศ.ดร.พิไลพันธ์ พุธวัฒนะ เป็นบรรณาธิการ

3.5 การจัดประชุมวิชาการ

3.5.1 จัดประชุมวิชาการเรื่อง “From Human to Avian and Swine-Origin Influenza Viruses” และบรรยายในหัวข้อเรื่อง Virology of influenza viruses และ Laboratory investigation of influenza virus infection ณ คณะแพทยศาสตร์ศิริราชพยาบาล วันที่ 31 พฤษภาคม 2552

3.5.2 จัดประชุมและเป็นวิทยากร ในหัวข้อเรื่อง “Pitfalls in laboratory investigation of influenza viruses” ในการประชุมเชิงวิชาการเรื่อง “Outbreak Situation of the 2009 Pandemic Influenza A (H1N1)” จัดโดยสมาคมไวรัสวิทยา (ประเทศไทย) ร่วมกับศูนย์ความร่วมมือการวิจัยไข้หวัดใหญ่ (Siriraj ICRC) ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล โครงการโรคติดเชื้ออุบัติใหม่ ศูนย์ความร่วมมือไทย-สหรัฐด้านสาธารณสุข (Thailand – US.CDC Collaboration, TUC) และทุนเมธีวิจัยอาวุโส สำนักงานกองทุนสนับสนุนการวิจัย (Thailand Research Fund for Senior Research Scholar) ณ โรงแรมรอยัลริเวอร์ กรุงเทพฯ วันที่ 9 สิงหาคม 2553

3.5.3 จัดการอบรมเชิงปฏิบัติการและเป็นวิทยากรบรรยาย 3 หัวข้อ ได้แก่ Immunofluorescence (IF) assay, Cell cultivation and virus isolation techniques และ Serology of influenza viruses: Hemagglutination-inhibition (HI) and microneutralization assays ในการอบรมเชิงปฏิบัติการเรื่อง “Workshop on Laboratory Investigation of Influenza Viruses” ซึ่งจัดโดยสมาคมไวรัสวิทยา (ประเทศไทย) ร่วมกับศูนย์ความร่วมมือการวิจัยไข้หวัดใหญ่ (Siriraj ICRC) ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล โครงการโรคติดเชื้ออุบัติใหม่ ศูนย์ความร่วมมือไทย-สหรัฐด้านสาธารณสุข (Thailand – US.CDC Collaboration, TUC) และทุนเมธีวิจัยอาวุโส สำนักงานกองทุนสนับสนุนการวิจัย (Thailand Research Fund for Senior Research Scholar) วันที่ 10-14 สิงหาคม 2553 ณ ตึกจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล ซึ่งมีจำนวนผู้รับการอบรม 19 คน จาก 15 สถาบัน

4. การผลิตนักศึกษาและนักวิจัย

4.1 นักศึกษาปริญญาโท

1. นายวรวัฒน์ แดงสกุล หัวข้อวิทยานิพนธ์คือ “การเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ตามฤดูกาลชนิดA(H1N1)ที่ดื้อต่อสารยับยั้งเอนไซม์นิวรามินิเดส, ประเทศไทย 2549 – 2552 (Surveillance for neuraminidase inhibitor resistant seasonal influenza A (H1N1) viruses, Thailand 2006-2009)”

2. นางสาวจารุณี ประเสริฐโสภณ หัวข้อวิทยานิพนธ์คือ “จลนศาสตร์การเพิ่มจำนวนและการเหนี่ยวนำการสร้างไซโตไคน์/คีโมไคน์ในเซลล์เยื่อบุเส้นเลือดดำจากสายสะดือทารก (Kinetics of influenza virus replication and cytokine/chemokine induction in human umbilical vein endothelial cells)”

3. นางสาวจุฑาทิพย์ พนอำพน หัวข้อวิทยานิพนธ์คือ “สารในซีรั่มซึ่งมีฤทธิ์ยับยั้งการติดเชื้อของไวรัสไข้หวัดใหญ่ (Serum inhibitory factors against influenza A virus infection)”

4. นางสาวจริง ชัยรัตน์ หัวข้อวิทยานิพนธ์คือ “ความสามารถของรีคอมบีแนนท์แวคซีเนียไวรัสที่มียีน H5 HA ของไวรัสไข้หวัดนกในการกระตุ้นระบบภูมิคุ้มกันของหนู (Efficiency of recombinant vaccinia virus containing H5 HA gene influenza in the induction of immune response in mice)

4.2 นักศึกษาปริญญาเอก

1. นางสาวหทัยรัตน์ เลิศสำราญ หัวข้อวิทยานิพนธ์คือ “ระบาดวิทยาทางซีรั่มของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 และความไวของเชื้อไข้หวัดใหญ่ต่อยาที่มีฤทธิ์ยับยั้งนิวรามินิเดส (Seroepidemiology of pandemic influenza A (H1N1) virus and susceptibility of influenza A viruses to neuraminidase inhibitors)”

2. นายภิรมย์ น้อยสำแดง หัวข้อวิทยานิพนธ์คือ “ภูมิคุ้มกันด้านสารน้ำและด้านเซลล์ชนิดจำเพาะและชนิดข้ามสายพันธุ์ต่อเชื้อไวรัสไข้หวัดนกชนิด เอ็ช 5 เอ็น 1 (Homosubtypic and heterosubtypic humoral and cellular immunity against highly pathogenic avian influenza A (H5N1) virus)”

4.3 นักวิจัย

1. นางสาวพิษณุ ภู่รักษ์ (นักวิจัยหลังปริญญาโท)
2. นางสาวกันทิมา แสงศิริวุฒิ (นักวิจัยหลังปริญญาโท ปฏิบัติงานนอกเวลา)
3. นางสาวณฐมน เงาสุวรรณกุล (นักวิจัยหลังปริญญาเอก)

ภาคผนวก

Research Paper

Immune response to 2009 H1N1 vaccine in HIV-infected adults in Northern Thailand

Volume 8, Issue 12 December 2012
Keywords: 2009 H1N1 vaccine, HIV, adults, seroconvertion rate, seroprotection rate
Authors: Nuntisa Chotirosniramit, Patcharaphan Sugandhavesa, Linda Aurpibul, Sunida Thetket, Natthapol Kosashunhanan, Taweewat Supindham, Panuwat Wongkulab, Quanhathai Kaewpoowat, Kanokporn Chaiklang, Oranitcha Kaewthip, Piyathida Sroysuwan, Antika Wongthanee, Hatairat Lerdsamran, Pilaipan Puthavathana and Khuanchai Suparatpinyo
View affiliations

Abstract:
Background: In late 2009, the Thai Ministry of Public Health provided two million doses of the monovalent pandemic influenza H1N1 2009 vaccine (Panenza® Sanofi Pasteur), which was the only vaccine formulation available in Thailand, to persons at risk of more severe manifestations of the disease including HIV infection. Several studies have shown poorer immune responses to the 2009 H1N1 vaccines in HIV-infected individuals. There are limited data in this population in resource-limited countries.

Results: At day 28 post-vaccination, seroconversion was found in 32.0% (95%CI 24.5 - 40.2) of the HIV-infected group and 35.0% (95%CI 15.4-59.2) of the healthy controls (p = 0.79). Seroprotection rate was observed in 33.3% (95%CI 25.8–41.6) and 35.0% (95%CI 15.4–59.2) of the HIV-infected group and the control group, respectively (p = 0.88). Among HIV-infected participants, the strongest factor associated with vaccine response was age 42 y or younger (p = 0.05).

Methods: We evaluated the immunogenicity of a single, 15µg/0.5ml dose of a monovalent, non-adjuvanted 2009 H1N1 vaccine in 150 HIV-infected Thai adults and 20 healthy controls. Immunogenicity was measured by hemagglutination inhibition assay (HI) at baseline and 28 d after vaccination. Seroconversion was defined as 1) pre-vaccination HI titer < 1:10 and post-vaccination HI titer ≥ 1:40, or 2) pre-vaccination HI titer ≥ 1:10 and a minimum of 4-fold rise in post-vaccination HI titer. Seroprotection was defined as a post-vaccination HI titer of ≥ 1:40.

Conclusions: A low seroconversion rate to the 2009 H1N1 vaccine in both study groups, corresponding with data from trials in the region, may suggest that the vaccine used in our study is not very immunogenic. Further studies on different vaccines, dosing, adjuvants, or schedule strategies may be needed to achieve effective immunization in HIV-infected population.

Provisional Full-Text corresponds to the article as it appeared upon acceptance. Fully formatted PDF and full text (HTML) versions containing any author galley corrections will be made available soon. When Provisional Full Text is displayed, it will always be open access.

Full Text

Introduction

Thailand was among the first countries in Southeast Asia hit hardest by the 2009 H1N1 influenza pandemic. From May 2009 to December 2010, approximately 226,000 influenza/influenza-like illnesses (ILI) with 47,000 cases of laboratory- confirmed pandemic 2009H1N1 and 347 deaths were reported to the surveillance center at the Bureau of Epidemiology, Ministry of Public Health, Thailand (MOPH).[1] In late 2009, the MOPH purchased two million doses of the monovalent pandemic influenza H1N1 2009 vaccine (Panenza® Sanofi Pasteur), which was the only vaccine formulation available in Thailand. The MOPH provided the vaccine free of charge to persons at risk of more severe manifestations of the disease (pregnant women, persons with obesity, diabetes, cardiopulmonary dysfunction, hematological malignancy, or HIV infection) as well as healthcare personnel.

Clinical studies have been conducted to evaluate the immunogenicity and safety of different types of 2009 H1N1 vaccines in different populations. Results from five studies showed that a single dose of 2009 H1N1 vaccine induced a robust immune response in most healthy adults.[2-6] However, several studies have shown poorer immune responses to the 2009 H1N1 vaccines in HIV-infected individuals.[7-14,16,17,19-21] There are limited data in the HIV-infected population in resource-limited countries. We, therefore, evaluated the seroconversion and seroprotection rate to a 2009 H1N1 vaccine (Panenza®) in HIV-infected and healthy individuals in Thailand.

Results

One participant in the HIV-infected group developed flu-like illness one day after vaccination. A throat swab for polymerase chain reaction (PCR) performed one day later was positive for Influenza A H1N1 2009. This participant was excluded from subsequent analysis.

Day 28 post-vaccination follow-up was completed in 147 HIV-infected participants and all 20 healthy controls. Baseline characteristics and vaccine response rate by HIV status are shown in **Table 1**. 39% of HIV-infected participants were male and the mean age was 42.1 ± 6.1 y. 98% were on combination antiretroviral therapy and 91.2% of participants had CD4+ cell count above 200 cell/mm3 at time of vaccination. The mean CD4+ cell count was 466 ± 206 cell/mm3. Among the 20 healthy volunteers, 45% was male and the mean age was 32.4 ± 6.3 y. The mean CD4+ cell count was 762 ± 283cell/mm3. At baseline, 3.4% (5/147) of HIV-infected participants and 5% (1/20) of controls had HI titers ≥ 1: 40.

Table 1. Baseline characteristics and vaccine response rates by HIV Status.

	HIV Infected (n = 147)	HIV Negative (n = 20)	*P-value*
Male gender N (%)	57 (38.8)	9 (45.0)	*0.59*
Mean age (SD)	42.1(6.1)	32.4(6.3)	*< 0.05*
Number of participant currently receiving HAART N (%)	144(98.0)	-	-
Absolute CD4 count(cell/mm^3)			
200 or less N (%)	13 (8.8)	-	-
More than 200 N (%)	134 (91.2)	20 (100)	-
Mean CD4(SD)	*465.52(206.09)*	*761.9(283.43)*	*< 0.05*
Pre-vaccination HI titer ≥ 1:40 N (%)	5(3.4)	1 (5.0)	*0.72*
Seroconversion rate [1] N (%) **95% CI**	47 (32.0) 24.5–40.2	7 (35.0) 15.4–59.2	*0.79*
Seroprotection rate [2] N (%) **95% CI**	49 (33.3) 25.8–41.6	7 (35.0) 15.4–59.2	*0.88*
Mean follow up days (SD)	26.43(1.48)	23.1(1.21)	

[1]Seroconversion was defined as: (1) pre-vaccination HI titer < 1:10 and post-vaccination HI titer ≥ 1:40 or (2) pre-vaccination HI titer ≥ 1:10 and a minimum of 4-fold rise in post vaccination HI titer. [2]Seroprotection was defined as a post-vaccination HI titer of ≥ 1:40

Seroconversion was found in 47 of 147 (32.0%, 95%CI 24.53 – 40.16) HIV-infected participants and 7 of 20 (35.0%, 95%CI 15.4–59.2) healthy controls (p = 0.79). Seroprotection rate was observed in 33.3% (95%CI 25.78–41.57) and 35.0% (95%CI 15.4–59.2) of the HIV-infected group and the control group, respectively (p = 0.88).

Factors associated with vaccine response among HIV-infected participants are shown in **Table 2**. In the univariate analysis, baseline HI titer ≥ 1:40 were significantly associated with seroconversion (p < 0.05). Age 42 y or younger and baseline CD4+ cell count above 200 cell/mm3 were borderline significant. However, in multivariate analysis, the only significant variable was age 42 y or younger (p = 0.05). Since the number of healthy participants was low, we did not analyze for the factor associated with seroconversion for this group.

Table 2. Univariate and multivariate analysis for factors associated with vaccine response among HIV-infected participants.

Characteristics	Number Seroconversion/total (%)	Odd Ratio (95% CI)	P-value	Adjusted Odd Ratio (95%CI)	P-value
Age in years					
≤ 42	32/83 (38.6)	**2.05**	**0.05**	**2.10**	**0.05**
> 42	15/64 (23.4)	**(0.94–4.58)**		**(0.99–4.45)**	
Gender					
Male	17/57 (29.8)	0.85	0.66	-	-
female	30/90 (33.3)	(0.39–1.84)			
Duration of HIV infection					
≤ 8 y	22/66 (33.3)	1.12	0.75	-	-
> 8 y	25/81 (30.9)	(0.53–2.38)			
Initiated Antiretroviral treatment					
Yes	46/144 (31.9)	0.94	0.96	-	-
No	1/3 (33.3)	(0.05–56.57)			
Baseline HIV RNA PCR (copies/ml)					
< 400	46/142 (32.4)	1.92	0.56	-	-
≥ 400	1/5 (20.0)	(0.18–96.40)			
Baseline CD4 count (cell/mm^3)					
> 200	46/134 (34.3)	**6.27**	**0.05**	**5.86**	**0.10**
≤ 200	1/13 (7.7)	**(0.87–273.83)**		**(0.73–47.05)**	
Symptomatic or AIDs indicator conditions at baseline					
Yes	2/11 (18.2)	0.45	0.31	-	-
No	45/136 (33.1)	(0.05–2.31)			
Baseline HI titer					
≥ 1:40	4/5(80)	**9.21**	**0.02**	**8.61**	**0.06**
< 1:40	43/142(30.3)	**(0.86–457.91)**		**(0.91–81.67)**	

Discussion

Our study demonstrated low seroconversion and seroprotection rates in response to the non- adjuvanted 2009 H1N1 vaccine in both HIV-infected and healthy participants. These overall response rates were much lower than the expectation since the majority of HIV-infected participants (91%) had CD4+ cell count > 200 cells/mm3 and all the healthy participants had normal immune status. Multiple studies have shown varying but generally high seroconversion rates (from 74 to 98%) to the 2009 H1N1 vaccine in HIV-negative individuals.[2-6] While studies in HIV-infected individuals found lower seroconversion rates (**Table 3**), despite an immune recovery indicated by increase in CD4+ cell count and full viral suppression indicated by an undetectable plasma HIV-1 RNA after combination antiretroviral therapy. The diversity of seroconversion rates may depend on different type of vaccines used among studies.[8] There is a trend that the ASO3 adjuvanted vaccine may elicit better immune response in HIV population than other types of vaccine.[15-18]

Table 3. Summary of studies of a single dose H1N1 vaccination

Type of vaccine	No. of HIV-infected / control	Age of HIV-infected vaccinees	CD4 count of HIV-infected vaccinees	Seroprotectionrate at baseline HIV/Control	Seroprotection rate post vaccination HIV/Control	Seroconversion rate HIV/Control	Author/ Country/ Reference number
Non-adjuvanted 15 μg							
	79/0	40(37–42)[a]	502(449–556)[a]	70/0	92/0	31/0	Lagler et al. / Austria/7
	182/42	47(13)[b]	411(178)[b]	13/12	50/86	39/ 86	Yanagisawa et al./ Japan/ 8
	CD4 < 200: 35/0 CD4 ≥ 200: 60/0	46(9)[b] 46(10)[b]	156(97)[b] 610(269)[b]	21/0 18/0	47/0 64/0	41/0 52/0	Sahly et al./ USA/9
	104/0	43(34 -53)[c]	373(256–520)[c]	12/0	56/0	50/0	Hatakeyama et al./Japan/10
	256/0	45 (22–75)[c]	Not evaluated	9/0	59/0	55/0	Miraglia et al. /Brazil/11
	120/0	46(40–53)[c]	502(307–640)[c]	25/0	69/0	56/0	Tebas et al./ USA/ 12
	65/66	36(26–45)[c]	581(476–814)[c]	20/33	65/85	68/83	CrumCianflone et al./ USA/ 13
	126/0	44 (37–51)[c]	530(400–685)[c]	39/0	87/0	67/0	Maruszak / Australia/14
	150/0	47(40–54)[c]	551(428–702)[c]	10/0	76/0	71/0	Launay et al./France/15
AS03adjuvanted 3.75 μg							
	155/0	47(39–54)[c]	522(387–752)[c]	8/0	93/0	89/0	Launay et al./France/15
	84/0	48(11)[b]	427(178)[b]	8/0	45/0	44/0	Tremblay et al./Canada/16
	160/0	46(11)[b]	514(246)[b]	14/0	75/0	69/0	Bickel et al./Germany/17
	252/0	47(10)[b]	570(266)[b]	26/0	92/0	83/0	Orlando et al./Italy/18
MF59adjuvanted 7.5 μg							
	44/148	45(no SD)[b]	563(505–621)[d]	80/35	98/97	36/79	Kajaste-Rudnitski et al./ Italy/ 19
	57/44	52(11)[b]	507(349–697)[c]	44/23	88/93	53/73	Soonawala et al./ Netherlands/20
	41/0	46(41–55)[c]	528(406–736)[c]	24/0	78/0	61/0	Fabbiani et al./Italy/ 21

[a] Median with 95% CI. [b]Mean with Standard deviation. [c] Median with Interquartile range. [d] Mean with 95% CI. Figures are rounded to the nearest whole number: rounded up for half or greater (≥ 0.5), rounded down for less than half (< 0.5).

It is important to investigate the factors responsible for our seroconversion rate being lowest among studies conducted in HIV-infected individuals. Possible explanations include: (1) the vaccine used in our study (Panenza®) is less immunogenic than the 2009 H1N1 vaccine used in other studies, (2) imperfect effectiveness of influenza vaccine in field condition caused by factors such as breakdown of the cold chain and improper vaccine management, and (3) the laboratory method used in our study is less sensitive than that employed in other studies in detecting seroconversion.

Results from previous studies using non-adjuvanted 2009 H1N1 vaccine in HIV-infected individuals also showed varying seroconversion rates ranging from 31% to 71% (Table3). However, the vaccine types used in those studies were different, for instances, inactivated Vero-cell-derived whole virion vaccine by Lagler et al.[7] and other non-adjuvanted recombinant vaccines produced by several pharmaceutical companies.[8-15] Therefore, comparison of differences in immunogenicity among those vaccine including that used in our study may not be possible. Nonrtheless, a high seroconversion rate in HIV uninfected group indicated that the immunogenicity of the vaccines employed in those studies were adequate. Conversly, our study failed to demonstrate a desire vaccine efficacy in HIV negative volunteers. This may support our hypothesis about the immunogenicity of Panenza® vaccine.

A literature review was done on the efficacy trials of the Panenza®. In the report authored by researchers affiliated with Sanofi Pasteur, two studies were conducted with single standard dose of Panenza® in 101 and 100 French healthy volunteers between August 2009 and October 2009 and between March 2010 and April 2010 respectively.[25] The seroconversion rate in the first study was 92% and that in the second study was 97%. However, the immunologic response to Panenza® was found to be less than that reported in French studies, involving healthy adults in Thailand and Hong Kong. First is a prospective cohort study of a single dose of Penenza® in hemodialysis patients and 149 healthy controls by Lertdumrongluk et al.[26]; the seroconversion rate was 63.1% and protective titers were obtained in 67.1% of the control group at 4 weeks post vaccination. A cross-sectional study conducted in Khon Kaen, Thailand in June 2010, 52.1% of 71 healthcare workers aged 21–75 y who had been vaccinated with Panenza® vaccine had HI titers ≥ 40.[27] A similar study conducted in 104 Hong Kong healthcare workers aged 19–64 y receiving Panenza® also showed seroprotection rate of 53.8% (95% CI 44.2%–63.2%).[28] One of the possible explanations for lower antibody response considered by the authors was imperfect effectiveness of Panenza®.

The only study of Panenza® in HIV-infected individuals in Thailand was done at Siriraj Hospital in Bangkok and Chiang Mai University Hospital in Chiang Mai, Thailand.[29] 119 children with a median age of 10.4 y (IQR 7.2–13.7) were given 2 doses of Panenza® 28 d apart. The seroconversion rates were 54.2% and 67.8% after the first and second doses, respectively. Our study is the first report of this particular vaccine in HIV-infected adults.

To answer the question of improper vaccine management, the vaccines used in our study were delivered from the Department of Disease Control, MOPH to our institute on January 14, 2010. Vaccine expiration date was September 30, 2010. Vaccinations were started from January 21, 2010 to March 2010. To ensure vaccine quality, all vaccines were stored and delivered under temperature controlled conditions in accordance with the Pharmacy guidelines and Instruction s for DAIDS Clinical Trials Networks Division of AIDS pharmaceutical Affairs Branch, July 2008 and the vaccine package insert. Each vaccine vial was only used within a day of first opening. After reviewing the vaccine management records, we did not find any errors that could explain the result of this study. It was also unlikely that cold chain breakdown and improper vaccine management had occurred concurrently in Hong Kong, Khon Kaen, Bangkok and Chiang Mai.

Lastly, considerable variability can be introduced into the laboratory assay used to measure HI antibodies including differences in viral strains and red blood cell types, and the presence of non-specific inhibitors in the assay medium.[24] In our study, the HI test was performed according to standard method,[30] the only exception is the use of goose erythrocyte instead of turkey erythrocyte. However, a study by Lerdsamran et al.[23] has demonstrated that goose and turkey erythrocytes yielded comparable HI antibody titers. A study by Miraglia JL et al.[11] also used the standard HI assay and demonstrated the low seroconversion rate (55%) to Sanofi-Pasteur non-adjuvanted H1N1 vaccine in HIV infected individuals. Therefore, the method used may not be a potential factor to the low response rate. The fact that the strain used in HI assay in our study, is not identical to the strain included in the vaccine could be a reason for a lower immunogenicity. The A/Thailand/104/2009(H1N1) strain which was also used to evaluate the vaccine response in the other two Thai studies was isolated from a confirmed case of pandemic H1N1 2009 who had recently traveled back from Mexico. The strain was submitted to the GenBank database on June 13, 2009 where full genomic sequence of A/Thailand/104/2009(H1N1) can be retrieved. Its HA gene is 99.7% identical to that of A/California/7/2009 pandemic virus.[23] The author's unpublished data (PP and HL) have shown that the antibody titers against these two viruses were comparable as assayed in 100 individuals without immune deficiency (patients and non-patients). . Therefore, the strain used in the HI assay would probably not have a significant influence on the vaccine response rate. To avoid the intra-laboratory variability of the test, we selected the HI assay instead of the viral neutralization assay that may have higher variability in results.[31] In addition, we performed the tests for all sera in a batch process using the same reagents and by the same lab personnel.

In conclusion, the unexpected low immune response to the single dose of non-adjuvanted 2009 H1N1 vaccine in our study together with similar results in the three other studies[26-28] suggest that the vaccine formulation Panenza® bought by the Hong Kong and Thai government in late 2009 may be the cause of this suboptimal response. Alternatively, there might be problems with cold chain, vaccine management, or the sensitivity of laboratory method. However, these are unlikely to happen in Hong Kong, Bangkok, Chiang Mai, and Khon Kaen concurrently. Further reports from countries that employed Panenza® and/or investigation by Sanofi Pasteur of the lot of Panenza® marketed in late 2009 are needed. Further studies on different vaccines, dosing, adjuvants, or schedule strategies may be needed to achieve effective immunization in HIV-infected population.

Limitations

Limitations of our study were a small number of HIV-negative controls which may have insufficient power to determine vaccine response in this population and lack of different type of vaccine to compare with Panenza® vaccine.

Methods

Participants

Our study was conducted after the first wave of pandemic influenza H1N1 2009 outbreak in Thailand. Between January 2010 and March 2010, we invited and enrolled, on a first-come-first-served basis, a total of 150 HIV-infected individuals aged 18–60 y from the Infectious Disease Clinic, Chiang Mai University Hospital, a 1500-beds tertiary care facility in Chiang Mai, Thailand, where a treatment-cohort of approximately 1,300 HIV-infected patients was under active follow-up. Exclusion criteria were an allergy to eggs or a history of Guillain-Barré Syndrome or family history of Guillain- Barré Syndrome. A total of 20 healthy volunteers were enrolled under the same protocol.

Clinical and laboratory Procedures

The vaccine, Panenza®, is a monovalent, non-adjuvanted vaccine formulated to contain 15 µg/0.5 mL of hemagglutinin (HA) of influenza A/California/07/2009 (H1N1) v-like virus produced by Sanofi Pasteur. A single 0.5 mL intramuscular dose of the vaccine was administered to all 170 participants. Clinical assessment was performed in HIV-infected individuals prior to vaccination for classification of CDC clinical category.[22] Baseline Laboratory evaluation included CD4+ cell count and hemagglutination inhibition (HI) antibody titer against 2009 H1N1 virus for both groups and plasma HIV-1 RNA measurement for HIV-infected group. The CD4+ cell count was performed using flow cytometry techniques and plasma HIV-1 RNA was measured by the COBAS Amplicor™ Analyzer, ROCHE Diagnostic System at the Research Institute for Health Sciences, Chiang Mai University.

The HI assay was performed at the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University. The methodology was described previously.[23] Briefly, 50 µl of the test serum were mixed with 150 µl of receptor destroying enzyme (RDE, Denka Seiken) and incubated overnight in water bath at 37°C. This step was followed by heat inactivation at 56°C for 30 min, and removal of nonspecific agglutinator by absorbing with goose erythrocytes for 1 h at 4°C. The replicating virus, A/Thailand/104/2009, at final concentration of 4 HA units/25 µl was used as the test antigen; and goose erythrocytes were used as the indicator. The treated serum was 2-fold serially diluted in duplicate wells of a microtiter V shaped plate at an initial dilution of 1:10; and 25 µl of the diluted serum were incubated with 25 µl of the test antigen for 30 min at room temperature. Thereafter, the reaction wells were added with 50 µl of 0.5% goose erythrocyte suspension and further incubated for 30 min at 4 C before the HI antibody titers were determined. HI antibody titer is defined as the reciprocal of the highest serum dilution that completely inhibits hemagglutination reaction. Reference/positive control serum with known HI titer, the serum control and back titration of virus antigen were included in each run. The reference human serum was obtained from the National Institute for Biological Standards and Control (NIBSC). The full genomic sequence of the virus, A/Thailand/104/2009, has been deposited with GenBank. Its HA gene is 99.7% identical to that of A/California/7/2009 pandemic virus.[23] This virus was the first strain isolated in Thailand in May 2009 from a case who traveled back from Mexico (PP, personal communication).

Evaluations and endpoints

Any participant who developed influenza-like illness was asked to come to the clinic within 72 h for respiratory specimen collection to confirm the diagnosis of 2009 H1N1 infection. The immunogenicity endpoint was the proportion of participants who had seroconversion and seroprotection from vaccination. Seroconversion was defined in accordance with the US FDA guidance[24] as 1) pre–vaccination HI titer < 1:10 and post-vaccination HI titer ≥ 1:40, or 2) pre–vaccination HI titer ≥ 1:10 and a minimum of 4–fold rise in post–vaccination HI titer. Seroprotection was defined as a post-vaccination HI titer of ≥ 1:40.

Ethics

The study protocol was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University.

Statistical methods

For baseline characteristics, continuous variables such as age and absolute CD4+ cell count are presented as mean ± standard deviation. Seroconversion rate and seroprotection rate with the corresponding 95% confidence interval (CI) were calculated. Univariate analyses and multivariate analysis by logistic regression were used to determine factors associated with seroconversion in HIV-infected group. Results were reported by presenting odds ratios and adjusted odds ratios with 95% confidence interval. Level of significance was defined as a p-value of < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors wish to express their deepest gratitude to the staff members of the Infectious Disease Clinic, Chiang Mai University hospital for their assistance in recruitment and retention of the participants in this study. This study was supported by the Thailand Research Fund for Senior Research Scholar (PP and HL), and the Research University Program, Chiang Mai University (all other authors). There is no conflict of interest to declare for all the authors.

References

1. Iamsirithaworn S, Akarasewi P, Yingyong T, Suthachana S, Pittayawonganon C, Ungchusak K.. Three waves of the 2009 H1N1 Influenza pandemic in Thailand. Siriraj Medical Journal ; 63.

2. Greenberg ME, Lai MH, Hartel GF, Wichems CH, Gittleson C, Bennet J, et al. Response to a monovalent 2009 influenza A (H1N1) vaccine. N Engl J Med 2009; 361:2405-13; PMID: 19745216; DOI: 10.1056/NEJMoa0907413.

3. Kubavat AH, Mittal R, Patel PM, Jarsaniya DH, Pawar PR, Group HNVS. A clinical trial to assess the immunogenicity and safety of Inactivated Influenza Vaccine (Whole Virion) IP (Pandemic Influenza (H1N1) 2009 Monovalent Vaccine; VaxiFlu-S™) in healthy Indian adult population. J Postgrad Med 2011; 57:102-8; PMID: 21654130; DOI: 10.4103/0022-3859.81860.

4. Ikematsu H, Nagai H, Kawashima M, Kawakami Y, Tenjinbaru K, Maeda A, et al. Immunogenicity and safety of a novel AS03(A)-adjuvanted H1N1 2009 pandemic influenza vaccine in adults in Japan. Hum Vaccin 2010; 6:888-93; PMID: 20980795; DOI: 10.4161/hv.6.11.12851.

5. Roman F, Vaman T, Gerlach B, Markendorf A, Gillard P, Devaster JM. Immunogenicity and safety in adults of one dose of influenza A H1N1v 2009 vaccine formulated with and without AS03A-adjuvant: preliminary report of an observer-blind, randomised trial. Vaccine 2010; 28:1740-5; PMID: 20034605; DOI: 10.1016/j.vaccine.2009.12.014.

6. Plennevaux E, Sheldon E, Blatter M, Reeves-Hoché MK, Denis M. Immune response after a single vaccination against 2009 influenza A H1N1 in USA: a preliminary report of two randomised controlled phase 2 trials. Lancet 2010; 375:41-8; PMID: 20018365; DOI: 10.1016/S0140-6736(09)62026-2.

7. Lagler H, Grabmeier-Pfistershammer K, Touzeau-Römer V, Tobudic S, Ramharter M, Wenisch J, et al. Immunogenicity and tolerability after two doses of non-adjuvanted, whole-virion pandemic influenza A (H1N1) vaccine in HIV-infected individuals. PLoS One 2012; 7:e36773; PMID: 22629330; DOI: 10.1371/journal.pone.0036773.

8. Yanagisawa N, Maeda K, Ajisawa A, Imamura A, Suganuma A, Ando M, et al. Reduced immune response to influenza A (H1N1) 2009 monovalent vaccine in HIV-infected Japanese subjects. Vaccine 2011; 29:5694-8; PMID: 21689709; DOI: 10.1016/j.vaccine.2011.06.003.

9. El Sahly HM, Davis C, Kotloff K, Meier J, Winokur PL, Wald A, et al. Higher antigen content improves the immune response to 2009 H1N1 influenza vaccine in HIV-infected adults: a randomized clinical trial. J Infect Dis 2012; 205:703-12; PMID: 22275399; DOI: 10.1093/infdis/jir837.

10. Hatakeyama S, Iwatsuki-Horimoto K, Okamoto K, Nukui Y, Yata N, Fujita A, et al. Unadjuvanted pandemic H1N1 influenza vaccine in HIV-1-infected adults. Vaccine 2011; 29:9224-8; PMID: 21978767; DOI: 10.1016/j.vaccine.2011.09.106.

11. Miraglia JL, Abdala E, Hoff PM, Luiz AM, Oliveira DS, Saad CG, et al. Immunogenicity and reactogenicity of 2009 influenza A (H1N1) inactivated monovalent non-adjuvanted vaccine in elderly and immunocompromised patients. PLoS One 2011; 6:e27214; PMID: 22087267; DOI: 10.1371/journal.pone.0027214.

12. Tebas P, Frank I, Lewis M, Quinn J, Zifchak L, Thomas A, et al. Poor immunogenicity of the H1N1 2009 vaccine in well controlled HIV-infected individuals. AIDS 2010; 24:2187-92; PMID: 20616698; DOI: 10.1097/QAD.0b013e32833c6d5c.

13. Crum-Cianflone NF, Eberly LE, Duplessis C, Maguire J, Ganesan A, Faix D, et al. Immunogenicity of a monovalent 2009 influenza A (H1N1) vaccine in an immunocompromised population: a prospective study comparing HIV-infected adults with HIV-uninfected adults. Clin Infect Dis 2011; 52:138-46; PMID: 21148532; DOI: 10.1093/cid/ciq019.

14. Maruszak H, Jeganathan S, Smith DE, Robertson P, Barnes T, Furner V. Improved serological response to H1N1 monovalent vaccine associated with viral suppression among HIV-1-infected patients during the 2009 influenza (H1N1) pandemic in the Southern Hemisphere. HIV Med 2012; 13:352-7; PMID: 22296264; DOI: 10.1111/j.1468-1293.2011.00987.x.

15. Launay O, Desaint C, Durier C, Loulergue P, Duval X, Jacomet C, et al. Safety and immunogenicity of a monovalent 2009 influenza A/H1N1v vaccine adjuvanted with AS03A or unadjuvanted in HIV-infected adults: a randomized, controlled trial. J Infect Dis 2011; 204:124-34; PMID: 21628666; DOI: 10.1093/infdis/jir211.

16. Tremblay CL, Rouleau D, Fortin C, Toma E, Sylla M, Cyr L, et al. Immunogenicity and tolerability of an inactivated and adjuvanted pandemic H1N1 influenza vaccine, in HIV-1-infected patients. Vaccine 2011; 29:1359-63; PMID: 21185423; DOI: 10.1016/j.vaccine.2010.12.023.

17. Bickel M, Wieters I, Khaykin P, Nisius G, Haberl A, Stephan C, et al. Low rate of seroconversion after vaccination with a split virion, adjuvanted pandemic H1N1 influenza vaccine in HIV-1-infected patients. AIDS 2010; 24:F31-5; PMID: 20559034; DOI: 10.1097/QAD.0b013e3283398da1.

18. Orlando G, Pariani E, Mazza F, Tanzi E, Meraviglia P, Gianelli E, et al. Pandemic influenza vaccine in adult HIV-1-infected patients. AIDS 2010; 24:2142-3; PMID: 20679764; DOI: 10.1097/QAD.0b013e32833cfcb0.

19. Kajaste-Rudnitski A, Galli L, Nozza S, Tambussi G, Di Pietro A, Pellicciotta G, et al. Induction of protective antibody response by MF59-adjuvanted 2009 pandemic A/H1N1v influenza vaccine in HIV-1-infected individuals. AIDS 2011; 25:177-83; PMID: 21150561; DOI: 10.1097/QAD.0b013e328341afa8.

20. Soonawala D, Rimmelzwaan GF, Gelinck LB, Visser LG, Kroon FP. Response to 2009 pandemic influenza A (H1N1) vaccine in HIV-infected patients and the influence of prior seasonal influenza vaccination. PLoS One 2011; 6:e16496; PMID: 21304982; DOI: 10.1371/journal.pone.0016496.

21. Fabbiani M, Di Giambenedetto S, Sali M, Farina S, Sansonetti P, Tamburrini E, et al. Immune response to influenza A (H1N1)v monovalent MF59-adjuvanted vaccine in HIV-infected patients. Vaccine 2011; 29:2836-9; PMID: 21349364; DOI: 10.1016/j.vaccine.2011.02.020.

22. . 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR Recomm Rep 1992; 41:1-19; PMID: 1361652.

23. Lerdsamran H, Pittayawonganon C, Pooruk P, Mungaomklang A, Iamsirithaworn S, Thongcharoen P, et al. Serological response to the 2009 pandemic influenza A (H1N1) virus for disease diagnosis and estimating the infection rate in Thai population. PLoS One 2011; 6:e16164; PMID: 21283570; DOI: 10.1371/journal.pone.0016164.

24. Department of Health and Human Services (US), Food and Drug Administration, Center for Biologics Evaluation and Research. Guidance for industry: clinical data needed to support the licensure of pandemic influenza vaccines. May 2007. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm091985.pdf

25. Van der Vliet D, Pepin S, Lambert M, Fauchoux N, Donazzolo Y, Dupuy M, et al. Similar immunogenicity of the A/H1N1 2009 pandemic influenza strain when used as a monovalent or a trivalent vaccine. Hum Vaccin 2010; 6:823-8; PMID: 20935517; DOI: 10.4161/hv.6.10.13600.

26. Lertdumrongluk P, Changsirikulchai S, Limkunakul C, Prachukthum P, Punpiput P, Buppanharun R, et al. Safety and immunogenicity of a 2009 influenza A (H1N1) vaccine in hemodialysis patients. Vaccine 2012; 30:1108-14; PMID: 22178515; DOI: 10.1016/j.vaccine.2011.12.023.

27. Prachayangprecha S, Makkoch J, Vuthitanachot C, Vuthitanachot V, Payungporn S, Chieochansin T, et al. Epidemiological and serological surveillance of human pandemic influenza A virus infections during 2009-2010 in Thailand. Jpn J Infect Dis 2011; 64:377-81; PMID: 21937818.

28. Zhou Y, Ng DM, Seto WH, Ip DK, Kwok HK, Ma ES, et al. Seroprevalence of pandemic H1N1 antibody among health care workers in Hong Kong following receipt of monovalent 2009 H1N1 influenza vaccine. PLoS One 2011; 6:e27169; PMID: 22102878; DOI: 10.1371/journal.pone.0027169.

29. Phongsamart W, Sirisanthana V, Wittawatmongkol O, Maleesatharn A, Sudjaritruk T, Chearskul P, et al. Immunogenicity and safety of monovalent influenza A (H1N1) 2009 in HIV-infected Thai children. Vaccine 2011; 29:8705-11; PMID: 21893147; DOI: 10.1016/j.vaccine.2011.08.101.

30. World Health Organization. WHO manual on animal influenza diagnosis and surveillance. Geneva: The World Health Organization 2002.

31. Wood JM, Major D, Heath A, Newman RW, Höschler K, Stephenson I, et al. Reproducibility of serology assays for pandemic influenza H1N1: collaborative study to evaluate a candidate WHO International Standard. Vaccine 2012; 30:210-7; PMID: 22100887; DOI: 10.1016/j.vaccine.2011.11.019.

OPEN ACCESS Freely available online

PLoS one

A Novel Pathogenic Mechanism of Highly Pathogenic Avian Influenza H5N1 Viruses Involves Hemagglutinin Mediated Resistance to Serum Innate Inhibitors

Jutatip Panaampon[1,2], Nathamon Ngaosuwankul[2,3], Ornpreya Suptawiwat[1,2], Pirom Noisumdaeng[1,2], Kantima Sangsiriwut[4], Bunpote Siridechadilok[5], Hatairat Lerdsamran[1,2], Prasert Auewarakul[1,2], Phisanu Pooruk[1], Pilaipan Puthavathana[1,2]*

1 Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, **2** Center for Emerging and Neglected Infectious Disease, Mahidol University, Bangkok, Thailand, **3** Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, **4** Department of Preventive Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, **5** National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand

Abstract

In this study, the effect of innate serum inhibitors on influenza virus infection was addressed. Seasonal influenza A(H1N1) and A(H3N2), 2009 pandemic A(H1N1) (H1N1pdm) and highly pathogenic avian influenza (HPAI) A(H5N1) viruses were tested with guinea pig sera negative for antibodies against all of these viruses as evaluated by hemagglutination-inhibition and microneutralization assays. In the presence of serum inhibitors, the infection by each virus was inhibited differently as measured by the amount of viral nucleoprotein produced in Madin-Darby canine kidney cells. The serum inhibitors inhibited seasonal influenza A(H3N2) virus the most, while the effect was less in seasonal influenza A(H1N1) and H1N1pdm viruses. The suppression by serum inhibitors could be reduced by heat inactivation or treatment with receptor destroying enzyme. In contrast, all H5N1 strains tested were resistant to serum inhibitors. To determine which structure (hemagglutinin (HA) and/or neuraminidase (NA)) on the virus particles that provided the resistance, reverse genetics (rg) was applied to construct chimeric recombinant viruses from A/Puerto Rico/8/1934(H1N1) (PR8) plasmid vectors. rgPR8-H5 HA and rgPR8-H5 HANA were resistant to serum inhibitors while rgPR8-H5 NA and PR8 A(H1N1) parental viruses were sensitive, suggesting that HA of HPAI H5N1 viruses bestowed viral resistance to serum inhibition. These results suggested that the ability to resist serum inhibition might enable the viremic H5N1 viruses to disseminate to distal end organs. The present study also analyzed for correlation between susceptibility to serum inhibitors and number of glycosylation sites present on the globular heads of HA and NA. H3N2 viruses, the subtype with highest susceptibility to serum inhibitors, harbored the highest number of glycosylation sites on the HA globular head. However, this positive correlation cannot be drawn for the other influenza subtypes.

Citation: Panaampon J, Ngaosuwankul N, Suptawiwat O, Noisumdaeng P, Sangsiriwut K, et al. (2012) A Novel Pathogenic Mechanism of Highly Pathogenic Avian Influenza H5N1 Viruses Involves Hemagglutinin Mediated Resistance to Serum Innate Inhibitors. PLoS ONE 7(5): e36318. doi:10.1371/journal.pone.0036318

Editor: Michael C. W. Chan, Centre of Influenza Research, The University of Hong Kong, Hong Kong

Received December 14, 2011; **Accepted** April 2, 2012; **Published** May 1, 2012

Funding: This study was supported by the Thailand Research Fund for Senior Research Scholar, Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative, scholarship for graduate students, Faculty of Medicine Siriraj Hospital, Mahidol University, and Mahidol University Research Grant. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: siput@mahidol.ac.th

Introduction

Human and H5N1 avian influenza viruses are different in terms of pathogenesis and severity of the disease. While the infections by influenza A(H1N1), A(H3N2) and influenza B viruses are confined mostly to the upper respiratory tract, the infection caused by highly pathogenic avian influenza (HPAI) H5N1 viruses frequently invades lower respiratory tract, induces cytokine storm, and causes severe pneumonia which progresses to acute respiratory distress syndrome and multi-organ failure [1,2]. Dissemination of H5N1 virus beyond the respiratory tract is well documented. The viral RNA could be detected in the autopsies of several organs [3], cerebrospinal fluid [4], and fetal tissues [5]. In addition, HPAI H5N1 virus could be isolated from a plasma sample of a Thai patient [6]. This information indicates that the propensity to undergo viremic phase is not uncommon for HPAI H5N1 virus infection. On the other hand, there has been just one recent report on viremia in patients infected with the 2009 pandemic A(H1N1) (H1N1pdm) virus [7]. Moreover, viremia associated with seasonal influenza H1N1 and H3N2 viruses is very rare [8–10]. These findings indicate that various influenza subtypes are different in the capability to exhibit viremic phase.

Sera and respiratory fluids of mammals contain several innate soluble factors that exhibit anti-influenza activity, for examples, members of the collectin superfamily such as surfactant protein A (SP-A), surfactant protein D (SP-D), conglutinin and mannose-binding lectin (MBL) [11–13], member of the pentraxin superfamily such as pentraxin 3 (PTX3) [14], and serum amyloid P component [15]. The binding of human SP-D and MBL to both hemagglutinin (HA) and neuraminidase (NA) can inhibit influenza

virus hemagglutinating activity, interfere with virus release through inhibition of viral neuraminidase activity, and hinder viral infection by preventing viral attachment to the cell receptor. The anti-influenza activities of human SP-D and MBL do not involve other complement factors [16]. Interestingly, the highly glycosylated seasonal H1N1 strains are sensitive to inhibition by both SP-D and MBL, while the poorly glycosylated A/Puerto Rico/8/1934(H1N1) (PR8) and H1N1pdm are resistant to either one of them [17]. The long chain pentraxin, PTX3 inhibits several strains of seasonal influenza A(H1N1), A(H3N2) and influenza B viruses [14], though the susceptibility to PTX3 may be strain-specific as some seasonal human influenza isolates including PR8 virus and the H1N1pdm viruses are resistant to PTX3 [17]. Complement, the major component of innate immunity, may play anti-influenza activity independently or in adjunct with other components. Activation of the complement system results in virus aggregation, virolysis or opsonization [18]. It can lead to increased vascular permeability and recruitment of phagocytic cells to destroy the pathogens. MBL, together with complement in guinea pig serum, exerted lytic activity on influenza virus infected-BHK-21 cells through the classical pathway [19]. MBL also binds influenza HA and activates complement through lectin pathway [20]. Moreover, complement together with natural IgM antibody can destroy virus through the classical pathway [21].

In the other system, non-specific inhibitors against influenza viruses present in normal sera of various animal species were classified into three types: α-, β-, and γ-inhibitors based on their chemical composition and several biological properties [22,23]. α-inhibitors are heat- stable, but receptor destroying enzyme (RDE)-sensitive glycoproteins. It inhibited influenza virus hemagglutination, but not influenza virus infectivity. The examples of α-inhibitors found in serum are soluble mucoproteins. β-inhibitors are non-sialylated, Ca^{2+} dependent and heat-labile but RDE-resistant. Study in bovine and mouse serum suggested that MBL and SP-D belonged to β-inhibitors [24]; and MBL was serologically cross reactive with human mannose-binding protein (MBP) [20]. γ-inhibitors are heat-stable and RDE-sensitive sialylated glycoproteins which compete with cellular receptor for binding with HA; these inhibitors neutralize viral infectivity by blocking the attachment step in the influenza virus replication cycle [23,25]. In horse, guinea pig and mouse sera, γ-inhibitor had been identified as α2-macroglobulin [25,26]. In addition, the study in murine model also demonstrated that the α2-macroglobulin could inhibit hemagglutination inhibition (HI) assay, but its activity on microneutralization (microNT) assay against various viruses expressing H3N2 HA was varied among H3N2 isolates [26].

For the influenza viruses to exhibit viremic phase, they should be able to overcome the inhibition by innate serum inhibitors. Therefore, the ability of HPAI H5N1 viruses to spread beyond the respiratory tract is suggestive of their ability to resist the serum inhibitors. Herein, guinea pig sera were used as the model to study serum innate immunity against various influenza viruses of human and avian origins, including seasonal H1N1, H3N2, H1N1pdm, and HPAI H5N1 viruses. The nature of inhibitors was characterized by their sensitivity to heat or RDE treatment. We demonstrated that HPAI H5N1 virus was the only subtype that could resist the inactivation by serum innate inhibitors. Using reverse genetics, the viral resistance to serum inhibitors could be mapped to HA. Our finding suggested a novel pathogenic mechanism on H5N1 virus dissemination beyond the respiratory system.

Results

Biological properties of the guinea pig sera

In order to determine role of serum inhibitors against influenza viruses, it is necessary to screen that the test guinea pig sera contained no specific antibodies against the study viruses. Otherwise, it would be difficult to differentiate between inhibitory effects mediated by the serum specific inhibitors and specific antibodies. Sera of three guinea pigs from different batches of animals were screened for specific antibody against all 14 influenza virus isolates and 4 reassorted viruses by HI and microNT assays. The result showed that none of the test sera contained pre-existing HI or NT antibody as screened at the initial serum dilution 1:10 (data not shown).

It has been known that some serum innate inhibitors are heat-labile and some are heat-stable. There is no control measure to determine stability of those unknown heat-labile components in the stored sera during the on going experiments. Therefore, our study used complement as the representative of heat-labile components based on its abundant amount in guinea pig sera and availability of the measurement method. We assumed that if complement in the test sera did not deteriorate, the other heat-labile components should remain in good condition. Each guinea pig serum was measured for complement titer by complement fixation test using hemolysin sensitized-sheep red blood cells (SRBCs) as the indicator system. We also showed that the three guinea pigs used in our experiments contained comparable serum concentrations of complement, i.e., 32–64 complement hemolytic units; in another word, the serum dilution of 1:32 or 1:64 contained one complement hemolytic unit. The native sera and heat-inactivated sera were assayed in parallel for their lytic activity on hemolysin sensitized- SRBCs. The result demonstrated that the native sera completely lysed the sensitized-SRBCs, while the lytic activity was eradicated after heat inactivation.

Furthermore, the sera were diluted to contain the complement concentrations of 0.25, 0.5, 1, 2, 4 and 8 hemolytic units and assayed against the seasonal A(H1N1) (SI-RA-TT/04), seasonal A(H3N2) (SI 03/04), H1N1pdm (Nonth/102/09) and HPAI H5N1 (KAN-1) viruses in order to determine the optimal dilutions that could exert anti-influenza activity. The result showed a slight increase in the percentages of viral inhibition when serum complement concentrations were greater than two hemolytic units (Figure 1). Therefore, the concentration of 2 complement hemolytic units was employed in subsequent experiments.

Determination for activity of serum innate inhibitors against various influenza subtypes

Guinea pig sera were used as source of innate inhibitors in the assay for infection inhibition against influenza viruses. The study viruses at concentration of 1,000 TCID50/50 μl (TCID50; 50% tissue culture infectious dose) were incubated with native guinea pig serum at working dilution which contained complement concentration of 2 hemolytic units. The virus-serum mixture in quadruplicate was inoculated onto Madin-Darby canine kidney (MDCK) cell monolayers for overnight. Infectivity of the viruses which were susceptible to serum innate inhibitors should be inhibited and resulted in the decreased amount of viral nucleoprotein produced in the infected MDCK cells as determined by enzyme-linked immunosorbent assay (ELISA). Based on the percentages of viral infection inhibition as compared to the virus control, H3N2 viruses were the subtype most susceptible to serum inhibitors (81–100% inhibition); and followed in order by H1N1pdm (34–89% inhibition) and seasonal H1N1 viruses (21–62% inhibition) (Table 1 and Figure 2). In contrast, all HPAI

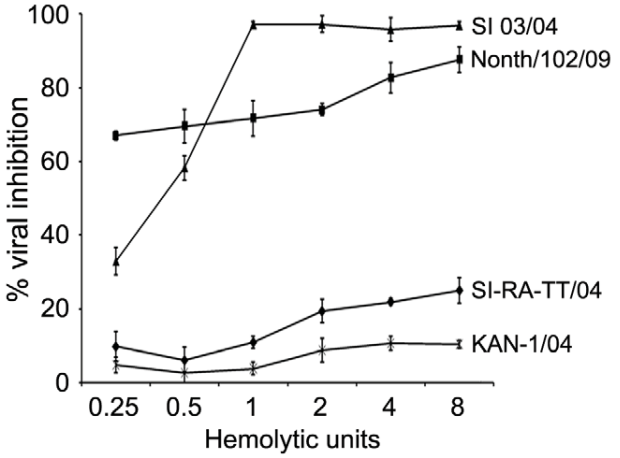


Figure 1. Anti-influenza activities of guinea pig sera at various complement hemolytic units. Native guinea pig sera at various complement hemolytic units were incubated with seasonal H1N1 (SI-RA-TT/04), H3N2 (SI 03/04), H1N1pdm (Nonth/102/09) or HPAI H5N1 (KAN-1/04) virus followed by inoculation the serum-virus mixture onto MDCK cell monolayers. After an overnight incubation, the viral inhibitory activity of the test serum was measured by determining the amount of viral nucleoprotein produced in the inoculated MDCK cells by ELISA. The results are shown as mean of the percentages of viral inhibition derived from quadruplicate experiments. Error bars represent the standard deviation.
doi:10.1371/journal.pone.0036318.g001

H5N1 viruses were resistant to serum inhibitors with the percentages of inhibition of less than 10, except one serum which exerted 19% inhibition against KAN-1, and 12% inhibition against NBL (feces in origin) viruses (Table 1).

Biochemical nature of serum innate inhibitors and the viral susceptibility

Heat-inactivated serum or RDE-treated+heat-inactivated serum was assayed for their viral inhibitory effect against various influenza subtypes/strains in comparison with the native serum control. Statistically significant difference in the percentage of inhibition was considered at $p \leq 0.05$. Each serum was assayed in quadruplicate and the percentages of viral inhibition of all 3 guinea pig sera were averaged. The viral inhibition effects exerted by native sera, heat-inactivated sera and RDE-treated+heat-inactivated sera are shown as the histograms in Figure 2.

Among H1N1 viruses (Figure 2A), PR8 virus was the most susceptible to serum inhibitors (62% viral inhibition) and followed in order by SI 3043/09 and SI-RA-TT/04. Nevertheless, the inhibitory activity against PR8 decreased to almost 4% when the viruses were treated with either heat-inactivated sera or RDE-treated+heat-inactivated sera. Thus, the PR8 virus was susceptible to heat-sensitive inhibitors only, as the addition of RDE (in the RDE-treated+heat-inactivated sera) did not further decrease the percentages of viral inhibitory activity. Similarly, SI 3043/09 was also inhibited by native serum; and percentages of viral inhibition decreased from 31% to 15% with heat-inactivated sera, and to 12% with RDE-treated+heat-inactivated sera. As the decrease in the percentages of inhibition from 15 to 12% was not statistically significant, it is interpreted that SI 3043/09 was susceptible to heat-labile inhibitors, but susceptibility to RDE-sensitive inhibi-

Table 1. Presence of inhibitory factors against influenza viruses in guinea pig sera.

			% inhibition of viral infection mediated by guinea pig sera		
Subtype	**Virus name**	**Abbreviations**	**No. 1**	**No. 2**	**No. 3**
Seasonal H1N1	A/PR/8/34 (reassorted virus)	PR8	47.7	56.9	62.1
	A/Thailand/Siriraj-Rama-TT/04 (A/New Caledonia/20/99-like virus)	SI-RA-TT/04	21.9	23.3	21.0
	A/Thailand/Siriraj3043/09 (A/Brisbane/59/07-like virus)	SI 3043/09	37.9	31.8	30.7
Seasonal H3N2	A/Thailand/Siriraj-08/98 (A/Sydney/05/97-like virus)	SI 08/98	82.6	82.2	80.9
	A/Thailand/Siriraj-06/02 (A/Moscow/10/99-like virus)	SI 06/02	88.7	87.0	100
	A/Thailand/Siriraj-02/03 (A/Fujian/411/02-like virus)	SI 02/03	99.5	97.1	96.2
	A/Thailand/Siriraj-03/04 (A/Fujian/411/02-like virus)	SI 03/04	94.9	97.2	96.9
H1N1pdm	A/Thailand/104/09	Thai/104/09	51.6	65.0	34.2
	A/Nonthaburi/102/09	Nonth/102/09	81.2	80.8	83.7
	A/California/07/09	CA/07/09	86.7	85.3	88.7
HPAI H5N1	A/Thailand/1(KAN-1)/04	KAN-1	8.9	18.6	5.5
	A/Thailand/676(NYK)/05	676(NYK)	0	2.9	6.0
	A/Thailand/NBL-1/06 (feces)	NBL feces	6.8	12.0	9.8
	A/Thailand/NBL-1/06 (lung)	NBL lung	7.3	6.5	5.2
	A/Laos/Nong Khai 1/07	Nong Khai 1	3.4	7.6	0.8
H5 reverse genetics	rgPR8-H5 HANA (KAN-1)	rgPR8-H5 HANA	1.9	7.1	6.4
	rgPR8-H5 HA (KAN-1)	rgPR8-H5 HA	0	4.6	1.6
	rgPR8-H5 NA (KAN-1)	rgPR8-H5 NA	80.0	41.6	84.9

doi:10.1371/journal.pone.0036318.t001

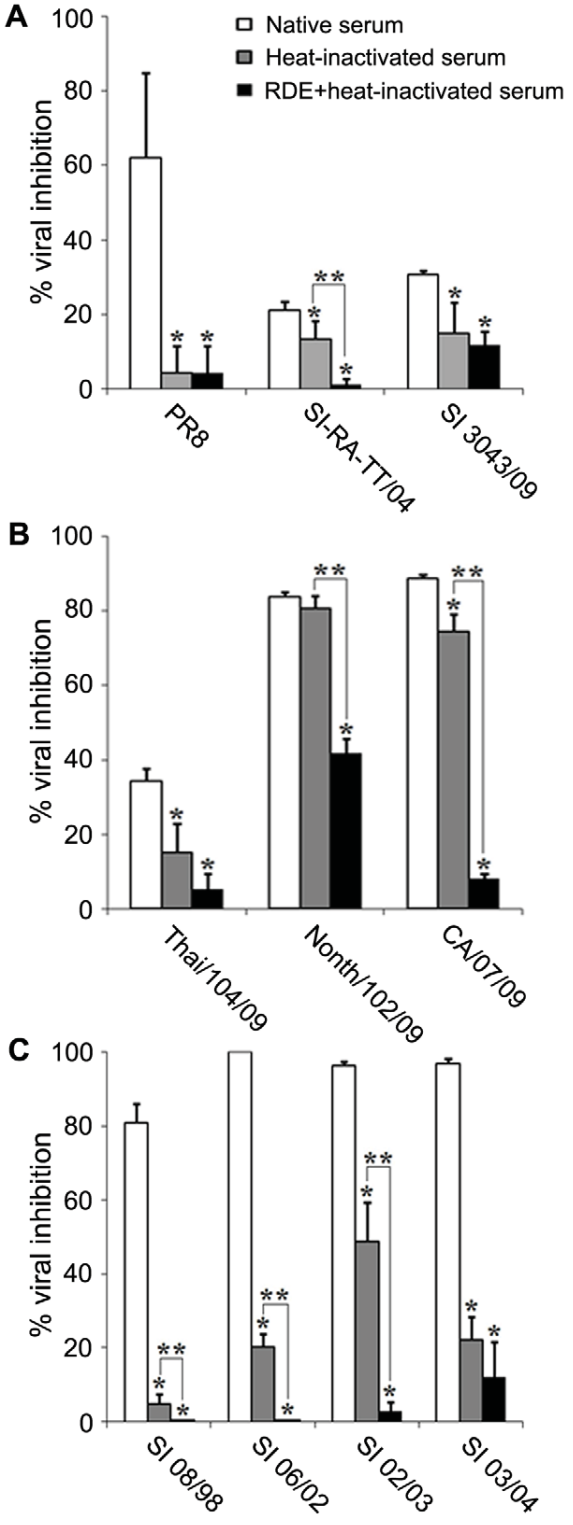


Figure 2. Inhibitory activities of native guinea pig serum, heat-inactivated serum, and RDE-treated+heat-inactivated serum against influenza viruses. Guinea pig sera were incubated with (A) H1N1 viruses, (B) H1N1pdm viruses, or (C) H3N2 viruses. The viral inhibitory activities of the test sera were determined in MDCK cells inoculated with the serum-virus mixtures by ELISA. The results are shown as mean of the percentages of viral inhibition derived from quadruplicate experiments. Error bars represent the standard deviation.

* There are statistically significant differences between the inhibitory activity of the native serum and the heat-inactivated serum or the native serum and the RDE-treated+heat-inactivated serum ($p \leq 0.05$).
** There are statistically significant differences in the inhibitory activity between heat-inactivated serum and RDE-treated+heat-inactivated serum ($p \leq 0.05$).
doi:10.1371/journal.pone.0036318.g002

tors, if any, should be very minute. On the other hand, SI-RA-TT/04 was susceptible to both heat-sensitive inhibitors and RDE-sensitive inhibitors as the percentages of viral inhibition dropped from 21% to 13% or 1% after treatment with heat-inactivated sera or RDE-treated+inactivated sera, respectively.

Among H1N1pdm (Figure 2B), percentages of inhibition of Thai/104/09 virus as assayed against native sera, heat-inactivated sera and RDE-treated+heat-inactivated sera were 34, 15 and 5%, respectively. This result showed that Thai/104/09 virus was susceptible to heat-labile serum inhibitors; however, its susceptibility to RDE-sensitive inhibitors could not be drawn statistically. On the other hand, percentages of inhibition of Nonth/102/09 virus as assayed with native sera, heat-inactivated sera and RDE-treated+heat-inactivated sera were 84, 81 and 42%, respectively. The data suggested that Nonth/102/09 virus was partially susceptible to RDE-sensitive inhibitors. The percentages of viral inhibition of CA/07/09 virus as assayed with native sera, heat-inactivated sera and RDE-treated+heat-inactivated were 89, 74, and 8%, respectively. The result suggested that CA/07/09 virus was slightly susceptible to heat-labile inhibitors, but highly susceptible to RDE-sensitive inhibitors.

Interestingly, all H3N2 viruses were highly susceptible to native serum with percentages of viral inhibition greater than 80% (Figure 2C). SI 08/98, SI 06/02 and SI 02/03 were susceptible to both heat-labile inhibitors and RDE-sensitive inhibitors. Such that the percentages of viral inhibition dropped from 81 to 5% for SI 08/98, from 100 to 20% for SI 06/02, and from 96 to 49% for SI 02/03, as the results obtained from the assays with native sera were compared with those employed heat-inactivated sera; and also, the percentages of viral inhibition dropped from 5 to 0% for SI 08/98, from 20 to 0% for SI 06/02, and from 49 to 3% for SI 02/03, as the results obtained from the assay with heat-inactivated sera were compared with those employed RDE-treated+heat-inactivated sera. The statistical analyses were significantly different for those comparisons. On the other hand, SI 03/04 was susceptible to heat-labile inhibitors alone as shown by the significant decrease in the percentages of viral inhibition from 97 to 22% as the assays with native sera were compared with those employed heat-inactivated sera. In contrast, the percentages of viral inhibition which decreased from 22% (as assayed with heat-inactivated sera) to 12% (as assayed with RDE-treated+heat-inactivated sera) was not significantly different.

Owing to low susceptibility of the HPAI H5N1 viruses to native guinea pig sera (the mean percentage of viral inhibition of 11, 3, 10, 6, and 4 for KAN-1, 676(NYK), NBL feces, NBL lung, and Nong Khai 1, respectively), the assays employing heat-inactivated serum as well as RDE-treated+heat-inactivated serum were not performed.

Role of HA on viral resistance to serum innate inhibitors

Owing to the difference in susceptibility to serum innate inhibitors among various influenza subtypes, it is speculated that HA and/or NA play a key role in this difference. To elucidate this speculation, reassorted PR8 virus together with three reverse genetic viruses harboring *HA* and/or *NA* genes from HPAI H5N1 (KAN-1) viruses in PR8 backbone, i.e., rgPR8-H5 HANA (6+2

virus), rgPR8-H5 HA (7+1 virus), and rgPR8-H5 NA (7+1 virus) were tested against guinea pig sera. The result showed that reassorted PR8 parental virus and rgPR8-H5 NA could be inhibited by serum innate inhibitors with 48–62% and 42–85% viral inhibition, respectively (Table 1). In contrast, rgPR8-H5 HA and rgPR8-H5 HANA could resist the serum inhibitors with percentages of viral inhibition of only 0–5% for rgPR8-H5 HA and 2–7% for rgPR8-H5 HANA, similarly to the H5N1 wild type viruses. When the viral inhibitory activity of native serum, heat-inactivated serum and RDE-treated+heat-inactivated serum were compared, the result showed that efficiency to inhibit reassorted PR8 parental virus and rgPR8-H5 NA viruses were almost completely lost simply by heat inactivation alone; and thus, the inhibitory effect from RDE could not be seen (Figure 3). No significant change in percentages of viral inhibition was observed with rgPR8-H5 HA and rgPR8-H5 HANA viruses when reacted with either heat-inactivated serum or RDE-treated+heat-inactivated serum. The results were reproducible with all three guinea pig serum samples tested (Table 1). In conclusion, it was the HA not NA that rendered HPAI H5N1 viruses the resistance to serum inhibitors.

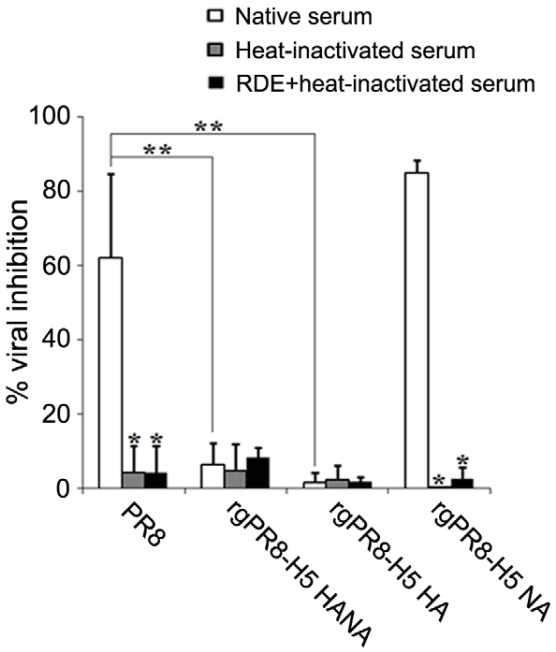


Figure 3. Inhibitory activities of native guinea-pig serum, heat-inactivated serum, and RDE-treated+heat-inactivated serum against reverse genetic viruses. Guinea pig sera were incubated with rgPR8 viruses that contain HPAI H5N1 HANA, HA alone or NA alone. The viral inhibitory activities of the test sera were determined in MDCK cells inoculated with the serum-virus mixtures by ELISA. The results are shown as mean of the percentages of viral inhibition derived from quadruplicate experiments. Error bars represent the standard deviation. * There are statistically significant differences between the inhibitory activity of the native serum and the heat-inactivated serum or the native serum and the RDE-treated+heat-inactivated serum ($p \leq 0.05$). ** There are statistically significant differences in the serum inhibitory activity as compared between the reassorted PR8 virus and the rgPR8-H5 HA virus or the reassorted PR8 virus and the rgPR8-H5 HANA ($p \leq 0.05$).
doi:10.1371/journal.pone.0036318.g003

Prediction of the potential N-linked glycosylation sites on HA and NA globular domains

Our previous experiments demonstrated that HA determined the viral susceptibility or resistance to serum innate inhibitors. Additionally, previous groups of investigators reported that an increase in glycosylation site in HA protein resulted in the increased susceptibility to innate inhibitory factors such as SP-D and collectins [27,28]. Therefore, our study viruses were predicted for their potential sites of glycosylation in HA and NA molecules by CountGS application in BioEdit program version 7.0.4.1 [29]. HA and NA sequences of the study viruses are retrieved from the GenBank database using the accession numbers as shown in Table 2. The potential N-linked glycosylation motif was defined as Asn-X-Ser/Thr, where X may represent any amino acids except proline. The present study determined number of glycosylation sites in the HA globular head of the study viruses, defining that the globular head is situated between the loop region flanked with a conserved disulfide bond in the cysteine bridge in HA1 domain [30]. With methionine as the start position, the globular heads are situated between amino acid position 59 to 291 (reassorted PR8 and seasonal H1N1 viruses), 59 to 292 (H1N1pdm viruses), 68 to 293 (seasonal H3N2 viruses), and 58 to 290 (HPAI H5N1 viruses).

The results showed that H3N2 viruses, the most susceptible subtype to serum inhibitors, contained highest number of glycosylation sites in HA. On the other hand, reassorted PR8 and H1N1pdm viruses which were moderately susceptible to serum innate inhibitors contained one glycosylation site on the HA globular head, while the resistant H5N1 viruses harbored 2 glycosylation sites (Table 2). A homology-based structural model of the HA molecules of seasonal H1N1 (SI-RA-TT/04), H1N1pdm (Thai/104/09), seasonal H3N2 (SI 03/04) and HPAI H5N1 (KAN-1) viruses were constructed using HA crystal structures of PR8, A/California/04/09, A/X-31 (H3N2) or A/HK/212/03 (H5N1) (PDB codes: 1RU7, 3LZG, 1HGF and 3FKU, respectively) as the template. All three-dimensional models are generated by Chimera program version 1.5.3 [31] as shown in Figure 4. Amino acid positions that are the potential *N*-linked glycosylation sites on globular head of HA were predicted for reasserted PR8 (Asn285), seasonal H1N1 (Asn71, Asn104, Asn142 and Asn176), H1N1pdm (Asn104), seasonal H3N2 (Asn79, Asn138, Asn142, Asn149, Asn160, Asn181 and Asn262, but Asn160 was not found in SI 08/98 (H3N2)), and H5N1 (Asn170 and Asn181).

Number of glycosylation sites on NA of the study viruses is shown in Table 2. Based on NA crystal structures of A/Brevig Mission/1/18 (H1N1) and A/Memphis/31/98 (H3N2) (PDB codes: 3BEQ and 2AEP, respectively) with methionine as the start position, the globular head domain of N1 NA starts at position 83 to 468 (N1 numbering) and that of N2 NA starts at position 82 to 469 (N2 numbering). Amino acid positions that are the potential *N*-linked glycosylation sites on globular head of NA were predicted for reassorted PR8 (Asn88 and Asn235), seasonal H1N1 (Asn88, Asn146, Asn235, Asn434 and Asn455), H1N1pdm (Asn88, Asn146, Asn235 and Asn385), seasonal H3N2 (Asn86, Asn146, Asn200, Asn234, Asn392 and Asn402, but additional site, Asn93, was found in SI 06/02), and H5N1 viruses (Asn126, Asn215, but additional site, Asn321, was found in 676(NYK), and Asn366 was found in Nong Khai 1 virus).

Discussion

Most of human sera contain pre-existing anti-influenza antibodies as the results of vaccinations or past infections with some influenza subtypes. Therefore, guinea pig sera instead of human sera were used as the model to study innate immunity

Table 2. Potential glycosylation sites on HA and NA proteins of the study viruses.

	Accession number		Number of glycosylation sites on			
Virus name	***HA***	***NA***	**HA**	**Globular head of HA**	**NA**	**Globular head of NA**
A/PR/8/34 (reassorted virus H1N1)	EF467821	EF467823	7	1	4	2
A/Thailand/Siriraj-Rama-TT/04 (H1N1)	JN676132	JN676133	10	4	9	5
A/Thailand/Siriraj3043/09 (H1N1)	JN676130	JN676131	10	4	9	5
A/Thailand/104/09 (H1N1)	GQ169382	GQ169381	8	1	8	4
A/Nonthaburi/102/09 (H1N1)	GQ150342	GQ150343	8	1	8	4
A/California/07/09 (H1N1)	FJ969540	HM138502	8	1	8	4
A/Thailand/Siriraj-08/98 (H3N2)	JN617980	JN617985	11	6	8	6
A/Thailand/Siriraj-06/02 (H3N2)	JN617982	JN617984	12	7	9	7
A/Thailand/Siriraj-02/03 (H3N2)	JN617979	JN617983	12	7	8	6
A/Thailand/Siriraj-03/04 (H3N2)	JN617981	JN617986	12	7	8	6
A/Thailand/1(KAN-1)/04 (H5N1)	AY555150	AY555151	8	2	3	2
A/Thailand/676(NYK)/05 (H5N1)	DQ360835	DQ360836	8	2	4	3
A/Thailand/NBL1/06 (H5N1) lung	GQ466176	GQ466177	8	2	3	2
A/Laos/Nong Khai 1/07 (H5N1)	EU499372	EU499378	8	2	4	3
rgPR8-H5 HANA (KAN-1)	AY555150	AY555151	8	2	3	2
rgPR8-H5 HA (KAN-1)	AY555150	-	8	2	4	2
rgPR8-H5 NA (KAN-1)	-	AY555151	7	1	3	2

doi:10.1371/journal.pone.0036318.t002

against influenza viruses in order to avoid interference effect from human specific antibody which might create miss-interpretation in the viral infection inhibition assay. Nevertheless, it has been shown that the serum components such as MBL and SP-D are presented in both guinea pig and human sera [11–13]. To some extents and with a careful interpretation, our results from guinea pig should be able to extrapolate to humans. Additionally, to ascertain that the guinea pig sera were also free from anti-influenza antibodies, the sera were tested negative against all study viruses at the screening dilution of 1:10 by HI and microNT assays.

Our viral infection inhibition assay demonstrated that among 10 human influenza viruses (reassorted PR8, 2 seasonal H1N1, 4 seasonal H3N2 and 3 H1N1pdm viruses) tested, 5 were susceptible to both heat-inactivated sera and RDE-treated+heat-inactivated sera, 4 were susceptible to heat-inactivated sera only and 1 was susceptible to RDE-treated+heat-inactivated sera only, with degree of inhibition varying to virus subtypes and strains within the same subtype. H3N2 subtype was the most susceptible to serum inhibitors, while seasonal H1N1 and H1N1pdm viruses were moderately susceptible. All HPAI H5N1 isolates tested were highly resistant to serum inhibitors. While the identities of the serum inhibitors were not determined in our study, the class of serum innate immunity could be estimated from the viral inhibition effects after the sera were treated with heat or RDE+heat. Since α-inhibitors can inhibit only hemagglutination but not virus infection [22,23], they are likely not contributing to the virus inhibition observed in our experiments. On the other hand, our results suggested that serum innate inhibitors against the non H5N1 influenza viruses could be β-inhibitors which are heat-labile and RDE-resistant, γ-inhibitors which are heat-stable and RDE-sensitive, and complement which is heat-labile.

While most of investigators utilized individual serum factors such as SP-D, SP-A, MBL or PTX-3 to study influenza innate immunity, serum factors are likely to work together to exert their anti-influenza activities. In addition, there might be several more anti-influenza factors in sera that have not been discovered yet; thus, complicating the interpretation of the results in broader picture of pathogenesis. The activity of individual factor may not represent overall inhibitory activity observed in the native serum. For example, investigators previously reported that PR8 virus was resistant to MBL [17], and SP-D [27]; however, the present study demonstrated that our reassorted PR8 virus was sensitive to native guinea pig sera with 48–62% viral inhibition activity. The effect of reverse genetics manipulation on susceptibility to serum inhibitors has been excluded as our rgPR8-H5 NA was sensitive while rgPR8-H5 HA and rgPR8-H5 HANA were resistant to the serum inhibitors. Therefore, our system of using whole serum to examine anti-influenza innate serum inhibitors provides a suitable platform to dissect how the HPAI H5N1 virus could disseminate to distal organs.

Number of glycosylation sites on the envelope glycoprotein, HA in particular, has been correlated to the susceptibility of influenza viruses to innate serum inhibitors [28]. Our study showed that H3N2, the most susceptible subtype, has the highest number of glycosylation sites on HA as compared to the other virus subtypes, with 11–12 sites on the H3N2 complete HA amino acid sequences and 6–7 sites on the globular head. A previous study demonstrated that high degree of *N*-linked glycosylation could attenuate H3N2 influenza viruses. The addition of oligosaccharide moieties to globular head of HA had been linked to increased sensitivity of H3N2 viruses to SP-D and MBL [32]. Glycosylation sites on HA have accumulated in the human influenza H3N2 virus since its appearance in 1968. This gain of sites and their long-term maintenance are presumed to be due to a selective advantage of glycosylation [33]. Extensive evidences indicated that glycosylation plays important roles in the life cycle of influenza viruses by conferring structural integrity and stability of virus particles as well as modulating the functions of HA and NA in the recognition of

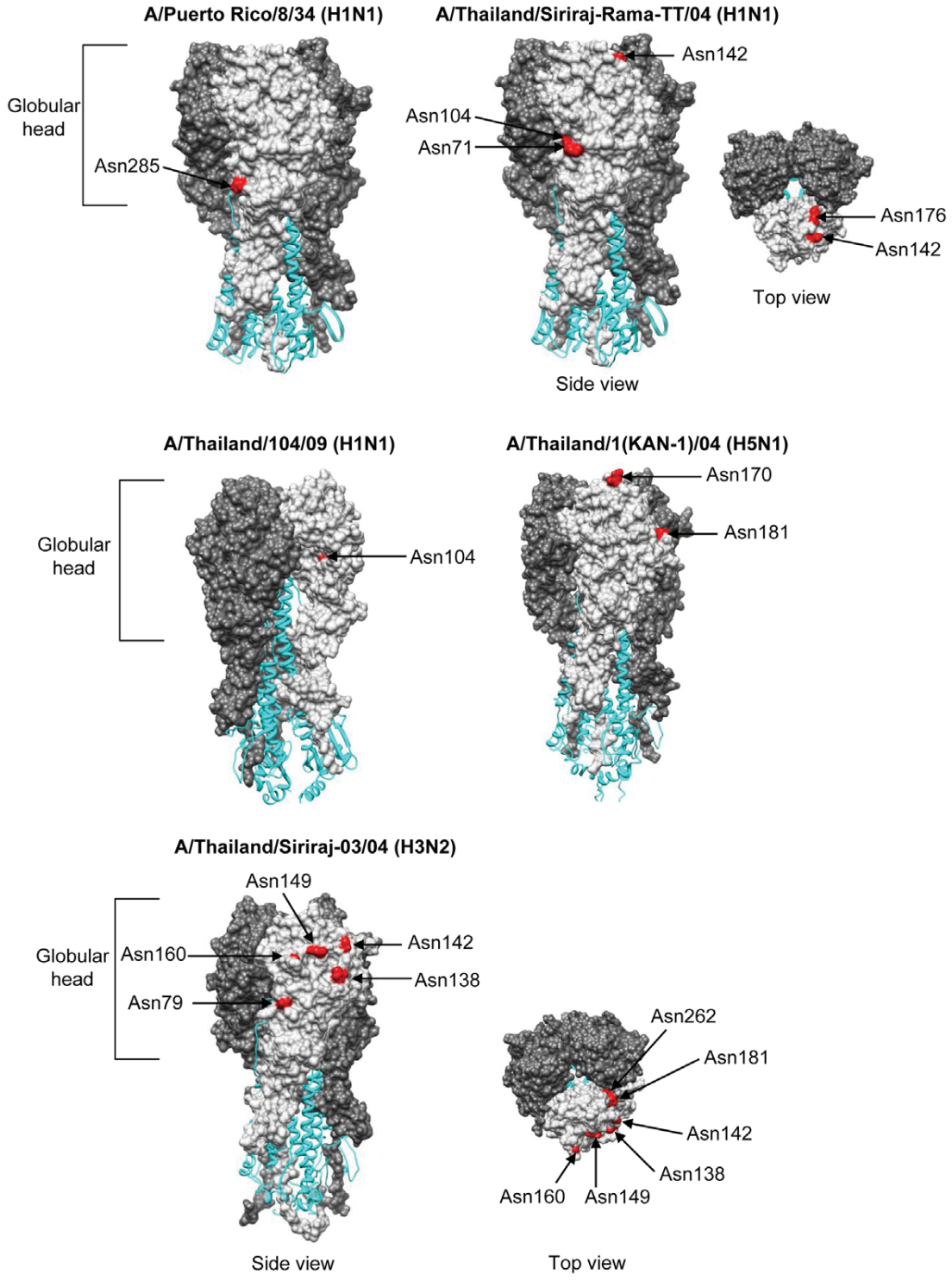


Figure 4. Potential *N*-linked glycosylation sites on HA globular domain. HA1 chains are displayed as space-filling models in HA trimer, while HA2 chains are displayed as ribbon. One of the three HA1 chains is highlighted in lighter shade than the other two HA1 chains. Model of H1N1pdm is related to models of PR8 by 60-degree counterclockwise rotation on vertical axis. The top view is related to the side view by 90-degree rotation on horizontal axis.
doi:10.1371/journal.pone.0036318.g004

host cell receptors, in particular [34]. Mannose-containing oligosaccharides on the virus envelope were shown to be a target for recognition and destruction by lectins of the innate immune system [35]. Based on the data obtained from H3N2 viruses, it is likely that the degree of viral neutralizing activity of our guinea pig sera was positively correlated to number of glycosylation sites

present on HA globular head. Nevertheless, our data from other human influenza subtypes does not support this hypothesis. Our reassorted PR8 and H1N1pdm viruses, which are moderately susceptible to serum inhibitors, contain only one glycosylation site on the HA globular head; while HPAI H5N1 viruses, which are the most resistant subtype, contain two sites. These findings suggested that the viral domains other than glycosylation sites on HA globular head could be the target for binding with serum innate inhibitors. In addition, our previous work also suggested that receptor binding site might not be the target of recognition by the serum inhibitors as 676(NYK) which preferred human type receptor (sialic acid-linked α2,6-galactose) was as resistant as the other H5N1 isolates which preferred avian type receptor (sialic acid-linked α2,3-galactose) [36].

Previous studies have shown genetic elements that contribute to the H5N1 viral virulence such as glutamine to lysine substitution at amino acid position 627 in PB2, the presence of multiple basic amino acids at the HA cleavage site, and the presence of a sequence motif, Glu-Ser-Glu-Val at the carboxyl terminus of NS1 protein [37]. Herein, our study suggests a novel mechanism to virulence of HPAI H5N1 viruses through their ability to resist serum innate immunity, enabling the viruses to disseminate beyond the respiratory tract, the primary organ of infection, to various visceral organs. The disseminated viruses may subsequently exert their immunopathological effect via an induction of cytokine storm and multi-organ failure, the hallmarks of HPAI H5N1 in humans.

Materials and Methods

Guinea pig sera

Guinea pig blood was purchased from the National Laboratory Animal Center, Mahidol University. When the blood clot retracted, serum was separated by spinning at 2,000 rpm in a refrigerated centrifuge, and then aliquoted and stored at −80°C. Guinea pig sera were negative for antibody to all study influenza viruses as determined by HI and microNT assays.

The viruses

Influenza A viruses in this study including seasonal H1N1 and H3N2, H1N1pdm and HPAI H5N1 viruses (Table 1), were propagated in MDCK cells (obtained from the American Type Culture Collection; CCL-34). These virus isolates were kindly provided by Siriraj Influenza Cooperative Research Center; and the routine service laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University.

Construction of influenza reassortants by reverse genetics

The pHW reverse genetic system [38] comprising 8 recombinant plasmids containing *PA*, *PB1*, *PB2*, *NP*, *HA*, *NA*, *M* or *NS* genomic segments derived from the parental PR8 virus were kindly provided by Prof. Robert Webster and Dr. Erich Hoffman, St. Jude Children Research Hospital, Memphis, Tennessee, USA. Three kinds of reverse genetic viruses harboring *HA*, *NA*, or both *HA* and *NA* genes derived from A/Thailand/1(KAN-1)/04 (H5N1) viruses were constructed. *HA* and *NA* genomic segments were amplified by RT-PCR using universal primers [39] and cloned individually into pHW2000 vector. *HA* segment (accession number AY555150) was engineered to eliminate the multiple basic amino acids at HA cleavage site (337-PQRERRRKKR-346 was changed to 337-PQ----IETR-346, H5 numbering) which mimics the cleavage site of the low pathogenic avian H6 virus before cloning; while the *NA* segment (accession number AY555151) was cloned unmodified. To construct the reverse genetic viruses, rgPR8-H5 HA (7+1), rgPR8-H5 NA (7+1) and rgPR8-H5 HANA (6+2) viruses, 1 μg of each of the 8 recombinant plasmids were transfected into HEK-293 cells co-culturing with MDCK cells using *Trans*IT®-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI). After 24 hours, the reassortants were rescued by adding with 1 ml of Opti-MEM® I Reduced Serum Medium (Invitrogen, Carlsbad, CA) containing 2 μg/ml of TPCK-treated trypsin (Sigma, St. Louis, MO). The culture plate was further incubated at 37°C for 24–48 hours, observed for cytopathic effect and screened for progeny viruses released in the culture supernatant by hemagglutination assay. In addition, all 8 pHW recombinant plasmids containing each gene of PR8 origin were transfected into HEK-293 cells co-culturing with MDCK cells to generate reassorted PR8 virus as the control of the experiments. The reassortants were propagated and titrated in MDCK cells.

HI assay

HI assay was performed based on the protocol as described in the WHO manual [40]. Goose RBCs were employed in the assay for antibody to HPAI H5N1 and H1N1pdm. On the other hand guinea pig RBCs were employed in the assay for antibody to the other human influenza viruses. Briefly, 50 μl of guinea pig serum was mixed with 150 μl of RDE (Denka Seiken Co. Ltd., Tokyo, Japan) and incubated at 37°C for 16–18 hours to eliminate the nonspecific inhibitors. The treated serum was inactivated at 56°C for 30 minutes and absorbed with the test RBCs to remove nonspecific agglutinators. The replicating influenza virus at the final concentration of 4 hemagglutination units/25 μl was used as the test antigen. The treated serum was serially two-fold diluted at an initial dilution of 1:10 into a final volume of 25 μl per well before 25 μl of the test antigen was added. After incubation at room temperature for 30 minutes, 50 μl of 0.5% goose RBCs (for HI antibody to H1N1pdm and H5N1 viruses) or 0.75% guinea pig RBCs (for HI antibody to seasonal H1N1 and H3N2 viruses) were added into every well and further incubated the reaction plate at 4°C 30 minutes (for goose RBCs) or 60 minutes (for guinea pig RBCs). HI titer was defined as the reciprocal of the last serum dilution that completely inhibited hemagglutination of RBCs.

MicroNT assay

Guinea pig sera were screened for the antibodies to the test influenza viruses by microNT assay using the protocol as described previously [40]. Guinea pig serum was treated with RDE before heat inactivation at 56°C for 30 minutes. The treated sera were serially two- fold diluted with 1X EMEM at an initial dilution of 1:10 in a microtiter plate. The diluted serum was incubated with the test virus at the final concentration of 100 TCID50/100 μl for 2 hours at 37°C. Then, 100 μl of each virus-antibody mixture was transferred onto the MDCK cell monolayer maintained in EMEM supplemented with trypsin TPCK (Sigma, St. Louis, MO), and incubated overnight at 37°C, 5% CO_2. The inoculated MDCK cell monolayers in the reaction plates were fixed with 80% acetone prior to determining for presence of viral nucleoprotein by ELISA. Antibody titer was defined as the highest serum dilution that yielded a 50% reduction in the amount of nucleoprotein as compared to the virus infected cell control.

Complement titration

Guinea pig serum contains many soluble factors, including complement factors that confer activity against influenza virus. The amount of complement present in each guinea pig serum can be measured by titration against hemolysin-sensitized SRBCs [41]. Equal volume of 2% SRBCs was mixed with appropriate

concentration of hemolysin (Sigma-Aldrich, St. Louis, MO) for 1 hour with shaking at intervals to obtain hemolysin sensitized SRBCs. The test guinea pig serum was serially two-fold diluted with veronal buffer to obtain a volume of 25 µl per well in a U-shaped microtiter plate. Then, the serum was incubated with hemolysin sensitized SRBCs for 45 minutes with shaking at intervals. In the presence of serum complement, the sensitized SRBCs would be lysed; while the SRBCs and sensitized SRBC controls appeared as a red button at the bottom of the well. One hemolytic unit was defined as the highest dilution of complement which yielded complete hemolysis of the sensitized SRBCs. Owing to heat-labile nature of the complement proteins, guinea pig sera were stored at -70°C; and complement titration was conducted prior to each use.

Assay for inhibition of influenza virus infection by soluble factors in guinea pig serum

Guinea pig serum at the working concentration of 2 complement hemolytic units was incubated with the test virus at a final concentration of 1,000 TCID50/50 µl at 37°C for 45 minutes in quadruplicate wells. Then, the mixtures were transferred onto MDCK cell monolayers and incubated overnight at 37°C prior to measuring the amount of viral nucleoprotein in the infected MDCK cell monolayers by ELISA. In the presence of serum inhibitors, viral infection was blocked, resulting in the decreased amount of viral nucleoprotein synthesized in the inoculated culture as compared to the infected MDCK cell controls. In the case where serum inhibitors could not inhibit viral infection, the high O.D. values indicating viral nucleoprotein synthesis would be observed. The percentages of viral inhibition were calculated based on the O.D. values as compared to the virus control without serum.

The assay employed native guinea pig serum was conducted in parallel with the heat-inactivated serum and together with the RDE-treated+heat-inactivated serum. Heat treatment to inactivate the β-inhibitors was performed by treating the guinea pig serum at 56°C for 30 minutes and then cooled on ice. RDE treatment was conducted to destroy α- and γ-inhibitors such that the guinea pig serum was mixed with RDE according to the protocol used for HI assay, followed by heat inactivation at 56°C for 30 minutes to get rid of RDE activity and β-inhibitors.

ELISA

ELISA for influenza viral nucleoprotein was performed with mouse specific monoclonal antibody against influenza nucleoprotein (Catalog number MAB8257, Millipore Corporation, Temecula, CA) as the primary antibody. Horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig (Catalog number 1010-05, Southern Biotech, Birmingham, AL) was used as the secondary antibody. The optical density was measured at dual wavelengths of 450/630 in a spectrophotometer.

Statistical analysis

Statistical analysis was done with PASW Statistics 18 software. The percentages of viral inhibition by native sera, heat-inactivated sera and RDE-treated+heat-inactivated sera were analyzed using analysis of variance (ANOVA). Difference between two data sets was examined using Student's *t*-test. The significance level was set at $p \leq 0.05$.

Acknowledgments

We thank Miss Malinee Chittaganpitch, the National Influenza Reference Laboratory, National Institute of Health, Department of Medical Science, Ministry of Public Health, Thailand for influenza strain identification.

Author Contributions

Conceived and designed the experiments: P. Puthavathana. Performed the experiments: JP NN OS KS P. Pooruk. Analyzed the data: NN PN BS HL. Contributed reagents/materials/analysis tools: P. Puthavathana PA. Wrote the paper: P. Puthavathana.

References

1. Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, et al. Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus (2008) Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med 358: 261–273.
2. Peiris JS, de Jong MD, Guan Y (2007) Avian influenza virus (H5N1): a threat to human health. Clin Microbiol Rev 20: 243–267.
3. Piwpankaew Y, Monteerarat Y, Suptawiwat O, Puthavathana P, Uipresertkul M, et al. (2010) Distribution of viral RNA, sialic acid receptor, and pathology in H5N1 avian influenza patients. APMIS acta pathologica microbiologica et immunologica Scandinavica 118: 895–902.
4. de Jong MD, Bach VC, Phan TQ, Vo MH, Tran TT, et al. (2005) Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med 352: 686–691.
5. Gu J, Xie Z, Gao Z, Liu J, Korteweg C, et al. (2007) H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 370: 1137–1145.
6. Chutinimitkul S, Bhattarakosol P, Srisuratanon S, Eiamudomkan A, Kongsomboon K, et al. (2006) H5N1 influenza A virus and infected human plasma. Emerg Infect Dis 12: 1041–1043.
7. Tse H, To KK, Wen X, Chen H, Chan KH, et al. (2011) Clinical and virological factors associated with viremia in pandemic influenza A/H1N1/2009 virus infection. PLoS One 6: e22534.
8. Naficy K (1963) Human influenza infection with proved viremia-Report of a case. N Engl J Med 269: 964–966.
9. Khakpour M, Saidi A, Naficy K (1969) Proved viraemia in Asian influenza (Hong Kong variant) during incubation period. Br Med J 4: 208–209.
10. Tsuruoka H, Xu H, Kuroda K, Hosaka Y (1997) Viremia in influenza: detection by polymerase chain reaction. Nihon Rinsho 55: 2714–2718.
11. Hartshorn KL, White MR, Shepherd V, Reid K, Jensenius JC, et al. (1997) Mechanisms of anti-influenza activity of surfactant proteins A and D: comparison with serum collectins. Am J Physiol 273(6 Pt 1): L1156–L1166.
12. Reading PC, Morey LS, Crouch EC, Anders EM (1997) Collectin-mediated antiviral host defense of the lung: evidence from influenza virus infection of mice. J Virol 71: 8204–8212.
13. Leth-Larsen R, Nordenbaek C, Tornoe I, Moeller V, Schlosser A, et al. (2003) Surfactant protein D (SP-D) serum levels in patients with community-acquired pneumonia. Clin Immunol 108: 29–37.
14. Reading PC, Bozza S, Gilbertson B, Tate M, Moretti S, et al. (2008) Antiviral activity of the long chain pentraxin PTX3 against influenza viruses. J Immunol 180: 3391–3398.
15. Horváth A, Andersen I, Junker K, Lyck Fogh-Schultz B, Holm Nielsen E, et al. (2001) Serum amyloid P component inhibits influenza A virus infections: in vitro and in vivo studies. Antiviral Res 52: 43–53.
16. Kase T, Suzuki Y, Kawai T, Sakamoto T, Ohtani K, et al. (1999) Human mannan-binding lectin inhibits the infection of influenza A virus without complement. Immunology 97: 385–392.
17. Job ER, Deng YM, Tate MD, Bottazzi B, Crouch EC, et al. (2010) Pandemic H1N1 influenza A viruses are resistant to the antiviral activities of innate immune proteins of the collectin and pentraxin superfamilies. J Immunol 185: 4284–4291.
18. Blue CE, Spiller OB, Blackbourn DJ (2004) The relevance of complement to virus biology. Virology 319: 176–184.
19. Reading PC, Hartley CA, Ezekowitz RA, Anders EM (1995) A serum mannose binding lectin mediates complement dependent lysis of influenza virus-infected cells. Biochem Biophys Res Commun 26: 1128–1136.
20. Anders EM, Hartley CA, Reading PC, Ezekowitz RA (1994) Complement-dependent neutralization of influenza virus by a serum mannose-binding lectin. J Gen Virol 75: 615–622.
21. Jayasekera JP, Moseman EA, Carroll MC (2007) Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. J Virol 81: 3487–3494.
22. Cohen A, Belyavin G (1961) The influenza virus hemagglutination inhibitors of normal rabbit serum: I. Separation of the inhibitory components. Virology 13: 58–67.

23. Krizanová O, Rathová V (1969) Serum inhibitors of myxoviruses. Curr Top Microbiol Immunol 47: 125–151.
24. Anders E, Hartley C, Jackson D (1990) Bovine and mouse serum beta inhibitors of influenza A viruses are mannose-binding lectins. Proc Natl Acad Sci U S A 87: 4485–4489.
25. Pritchett TJ, Paulson JC (1989) Basis for the potent inhibition of influenza virus infection by equine and guinea pig α2-macroglobulin. J Biol Chem 15: 9850–9858.
26. Cwach KT, Sandbulte HR, Klonoski JM, Huber VC (2012) Contribution of murine innate serum inhibitors toward interference within influenza virus immune assays. Influenza Other Respi Viruses 6: 127–135.
27. Tate MD, Brooks AG, Reading PC (2011) Specific sites of *N*-linked glycosylation on the hemagglutinin of H1N1 subtype influenza A virus determine sensitivity to inhibitors of the innate immune system and virulence in mice. J Immunol 187: 1884–1894.
28. Tate MD, Job ER, Brooks AG, Reading PC (2011) Glycosylation of the hemagglutinin modulates the sensitivity of H3N2 influenza viruses to innate proteins in airway secretions and virulence in mice. Virology 413: 84–92.
29. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98.
30. Steel J, Lowen AC, Wang TT, Yondola M, Gao Q, et al. (2010) Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio 1: e00018–10.
31. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, et al. (2004) UCSF Chimera–a visualization system for exploratory research and analysis. J Comput Chem 25: 1605–1612.
32. Vigerust DJ, Ulett KB, Boyd KL, Madsen J, Hawgood S, et al. (2007) *N*-linked glycosylation attenuates H3N2 influenza viruses. J Virol 81: 8593–8600.
33. Cherry JL, Lipman DJ, Nikolskaya A, Wolf YI (2009) Evolutionary dynamics of *N*-glycosylation sites of influenza virus hemagglutinin. PLoS Curr 1: RRN1001.
34. Vigerust DJ, Shepherd VL (2007) Virus glycosylation: role in virulence and immune interactions. Trends Microbiol 15: 211–218.
35. Reading PC, Tate MD, Pickett DL, Brooks AG (2007) Glycosylation as a target for recognition of influenza viruses by the innate immune system. Adv Exp Med Biol 598: 279–292.
36. Auewarakul P, Suptawiwat O, Kongchanagul A, Sangma C, Suzuki Y, et al. (2007) An avian influenza H5N1 virus that binds to a human-type receptor. J Virol 81: 9950–9955.
37. Krug RM (2006) Virology. Clues to the virulence of H5N1 viruses in humans. Science 311: 1562–1563.
38. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A 97: 6108–6113.
39. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146: 2275–2289.
40. World Health Organization (2002) WHO manual on animal influenza diagnosis and surveillance. Available: http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf. Accessed 13 October 2011.
41. Hawkes RA (1979) General principle underlying laboratory diagnosis of viral infection. In: Lennette EH, Schmidt NJ, eds. Diagnostic procedures for viral, rickettsial and chlamydial infection, 5th ed. Washington DC: American Health Association Inc. pp 32–41.

The difference in IL-1β, MIP-1α, IL-8 and IL-18 production between the infection of PMA activated U937 cells with recombinant vaccinia viruses inserted 2004 H5N1 influenza HA genes and NS genes

Molvibha Vongsakul,[1] Jitra Kasisith,[1] Pirom Noisumdaeng[2] and Pilaipan Puthavathana[2]

Summary

***Background*: The severity of avian influenza H5N1 disease is correlated with the ability of the virus to induce an over production of pro-inflammatory cytokines from innate immune cells. However, the role of each virus gene is unknown. To elaborate the function of each virus gene, the recombinant vaccinia virus inserted HA and NS gene from the 2004 H5N1 virus were used in the study.**

***Methods*: U937 cells and PMA activated U937 cells were infected with recombinant vaccinia virus inserted with HA or NS gene. The expressions of HA and NS proteins in cells were detected on immunofluorescence stained slides using a confocal microscope. The cytokine productions in the cell supernatant were quantitated by ELISA.**

***Results*: The recombinant vaccinia virus inserted with HA genes induces the production of IL-1β, MIP-1α, IL-8 and IL-18 cytokines from PMA activated U937 cells significantly more than cells infected with wild type vaccinia, whereas the recombinant vaccinia virus inserted with NS genes it was similar to that with the wild type vaccinia virus. However, there was no synergistic nor antagonistic effect of HA genes and NS genes in relation to cytokines production.**

Conclusion*: Only the HA gene from the 2004 H5N1 virus induces IL-1β, MIP-1α, IL-8 and IL-18 cytokine productions from activated U937 cells. The same HA gene effect may or may not be the same in respiratory epithelial cells and this needs to be explored. *(Asian Pac J Allergy Immunol 2011;29:349-56)

Key words: *H5N1 influenza viruses, cytokines, U937 cells, HA, NS*

Abbreviations

H5N1 influenza	=	Influenza A virus subtype of hemagglutinin type 5 and neuraminidase type 1 surface antigens
HA	=	Hemagglutinin gene
NS	=	Non-structural gene
PMA	=	Phorbol myristate acetate
r-HA	=	Recombinant hemagglutinin gene
r-NS	=	Recombinant non-structural gene

Introduction

Avian influenza in humans is severe and fatal. The severity of the disease correlates with an ability of the virus to induce an over production of pro-inflammatory cytokines.[1-5] The cytokine response occurs, whereas a specific immune response has hardly been detected. Thus, a cytokine storm is the immunopathology mediated by innate immunity in H5N1 infection. Pro-inflammatory cytokines are produced in bronchial epithelial cells infected with H5N1 viruses[6] and it is also well documented that innate mononuclear cells, monocytes and macrophages, are the major cell types that release a variety of cytokines.[1,7] Information elucidated from these studies was derived from using whole virions, either wild type or reverse genetic viruses. It can not be ruled out that the interplay between different genes or their products, either RNA-RNA interaction or RNA-protein interaction, may influence certain

From the [1]Department of Microbiology, Faculty of Science Mahidol University, Bangkok, Thailand
[2]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
Corresponding author: Molvibha Vongsakul
E-mail: scmvs@mahidol.ac.th
Submitted date: 5/7/2011
Accepted date: 7/10/2011

gene expression and function. The only way to study individual gene function is to create a recombinant virus containing the gene of interest.

This study aimed to investigate cytokine released in culture supernatants of PMA activated U937 cells infected with recombinant vaccinia virus inserted with HA (r-HA vaccinia) or NS (r-NS vaccinia) genes from the 2004 H5N1 virus by using the ELISA technique. Confocal microscopy of immunofluorescence staining for HA or NS protein and RT-PCR for mRNA in infected cells was performed. The results show that (compared to the wild type vaccinia) chemokines; MIP-1α and IL-8, antiviral cytokine; IL-18 and proinflammatory cytokine; IL-1β are significantly released in the supernatant of PMA activated U937 cell infected with r-HA vaccinia whereas TNFα, another pro-inflammatory cytokine, is not. This effect on cytokines production is not observed for r-NS infection. This study demonstrates the direct effect of either HA or NS without any interference by other influenza genes in cytokines production in activated monocytes. Moreover, the results suggest the absence of NS gene suppressive effect and an antagonistic effect on cytokines production induced by HA gene expression.

Methods

Cell preparation

U937, a human monocyte cell line, was cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum, 2.5 mM glutamine, 10mM HEPES, 10,000U/ml penicillin and 10,000µg/ml streptomycin. The cells were activated by incubating them with 10 nM of phorbol myristate acetate (PMA) (Sigma, Mo. U.S.A) for 24 hours (activated U937 cell).

Viruses

The viruses used in this study were: Vaccinia virus Lister strain (wild type control), recombinant vaccinia virus inserted with HA (r-HA vaccinia) and recombinant vaccinia virus inserted with NS (r-NS vaccinia). Both the HA and NS genes of the two recombinant viruses were derived from A/Thailand/1(KAN-1)/04 (H5N1) (sequences of both genes can be accessed from GenBank with accession numbers AY555150 and AY626146 for HA and NS respectively). These viruses were propagated and titrated in TK cells. Titers were presented as plaque forming units /ml.

Virus infection in U937cell culture

Resting (no PMA) or activated (with PMA) U937 cells at a concentration of $1x10^6$ cells/ml in 2% FBS RPMI 1640 were infected in triplicate with wild type vaccinia or r-HA vaccinia or r-NS vaccinia at a multiplicity of infection (M.O.I.) of 0.05 in a 96 well- tissue culture plate. An M.O.I. of 0.1 was obtained by double infection with both types of the recombinant viruses. Infection was carried out for 24 hours at 37°C in a humidifying CO_2 incubator. Thereafter, culture supernatants were harvested and kept at -80°C for cytokine assays by ELISA. Meanwhile; cell pellets were washed once with PBS and divided into two portions; one portion was used for RNA extraction by TRIzol® Reagent (Invitrogen, Carlsbad, CA., USA.) and another portion was smeared on microscopic slides for immune-fluorescence staining.

Semiquantitation for HA or NS mRNA by RT-PCR

Total RNA extracts were reverse transcribed into cDNA by using AMV reverse transcriptase™ first-strand kit (Invitrogen, Carlsbad, CA., USA.). The cDNA was further used as template in polymerase chain reactions (PCR). The primer pairs were: HA-H5f:5'-ACTCCAATGGGGGCGATAAA-3', HA-H5r:5'-CAACGGCC TCAAACTGAGTGT-3'and SNSF:5'-GATAAGGCACTTAAAATGCCG-3'and SNSR: 5'-ACGGTGAGATTTCTCCCACG-3'. The β-actin gene was amplified as the internal control 5'-ATC TGG CAC CAC ACT TCT ACA-3' as the forward primer and 5'- GTT TCG TGG ATG CCA CAG GAC T-3'as the reverse primer. Amplicons were analysed by densitometer (Amersham Biosciences, England) using Image Scanner Software (Image MasterTotal Lab version 2.01) analysis. The percentage of gene expression originating from HA and NS mRNA were semiquantitated by comparing them to that of the β-actin gene.

Immunofluorescence assay for HA or NS protein

Slides of cell deposits were air-dried and fixed in pre-cooled acetone at -20°C for 10 minutes. The slides were stained immediately, or otherwise kept at -70°C until staining by the indirect immunofluorescence technique. Goat antiserum against H5N1 HA (kindly provided by Dr. Richard Webby, St Jude Children Research Hospital) or goat anti-NS peptides antiserum (Santa Cruz Biotechnology Inc., CA., USA) was used as the primary antibody, FITC conjugated rabbit anti-goat Ig was used as the second antibody. Uninfected cells and cells infected

with wild type vaccinia virus were used as negative controls. The stained slides were observed for fluorescence using a Confocal Laser Scanning Biological Microscope (FV1000 Fluoview, Olympus, Tokyo, Japan).

Cytokine quantitation by ELISA

Culture supernatants were assayed for pro-infammatory cytokines: TNFα and IL-1β, chemokines: MIP-1α and IL-8, and antiviral cytokines: IFNα and IL-18 by ELISA kits (R&D Systems Inc., Minneapolis, USA.). The test protocols followed those described in the kit instructions. The reproducibility of the results was confirmed by repeating the experiments in culture supernatants collected from the three separate experiments.

Statistical analysis

The cytokine concentrations from ELISA (in triplicate) were analysed using Prism software (GraphPad prism 4 Software). Nonparametric, one tailed Mann Whitney u test was used; a p value < 0.05 was considered statistically significant.

Results

HA or NS proteins are more strongly expressed in PMA activated U937 cells than in resting/non-activated U937 cells

Compared to the results of semi-quantitation of mRNA for HA and NS band density of RT-PCR (% band density for HA from PMA activated U937 is 152.5 and non-activated is 96), the amount of positive immunofluorescence staining in PMA activated U937 cells was higher and more intense than in non-activated U937 cells as shown in Figure 1A and 1B for anti HA staining, whereas there was negative staining in wild type vaccinia infected cells.

A similar pattern is observed for anti NS staining as shown in Figure 2A and 2B (% band density for NS from PMA activated U937 is 41.7 and non-activated is 12.4).

Cytokine productions in resting/non-activated U937 cell

There were no differences in cytokines production from non activated U937 cells infected with r-HA vaccinia or r-NS vaccinia or both or wild vaccinia. Three groups of cytokine effector functions were investigated; these are pro-inflammatory cytokines (TNFα and IL-1β), chemokines (MIP-1α and IL-8) and antiviral cytokines (IFNα and IL-18). The concentrations of these 6 cytokines in the

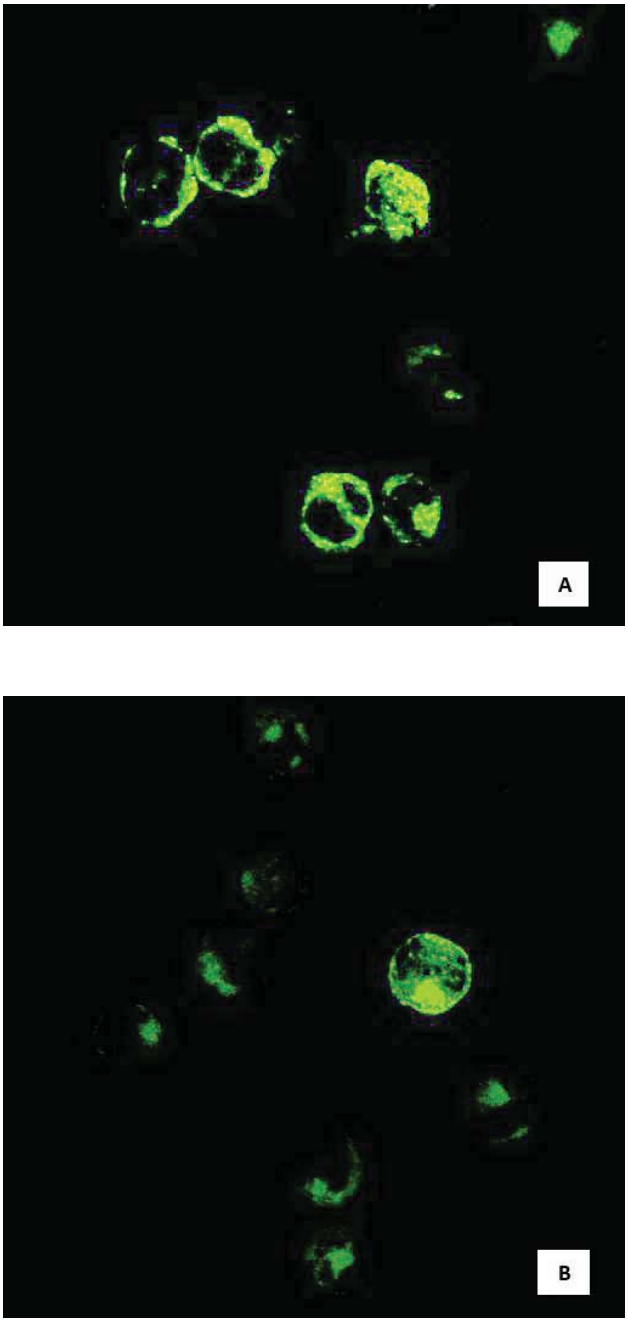


Figure 1. A. Anti HA immunofluorescence staining of PMA activated U937 cells infected with recombinant HA vaccinia (r-HA vaccinia), observed by UV light under a confocal microscope (400 magnification).
B. Anti HA immunofluorescence staining of U937 cells infected with recombinant HA vaccinia (r-HA vaccinia), observed by UV light under a confocal microscope (400 magnification).

supernatants of non activated U937 cells were not different in all infections, the average cytokine concentrations from 3 separate infections (n=3) of r-HA, r-NS and dual vaccinia infections were not different from wild type vaccinia infection.

Cytokine production in activated U937 cell

Cytokine production from r-HA vaccinia or r-NS vaccinia or both infected PMA activated U937 cells were different. The cytokine concentrations in the supernatants of infected PMA activated U937 cells were different in rHA vaccinia, rNS vaccinia or dual infections. rHA vaccinia infection induces increased production of TNF α, IL-1 β (significantly), MIP-1 α (significantly), IL-8 (significantly) and IL-18, compared to wild vaccinia infection. On the contrary, the rNS vaccinia infection did not induce any effect. The cytokine concentrations in rNS vaccinia infection supernatants were the same as in wild vaccinia infection. Furthermore, in supernatants of the dual infections with rHA and rNS vaccinia, the TNF α, IL-1 β, MIP-1 α and IL-18 levels were significantly higher than in the supernatant of wild vaccinia infection, but the levels of the cytokine concentrations were almost the same as observed in supernatants of rHA vaccinia infections, as shown in Figure 3A-F.

Discussion

Cytokine dysregulation is proposed to be a mechanism explaining the unusual severity of avian influenza. High levels of inflammatory cytokines such as TNFα , IL-1 and IL-6 or chemokines such as IL-8 and CCL2 were detected in the patients' serum.[2,3,5] *In vitro* studies had demonstrated hyper-production of pro-inflammatory cytokines in macrophages infected with H5N1 viruses.[1] Excessive infiltration of macrophages and neutrophils together with significantly higher levels of pro-inflammatory cytokines were noted in the lungs of mice infected with 1918 H1N1 virus and in those had had recent H5N1 virus infection with A/Thailand/SP/83/2004 and A/Thailand/16/2004.[8] Based on the knowledge that macrophages could be infected by H5N1 viruses and macrophage is the major source of innate cytokine, the present study used U937 as the cell source to explore the effect of the H5N1 HA gene and the NS gene, and their interaction, on cytokine production. An advantage of this study was the use of recombinant vaccinia viruses, so that the influence of the HA or NS gene could be studied

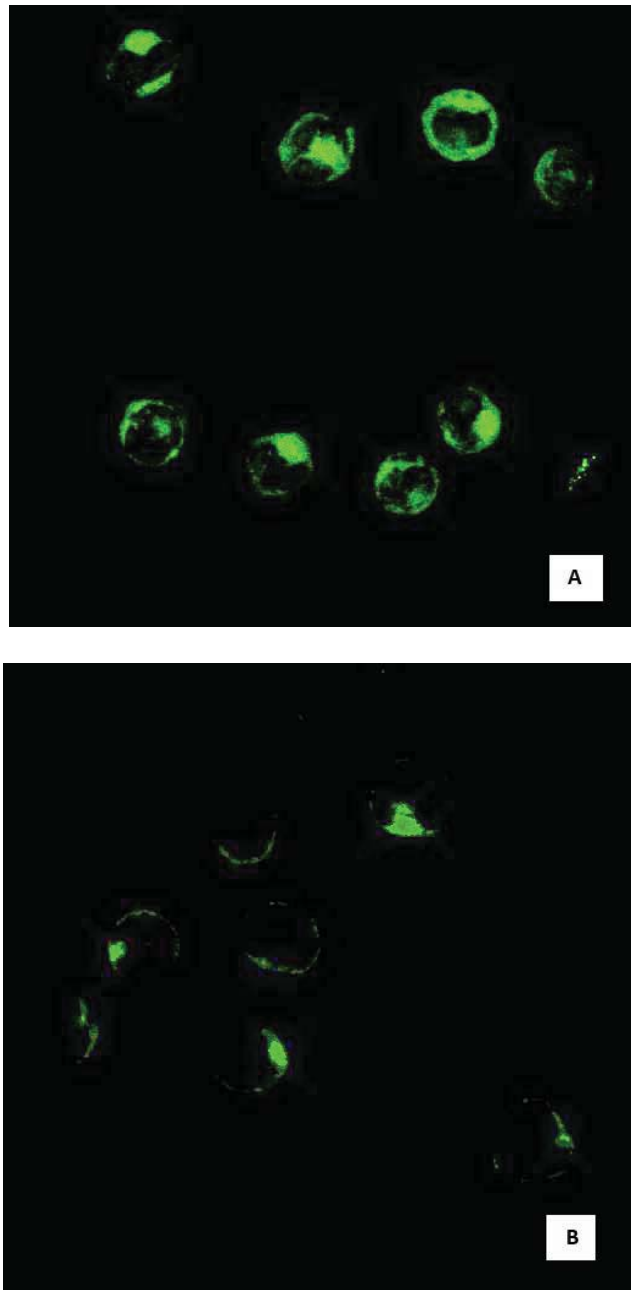
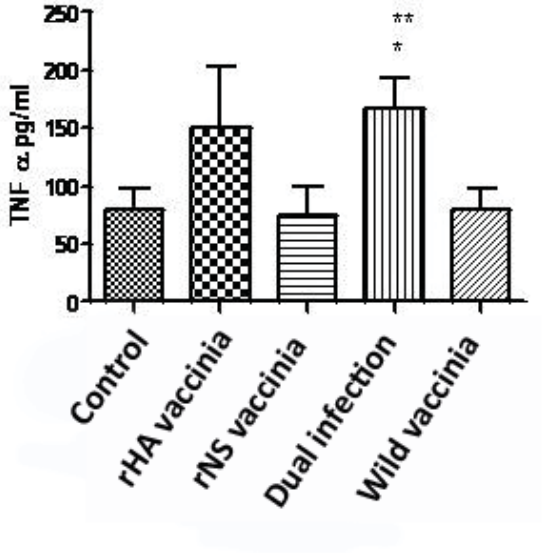


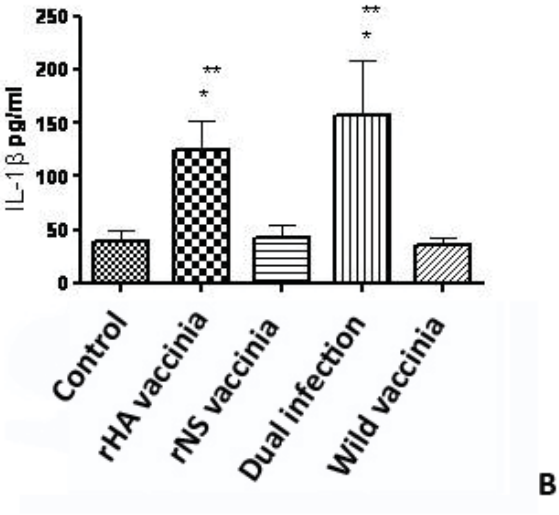
Figure 2. A. Anti NS immunofluorescence staining of PMA activated U937 cells infected with recombinant NS vaccinia (r-NS vaccinia), observed by UV light under a confocal microscope (400 magnification).
B. Anti NS immunofluorescence staining of U937 cells infected with recombinant NS vaccinia (r-NS vaccinia), observed by UV light under a confocal microscope (400 magnification).

without interference from the other influenza genes. Wild type vaccinia virus, as the parent of recombinant virus, provided the background levels of the cytokines released. In addition, using U937 cells provided advantages as well, since the essentials of the cell condition in HA or NS gene expression and cytokine production can be elucidated. The PMA stimulated U937 cells were demonstrated to exhibit increased expression of a variety of cellular molecules, including the ability to mediate chemotaxis and phagocytosis, i.e., the stimulated cells became activated and mature.[9] The present study showed that viral infection in activated U937 cells induced higher levels of influenza specific mRNA, protein expression and of all the cytokines studied, when compared with the infection in resting cells. No cell death was observed either in resting or activated cells under the conditions of infection at an M.O.I. of 0.05 or 0.1 in case of double infection for 24 hours. This study suggests that activation of U937 cells or the monocyte maturation may provide biochemical conditions that allow the HA or NS gene to exert their influence on the cytokines synthesis. This effect may or may not be the same as that of dendritic cells, which was reported by other investigators.[10] A low amount of the chemokine, MIP-1α, was detected in the cell supernatant of rHA-vaccinia infected non-activated U937 cells, which was the same level in cell supernatant of wild vaccinia infection. This is normally detected in virus infection such respiratory virus infection.[11] However, significantly increased production of both detected chemokines, MIP-1 α and IL-18 levels was observed in the cell supernatant of rHA vaccinia infected activated U937 cells (Figure 3C and 3D). This may suggest that chemokines are cytokines produced to influence the immune cell recruitment to influenza infected cells. TNFα is a cytokine that plays this role or functions as "intracrine".[12] TNFα probably plays this role in H5N1 influenza infection too. Others have reported the hyper-production of TNFα in human macrophages infected with the 1997 H5N1 virus and the high production of MIP-1α, IL-1α, KC (mouse equivalent to human IL-8), IL6, MCP-1 and IFN-γ lung of mice infected with 1918 influenza virus or recent H5N1 virus.[8] Our results show non-significant production of TNFα in 24 hours the supernatant of rHA-vaccinia infection in activated U937 cells but significant production of MIP-1α and IL-8, Figure 3A, 3C, 3D. This may indicate that during the 24 hr period of rHA-vaccinia infection TNFα effected chemokines induction and was then used as an autocrine. The cytokine storm is hypothesized to be the major cause of the unusual disease severity in avian influenza and during that period of time TNFα should be produced from dendritic cells; however, cytokine inhibition did not protect mice against lethal challenge with H5N1 virus.[13]

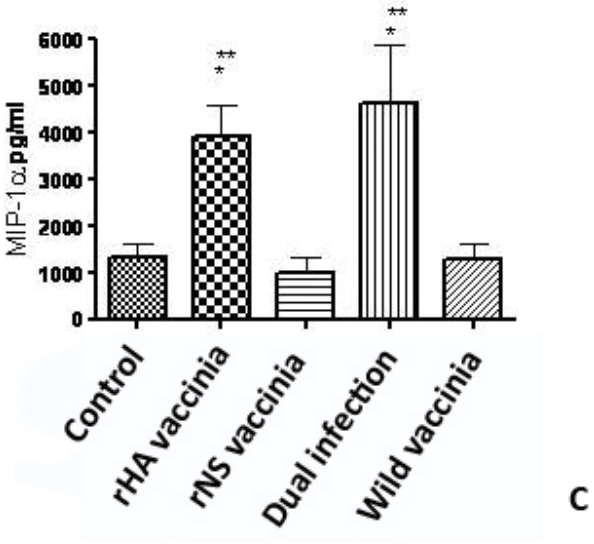
In contrast to the increase in cytokines production in rHA-vaccinia infection, this study demonstrated that rNS-vaccinia was a poor cytokine inducer. Levels of cytokines induced by rNS-vaccinia were not significantly different from those induced by wild type vaccinia virus. Moreover, the levels of cytokines synthesized in cells infected with both rHA-vaccinia and rNS-vaccinia, the dual infection, were not different from those released from cells infected with rHA-vaccinia. This result implies that the NS gene transcription and translation processes do not exert any antagonistic nor synergistic effect in cytokine induction mediated by processes in the HA gene, at least in activated U937 cells. The first H5N1 avian influenza outbreak in 1997 caused the disease in 18 human cases with six deaths, or a fatality rate of 33%.[14] The current H5N1 avian influenza epidemic is even more disastrous and covers a wide geographical area. Even though cytokine dysregulation is proposed to be the mechanism of disease severity in patients from both outbreaks, the NS protein of H5N1 viruses that caused the recent outbreak does not possess Glu92, but Asp92 instead. Nevertheless, the recent viruses possess Ala149.[14] Role of Glu92 and Asp149 on the virulence and pathogenicity of avian influenza are in doubt as a result of the study done by Perrone, et al.[8] The study involved two recent isolates from fatal cases: A/Thailand/16 and A/Thailand/SP/83, which both possess Glu92 and Asp149.[8,15] A/Thailand/16 was highly virulent in the mouse model with a mouse lethal dose 50 (MLD50) of 1.7, while A/Thailand/SP/83 was of low virulence with an MLD50 of 5.5.[8] These previous studies together with our results have led to the suggestion that the NS1 gene alone is not enough to elicit cytokine dysregulation. Moreover, the interplay between HA and NS genes in double infected cultures did not lead to an enhancement of cytokine synthesis. The results of the present study suggests that the severity of avian influenza might be derived from the interplay between genes, apart from HA and NS genes, within cassette of the viral genome. Essentially, this study demonstrated an important role for HA in certain cytokine inductions.



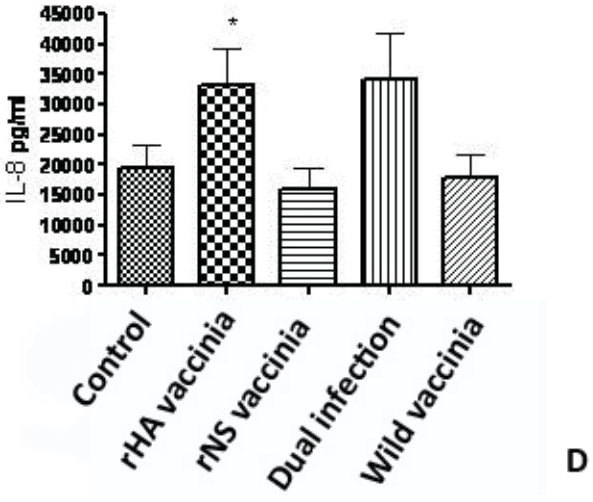
A



B



C



D

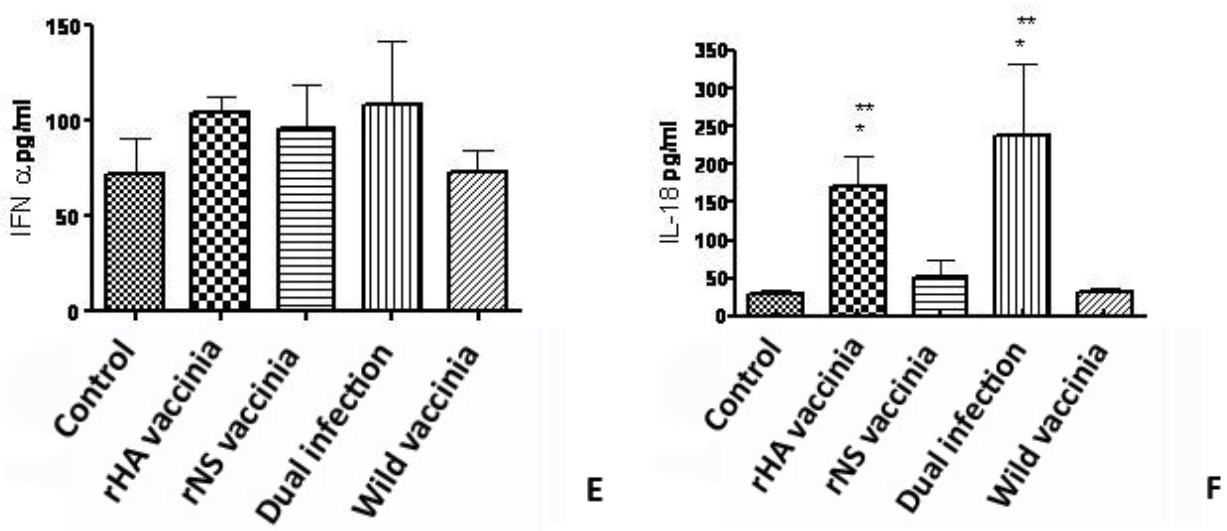


Figure 3. A. Histogram shows TNFα cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

B. Histogram shows IL-1β cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

C. Histogram shows MIP-1α cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

D. Histogram shows IL-8 cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

E. Histogram shows IFNα cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

F. Histogram shows IL-18 cytokine levels (from 3 separate experiments) in supernatants of infected PMA activated U937 cells with r-HA Vaccinia, r-NS Vaccinia, r-HA and r-NS vaccinia (dual infection), Wild vaccinia and non-infected (control).

*statistically significant against wild vaccinia infection,

**statistically significant against non-infected control (PMA activated U937 cell).

Acknowledgment

This study was supported by the National Institute for Genetic Engineering and Biotechnology, and the Thailand Research Fund for Senior Research Scholar. The authors are grateful to Prof. Bernard Moss, the National Institute of Health, U.S.A. for providing pSC11 plasmid used in the construction of recombinant vaccinia viruses.

References

1. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet. 2002;360:1831-7.
2. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med. 2006;12:1203-7.

3. The Writing Committee of the World Health Organization (WHO) Consultation on Human Influenza A/H5, Avian influenza A (H5N1) infection in humans. N Engl J Med. 2005;353:1374-85.
4. Wong SS, Yuen KY. Avian influenza virus infections in humans. Chest. 2006;129: 156-68.
5. La Gruta NL, Kedzierska K, Stambas J, Doherty PC, A question of self- preservation: immunopathology in influenza virus infection. Immunol Cell Biol. 2007;85:85-92.
6. Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO , et al. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir Res. 2005;6:135.
7. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol. 2008;26:421-52.
8. Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS pathogens. 2008 4 e1000115.
9. Minta JO, Pambrun L. *In vitro* induction of cytologic and functional differentiation of the immature human monocyte like cell line U-937 with phorbol myristate acetate. Am J Pathol. 1985;119:111-26.
10. Nargorsen D, Marincola FM, Panelli MC. Cytokine and chemokine expression profiles of maturating dendritic cells using multiprotein platform array. Cytokine 2004;25:31-5.
11. Neuzil KM, Graham BS. Cytokine release innate immunity in respiratory viral infection. Semin Virol. 1996;7:255-264.
12. Oppenheim JJ, Zachariae COC, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene " Intracrine" cytokine family. Annu Rev Immunol. 1991;9:617-48.
13. Salomon R , Hoffmann E , Webster RG. Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. Proc Natl Acad Sci. USA. 2007;104:12479-81.
14. Claas ECJ, Osterhaus ADME, Van Beek R, De Jong J C, Rimmelzwaan G F, Senne D A, Krauss S, Shortridge KF, Webster RG. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet. 1998;351:472-7.
15. Puthavathana P, Auewarakul P, Chor Charoenying P, Sangsiriwut K, Pooruk P, Boonak K, et al. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J Gen Virol. 2005;86:423-33.

Journal of Public Health and Epidemiology Vol. 3(4), pp. 187-193, April 2011
Available online at http://www.academicjournals.org/jphe
ISSN 2141-2316 ©2011 Academic Journals

Full Length Research Paper

Monitoring the influenza pandemic of 2009 in Thailand by a community-based survey

Chakrarat Pittayawonganon[1]*, Hathaikan Chootrakool[2], Sopon Iamsirithaworn[1], Pilaipan Puthavathana[3], Sukhum Chaleysub[2], Prasert Auewarakul[4], Somkid Kongyu[1], Kumnuan Ungchusak[1] and Pasakorn Akarasewi[1]

[1]Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand.
[2]Suan Dusit Poll, Suan Dusit Rajabhat University, Bangkok, Thailand.
[3]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.
[4]National Science and Technology Development Agency, Ministry of Science and Technology, Bangkok, Thailand.

Accepted March 7, 2011

As an international traveling hub of South-East Asia, Thailand was one of the countries hardest and earliest hit by the influenza A (H1N1) 2009 pandemic. In order to understand the epidemic spread in the country, we conducted community-based surveys in metropolitan, urban, and rural areas using questionnaire interviews. We also determined sero-positive rates from randomly selected samples within the surveyed population. Recalled incidences of fever and acute respiratory symptoms in the survey correlated well with systematic reports of 2009 pandemic influenza cases from hospitals in the same areas, giving a ratio of total cases extrapolated from the surveyed data for persons who sought medical attention reported in the hospital-based surveillance system at 275:1. Conducting a large scale survey of the influenza outbreak is time consuming and also can be difficult to complete in a short time. Therefore, we used the survey for monitoring the outbreak of respiratory disease in the early pandemic phase. The seroprevalence rate was 8 to 10%, with higher rate for younger age groups, and suggests that sufficient herd immunity may have been reached in Thailand, especially in urban areas, while others may still be vulnerable to the second wave of the pandemic.

Key words: Pandemic, influenza, survey, Thailand.

INTRODUCTION

Influenza epidemics can be unpredictable and vary enormously in severity (Bramley et al., 2009). Novel influenza A (H1N1) 2009 was first reported in the USA and Mexico in April 2009 (CDC, 2009). The World Health Organization announced Phase 6 of the influenza pandemic on June 11, 2009. The virus is new to humans, so there are uncertainties about transmission efficiency and disease severity as pandemic influenza continues to evolve rapidly (AlMazroa et al., 2009).

As an international traveling hub of South-East Asia, Thailand was one of the countries hardest and earliest hit by the influenza A (H1N1) 2009 pandemic. The initial cases of laboratory-confirmed influenza A (H1N1) 2009 in Thailand were among travelers and students returning from epidemic area of the American Continent, The Thai Ministry of Public Health (MOPH) reported. In Thailand, locally acquired epidemics of pandemic influenza were first detected in June 2009. In the early epidemic, wide and rapid spreads of influenza transmission primarily occurred in schools in Bangkok metropolitan and major tourist cities (Apisarnthanarak, 2009; Suchada, 2009; Jongcherdchootrakul, 2010).

Having accurate and timely information on the extent of spread of outbreaks is crucial to informed decisions, and to deployment of proper interventions and mitigating measures. Thailand has a well-developed public health infrastructure, however, getting accurate information on numbers of cases and their distribution is often difficult in a widespread outbreak situation (Fraser et al., 2009).

Most public health authorities have relied on systematic reporting of laboratory-confirmed cases, influenza-like illnesses in out-patient settings, hospitalized patients with severe cases, and deaths (Kitler et al., 2002; Rao, 2003;

*Corresponding author. E-mail: chakrarat@gmail.com. Tel: (662) 590-1734. Fax: (662) 591-8581.

Flahault, 2006). Having estimated the total cases based on available data, one has to assume fixed proportions of total cases to the surveyed population, which can be acquired from studies at the beginning of the outbreak when the number of total cases is small enough to be tracked.

In addition, the proportion of patients who seek medical attention can be strongly affected by perception about disease severity, for instance, information gathering through the media, however, this perception can vary with time depending on several factors. The proportion of admitted cases and deaths can also vary as the severity of the virus changes while it evolves. Community-based active survey seems reasonable alternative (Ghosh, 2008; Levy-Bruhl, 2009). However, it is difficult to get accurate data from a large-scale clinical-based survey, because influenza symptoms are mostly non-specific, and other infectious and non-infectious illnesses can confound the findings. To overcome this problem, we designed a community-based survey to monitor the situation of influenza-like illnesses and tested its reliability to detect pandemic influenza infections by serological testing.

METHODS

Questionnaire and survey

The tool we used in the survey was a structured questionnaire, which was tested for feasibility and practicality in a district of the Bangkok Metropolitan Administration (BMA) called Dusit. The set of questions on demographic information of respondents and their families included gender, age, education level, religion, number of household members, and type of property resided in. Also, questions about the trend of the influenza outbreak comprised the number of household members who had influenza-like symptoms in the immediately preceding 2 weeks and in the 3 months prior to the survey; details of influenza-like illness and chronic diseases of each household member; behavior of sick individuals to prevent transmission to others; behavior to prevent oneself from getting influenza; and willingness to receive vaccination. For quality control of the interview process, a group of interviewers were trained, educated, and observed by an investigator as they moved step-by-step to follow the guideline for the survey.

Population and sampling method

The initial outbreaks of influenza A (H1N1) 2009 occurred at Thailand at different times. Areas of the country can be categorized into four groups on the basic of timing of outbreaks as May, June, July and August. We selected the province that first reported an outbreak of pandemic influenza in each of the four time-based groups for our survey including Bangkok Metropolitan Administration, NakhonRatchasima, ChiangMai, and Nakhonsrithammarat, respectively. The household survey by poll method (Poll-1), cluster sampling technique, in urban and suburban areas of BMA was conducted by a poll conducted by experienced interviewers in October 2009. For the other three provinces, their cities were purposively chosen, and rural areas were simply randomized to be surveyed with similar periods and techniques.

We also conducted a serologic survey based on a proportional-cluster sampling technique classified by number of households in sub-districts of ChiangMai and Nakhonsrithammarat. Since the prevalence of Influenza A (H1N1) 2009 in the Thai population was estimated at 20% with 5% as worst acceptable value, a total of 246 respondents should be tested. Face-to-face interviews and blood sample collection was done by an investigator and a team of health professionals. Exclusion criteria for recruitment for blood sample collection was a person who was <5 years old, and when fewer than 75% of family members in the household agreed to provide blood samples.

Case definition

An acute respiratory illness (ARI) case was defined as one affecting a person who had history of at least two of the following symptoms; fever, cough, sore throat, and running noses within recent three months prior to the survey.

Serologic testing

Hemagglutination-inhibition assay (HI assay) was performed as previously described (Iconic et al., 2009). The protocol called for A/Thailand/104/2009(H1N1) live virus as the test antigen; and 0.5% goose erythrocytes were used as the detector. The test sera were rid of non-specific inhibitors by pre-treatment with receptor destroying enzyme (Denka Seiken, Tokyo, Japan), at 37°C overnight, followed by heat inactivation at 56°C for 30 min; nonspecific agglutinator was removed by addition of 50% goose erythrocytes and incubated at 4°C for 1 h. Two-fold serial dilutions of test sera were prepared in duplicate, followed by incubation with the test antigen at a working concentration of 4 HA-units, the highest dilution of antigen that gives complete haemagglutination of cells, for 30 min at room temperature. Erythrocyte suspension was added to the reaction plates, and further incubation at 4°C for 30 min was performed before the result was read. HI antibody titer was defined as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

A microneutralization assay (MicroNT) was performed as previously described (Kitphati et al., 2009). The assay was based on a reduction in the amount of nucleoprotein produced in the virus-infected Madin-Darby Canine Kidney Cells (MDCK) monolayer as infectivity of the test virus is neutralized by specific antibody. A/Thailand/104/2009 pandemic strain was used as the test virus. The test sera were two-fold serially diluted, added with the test virus at a final concentration of 100TCID50 for 2 h at 37°C. The serum-virus mixture was then added onto the MDCK cell monolayer and further incubated for 24 h at 37°C. The reaction plate was fixed and tested by enzyme-linked immunosorbent assay (ELISA) for presence of the viral nucleoprotein using mouse monoclonal antibody (Chemicon, Temecula, CA) as the primary antibody and goat anti-mouse Igs (Southern Biotech, Birmingham, AL) as the secondary antibody. Antibody titer was defined as reciprocal of the highest serum dilution that could reduce ≥50% of the amount of nucleoprotein when compared with the virus control.

RESULTS

The surveyed incidence of ARI correlated with systematically reported ARI

We initially conducted a small exploratory survey to test tools in a district of Bangkok in August 2009. A total of 90 households were recruited with data for each household member obtained in an interview with a family member

Table 1. Demographic characteristics and practices of prevention measures in community based survey (Poll-1) in 8 areas, Thailand, July - September 2009

Characteristics	Bangkok		ChiangMai		NakornRatchasima		Nakornsrithammarat	
	City	Suburb	City	Rural	City	Rural	City	Rural
Population* (mid-year 2009)	99,994	150,166	238,460	21,405	329,531	83,639	254,261	30,816
Interviewed family (n = 826)	100	115	101	106	103	98	101	102
Family members (n = 3,351)	479	450	352	373	429	434	408	426
- Median of member in family [range]	4 [2-25]	3 [1-20]	3 [1-15]	3 [1-8]	4 [1-10]	4 [1-12]	4 [2-7]	4 [2-8]
Any member developed ARI between July and September 2009 (n = 460) (%)	48 (10)	62 (14)	36 (10)	29(8)	101 (24)	60 (14)	66 (16)	58 (14)
- Male (%)	16 (33)	34 (55)	13 (36)	15 (52)	42 (42)	32 (53)	28 (42)	30 (52)
- Median of age (year) [range]	21 [1-67]	25 [1-55]	24 [2-65]	23 [1-71]	18 [1-76]	7 [1-62]	16 [1-70]	12 [1-52]
Interviewee developed ARI (n = 128) (%)	18 (18.0)	22 (19.1)	14 (13.9)	6 (5.7)	35 (34.0)	6 (6.1)	18 (17.8)	9 (8.8)
- Mask use by interviewee (%)	38.9	63.6	71.4	83.3	31.4	16.7	11.1	22.2
- Mask use by family members (%)	5.6	22.7	0.0	50.0	11.4	33.3	5.6	0.0
- Take a sick leave (%)	33.3	59.1	57.1	50.0	60.0	50.0	83.3	55.6
- Hand covers mouth when cough (%)	77.8	63.6	100.0	83.3	82.9	66.7	94.4	88.9
- Not sharing bedroom (%)	22.2	31.8	21.4	50.0	25.7	0.0	33.3	44.4
- Frequent hand washing (%)	83.3	59.1	92.9	100.0	77.1	50.0	100.0	88.9

* Mid-year 2009 Thai population from Department of Provincial Administration, Ministry of Interior, Thailand.

whose age was higher than 15 years. Report for those households indicated that 62.2% had experienced symptoms of acute respiratory infections, example, fever and/or respiratory tract symptoms during the previous 3-month period. The highest frequency of illness was reported for children and young adults (ages 0 to 19 years).

We subsequently conducted surveys (Poll-1) in two areas each of ChiangMai, Nakhonsrithammarat, Nakhon-ratchasima, and Bangkok Metropolitan Administration (BMA), simultaneously in October 2009. These eight areas represented different levels of impact by pandemic influenza according to the national surveillance system for ARI. A total of 826 families were recruited in the eight survey sites. Among those, a member of each family was randomly selected for interview. Table 1 shows popu-lation density and characteristics of the surveyed areas, as well as demographic data of the surveyed subjects. Of all 460 persons who reported having ARI in the surveyed households, 128 (28%), one from each household, were interviewed for their practice of personal hygiene and their family members' preventive measures, example, a face mask wearing, home rest, personal hygiene practice, bedroom separation, and frequent hand washing (Table 1). The survey showed a cumulative incidence of ARI since the beginning of the epidemic to be just under 14%. Concurrent illnesses of two or more members in the same household were reported in 39% of all reported illnesses. Younger age groups had higher incidence of ARI, with cumulative incidence of ARI up to 43% for children <5 years old (Figure 1). The surveyed ARI incidence was extrapolated to the total population in the eight survey areas. Total numbers of ARI by age group correlate well with numbers of reported pandemic influenza A (H1N1) 2009 by age group in the MOPH system (r^2 = 0.733, $p<0.005$) (Figure 2) giving a ratio of total community-based ARI cases to the reported pandemic H1N1 cases seeking medical attention in the MOPH system was estimated to be 275:1.

We repeated the survey in ChiangMai city and

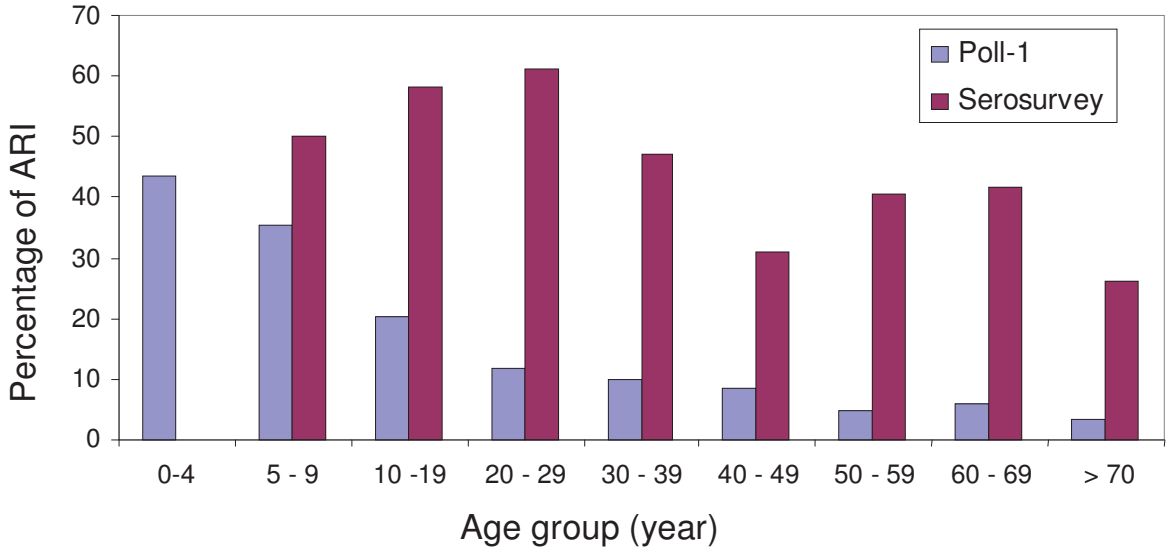


Figure 1. Prevalence of acute respiratory illnesses (ARI) by age-group, community based survey. (Poll-1) in October 2009 (n=3,351) and sero-survey in December 2009 (n=222).

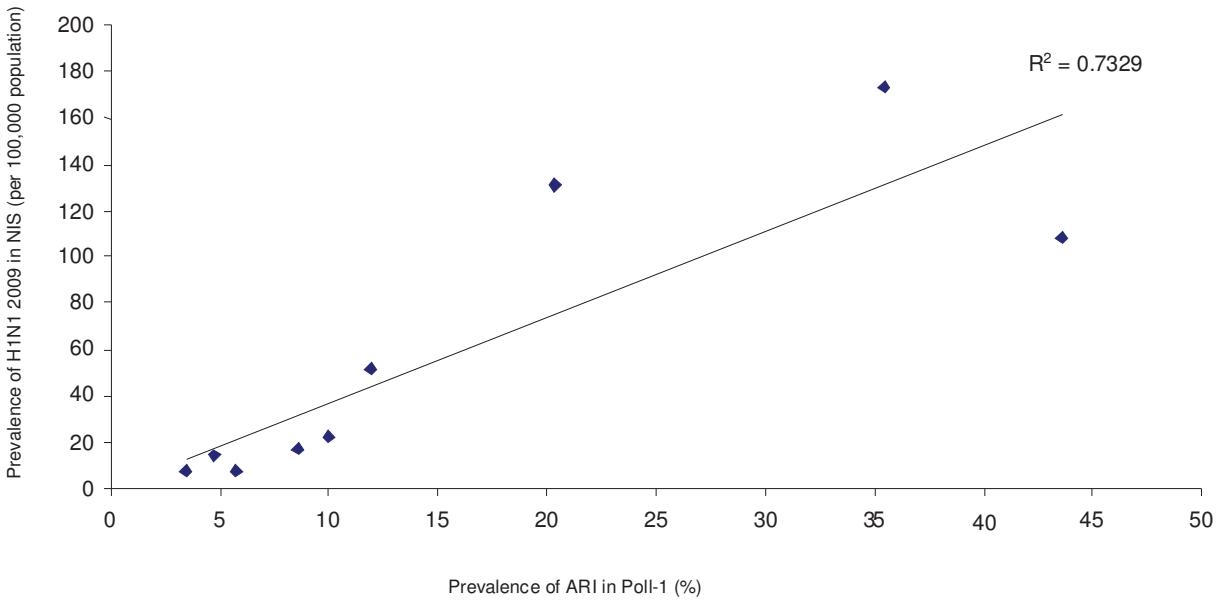


Figure 2. Correlation between prevalence of ARI and prevalence of reported pandemic H1N1 2009 infections by age group in 4 provinces of Thailand, July - September 2009.

Nakhonsrithammarat city in December 2009. The cumulative incidence of ARI increased to 43%, but the incidence of ARI during the 2-week period prior to the survey was 16%.

Antibody detection

In the second survey, we performed serologic tests for antibody to the influenza A (H1N1) 2009 targeted to 246 subjects. Only 222 (90%) subjects from 40 households in ChiangMai city and 44 households in Nakhonsrithammarat city had blood samples taken. Among the 222 subjects, a total of 20 serologically positive cases were detected with HI and MicroNT assays, and the proportion of serologically positive subjects was 9% (95%CI: 5.6 to 13.6%). Among the 96 ARI cases, the proportion of serologically positive cases was 14.6% (95%CI: 8.2 to

Table 2. Characteristics of respondents in sero-survey for a novel influenza H1N1, Thailand, December 2009

Factor	All respondents (n=222)	ILI cases (n=49)	ARI cases (n=96)	Seropositive cases (n=20)
Study area				
- ChiangMai city (%)	110 (50)	23 (47)	52 (54)	11 (55)
- Nakornsrithammarat city (%)	112 (50%)	26 (53%)	44 (46%)	9 (45%)
Median of age (years)	45 (range 5 - 89)	35 (range 7 - 70)	39 (range 7 - 89)	12 (range 7 - 57)
Male : Female	0.57 : 1	0.75 : 1	0.58 : 1	0.43 : 1
Education				
- Primary school (%)	86 (39)	17 (35)	32 (33)	12 (60)
- Secondary school (%)	73 (33)	16 (33)	29 (30)	4 (20)
Had history of any chronic illness (%)	85 (38)	16 (33)	40 (42)	4 (20)
Type of house				
- Separated house (%)	154 (69)	32 (65)	64 (67)	13 (65)
- Dormitory / Apartment (%)	64 (29)	17 (35)	32 (33)	7 (35)
Got seasonal influenza vaccine within 6 months (%)	11 (5)	0	3 (3%)	1 (5)

23.3%). Among 126 non-ARI cases, the proportion of serologically positive cases was 4.8% (95%CI: 1.8 to 10.1%).

The demographic findings for all respondents with reported ARI cases, and with sero-positive cases are shown in Table 2. None of the 222 individuals had a history of pneumonia, hospitalization, and Oseltamivir administration. Only 10 (4.5%) individuals had a history of seasonal influenza A vaccination in the previous year, and 162 (73%) of them stated that they would like to get a novel influenza A (H1N1) vaccination. Age-distribution of the sero-positive cases indicates that children and young adults were most affected by the virus. Subjects living in households with more family members had higher of seropositivity rates, indicating the importance of intra-household transmission. Among 20 case families, 11 families had 1 case each, 3 families had 2 cases each, and 1 family had 3 cases. Among the 11 families with 1 case each, the mean attack rate was 30%. Among the other 4 families, the mean attack rate was 58%. The difference in seropositivity rates among the areas is consistent with the survey data (Table 2). Using serologic data as a gold standard, our survey showed that ARI has a sensitivity of 70%, a specificity of 59%, and a positive predictive value of 15%.

DISCUSSIONS AND CONCLUSIONS

In Thailand, the first wave of the 2009 H1N1 influenza pandemic peaked in mid-July, and the low level of transmission activities lasted until October 2009. During late December 2009 to early January 2010, the rising trend in the influenza pandemic signaled an upcoming second wave of the pandemic in Thailand (Figure 3). In all the studied areas, the outbreaks subsided to an insignificant level by the time blood samples were obtained. So the seroprevalence rate reflected the cumulative incidence of the novel H1N1 influenza infections toward the end of the first wave.

It is not clear why the epidemic declined with herd immunity levels of as low as <10%. Behavioral changes caused by increased awareness and public campaigns may have contributed to this pattern (Neumann et al., 2009; Wiwanitkit, 2009). Weather changes, including reduced rainfall, which usually synchronizes with reduction in seasonal influenza outbreaks, may have also played a role. Another possibility is that the H1N1 pandemic may not have been able to sustain itself in the general population but required continuous sources with higher transmission rates and reproduction numbers of greater than one within the subpopulations. With enough herd immunity within these subpopulations, outbreaks might have been interrupted. Our data that show higher sero-positive rates among children support this hypothesis. However, pharmaceutical and non-pharmaceutical preventions and controls were implemented countrywide during the first pandemic wave. The different levels of saturated infection and immunity in different areas may reflect different timing of deployment of interventions, as areas with delayed onset of outbreak could start interventions earlier in the outbreak, or they may reflect different contact rates and transmission rates, thereby required different levels of herd immunity to stop

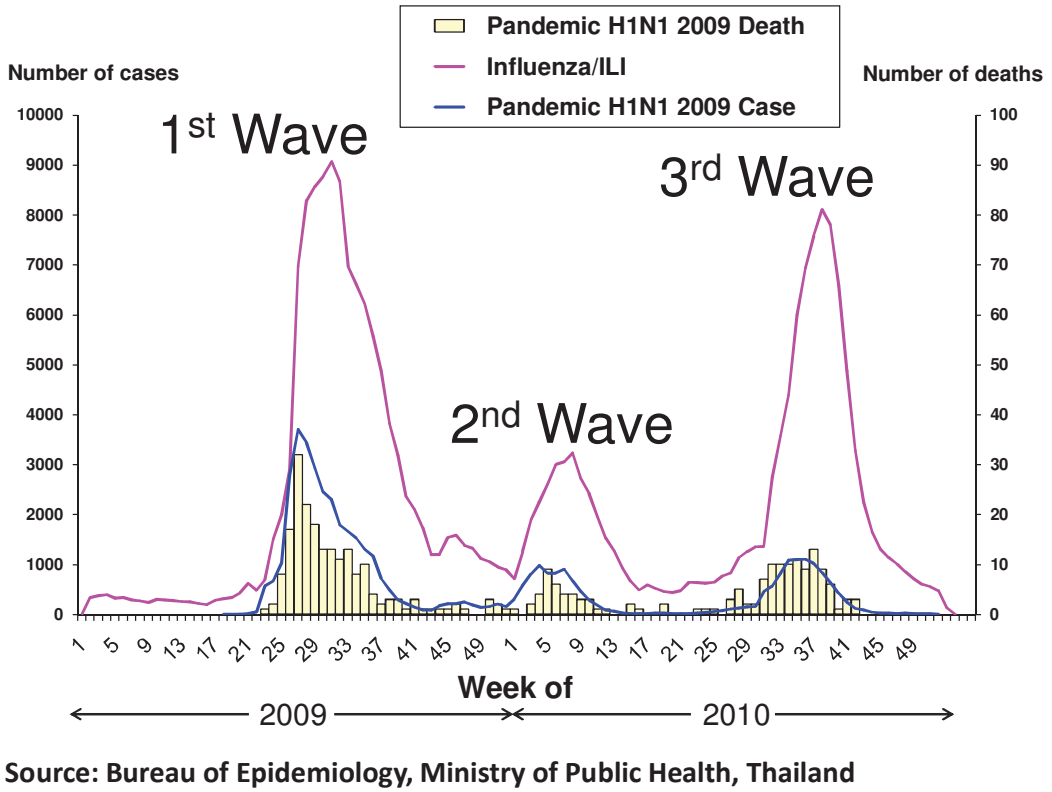


Figure 3. Three waves of the 2009 H1N1 influenza pandemic in Thailand. Source: Bureau of Epidemiology, Ministry of Public Health. Thailand.

an outbreak.

Our study showed that the rural areas had smaller proportion of ARI cases than cities did. This finding seems to support the pattern of a spread of pandemic influenza from cities to rural area in the early phase of pandemic, and similar patterns have been observed in many countries (Hien et al., 2009; Lopez-Cervantes et al., 2009; Yasuda and Suzuki, 2009). However, the different perceivable to ARI symptoms between city and rural people and the survey without serologic confirmation in countrywide were not allowed to conclude the spread of pandemic influenza A (H1N1) 2009 from cities to rural area. In addition, we found inadequacy of non-pharmaceutical prevention measures in the families that reported at least one ARI case. Therefore household transmission of influenza virus was inevitable, and sick children served as effective spreaders in families (Appuhamy et al., 2009; Health Protection Agency, 2009).

The limited sensitivity of a questionnaire to detect pandemic influenza infection was likely due to asymptomatic infections or mild infections, which may have been discountenanced. In an outbreak in a military camp in Thailand, in which all subjects were tested for H1N1 pandemic influenza-specific antibody response, we found an asymptomatic infection rate to be just under 30% of all reported infections (Wattanasak, 2010). Nevertheless, survey results are predictive enough to show good correlation with data in official reporting system. This gave a rather constant ratio of extrapolated total cases to reported cases of 275:1. This ratio is very useful for estimation of total cases and of the impact of the outbreak from the existing reporting system all over the country. This information is important for policy makers and for strategic implementation of outbreak control measures.

ACKNOWLEDGEMENTS

This research has been a collaboration between Bureau of Epidemiology; Department of Disease Control, Ministry of Public Health, Suan Dusit Poll; Suan Dusit Rajabhat University and National Science and Technology Development Agency (NSTDA); Ministry of Science and Technology. Additionally, we were financially supported by NSTDA and Science and Technology and the Thailand Research Fund for Research Scholar. The authors thank the Bureau of Epidemiology for sharing information of Thai influenza surveillance. Finally, we would like to acknowledge Mrs. Suwannee Thaenthanee

who was responsible for project coordination in the National Science and Technology Development Agency.

REFERENCES

(CDC), CfD, CaP (2009). Update: novel influenza A (H1N1) virus infection - Mexico, March-May, 2009. MMWR Morb Mortal Wkly Rep., 58(21): 585-589.

AlMazroa MA, Memish ZA, Ali MA (2009). Pandemic influenza A (H1N1) in Saudi Arabia: description of the first one hundred cases. Ann. Saudi. Med., 30(1): 11-14.

Apisarnthanarak A, Apisarnthanarak P, Cheevakumjorn B, Mundy LM (2009). Implementation of an infection control bundle in a school to reduce transmission of influenza-like illness during the novel influenza A 2009 H1N1 pandemic. Infect. Control. Hosp. Epidemiol., 31(3): 310-311.

Bramley AM, Bresee J, Finelli L (2009). Pediatric influenza. Pediatr. Nurs., 35(6): 335 -345.

Flahault A (2006). Global monitoring of influenza: potential contribution of national networks from a French perspective. Expert. Rev. Anti. Infect. Ther., 4(3): 387-393.

Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F, Ghani AC, Ferguson NM, Rambaut A, Pybus OG, Lopez-Gatell H, Alpuche-Aranda CM, Chapela IB, Zavala EP, Guevara DM, Checchi F, Garcia E, Hugonnet S, Roth C (2009). Pandemic potential of a strain of influenza A (H1N1): early findings. Science, 324(5934): 1557-1561.

Gabriele N, Takeshi N, Yoshihiro K (2009). Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature, 459(7249): 931-939.

Ghosh TS (2008). Active influenza surveillance at the local level: A model for local health agencies." Am. J. Public Health, 98(2): 213-215.

Health Protection Agency, Health Protection Scotland, National Public Health Service for Wales, HPA Northern Ireland Swine influenza investigation teams (2009). Epidemiology of new influenza A (H1N1) virus infection, United Kingdom, April-June 2009. Euro Surveill, 14(22).

Hien TT, Boni MF, Bryant JE, Ngan TT, Wolbers M, Nguyen TD (2009). Early pandemic influenza (2009 H1N1) in Ho Chi Minh City, Vietnam: a clinical virological and epidemiological analysis. PLoS. Med., 7(5): e1000277.

Ikonen N, Strengell M, Kinnunen L, Österlund P, Pirhonen J, Broman M, Davidkin I, Ziegler T, Julkunen I (2009). High frequency of cross-reacting antibodies against 2009 pandemic influenza A (H1N1) virus among the elderly in Finland. Euro Surveill, 15(5).

Jongcherdchootrakul K, Sukkhawee R, Khaopravej K, Silaporn P, Jiamsiri S, Wattanarangsan R, Ayut P, Iamsirithavorn S, Siriarayaporn P (2010). First School Outbreak of Novel Influenza A (H1N1) Infection in Thailand, June- August 2009). Weekly Epidemiological Surveillance Report-Thailand (WESR), 41: 49-56.

Kitler ME, Gavinio P, Lavanchy D (2002). Influenza and the work of the World Health Organization. Vaccine, 20(Suppl 2): S5-14.

Kitphati R, Pooruk P, Lerdsamran H, Poosuwan S, Louisirirotchanakul S, Auewarakul P, Chokphaibulkit K, Noisumdaeng P, Sawanpanyalert P, Puthavathana P (2009). Kinetics and longevity of antibody response to influenza A H5N1 virus infection in humans. Clin. Vaccine. Immunol., 16(7): 978-981.

Levy-Bruhl D (2009). Modified surveillance of influenza A(H1N1)v virus infections in France." Euro Surveill, 14 (29).

López-Cervantes M, Venado A, Moreno A, Pacheco-Domínguez RL, Ortega-Pierres G (2009). On the spread of the novel influenza A (H1N1) virus in Mexico. J. Infect. Dev. Ctries., 3(5): 327-330.

Ranil DA, Frank HB, Hai NP, Christine ES, Frances AB, Terry HC (2009). The changing phases of pandemic (H1N1) 2009 in Queensland: An overview of public health actions and epidemiology. Med. J. Aust., 192(2): 94-97.

Rao BL (2003). Epidemiology and control of influenza. Natl. Med. J. India., 16(3): 143-149.

Suchada J, MacKenzie A, Waller M, Brundage JF (2009). Outbreak of Influenza A (H1N1) Pandemic in Night-Pub, Pattaya, Chonburi Province, June 2009. Weekly Epidemiological Surveillance Report-Thailand (WESR), 40: 601-606.

Wattanasak A (2010). Infection Rate, Duration of Viral Shedding and Viral Load in an Outbreak of Novel Influenza A (H1N1) 2009. Infections among Military Conscripts in a Training Center, Thailand, June 2009. Weekly Epidemiological Surveillance Report-Thailand (WESR), 41: 209-212.

Wiwanitkit V (2009). Swine flu infection among medical students: an issue of concern. Am. J. Infect. Control., 37(10): 868.

Yasuda H, Suzuki K (2009). Measures against transmission of pandemic H1N1 influenza in Japan in 2009: simulation model. Euro Surveill, 14(44).

OPEN ACCESS Freely available online

PLoS one

Serological Response to the 2009 Pandemic Influenza A (H1N1) Virus for Disease Diagnosis and Estimating the Infection Rate in Thai Population

Hatairat Lerdsamran[1], Chakrarat Pittayawonganon[2], Phisanu Pooruk[1], Anek Mungaomklang[3], Sopon Iamsirithaworn[2], Prasert Thongcharoen[1], Uraiwan Kositanont[1], Prasert Auewarakul[1], Kulkanya Chokephaibulkit[4], Sineenat Oota[5], Warin Pongkankham[6], Patummal Silaporn[2], Supaloek Komolsiri[1], Pirom Noisumdaeng[1], Tawee Chotpitayasunondh[7], Chariya Sangsajja[8], Witthawat Wiriyarat[9], Suda Louisirirotchanakul[1], Pilaipan Puthavathana[1]*

1 Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, **2** Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand, **3** Maharat Nakhonratchasima Hospital, Ministry of Public Health, Nakhonratchasima, Thailand, **4** Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, **5** The National Blood Center, The Thai Red Cross Society, Bangkok, Thailand, **6** Division of Preventive Medicine, Naval Medical Department, Royal Thai Navy, Chon Buri, Thailand, **7** Queen Sirikit National Institute of Child Health, Ministry of Public Health, Bangkok, Thailand, **8** Bamrasnaradura Infectious Disease Institute, Ministry of Public Health, Nonthaburi, Thailand, **9** Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand

Abstract

Background: Individuals infected with the 2009 pandemic virus A(H1N1) developed serological response which can be measured by hemagglutination-inhibition (HI) and microneutralization (microNT) assays.

Methodology/Principal Findings: MicroNT and HI assays for specific antibody to the 2009 pandemic virus were conducted in serum samples collected at the end of the first epidemic wave from various groups of Thai people: laboratory confirmed cases, blood donors and health care workers (HCW) in Bangkok and neighboring province, general population in the North and the South, as well as archival sera collected at pre- and post-vaccination from vaccinees who received influenza vaccine of the 2006 season. This study demonstrated that goose erythrocytes yielded comparable HI antibody titer as compared to turkey erythrocytes. In contrast to the standard protocol, our investigation found out the necessity to eliminate nonspecific inhibitor present in the test sera by receptor destroying enzyme (RDE) prior to performing microNT assay. The investigation in pre-pandemic serum samples showed that HI antibody was more specific to the 2009 pandemic virus than NT antibody. Based on data from pre-pandemic sera together with those from the laboratory confirmed cases, HI antibody titers $\geq$40 for adults and $\geq$20 for children could be used as the cut-off level to differentiate between the individuals with or without past infection by the 2009 pandemic virus.

Conclusions/Significance: Based on the cut-off criteria, the infection rates of 7 and 12.8% were estimated in blood donors and HCW, respectively after the first wave of the 2009 influenza pandemic. Among general population, the infection rate of 58.6% was found in children *versus* 3.1% in adults.

Citation: Lerdsamran H, Pittayawonganon C, Pooruk P, Mungaomklang A, Iamsirithaworn S, et al. (2011) Serological Response to the 2009 Pandemic Influenza A (H1N1) Virus for Disease Diagnosis and Estimating the Infection Rate in Thai Population. PLoS ONE 6(1): e16164. doi:10.1371/journal.pone.0016164

Editor: Ralph Tripp, University of Georgia, United States of America

Received September 24, 2010; **Accepted** December 7, 2010; **Published** January 25, 2011

Funding: This study was supported by the National Science and Technology Development Agency, the Thailand Research Fund for Senior Research Scholar and the Mahidol University Research grant. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: siput@mahidol.ac.th

Introduction

There were 3 influenza pandemics occurring in the last century, i.e., Spanish influenza A (H1N1) in 1918, Asian influenza A (H2N2) in 1957 and Hong Kong influenza A (H3N2) in 1968 [1]. The influenza pandemic phase of this century, as declared by the World Health Organization (WHO) on 11th June 2009, was caused by A (H1N1) virus [2], a reassortant derived from influenza viruses of 4 origins: classical swine, European swine, avian, and human influenza viruses [3]. Epidemiological studies of the 2009 pandemic showed that the disease is more common in children [4,5]. Death mostly occurred in patients with underlying conditions, such as pregnancy, obesity, diabetes, hematological malignancy and cardiopulmonary dysfunction [6–9].

It is necessary to estimate and predict the magnitude of the pandemic in various regions worldwide, either by case based or serological based surveillance. However, the serological surveys were estimated to be approximately 10 times more sensitive than the clinical surveillance for determining infection rate of the pandemic virus [10,11]. HI assay employing turkey erythrocytes

was conducted; and the HI antibody titers ≥32 or ≥40 were established as the cut-off levels to estimate the infection rates in populations by various groups of investigators [10–13]. This cut-off titer was established based on the WHO guideline for vaccine evaluation which suggested HI antibody titers ≥40 as the levels indicating 50% protection [14,15]. Moreover, microNT assay had been conducted in parallel in order to determine the protection correlation, and it was suggested that the HI antibody titer 40 was correlated to the NT titer 160 in adults or 40 in children [13].

On 10th August 2010, WHO announced the beginning of the post-pandemic phase of the 2009 pandemic influenza. Nevertheless, epidemiological data from the Bureau of Epidemiology of Thailand suggested that only one fourth of the Thai population had been infected by this novel virus after it was introduced into Thailand at the beginning of May 2009 until December 2009. The data suggested that Southeast Asian countries and some other parts of the world might still be vulnerable to the new attack by that time.

The present study aimed to establish the cut-off HI and NT antibody titers that could differentiate between individuals with or without past infection by the 2009 pandemic influenza. We demonstrated that erythrocytes from goose yielded comparable HI antibody titers as those from turkey, an animal species that is not common in Southeast Asian countries. Moreover, we showed that it is necessary to treat human sera with receptor destroying enzyme (RDE) before running microNT assay. This RDE treatment is usually not included in the microNT protocol generally employed for testing human sera in most laboratories [10,13,16,17]. Our established cut-off titers were applied in the seroepidemiological surveillance to estimate the infection rate in different groups of the Thai populations after subsidence of the first epidemic wave.

Materials and Methods

Ethical issues

This study was approved by two Ethical Committees: Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University and the Ministry of Public Health Review Board. Adult subjects signed in consent form for participation. With ascent from children subjects, their parents signed the consent form for them.

Subjects

Serum samples tested in this study were collected from 5 groups of subjects. The first group comprised 80 patients with 2009 pandemic influenza as confirmed by real time reverse transcription- polymerase chain reaction employing the protocol of the US, Centers for Disease Controls [18]. Part of these patients were sent for disease diagnosis by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health under public health emergency service; and part of them were sent anonymously from the clinic sites under the Southeast Asia Infectious Disease Clinical Research Network, Thailand. The second group comprised 100 anonymous blood donors of the National Blood Center, the Thai Red Cross Society, Bangkok. All were bled within the same day in September 2009. Small aliquots of blood were subjected to anti-HIV testing; and the leftovers were provided for this study. The third group comprised 258 healthcare workers (HCW) from two hospitals: Siriraj Hospital, Bangkok and Thammasat University Hospital in Pathum Thani, the neighboring province of Bangkok. These HCW were exposed to patients suspected of the 2009 pandemic influenza and/or to the laboratory confirmed cases during their duty. The fourth group comprised 222 general population from two provinces, Chiang Mai (696 km. north from Bangkok) and Nakhon Si Thammarat (780 km. south from Bangkok), whose ages were older than 5 years. These two provinces were selected based on highest numbers of reported cases in the region; and in each province, the random samples were collected in the community-based setting during the opinion survey in a district that high number of cases was reported. The test samples also included anonymously archival sera collected during pre-pandemic period from vaccinees whose ages were at range of 21–49 years. The vaccinees received inactivated influenza vaccine of the 2006 season which contained 15 μg of hemagglutinin antigen of A/New Caledonia/20/1999(H1N1)-like strain (A/New Caledonia), A/Wellington/1/2004(H3N2)-like strain, and B/Shanghai/361/2002-like strain (Government Pharmaceutical Organization-Merieux Biological Products Co., Ltd., Bangkok). Details regarding subjects who participated in this study are shown in Table 1.

Blood samples

Paired bloods were collected from the patients, and single blood samples were collected from the other groups of subjects. Acute blood samples were collected mostly within 7 days; meanwhile, the convalescent samples were collected at between 11 to 54 days after onset of illness. Serum was separated, aliquot and kept frozen at -20 C until tested.

Regarding archival sera, the pre-vaccinated blood samples were collected just before vaccination; and the post-vaccinated blood samples were collected at one month later.

The study virus

A/Thailand/104/2009(H1N1) propagated in MDCK cells was used as the test virus for both HI and microNT assays. Full genomic sequence of this isolate can be retrieved from the GenBank database. The H genomic sequence of this virus was 99.7% identity to that of A/California/7/2009 pandemic virus (data not shown).

Reference serum

A reference human serum from the National Institute for Biological Standards and Control (NIBSC), UK was used for standardizing our serological methods. Based on the investigation performed by various laboratories under the International Collaborative Study, this reference serum had the overall geometric mean titer (GMT) of 183 by HI and 516 by microNT assays (NIBSC package insert). The HI GMT titer 183 implies that the results of HI titers obtained from those laboratories varied between 160 and 320. Similarly, the NT GMT titer 516 implies that the results of NT titers varied between 320 and 640.

Hemagglutination (HA) assay

HA assay was performed in order to measure the amount of hemagglutinin antigen present in the test virus suspension prior to running HI assay [17]. The test virus was serially twofold diluted with phosphate buffered saline (PBS) in a volume of 50 μl/well in duplicate. Fifty μl of 0.5% goose or 0.5% turkey erythrocyte suspension was added into the test wells and incubated for 30 minutes at 4 C before hemagglutinating result was determined. One HA unit of the test virus was defined as the highest virus dilution that displayed complete hemagglutinating activity.

Hemagglutination inhibition (HI) assay

HI assay was performed as previously described [17,19–21]. Fifty μl of the test serum were mixed with 150 μl of RDE (Denka

Table 1. Subjects and time of specimen collection.

Subjects	No. of subjects	Age (years)			Time at specimen collection
		Mean	Median	Range	
Vaccinees who received seasonal influenza	71	33.6	31	21–49	Dec 2005 - Mar 2006 (pre-vaccination) Jan - Apr 2006 (post-vaccination)
Patients					
- Pediatrics	36	10.6	12.5	2–15	June 2009 – Feb 2010
- Adults	44	23.6	21	18–62	June 2009 – Feb 2010
Blood donors	100	35.7	34.5	17–60	Sep 2009
Health care workers	258	35.3	34	20–61	Oct 2009
General population (Chiang Mai)					
- Children	11	9.8	10	7–13	Dec 2009
- Adults	99	49.3	52	15–89	Dec 2009
General population (Nakhon Si Thammarat)					
- Children	18	10.1	10.5	5–14	Dec 2009
- Adults	94	49.5	48	15–87	Dec 2009

doi:10.1371/journal.pone.0016164.t001

Seiken, Tokyo, Japan) and incubated overnight in water bath at 37 C for eliminating the nonspecific inhibitors. This step was followed by heat inactivation at 56 C for 30 minutes, and removal of nonspecific agglutinator by absorbing with the test erythrocytes for 1 hour at 4 C. The replicating virus at final concentration of 4 HA units/25 µl was used as the test antigen; and goose or turkey erythrocytes were used as the indicator. The treated serum was twofold serially diluted in duplicate wells of a microtiter V shaped plate at an initial dilution of 1:10; and 25 µl of the diluted serum were incubated with 25 µl of the test antigen for 30 minutes at room temperature. Thereafter, the reaction wells were added with 50 µl of 0.5% goose or 0.5% turkey erythrocyte suspension and further incubated for 30 minutes at 4 C before the HI antibody titers were determined. HI antibody titer is defined as the reciprocal of the highest serum dilution that completely inhibits hemagglutination reaction. Reference/positive control serum with known HI titer, the serum control and back titration of virus antigen were included in each run. For calculating GMT, the antibody titer <10 was assigned as 5, and the titer ≥ 2560 was assigned as 2560.

Microneutralization (microNT) assay

ELISA based microNT assay was performed as described previously [17,19–21]. The test sera were treated by any of the following two protocols. The first one was the standard protocol employing only heat inactivation of the native sera at 56 C for 30 minutes; and the second one employed RDE treatment similar to that mentioned above for HI assay. Briefly, 50 µl of the test serum were mixed with 150 µl of RDE and incubated overnight in water bath at 37 C followed by heat inactivation at 56 C for 30 minutes. The treated sera were twofold serially diluted in duplicate and incubated with the test virus at final concentration of 100TCID50/100 µl for 2 hours at 37 C. The serum-virus mixture was transferred onto MDCK monolayer maintained in minimum essential medium supplemented with trypsin TPCK (Sigma, St.Louis, MO.) for 24 hours. The reaction plate was tested by ELISA for presence of the viral nucleoprotein using mouse specific monoclonal antibody (Chemicon, Temecula, CA.) as the primary antibody and goat anti-mouse Igs (Southern Biotech, Birmingham, AL.) as the secondary antibody. Antibody titer is defined as reciprocal of the highest serum dilution that reduces $\geq 50\%$ of the amount of viral nucleoprotein in the reaction wells as compared to the virus control wells. For calculating GMT, the antibody titer <10 was assigned as 5, and the titer ≥ 2560 was assigned as 2560.

Results

Goose and turkey erythrocytes yielded comparable HI antibody titers

In order to determine that goose and turkey erythrocytes yielded comparable HI antibody titers, the reference human serum from the NIBSC which contains HI antibody at GMT 183 was assayed in duplicate by 6 scientists using goose and turkey erythrocytes in parallel experiments. The HI antibody titer 160 was obtained from all 6 scientists as using either one of both erythrocyte species. The comparison was further extended to include the acute and convalescent serum samples from 53 patients as well as single serum samples from 100 HCW. The analysis on the total number of 206 serum samples showed that goose and turkey erythrocytes yielded comparable HI titers with $r = 0.96$ (Spearman's rank, $p < 0.0001$) (Figure 1). The number of samples with HI antibody titers ≥ 40, as well as the ratio between convalescent to acute antibody titers, and the number of samples showing a fourfold or greater rise in HI antibody titer, were similar when goose or turkey erythrocytes were used (Table 2). Based on comparable HI titers obtained by the two erythrocyte species as well as our convenience to obtain goose erythrocytes; therefore, goose erythrocytes were employed in the subsequent experiments of our HI assay.

RDE treated serum was required for microNT assay

We recognized that microNT assay using RDE untreated sera yielded an unusually high level of NT antibody to the 2009 pandemic virus in the test sera which had no HI antibody. Therefore, the serum samples were treated with RDE and retested again. The result showed that the RDE treated sera from all serum settings showed a marked decrease in level of NT antibody titer when compared to the RDE untreated samples (Wilcoxon Signed

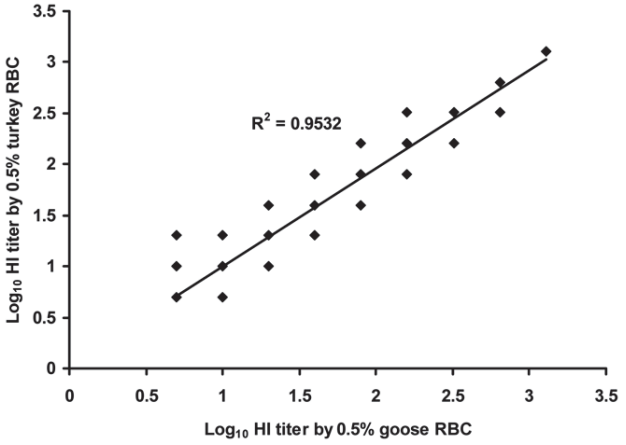


Figure 1. Correlation between HI antibody titers obtained from goose and turkey erythrocytes by HI assays.
doi:10.1371/journal.pone.0016164.g001

Ranks test, $p<0.05$) (Table 3). Therefore, the RDE treated sera were employed subsequently.

Moreover, the reference human serum from NIBSC which harbored NT antibody at GMT 516 was assayed in parallel by our two scientists using RDE treated serum as well as the untreated serum control in triplicate experiments. The GMT 640 was obtained either with the RDE treated or untreated serum.

Cross-reactive antibody to the 2009 pandemic virus in pre-pandemic serum samples

Information about serological response in our vaccinees who received influenza vaccines during the pre-pandemic period had been published previously [22]. Among 71 tested sera, 98.6% developed a fourfold or greater rise in HI antibody titer against A/New Caledonia, a component of the immunizing vaccine (Table 4). Herein, those serum samples were investigated for serological response against the 2009 pandemic virus; and it was found that 16.9% of the vaccinees developed seroconversion as determined by HI assay. Six (8.4%) subjects seroconverted with HI titer rising from ≤10 to ≥40. Cross-reactive HI antibody titer 80 was found in one (1.4%) pre-vaccinated serum sample; nevertheless, post-vaccinated serum from this subject did not increase in antibody titer against the 2009 pandemic virus. At post-vaccination, number of subjects with the cross-reactive HI antibody ≥40 increased to 8 (11.3%). Regarding microNT assay, 16 (22.5%) developed a fourfold or greater rise in antibody titer (convalescent titer ≥40) in post-vaccination sera. Our study demonstrated broader cross-reactivity of NT antibody than HI antibody.

HI and NT antibody response in patients infected with the 2009 pandemic influenza virus

HI and NT antibody response in the patients by days after onset of illness is shown in Figure 2. Positive correlation with $r = 0.85$ (Spearman's rank, $p<0.0001$) was found between the two assays. Seroconversion or a fourfold or greater rise in HI or NT antibody titers could be demonstrated when convalescent blood samples were collected at 11 days earliest after disease onset. Among 36 pediatric cases, 32 (88.9%) developed seroconversion (Table 5). The other 4 cases already contained high HI titers ≥40 in their first blood samples. And among 32 seroconverters, 5 cases (13.9%) seroconverted with HI titers rising from <10 to 20; and the remaining 29 cases seroconverted with convalescent titers ≥40. Therefore, the HI titers ≥20 were found in 100%, and HI titer ≥40 were found in 86.1% of the pediatric patients. Similarly, the NT titers ≥20 were found in 97.2% and NT titer ≥40 were found in 94.4%.

Among 44 adult patients, 43 (97.7%), developed HI titers ≥40 in their convalescent sera. There was one adult patient who could not develop significant HI antibody response (HI titer <10 and 10), although he possessed NT antibody titer of 20 and 80. The convalescent NT titers ≥80 were found in all adult patients.

Estimation of the infection rate of the 2009 pandemic influenza after the first epidemic wave

Regarding titers of HI antibody found in the patients together with the data showing the absence of HI antibody in all except one pre-pandemic serum samples, the cut-off HI antibody titers ≥20 for pediatric cases and ≥40 for adult patients were established to indicate past infection by the 2009 pandemic virus. The established criteria had been used to estimate the infection rate of the 2009 pandemic influenza in various groups of populations;

Table 2. Comparison between HI antibody titers obtained from goose and turkey erythrocytes.

Subjects	HI assay with	Number of sera test	Blood	GMT (95% CI)	No. with HI titers ≥40	No. with 4-fold rising Ab titer (%)	Ratio Conv./ Acute
Patients	0.5% turkey RBC	Children = 29	Acute	14 (8–25)	7		
			Convalescent	111 (78–158)	28	23 (79.3)	7.6
		Adults = 24	Acute	15 (7–29)	6		
			Convalescent	87 (53–141)	22	19 (79.2)	5.8
	0.5% goose RBC	Children = 29	Acute	15 (8–27)	8		
			Convalescent	106 (72–157)	27	24 (82.8)	6.9
		Adults = 24	Acute	14 (7–28)	6		
			Convalescent	89 (55–145)	22	19 (79.2)	6.4
Health care workers	0.5% turkey RBC	Adults = 100	Single blood	9 (7–10)	14	NA	NA
	0.5% goose RBC	Adults = 100	Single blood	9 (7–11)	15	NA	NA

Note: A/Thailand/104/2009(H1N1) was used as the test virus. GMT = geometric mean titer, CI = confidence intervals, RBC = red blood cells, NA = Not applicable
doi:10.1371/journal.pone.0016164.t002

Table 3. Comparison between NT antibody titers obtained from RDE treated and RDE untreated sera.

				Number of cases at NT antibody of									
Subjects	**No. of subjects**	**Blood samples**	**RDE treatment**	**<10**	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**≥1280**	**GMT* (95% CI)**
Patients	80	Acute	Yes	9	30	8	3	8	12	3	4	3	33 (23–47)
			No	0	0	0	7	18	21	22	5	7	191 (155–236)
	80	Convalescent	Yes	0	1	1	3	14	23	22	9	7	214 (172–267)
			No	0	0	0	1	2	11	36	22	8	380 (326–443)
General population	222	Single blood	Yes	15	87	46	33	24	10	5	2	0	21 (18–24)
			No	0	0	0	103	88	22	5	3	1	66 (61–72)
Vaccinees	71	Pre-vaccination	Yes	22	34	6	7	0	1	0	1	0	10 (8–13)
			No	0	0	1	5	33	27	2	3	0	110 (95–127)
	71	Post-vaccination	Yes	3	19	24	16	6	1	1	1	1	23 (18–28)
			No	0	0	0	0	24	34	8	4	1	152 (131–176)
Blood donors	100	Single blood	Yes	10	40	19	16	6	7	2	0	0	19 (16–24)
			No	0	0	0	1	29	60	10	0	0	138 (126–150)
Health care workers	258	Single blood	Yes	14	116	61	35	15	12	1	4	0	19 (16–21)
			No	0	0	0	10	81	127	34	5	1	138 (129–148)

*There are significant differences between GMT of NT antibodies in RDE treated and untreated sera from all 7 serum settings (Wilcoxon Signed Ranks test, $p<0.05$).
doi:10.1371/journal.pone.0016164.t003

and it was found that 7.0% of blood donors and 12.8% of HCW had been infected with the pandemic virus by the end of the first epidemic wave (Table 6). Magnitude of the infection in the general population residing in the North and the South of Thailand was similar. In these two populations, the infection rates were much higher in children (17 of 29) than adults (6 of 193), i.e., 58.6 *versus* 3.1%, respectively.

It was not easy to establish the cut-off NT antibody titer owing to presence of cross-reactive NT antibody at high titers in the pre-pandemic serum samples collected from vaccinees whose ages were under 50 years, and also in general people who had no HI antibody against the 2009 pandemic virus. Poorer correlation between HI and NT antibody was found in this group of subject ($r = 0.32$: Spearman's rank, $p<0.0001$) (data not shown).

Discussion

Herein, HI and microNT assays that were suitable for a Southeast Asian country had been established to estimate the infection rate of the 2009 pandemic influenza in Thai people after subsidence of the first epidemic wave. HI assay has long been used for serodiagnosis of influenza virus infection, vaccine evaluation, and vaccine strain selection [16,23,24]. It was noted that sensitivity of the HI assay could be affected by the erythrocyte species

Table 4. Cross-reactive antibody to the 2009 pandemic A (H1N1) influenza virus in vaccinees who received trivalent influenza vaccine of the 2006 season (N = 71).

		Number of cases with antibody titer of										
Test viruses	**Assays**	**<10**	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**GMT (95% CI)**	**Post- to pre-vaccination ratio**	**No. with ≥4 folded rise in Ab titer [a] (%)**
A/New Caledonia/20/99-like (H1N1)												
Pre-vaccination	HI	43	8	9	10	0	0	1	0	9 (7–11)		
Post-vaccination	HI	0	0	1	1	8	13	15	33	310 (254–379)	34	70 (98.6) [b]
A/Thailand/104/09 (H1N1)												
Pre-vaccination	HI	64	5	1	0	1	0	0	0	5 (5–6)		
Post-vaccination	HI	43	12	8	2	3	2	1	0	9 (7–11)	2	6 (8.4)
Pre-vaccination	microNT	22	34	6	7	0	1	0	1	10 (8–13)		
Post-vaccination	microNT	3	19	24	16	6	1	1	1	23 (18–28)	2	16 (22.5)

[a]Seroconversion with post-vaccination antibody titer ≥40 to the 2009 pandemic virus.
[b]A/New Caledonia/20/99 was used as the test antigen.
doi:10.1371/journal.pone.0016164.t004

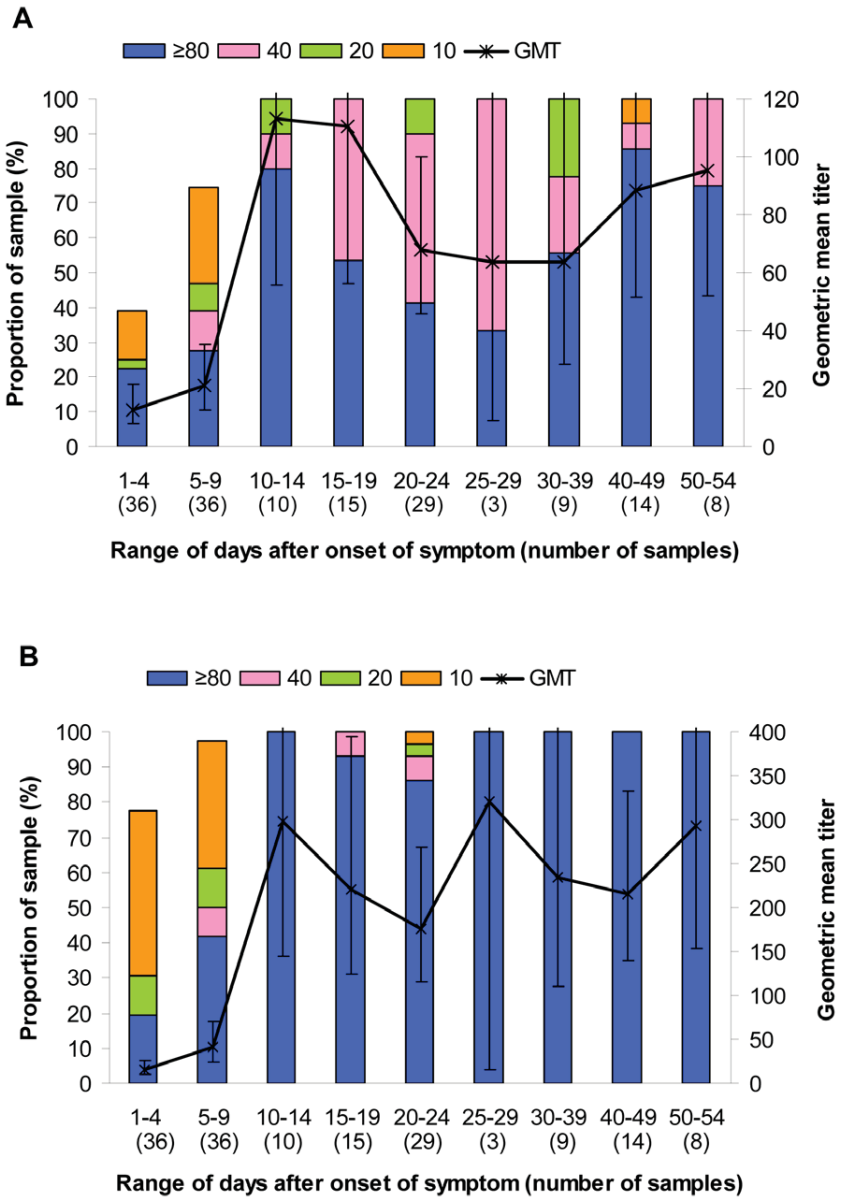


Figure 2. Antibody titers by date after onset of symptom. (A) HI antibody titer; (B) NT antibody titer. Colored stacked bars give the proportion with titers of 10, 20, 40 and ≥80 while the line denotes the geometric mean titer with error bars indicating 95% confidence intervals.
doi:10.1371/journal.pone.0016164.g002

employed. Stephensen, et al [25] previously reported that horse erythrocytes were more sensitive than turkey erythrocytes in the detection of HI antibody to H5N1 highly pathogenic avian influenza (HPAI) virus. Since there was difficulty in accessing both horse and turkey erythrocytes in Thailand, we previously looked for the alternative erythrocyte species and found that goose erythrocytes yielded comparable results in both HA and HI assays [19]. Similarly, the present study showed that goose erythrocytes could replace turkey erythrocytes for detection of HI antibody to the 2009 pandemic influenza virus. Our preliminary study on 206 serum samples as well as the reference serum from NIBSC demonstrated good correlation between HI titers employing either goose or turkey erythrocytes. This finding is an advantage for laboratories in Southeast Asian countries where goose erythrocytes have long been used in HI assay for diagnosis of dengue, chikungunya and Japanese encephalitis, the endemic diseases in this region. It was also demonstrated that the hemagglutination pattern of goose was also clearer than that of turkey erythrocytes.

A number of laboratories performed microNT in adjunct with HI assay in a seroepidemiological study, but the step of serum treatment with RDE was not included [10,13]. In contrast, our study showed that NT GMT titer in the test sera without RDE treatment was significantly higher than that employed the RDE treated sera. Collectively, RDE is used for removal of nonspecific inhibitor from the test sera, in which its presence may lead to false positive result in HI as well as microNT assays as shown by this study. On the other hand, the presence of this nonspecific inhibitor did not affect our result on using RDE untreated sera in microNT assay for antibody against H5N1 HPAI virus. Cross-reactive H5N1 antibody, even at low level, was rare [20,21,26]. Therefore,

Table 5. HI and NT antibody response in patients infected with the 2009 pandemic influenza virus.

			No. of cases at antibody titer of												
Patients	**Assays**	**Blood samples**	**<10**	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	**2560**	**GMT (95% CI)**	**Ratio of Conv./Acute**	**No. with ≥4 folded rise in Ab titers (%)**
Children (N = 36)	HI	Acute	19	8	1	2	1	3	2	0	0	0	12 (7–19)		
		Conv.	0	0	5	12	10	3	1	4	1	0	78 (53–114)	6.3	32 (88.9%)
	microNT	Acute	5	20	2	1	5	3	0	0	0	0	16 (11–23)		
		Conv.	0	1	1	3	9	6	8	4	4	0	179 (119–269)	10.9	31 (86.1%)
Adults (N = 44)	HI	Acute	15	4	4	3	8	5	2	2	1	0	29 (17–48)		
		Conv.	0	1	0	16	15	6	3	2	1	0	83 (63–111)	38.1	24 (54.5%)
	microNT	Acute	4	10	6	2	3	9	3	4	2	1	58 (33–100)		
		Conv.	0	0	0	0	5	17	14	5	3	0	248 (199–310)	4.3	23 (52.3%)

Note: Number of pediatric patients with convalescent HI titers ≥20 = 36/36 (100.0%).
Number of adult patients with convalescent HI titers ≥40 = 43/44 (97.7%).
doi:10.1371/journal.pone.0016164.t005

it is postulated that HPAI H5N1 and the 2009 pandemic viruses bind to different species of nonspecific inhibitor.

Investigation in pre-pandemic serum samples demonstrated that NT antibody is broader in activity than HI antibody; however, both cross-reactive HI and NT titers could be increased by seasonal influenza vaccination. Similarly, Hancock, et al. [13] previously reported a fourfold or greater increase in cross-reactive antibody to the 2009 pandemic virus in archival serum samples from adult recipients of trivalent inactivated influenza vaccines during 2007–2009 seasons. A fourfold or greater increase in cross-reactive HI antibody was found in 7% among vaccinees of age 18–64 years, but it was as high as 22% for cross-reactive NT antibody. This cross- reactive antibody was rarely found in young children in their study. Our group previously reported that 5.2% of the elderly who received seasonal influenza vaccine seroconverted to HPAI H5N1 virus as determined by microNT assay [27]. Nevertheless, the studies from U.S. and Australia concluded that vaccination with seasonal influenza vaccine did not protect against the current pandemic [13,28]. In contrast, partial protection conferred through seasonal influenza vaccination was reported by the other group of investigators [29]. Frequency of cross-reactive NT antibody was high in Thai and U.S. population [13]; and it was as low as 0.3% in Chinese [12]. However, it is well accepted that NT antibody activity is broader than HI antibody. HI antibody recognizes small epitopes in erythrocyte binding site, while NT antibodies recognize the epitopes in HA1 variable domain of the

Table 6. Estimation on the infection rates of the 2009 pandemic influenza in different groups of subjects after the first epide mic wave.

		No. of cases at antibody titer of									
Subjects	**Assays**	**<10**	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**Infection rate (%)**	**GMT (95% CI)**
Blood donors (N = 100)	HI	81	7	5	7	0	0	0	0	7 (7.0)	6 (5–7)
	microNT	10	40	19	16	6	7	2	0		19 (16–24)
Health care workers (N = 258)	HI	177	26	22	16	12	5	0	0	33 (12.8)	8 (7–9)
	microNT	14	116	61	35	15	12	1	4		19 (16–21)
General population (Chiang Mai, N = 110)											
- Children (N = 11)	HI	2	0	1	4	2	2	0	0	9 (81.8)	37 (17–82)
- Adults (N = 99)	HI	86	8	2	3	0	0	0	0	3 (3.0)	5 (5–6)
- Children (N = 11)	microNT	0	1	1	0	2	2	3	2		141 (57–348)
- Adults (N = 99)	microNT	2	40	24	16	11	4	2	0		22 (18–26)
General population (Nakhon Si Thammarat, N = 112)											
- Children (N = 18)	HI	10	0	2	2	4	0	0	0	8 (44.4)	13 (7–24)
- Adults (N = 94)	HI	81	6	4	3	0	0	0	0	3 (3.2)	5 (5–6)
- Children (N = 18)	microNT	2	7	4	2	1	2	0	0		19 (11–32)
- Adults (N = 94)	microNT	11	39	17	15	10	2	0	0		17 (14–20)

Infection rate is determined by HI titer ≥40 in adults or ≥20 in children.
doi:10.1371/journal.pone.0016164.t006

hemagglutinin molecule as well as the epitopes in HA2 domain which is conserved across influenza A subtypes [30–32].

Only one (2.3%) of our 44 adult patients failed to mount HI antibody titers ≥40 in their convalescent blood. Meanwhile, Miller, et al [10] demonstrated that HI assay failed to diagnose 10.9% of laboratory confirmed cases in their setting as the cut-off point ≥32 was employed. According to Millers, et al, the adult and pediatric patients were not separately analyzed; and the HI titers 32 or greater were found in 89.1% of their patients. In our study, if the data from pediatric and adult patients was pooled and analyzed together, the HI titer 40 or greater will be found in 92.5% of our patients. Regarding the study by Chen, et al [33], the HI titers 40 or greater were found in 93% of their adult patients if the convalescent blood samples were collected at peak between 25 and 29 days after onset of symptom.

Our study decided to use the HI antibody at cut-off titers ≥40 for adults and ≥20 for children to differentiate between individuals with and without past infection by the 2009 pandemic influenza. The cut-off titer for NT antibody could not be established because frequency of the cross-reactive NT titers was high as shown from the result of investigation in pre-pandemic sera obtained from vaccinees of age younger than 50 years, and additionally, from the high number of general adult population who had no HI antibody, but possessed NT titer ≥40. Based on our criteria, magnitudes of the 2009 pandemic influenza after the first epidemic wave were around 7% in blood donors and 12.8% in HCW. Eventually, the infection rate in general population was much higher in children than adults, i.e., 58.6% (17/29) *versus* 3.1% (6/193), which is suggestive of susceptibility of children and partial protection from pre-existing immunity in Thai adults. In United Kingdom, seroincidence rate of the 2009 pandemic influenza was also high in children [10]. Seroepidemiological data from Pittsburgh, U.S. showed that approximately 21% of population was infected following the second epidemic wave [34]. Estimation on the infection rates based on serological data may be affected by confounding factor of cross-reactive antibody-rising from seasonal influenza vaccination; and probably from pre-existing antibody against the 1957 influenza A (H1N1) virus. Nevertheless, this effect may be not drastic in the Thai population owing to less than 1% coverage of seasonal influenza vaccination among the Thai population. In addition, most of our adult subjects were younger than 50 years, therefore, cross-reactive antibody owing to previous infection by the 1918 influenza A (H1N1) virus was excluded.

Thailand reported the first two imported cases from Mexico in the beginning of May 2009. Subsequently, the virus was re-introduced into the country both by groups of tourists and Thai students who returned from Europe and America. The first epidemic wave began in late May, peaked in July and almost disappeared in November 2009. The first wave was followed by a short period of the second epidemic wave during December 2009 to April 2010 with peak in February. The third epidemic wave which lasted between June and October with peak in August 2010 was more serious than the second one. Serosurveillance nation-wide will help the estimation for number of vulnerable people and immunity of the population to this pandemic virus. The 2009 pandemic monovalent vaccine was introduced into Thailand in December 2009; and trivalent vaccine containing the 2009 pandemic virus as a component has been introduced into the country in June 2010. Nevertheless, the vaccine coverage was less than 3% of the Thai population. Therefore, cross-reactive HI antibody due to the pandemic virus might have least effect on the estimated infection rate in the present study.

Acknowledgments

The authors would like to thanks the Southeast Asian Infectious Disease Clinical Research Network and the US. National Institute of Health for some serum samples from the 2009 pandemic influenza patients. Thanks are also to Dr. Anucha Apisarnthanarak, Thammasat University Hospital, Pathum Thani, Thailand for part of serum samples from health care workers.

Author Contributions

Conceived and designed the experiments: P. Puthavathana. Performed the experiments: HL P. Pooruk SK PN. Analyzed the data: HL P. Puthavathana. Wrote the paper: P. Puthavathana HL SI. Specimen providers: CP AM SI PT UK PA KC SO WP PS TC CS SL. Processing on ethical issue: PA. Veterinarian: WW.

References

1. Morens DM, Fauci AS (2007) The 1918 influenza pandemic: insights for the 21st century. J Infect Dis 195: 1018–1028.
2. Chan M World now at the start of 2009 influenza pandemic. Available: http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html. Accessed 26 June 2009.
3. Neumann G, Noda T, Kawaoka Y (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459: 931–939.
4. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, et al. (2009) Pandemic potential of a strain of influenza A (H1N1): early findings. Science 324: 1557–1561.
5. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 360: 2605–2615.
6. Hajjar LA, Mauad T, Galas FR, Kumar A, da Silva LF, et al. (2010) Severe novel influenza A (H1N1) infection in cancer patients. Ann Oncol doi: 0.1093/annonc/mdq254.
7. Jamieson DJ, Honein MA, Rasmussen SA, Williams JL, Swerdlow DL, et al. (2009) H1N1 2009 influenza virus infection during pregnancy in the USA. Lancet 374: 451–458.
8. Miller M, Viboud C, Simonsen L, Olson DR, Russell C (2009) Mortality and morbidity burden associated with A/H1N1pdm influenza virus: Who is likely to be infected, experience clinical symptoms, or die from the H1N1pdm 2009 pandemic virus? Version 2. PLoS Curr 1: RRN1013. doi:10.1371/currents.RRN1013.
9. World Health Organization (2009) Assessing the severity of an influenza pandemic. Available: http://www.who.int/csr/disease/swineflu/assess/disease_swineflu_assess_20090511/. Accessed 24 Apr 2010.
10. Miller E, Hoschler K, Hardelid P, Stanford E, Andrews N, et al. (2010) Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study. Lancet 375: 1100–1108.
11. Reed C, Katz JM (2010) Serological surveys for 2009 pandemic influenza A H1N1. Lancet 375: 1062–1063.
12. Chen H, Wang Y, Liu W, Zhang J, Dong B, et al. (2009) Serologic survey of pandemic (H1N1) 2009 virus, Guangxi Province. China Emerg Infect Dis 15: 1849–1850.
13. Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, et al. (2009) Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N Engl J Med 361: 1945–1952.
14. de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, et al. (2003) Haemagglutination-inhibiting antibody to influenza virus. Dev Biol (Basel) 115: 63–73.
15. Eichelberger M, Golding H, Hess M, Weir J, Subbarao K, et al. (2008) FDA/NIH/WHO public workshop on immune correlates of protection against influenza A viruses in support of pandemic vaccine development, Bethesda, Maryland, US, December 10–11, 2007. Vaccine 26: 4299–4303.
16. Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, et al. (1999) Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol 37: 937–943.
17. World Health Organization (2002) WHO manual on animal influenza diagnosis and surveillance. WHO/CDS/CSR/NCS/2002.5. Available: http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf. Accessed 22 Feb 2010.
18. World Health Organization (2009) CDC protocol of realtime RT-PCR for influenza A(H1N1). Available from: http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf. Accessed 24 Apr 2010.
19. Louisirirotchanakul S, Lerdsamran H, Wiriyarat W, Sangsiriwut K, Chaichoune K, et al. (2007) Erythrocyte binding preference of avian influenza H5N1 viruses. J Clin Microbiol 45: 2284–2286.

20. Dejpichai R, Laosiritaworn Y, Phuthavathana P, Uyeki TM, O'Reilly M, et al. (2009) Seroprevalence of antibodies to avian influenza virus A (H5N1) among residents of villages with human cases, Thailand, 2005. Emerg Infect Dis 15: 756–760.
21. Kitphati R, Pooruk P, Lerdsamran H, Poosuwan S, Louisirirotchanakul S, et al. (2009) Kinetics and longevity of antibody response to influenza A H5N1 virus infection in humans. Clin Vaccine Immunol 16: 978–981.
22. Auewarakul P, Kositanont U, Sornsathapornkul P, Tothong P, Kanyok R, et al. (2007) Antibody responses after dose-sparing intradermal influenza vaccination. Vaccine 25: 659–663.
23. Ndifon W, Dushoff J, Levin SA (2009) On the use of hemagglutination-inhibition for influenza surveillance: surveillance data are predictive of influenza vaccine effectiveness. Vaccine 27: 2447–2452.
24. Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, et al. (2008) Influenza vaccine strain selection and recent studies on the global migration of seasonal influenza viruses. Vaccine 26 (Suppl 4): D31–4.
25. Stephenson I, Wood JM, Nicholson KG, Zambon MC (2003) Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J Med Virol 70: 391–398.
26. Hinjoy S, Puthavathana P, Laosiritaworn Y, Limpakarnjanarat K, Pooruk P, et al. (2008) Low frequency of infection with avian influenza virus (H5N1) among poultry farmers, Thailand, 2004. Emerg Infect Dis 14: 499–501.
27. Kositanont U, Wongsurakiat P, Pooruk P, Maranetra N, Puthavathana P (2010) Induction of cross-neutralizing antibody against H5N1 virus after vaccination with seasonal influenza vaccine in COPD patients. Viral Immunol 23: 329–334.
28. Kelly H, Grant K (2009) Interim analysis of pandemic influenza (H1N1) 2009 in Australia: surveillance trends, age of infection and effectiveness of seasonal vaccination. Euro Surveill 14: pii: 19288.
29. Garcia-Garcia L, Valdespino-Gómez JL, Lazcano-Ponce E, Jimenez-Corona A, Higuera-Iglesias A, et al. (2009) Partial protection of seasonal trivalent inactivated vaccine against novel pandemic influenza A/H1N1 2009: case-control study in Mexico City. BMJ 339: b3928.
30. Gocník M, Fislová T, Sládková T, Mucha V, Kostolanský F, et al. (2007) Antibodies specific to the HA2 glycopolypeptide of influenza A virus haemagglutinin with fusion-inhibition activity contribute to the protection of mice against lethal infection. J Gen Virol 88: 951–955.
31. Gocník M, Fislová T, Mucha V, Sládková T, Russ G, et al. (2008) Antibodies induced by the HA2 glycopolypeptide of influenza virus haemagglutinin improve recovery from influenza A virus infection. J Gen Virol 89: 958–967.
32. Sui J, Hwang WC, Perez S, Wei G, Aird D, et al. (2009) Structure and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 16: 265–73.
33. Chen MI, Barr IG, Koh GCH, Lee VJ, Lee CPS, et al. (2010) Serological Response in RT-PCR Confirmed H1N1-2009 Influenza A by Hemagglutination Inhibition and Virus Neutralization Assays: An Observational Study. PLoS One 5: e12474.
34. Ross T, Zimmer S, Burke D, Crevar C, Carter D, et al. (2010) Seroprevalence following the second wave of pandemic 2009 H1N1 influenza. PLoS Curr 2: RRN1148. doi:10.1371/currents.RRN1148.

Veterinary Microbiology 146 (2010) 346–349

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Erythrocyte binding preference of 16 subtypes of low pathogenic avian influenza and 2009 pandemic influenza A (H1N1) viruses

Witthawat Wiriyarat [a], Hatairat Lerdsamran [b], Phisanu Pooruk [b], Robert G. Webster [c], Suda Louisirirotchanakul [b], Parntep Ratanakorn [a], Kridsada Chaichoune [a], Kannika Nateerom [b], Pilaipan Puthavathana [b,*]

[a] Faculty of Veterinary Science, Mahidol University, Nakhonpathom 73170, Thailand
[b] Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkok-noi, Bangkok 10700, Thailand
[c] St. Jude Children Research Hospital, TN, USA

ARTICLE INFO

Article history:
Received 1 November 2009
Received in revised form 11 May 2010
Accepted 21 May 2010

Keywords:
Low pathogenic avian influenza viruses
2009 pandemic influenza A (H1N1) virus
Erythrocytes
Hemagglutination assay

ABSTRACT

All 16 subtypes of avian influenza viruses of low pathogenicity (LPAIV) as well as their hemagglutinin (H) antigens, and four 2009 pandemic influenza A (H1N1) virus isolates were assayed for hemagglutinating activity against 5 erythrocyte species: goose, guinea pig, human group O, chicken and horse. Of all viruses and antigens assayed, the highest hemagglutination (HA) titers were obtained with goose and guinea pig erythrocytes. Hemagglutinating activity of replicating LPAIV and LPAIV antigens decreased, in order, with chicken and human group O; meanwhile, horse erythrocytes yielded lowest or no HA titer. Moreover, the 2009 pandemic viruses did not agglutinate both horse and chicken erythrocytes. Our study concluded that goose and guinea pig erythrocytes are the best in HA assay for all subtypes of influenza viruses.

1. Introduction

Hemagglutinating activity of influenza viruses is mediated by binding of hemagglutinin surface glycoprotein and sialic acid receptors on erythrocyte surface membrane; that can be measured by HA assay which is the basis for recognition of the virus isolates (Ito et al., 1997). Additionally, adhering of erythrocytes on the cell monolayer inoculated with clinical sample is also suggestive of positive virus isolation; this reaction is known as hemadsorption assay. HA assay is performed for virus titration prior to running hemagglutination-inhibition (HI) assay for specific antibody detection (Webster et al., 2002). The importance of erythrocyte species in HA and HI assays has come into consideration after several occasions of cross-species transmission of avian influenza viruses to human populations (Subbarao and Shaw, 2000).

At present, 16 H subtypes and 9 neuraminidase (N) subtypes of influenza A viruses have been discovered. All of these subtypes are found in aquatic birds; therefore, it is believed that these viruses are ancestors of all those that cause infection in man and other animal species (Webster, 1998). However, most of avian viruses are of low virulence, with exception of some isolates in H5 and H7 subtypes that are recognized as highly pathogenic avian influenza viruses (HPAIV). Both subtypes were evidenced for cross-species transmission from avian to man (Liu, 2006; Myers et al., 2007). However, the subtypes that are currently known as human viruses comprise only H1N1 and H3N2 subtypes (Brockwell-Staats et al., 2009). Humans are seldom vulnerable to LPAIV. Nevertheless, sporadic human infections with LPAIV H7N2, H7N3, H7N7, H9N2 and H10N7 had been reported without death (Kalthoff et al., 2010). On the other hand, it is well documented that LPAIV was the ancestors of HPAIV.

* Corresponding author. Tel.: +66 2 419 7059/418 2663; fax: +66 2 418 4148/418 2663.
E-mail address: siput@mahidol.ac.th (P. Puthavathana).

0378-1135/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2010.05.031

Through reassortment events, the avian virus H5N1 which originated in Hong Kong in 1997 received *H* gene from A/goose/Guangdong/1/1996 (H5N1) (A/Gd), *N* gene from A/teal/Hong Kong/W312/1997 (H6N1)-like viruses and the 6 internal genes from A/quail/Hong Kong/G1/1997 (H9N2)-like or A/teal/Hong Kong/W312/1997 (H6N1)-like viruses (Hatta and Kawaoka, 2002; Subbarao and Shaw, 2000). A/Gd was also the gene donor for *H* and *N* genomic segments of the current H5N1 virus.

Our group previously investigated hemagglutinating activity of HPAIV H5N1 against 5 erythrocyte species: horse, goose, chicken, guinea pig and human O cells and found that goose cells yielded the highest HA titer, and was followed in order by chicken, guinea pig, human O and horse cells. Meanwhile, human viruses H1N1 and H3N2 could agglutinate guinea pig erythrocytes at highest titers, but none could agglutinate horse erythrocytes. Additionally, human virus H3N2 isolated in 1998 could agglutinate chicken erythrocytes, but this activity was lost with the 2003 and 2004 H3N2 viral isolates (Louisirirotchanakul et al., 2007; Medeiros et al., 2001; Nobusawa et al., 2000). The present study, then, further explored the hemagglutinating activity of all 16 subtypes of replicating LPAIV, LPAIV antigens as well as the replicating 2009 pandemic A (H1N1) viruses against all 5 erythrocyte species mentioned above.

2. Materials and methods

2.1. Viruses

All 16 subtypes of LPAIV investigated in this study were kindly provided by St. Jude Children Research Laboratory, Tennessee, U.S.A. These viruses were further propagated in embryonated eggs in Thailand; and the replicating viruses were used as the test antigens in HA assay. H antigens from LPAIV used in the assay were purchased from the Veterinary Laboratory Agency, Weybridge, UK. The four 2009 pandemic A (H1N1) viruses were isolated and propagated in MDCK cells at the Siriraj Cooperative Research Center, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University.

2.2. Erythrocyte species

Five erythrocyte species: goose, guinea pig, chicken, horse and human blood group O, were employed in the study. Fresh blood was mixed with Alsever's solution at equal volumes and kept at 4 °C until used within 2 weeks.

2.3. Hemagglutination assay

The procedure was performed as described previously (Louisirirotchanakul et al., 2007; Medeiros et al., 2001; Webster et al., 2002). Different erythrocyte species were tested at different concentrations, i.e., 1% horse, 0.5% goose, 0.5% chicken, 0.75% guinea pig and 0.75% human blood group O cells. These erythrocyte species were suspended in phosphate buffered saline (PBS), pH 7.2, except for that of horse erythrocytes which were suspended in PBS plus 0.5% bovine serum albumin. The same aliquot of each virus subtype was titrated against all 5 erythrocyte species. HA assays employing goose and chicken erythrocytes were performed in V-shaped bottom microtiter plates, while those employing guinea pig, human blood group O and horse erythrocytes were performed in U-shaped bottom microtiter plates. The test viruses/antigens were twofold serially diluted, starting from the dilution 1:2/1:8 and proceeding to 1:1024. A reaction well consisting of 50 μl of test viruses/antigens and 50 μl of erythrocyte suspension was incubated at 4 °C for approximately 45 min before determining the HA titer which is defined as the highest virus dilution that yielded complete hemagglutination of the test erythrocytes.

3. Results

With each LPAIV subtype, three erythrocyte donors from each species were employed in three separate experiments, except that two separate experiments were

Table 1
Hemagglutinating activity of replicating LPAIV.

Viruses	HA titers as determined by erythrocytes from				
	Goose	Guinea pig	Human	Chicken	Horse
A/aquatic bird/Hong Kong/DI25/2002 (H1N1)	256	256	128	128	64
A/wild duck/Shan Tou/992/2000 (H2N8)	256	256	128	128	64
A/duck/Shan Tou/1283/2001 (H3N8)	256	256	128	64	128
A/duck/Shan Tou/461/2000 (H4N9)	512	512	256	256	128
A/duck/Jiangxi/6151/03 (H5N3)	256	256	128	256	64
A/heron/Hong Kong/LC10/2002 (H6N8)	1024	1024	256	256	128
A/ostrich/Zimbabwe/222/1996 (H7N1)	512	512	256	256	128
A/mallard/Alberta/242/2003 (H8N4)	512	512	256	256	256
A/quail/Hong Kong/G1/1997 (H9N2)	1024	512	256	1024	<2
A/chicken/Hong Kong/G9/1997 (H9N2)	512	512	256	512	<2
A/duck/Hong Kong/Y280/1997 (H9N2)	1024	1024	512	512	<2
A/duck/Shan tou/1796/2001 (H10N8)	256	256	128	128	128
A/duck/Shan tou/1411/2000 (H11N2)	256	512	128	128	128
A/red-necked stint/Australia/5745/1981 (H12N9)	512	512	128	256	128
A/gull/MD/704/1977 (H13N6)	128	128	64	128	<2
A/mallard/Gurjev/263/1982 (H14N5)	1024	1024	256	512	256
A/duck/Australia/341/83(H15N8)	128	256	128	128	32
A/shore bird/DE/172/2006 (H16N3)	128	128	64	128	64

Table 2
Hemagglutinating activity of hemagglutinin antigen of LPAIV.

Viruses[a]	HA titers as determined by erythrocytes from				
	Goose	Guinea pig	Human	Chicken	Horse
A/duck/Alberta/35/1976 (H1N1)	512	512	256	256	<8
A/duck/Germany/1215/1973 (H2N3)	512	512	512	512	128
A/turkey/England/1969 (H3N2)	1024	1024	512	512	256
A/duck/Czech/1956 (H4N6)	512	512	256	512	128
A/ostrich/Denmark/72420/1996 (H5N2)	512	512	256	512	64
A/turkey/England/647/1977 (H7N7)	512	512	256	512	64
A/turkey/Ontario/6118/1968 (H8N4)	64	128	32	64	16
A/duck/England/1956 (H11N6)	32	64	32	64	<8
A/duck/Alberta/60/1976 (H12N5)	256	512	256	256	16
A/mallard/Astrakhan/244/1982 (H14N6)	64	128	64	32	<8
A/she/WA/2576/1979 (H15N6)	512	512	256	512	128
A/gull/Denmark/68110/2002 (H16N3)	16	16	8	16	<8

[a] Hemagglutinin titers of some subtypes are too low for performing HA assay.

Table 3
Hemagglutinating activity of the 2009 pandemic A (H1N1) viruses.

Virus name	Passage history	HA titers as determined by erythrocytes from				
		Goose	Guinea pig	Human	Chicken	Horse
A/Nonthaburi/102	MDCK4	128	128	32	<2	<2
A/Thailand/104	MDCK4	64	64	32	<2	<2
A/Bangkok/SI 614	MDCK2	16	16	8	<2	<2
A/Bangkok/SI 618	MDCK2	16	16	8	<2	<2

conducted for the 2009 pandemic viruses. Each experiment was run in duplicate. In total, 4 or 6 titer values were obtained for each virus/antigen; these values were equal or were within twofold differences. Therefore, the HA titer shown in Tables 1 and 2 represented the result for each erythrocyte species. The highest HA titers were obtained with goose and guinea pig erythrocytes for most of the LPAIV subtypes assayed. Chicken erythrocytes were slightly more sensitive than human group O erythrocytes, and horse erythrocytes were least sensitive. Particularly, it was demonstrated that quail H9N2, chicken H9N2, duck H9N2 and H13N6, could not agglutinate horse erythrocytes (Table 1).

With regard to usage of commercial H antigen, the amount supplied for some subtypes were inadequate for running an assay. The HA titers using H antigens from 12 subtypes are shown in Table 2. Again, the result showed that goose and guinea pig erythrocytes yielded the highest HA titers; chicken erythrocytes were more sensitive than human group O erythrocytes, while horse erythrocytes yielded the lowest titers.

Additionally, four 2009 pandemic influenza A (H1N1) virus isolates were assayed against these 5 erythrocyte species. It was similarly demonstrated that goose and guinea pig erythrocytes were the most sensitive, followed by human O erythrocytes. Meanwhile, the viruses did not agglutinate either chicken or horse erythrocytes (Table 3).

4. Discussion

Upon the isolation of influenza viruses in cell cultures or embryonated eggs, various erythrocyte species have been applied for recognition of the isolated viruses by HA assay. However, no rigid data has discovered differences in sensitivity of the assay when different erythrocyte species are employed. Even though turkey erythrocyte has been recommended, this animal species is seldom found in Thailand; and thus, it is not included in this study. AI viruses preferentially bind sialic acid-α2,3-galactose (SAα2,3Gal) receptor, while human viruses prefer SAα2,6-Gal receptor, and swine viruses have a propensity for both (Ito et al., 1997). Some mutational change in the receptor binding site might lead to change in binding property of the viruses to certain erythrocyte species (Auewarakul et al., 2007; Nobusawa et al., 2000). Of 5 erythrocyte species, all 16 subtypes of LPAIV preferentially bound guinea pig and goose erythrocytes and yielded the highest HA titers. Meanwhile, horse erythrocyte was least sensitive in recognizing the test viruses or H antigens (Table 1). Interestingly, H9N2 virus, regardless of host of origin (quail, chicken or duck), as well as H13N6 virus, could not agglutinate horse erythrocytes. Binding between influenza viral hemagglutinin and cellular receptor is influenced not only by the type of galactosyl linkage, but also the species of sialic acid, i.e., *N*-acetylneuraminic acid (NeuAc) or *N*-glycolylneuraminic acid (NeuGc). While the other erythrocyte species contain both NeuAcα2,3Gal and NeuAcα2,6Gal, horse erythrocytes contain only NeuGcα2,3Gal (Stephenson et al., 2003). As avian viruses preferentially bind SAα2,3Gal, failure of some avian virus subtypes to agglutinate horse erythrocytes might possibly be explained by a loss of ability to recognize sialic acid residue.

Our study with the 2009 pandemic A (H1N1) virus demonstrated that all 4 isolates could not agglutinate both chicken and horse erythrocytes; whereas, highest HA titers

were observed with goose and guinea pig erythrocytes. Genetic analysis revealed that the *H* genomic segment of the 2009 pandemic viruses was derived from classic American swine influenza A (H1N1) virus (Smith et al., 2009). Complete genomic sequences of two pandemic strains in our study (A/Nonthaburi/102/2009 and A/Thailand/104/2009) can be accessed through GenBank.

Our study also demonstrated that the HA titers for each virus as obtained from three different donors within the same species were comparable; therefore, we can draw the conclusion that different genetic allotypes within species do not affect hemagglutinating reaction.

Collective data obtained with our LPAIV and 2009 pandemic A (H1N1) viruses as well as our previous work on HPAIV H5N1, demonstrated that goose and guinea pig erythrocytes are the best erythrocyte species in HA assay, and are then followed by chicken erythrocytes for LPAIV or human O cells for the pandemic A (H1N1) viruses. Our study thus exerts its importance on an increase in sensitivity for recognizing the virus isolates in the virus isolation technique when the appropriate erythrocyte species is chosen. Viruses that replicate slowly and yield low levels of viral progenies could be picked-up. Additionally, the sensitive HA assay will consequently result in the sensitive HI assay. When a smaller amount of viruses is needed to prepare the working dilution of H antigen, a smaller amount of molecules of HI antibody will be required to completely inhibit the hemagglutinating activity of the test virus.

Acknowledgements

This study was a part of the project entitled ''Avian Influenza Surveillance in Thailand-Studies at Human-Animal Interface'' supported by Cooperative Agreement Number 1U19CI00399-01 from Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the authors and do not necessarily represent the official view of CDC. The study was also partially supported by Thailand Research Fund for Senior Research Scholars through P.P. The authors would like to express sincere thanks to Drs. Dianne Gross and Jacquerine Katz, U.S., CDC for their kind support; and for Mekkla Thomson and Steve Durako, Westat, for coordinating this study.

References

Auewarakul, P., Suptawiwat, O., Kongchanagul, A., Sangma, C., Suzuki, Y., Ungchusak, K., Louisirirotchanakul, S., Lerdsamran, H., Pooruk, P., Thitithanyanont, A., Pittayawonganon, C., Guo, C.T., Hiramatsu, H., Jampangern, W., Chunsutthiwat, S., Puthavathana, P., 2007. An avian influenza H5N1 virus that binds to a human-type receptor. J. Virol. 81, 9950–9955.

Brockwell-Staats, C., Webster, R.G., Webby, R.J., 2009. Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). Influenza Other Respi. Viruses 3, 207–213.

Hatta, M., Kawaoka, Y., 2002. The continued pandemic threat posed by avian influenza viruses in Hong Kong. Trends Microbiol. 10, 340–344.

Ito, T., Suzuki, Y., Mitnaul, L., Vines, A., Kida, H., Kawaoka, Y., 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology 227, 493–499.

Kalthoff, D., Globig, A., Beer, M., 2010. (Highly pathogenic) avian influenza as a zoonotic agent. Vet. Microbiol. 140, 237–245.

Liu, J.P., 2006. Avian influenza – a pandemic waiting to happen? J. Microbiol. Immunol. Infect. 39, 4–10.

Louisirirotchanakul, S., Lerdsamran, H., Wiriyarat, W., Sangsiriwut, K., Chaichoune, K., Pooruk, P., Songserm, T., Kitphati, R., Sawanpanyalert, P., Komoltri, C., Auewarakul, P., Puthavathana, P., 2007. Erythrocyte binding preference of avian influenza H5N1 viruses. J. Clin. Microbiol. 45, 2284–2286.

Medeiros, R., Escriou, N., Naffakh, N., Manuguerra, J.C., van der Werf, S., 2001. Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology 289, 74–85.

Myers, K.P., Setterquist, S.F., Capuano, A.W., Gray, G.C., 2007. Infection due to 3 avian influenza subtypes in United States veterinarians. Clin. Infect. Dis. 45, 4–9.

Nobusawa, E., Ishihara, H., Morishita, T., Sato, K., Nakajima, K., 2000. Change in receptor-binding specificity of recent human influenza A viruses (H3N2): a single amino acid change in hemagglutinin altered its recognition of sialyloligosaccharides. Virology 278, 587–596.

Smith, G.J., Vijaykrishna, D., Bahl, J., Lycett, S.J., Worobey, M., Pybus, O.G., Ma, S.K., Cheung, C.L., Raghwani, J., Bhatt, S., Peiris, J.S., Guan, Y., Rambaut, A., 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459, 1122–1125.

Stephenson, I., Wood, J.M., Nicholson, K.G., Zambon, M.C., 2003. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J. Med. Virol. 70, 391–398.

Subbarao, K., Shaw, M.W., 2000. Molecular aspects of avian influenza (H5N1) viruses isolated from humans. Rev. Med. Virol. 10, 337–348.

Webster, R., 1998. Influenza: an emerging disease 1998. Emerg. Infect. Dis. 4, 436–441.

Webster, R., Cox, N., Stohr, K., 2002. WHO manual on animal influenza diagnosis and surveillance, WHO/CDS/CSR/NCS/2002.5, available at http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf.

VIRAL IMMUNOLOGY
Volume 23, Number 3, 2010

Pp. 329–334
DOI: 10.1089/vim.2009.0082

Induction of Cross-Neutralizing Antibody Against H5N1 Virus After Vaccination with Seasonal Influenza Vaccine in COPD Patients

Uraiwan Kositanont,[1] Phunsup Wongsurakiat,[2] Phisanu Pooruk,[1]
Nanta Maranetra,[2] and Pilaipan Puthavathana[1]

Abstract

Archival serum samples from elderly individuals with underlying chronic obstructive pulmonary disease (COPD) who were enrolled in a double-blind case-control study of seasonal influenza vaccine efficacy were assayed for cross-neutralizing antibody formation to avian influenza A (H5N1) virus. Of 118 serum samples, 58 were collected from influenza vaccinees (mean age 68.5 y), and 60 from placebo controls (mean age 68.4 y) who received vitamin B injections. Blood samples were collected before and at 1 mo after seasonal influenza vaccination from all subjects; in addition, for a longitudinal follow-up period of 1 y paired-blood samples were collected again from subjects who developed acute respiratory illness. Hemagglutination inhibition assay for antibodies to influenza A (H1N1), influenza A (H3N2), and influenza B viruses was carried out to determine the serological response to vaccination, and to diagnose influenza viral infection, while microneutralization assays were performed to detect cross-reactive antibody to H5N1 virus. Pre-existing cross-reactive H5N1 antibody at reciprocal titer 10 was found in 6 (10.3%) vaccinees and 4 (6.7%) placebo controls. There was no change in H5N1 antibody titer in these subjects after vaccination. On the other hand, 3 (5.2%) vaccinees developed seroconversion to H5N1 virus at 1 mo after vaccination, even though they had no pre-existing H5N1 antibody in their first blood samples. No cross-neutralizing antibody to H5N1 virus was detected in the placebo controls or in the 22 influenza patients, suggesting that influenza vaccination, but not influenza virus infection, induces cross-neutralizing antibody against avian influenza H5N1 virus.

Introduction

Direct transmission of highly pathogenic avian influenza (HPAI) H5N1 virus from poultry to humans was first reported in Hong Kong in 1997, and it caused 18 cases with 6 deaths, for a 33% fatality rate (3). Its re-emergence since 2003 has spread across continents, producing a higher fatality rate of about 60% in infected humans (24). Thailand reported the first human case on January 23, 2004, and the last case occurred in July 2006 (9). Nevertheless, human cases of avian influenza were seen in the People's Republic of China, Vietnam, Indonesia, and Egypt in 2009 (24). The elderly succumbed to avian influenza to a lesser extent than young children, which may be a result of cross-immunity elicited by repeated infection with other human influenza A virus subtypes (26).

A few pieces of evidence have suggested that heterosubtyping immunity may be able to confer protection across influenza subtypes, but the mechanism of this cross-protection, if it is mediated by cellular or humoral immunity, and from which antigenic domain, is not well defined (12,13). Theoretically, cross-immunity can be induced either by hemagglutinin (HA) or neuraminidase (NA) antigen. The hypothesis of cross-protection mediated by NA immunity was derived from the observation that the 1968 pandemic influenza caused by the H3N2 virus, which came after the 1957 pandemic caused by the H2N2 virus, produced fewer deaths (8,21). Increasing levels of anti-NA antibody have been associated with decreasing frequency of viral infection and suppression of clinical manifestations (11). In an animal model, mice immunized with DNA vaccine containing a human N1 NA gene survived lethal challenge with H5N1 virus (18). On the other hand, HA antigen might also play a role in heterosubtypic immunity. In a previous study researchers reported that children with primary infection by H1N1 or H3N2 virus developed HA antibody to the

[1]Department of Microbiology, and [2]Department of Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

contemporary subtypes, as well as cross-reactive antibody to H8 as assayed by ELISA (1).

During 1997 and 1998, our group conducted a double-blind controlled trial to demonstrate the efficacy of human influenza vaccination in patients with chronic obstructive pulmonary disease (COPD) aged >60 y (10,22). Serum samples were collected from these subjects before and at 1 mo after vaccination to determine the serological response to influenza vaccine. These vaccinees were longitudinally followed up for a year, and paired-blood samples were collected from the individuals who developed acute respiratory illness (ARI) for serodiagnosis of influenza by hemagglutination inhibition (HI) assay. In the present study we aimed to detect neutralizing antibody against HPAI H5N1 virus in the aforementioned serum samples by microneutralization (microNT) assay. In this study we demonstrated that the elderly subjects developed cross-neutralizing H5N1 antibody as the result of vaccination, but not from influenza illness. Since our archival serum samples were collected before the occurrence of the avian influenza outbreak, H5N1 infection in the subjects was excluded.

Materials and Methods

Subjects

A total of 118 COPD patients who attended the COPD clinic at Siriraj Hospital, Bangkok, Thailand, were enrolled in a double-blind case-control study to evaluate the efficacy of seasonal influenza vaccine between 1997 and 1998. Fifty-eight subjects (mean age 68.5 y) received vaccine, whereas 60 subjects (mean age 68.4 y) received vitamin B injection as the placebo control. Venous blood samples (10 mL) were collected from each subject prior to vaccination or vitamin B injection; the second blood samples were collected 1 mo thereafter to determine the serological response to vaccination. No subject developed influenza-like illness during this 1-mo period. Furthermore, the subjects were longitudinally followed-up for 1 y for ARI. In those cases, paired-blood samples at 4- to 6-wk intervals were collected for serodiagnosis of influenza by HI assay using the vaccine strains as the test antigens. Serum samples were kept frozen at −20°C until testing. The study was approved by the Institutional Review Board of the Committee on Ethics, Faculty of Medicine, Siriraj Hospital, Mahidol University.

Vaccines

The seasonal influenza vaccine used in this study was the split-typed trivalent vaccine from Pasteur Merieux, Lyon, France. A 0.5-mL dose of vaccine contained influenza A/Texas/36/91 (H1N1), A/Nanchang/933/95 (H3N2), and B/Harbin/07/94, at concentration of 15 μg of HA for each virus. A 0.5-mL dose of vitamin B_1 was used as placebo.

Hemagglutination inhibition test

The procedure for the HI assay was as described elsewhere (10,23). Briefly, non-specific inhibitor in the test sera was eliminated by treatment with receptor-destroying enzyme from *Vibrio cholerae* (Denka Seiken, Niigata, Japan) overnight at 4°C, followed by inactivation at 56°C for 30 min. Then non-specific agglutinator was removed by absorption with 50% chick red blood cells. The test antigen panel, influenza A/H1N1, influenza A/H3N2, and influenza B viruses, was kindly provided by the World Health Organization (WHO). Serum with HI antibody titer ≥1:10 was considered positive for influenza antibody. A positive seroresponse to influenza vaccine was obtained when paired-blood samples collected before and after vaccination showed a fourfold or greater rise in HI antibody titer against any of the test antigens. Similarly, influenza diagnosis by HI assay was given based on the same criteria.

Microneutralization assay

The ELISA-based microNT assay protocol was based on that described in Kitphati *et al.* (9) and the WHO manual (23). The experiments were conducted in a laboratory with biosafety level 3. Influenza A/Thailand/1 (KAN-1)/2004 (H5N1) was used as the test virus. Briefly, a serum sample was twofold serially diluted from the dilution of 10 to 1280; then 60 μL of each serum dilution was mixed with an equal volume of the test virus at a concentration of 200 $TCID_{50}$/100 μL and incubated at 37°C for 2 h. A 100-μL volume of the virus-serum mixture was inoculated onto a Madin-Darby canine kidney (MDCK) cell monolayer in a well of a microtiter plate and further incubated for 18–20 h in a CO_2 incubator. The assays were run in duplicate. The reaction plate was tested by ELISA to determine the amount of influenza nucleoprotein produced in the infected MDCK cells. Mouse monoclonal antibody (Chemicon International, Temecula, CA) was used as the primary antibody, anti-mouse Ig conjugated with horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL) was used as the second antibody, and TMB (KPL Inc., Gaithersburg, MD) was used as the chromogenic substrate. The reaction plate was read under a spectrophotometer at the wavelengths of 450 and 630 nm. Wells with uninfected cells and virus back-titration were included as the control sets in every test plate. The serum dilution that reduced more than 50% of the amount of viral nucleoprotein compared to the virus control was considered to be positive for anti-H5N1 antibody. The cut-off titer for positive H5N1 antibody was set at 10.

Statistical analysis

Data were analyzed by SPSS version 11.5 software (SPSS, Inc., Chicago, IL). Comparison of geometric mean titer (GMT) values and fold increase in titers between the two groups was performed by the Mann-Whitney *U* test. Comparison of prevalence between two groups was performed by chi-square testing. The level of statistical significance was set at $p < 0.05$.

Results

H5N1-neutralizing antibody after seasonal influenza vaccination

Elderly subjects with COPD were immunologically intact, as shown by the fact that 85% of the vaccinees, but not the placebo controls, developed a fourfold or greater rise in HI antibody titer to the influenza A/H1N1 and H3N2 vaccine strains (Table 1). GMTs of H5N1-neutralizing antibodies prior to vaccination in both groups were not statistically significantly different (5.37 versus 5.24; $p = 0.558$). Meanwhile, pre-existing H5N1-neutralizing antibody at titer 10 was found in 6 (10.3%) of 58 vaccinees, and in 4 (6.7%) of 60

Table 1. Distribution of Antibody Response to H1N1 and H3N2 Vaccine Strains in Paired-Blood Samples from Vaccine and Placebo Groups

			Antibody titer									
Virus	*Group*	*Vaccination*	*<10*	*10*	*20*	*40*	*80*	*160*	*320*	*640*	*1280*	*% Vaccine responders*[a]
H1N1	Vaccine	Pre	33	5	9	8	2	0	1	0	0	
	(n = 58)	Post	3	3	8	5	10	9	7	4	9	85
	Placebo	Pre	24	12	8	10	4	1	0	1	0	
	(n = 60)	Post	23	14	7	11	2	2	0	1	0	0
H3N2	Vaccine	Pre	27	10	5	7	5	2	1	1	0	
	(n = 58)	Post	3	1	7	7	10	13	4	2	11	85
	Placebo	Pre	28	8	7	4	5	4	1	0	3	
	(n = 60)	Post	28	8	7	4	6	4	0	0	3	0

[a]A responder is defined by at least a fourfold rise in antibody titers in the paired serum samples.

placebo controls (Table 2). The numbers of subjects who had pre-existing H5N1 antibody in the two groups were also not statistically significantly different ($p = 0.637$). After influenza vaccination, 3 (5.2%) vaccinees who had no pre-existing H5N1 antibody in their initial blood samples did develop a fourfold or greater rise in H5N1-neutralizing antibody titers. The increase in GMT of H5N1 antibody in the vaccine group was not statistically significantly different (5.37 versus 6.05; $p = 0.10$). However, a slight increase in GMT was observed in the vaccine group, but not in the placebo group (Table 2). Two of these three subjects seroconverted to both H1N1 and H3N2, while the third one seroconverted to H1N1 virus only (Table 3).

Lack of H5N1-neutralizing antibody after natural influenza illness

During 1 y of longitudinal follow-up, 102 episodes of ARI occurred among vaccinees and placebo controls. Influenza was diagnosed in 5 (8.6%) vaccinees and 17 (28.3%) placebo controls (Table 4). Nevertheless, no increase in cross-reactive neutralizing H5N1 antibody was detected in these influenza patients. There was one placebo control that had an H5N1 antibody titer of 20 in paired sera. The results demonstrated that acute influenza virus infection did not elicit detectable cross-H5N1 antibody in the study subjects.

Discussion

In the present study we demonstrated pre-existing cross-neutralizing antibody against H5N1 virus in 10 (8.5%) of 118 subjects, as well as induction of a fourfold or greater rise of this cross-reactive antibody in 5.2% of elderly individuals with underlying COPD who were vaccinated with seasonal influenza vaccine. The data on the induction of cross-neutralizing antibody against H5N1 virus by seasonal influenza vaccine are controversial. In our previous unpublished work, cross-neutralizing antibody against H5N1 virus was not detected in 42 young people aged 25–40 y with a history of seasonal influenza vaccination, and also not in 140 healthy subjects aged 20–45 y, of which approximately 70% had neutralizing antibody to A/New Caledonia/20/99 (H1N1) and A/Fujian/411/02 (H3N2). Gioia *et al.* (4) demonstrated a rise in H5N1-neutralizing antibody titer to >20-fold over baseline in 13 (34.2%) of 38 vaccinees (aged 27–59 y, with an average age of 43 y), while their H5N1 HI antibody titers remained at undetectable levels. In contrast, Tang *et al.* (19) did not detect cross-reactive antibody to influenza H5N1 virus, either by microNT or HI assay, in serum samples collected at intervals from 10 vaccinees (aged 20–40 y) who received seasonal influenza vaccine, even though they could elicit a serological response with high HI antibody titers to the vaccine strains. During the 1997 avian influenza outbreak, Rowe *et al.* (17) found that an H5N1-neutralizing antibody titer of 80 was suggestive of avian influenza infection. However, this appeared to apply only to people aged <50 y. Moreover, the H5N1 microNT assay may be less specific in adults aged 60 y and older (17). Results of that study and ours suggest that elderly persons may have cross-reactive antibody to avian H5N1 virus as a result of seasonal

Table 2. Distribution of H5N1 NT Antibodies in Paired-Blood Samples from Vaccine and Placebo Groups

		No. of positive samples at NT antibody titers						
Group		*<10*	*10*	*20*	*40*	*80*	*GMT*	*Number of subjects with pre-existing antibody*
Vaccine	B1	52	6	0	0	0	5.37[a,c]	6[b]
(n = 58)	B2	49	6	0	2[d]	1[d]	6.05[c]	
Placebo	B1	56	4	0	0	0	5.24[a]	4[b]
(n = 60)	B2	56	4	0	0	0	5.24	

[a]$p = 0.558$ for the comparison of pre-vaccination GMT.
[b]$p = 0.637$ for the comparison of frequencies of subjects with pre-exiting H5N1 antibodies.
[c]$p = 0.100$ for comparison of GMT between pre- and post-vaccination in the vaccine group.
[d]Without acute respiratory infection.
Abbreviations: B1, pre-vaccination; B2, post-vaccination; GMT, geometric mean titer; NT, neutralizing antibody.

Table 3. Antibody Responses to H1N1, H3N2, and B Viruses in Vaccinees Who Developed A Fourfold Rise in Antibody to H5N1 Virus

			Antibody titers to influenza viruses	
Code No.	*Group*	*Antibody to subtype*	*Pre-vaccination (B1)*	*Post-vaccination (B2)*
15	V	H5N1 NT	<10	80
		H1N1 HI	<10	40
		H3N2 HI	<10	160
		B HI	<10	20
29	V	H5N1 NT	<10	40
		H1N1 HI	<10	160
		H3N2 HI	<10	320
		B HI	<10	<10
37	V	H5N1 NT	<10	40
		H1N1 HI	10	640
		H3N2 HI	160	160
		B HI	20	80

Abbreviations: NT, neutralizing antibody; HI, hemagglutination inhibition antibody; V, vaccination; B1, pre-vaccination; B2, post-vaccination; H5N1, influenza A/Thailand/1 (KAN-1)/2004; H1N1, influenza A/Texas/36/91; H3N2, influenza A/Nanchang/933/95; B, influenza B/Harbin/07/94.

influenza vaccination or repeated infection by human influenza viruses. The inducible H5N1 antibody may be the result of immunologic priming that occurred in children infected with seasonal influenza viruses bearing common epitopes to the H5N1 strain, as previously reported (16). Preschool-age children were more susceptible due to their lack of immunity against influenza virus infections (2). During the 2003–2004 influenza season approximately 43% of cases occurred in 10-year-old children, while only 12% occurred in those aged 65 and older (14). The attack rate of seasonal influenza is approximately 5–10% per year (25). Therefore elderly persons have most likely experienced natural influenza virus infections several times in their lives. Data from the current outbreak also indirectly support the theory that the elderly, who may have experienced influenza disease several times in their lives, died from H5N1 infection at lower rate than younger people (26).

Based on the *in-vitro* microNT assay results, it is possible that the H5N1-neutralizing antibodies detected may be induced by the common epitopes present in HA proteins, but less likely to those in NA proteins. It is generally accepted that antibody to HA blocks viral entry and confers neutralizing activity and protective immunity, whereas antibody to NA blocks virus release and confers only partial protective immunity. Cross-neutralizing antibodies induced by common epitopes in HA from different influenza subtypes has been reported. Monoclonal antibodies against conserved antigenic sites on H1 or H2 could neutralize both the H1 and H2 subtypes (15). Using the phage display library technique together with panning by recombinant H5 HA, two sets of researchers showed that gene pools from peripheral blood mononuclear cells of healthy subjects could produce heterosubtypic monoclonal antibodies directed against epitopes in the HA2 domain of the HA molecule that were able to

Table 4. Distribution of H5N1 NT Antibodies in Paired-Blood Samples from Patients with Acute Respiratory Illness

			No. of positive samples at NT antibody titers					
Illness	*Group (no. episodes/no. case)*	*Sample*	*<10*	*10*	*20*	*40*	*80*	*GMT*
Flu	Vaccine	BA	5	0	0	0	0	5.00
	(5/5)	BC	5	0	0	0	0	5.00
	Placebo	BA	16	1	0	0	0	5.21
	(17/17)	BC	16	1	0	0	0	5.21
	Total	BA	21	1	0	0	0	5.16
	(22/22)	BC	21	1	0	0	0	5.16[a]
Non-Flu	Vaccine	BA	34	4	1	0	0	5.56
	(39/35)	BC	34	4	1	0	0	5.56
	Placebo	BA	40	1	0	0	0	5.09
	(41/33)	BC	40	1	0	0	0	5.09
	Total	BA	74	5	1	0	0	5.31
	(80/68)	BC	74	5	1	0	0	5.31[a]

[a] $p = 0.952$ for comparison of GMT between influenza and non-influenza patient groups.
Abbreviations: BA, acute blood sample; BC, convalescent blood sample; GMT, geometric mean titer; NT, neutralizing antibody.

neutralize HPAI H5N1 virus, as well as other influenza virus subtypes (5,6). Although seasonal influenza vaccine induced a fourfold or greater rise in cross-neutralizing H5N1 antibody in the three vaccinees who did not have pre-existing H5N1 antibody in their initial blood samples, the vaccine failed to boost the anamnestic response in vaccinees who had pre-existing H5N1 antibody. It is unclear whether the population of memory lymphocytes and the common epitopes present in vaccines are well matched; perhaps pre-existing H5N1 antibody had bound the common epitopes in the vaccine and diminished its boosting effect.

Regarding serologic surveys for H5N1 virus infection in Hong Kong, Treanor *et al.* (7,20) reported that the an H5N1-neutralizing antibody titer of 40 was the primary immunogenic threshold to distinguish between infected and uninfected persons. Neutralizing H5N1 antibody titers of 40–80 were also found in our three vaccinees. Since this study was carried out on archival blood samples collected before the avian influenza outbreak, it appears that the cross-H5N1-neutralizing antibody that developed in our subjects was induced by vaccination, not by natural H5N1 infection.

Acknowledgments

This work was supported by grants from the National Research Council of Thailand, the Thailand Research Fund for Senior Research Scholars, and the Chalermphrakiat Grant, Faculty of Medicine, Siriraj Hospital, Mahidol University. The authors thank the WHO for kindly providing the influenza antigens, and special thanks are due to all of the subjects who participated in this study.

References

1. Burlington DB, Wright PF, van Wyke KL, Phelan MA, and Mayner RE: Development of subtype-specific and heterosubtypic antibodies to the influenza A virus hemagglutinin after primary infection in children. J Clin Microbiol 1985;21: 847–849.
2. Brownstein JS, Kleinman KP, and Mandl KD: Identifying pediatric age groups for influenza vaccination using a real-time regional surveillance system. Am J Epidemiol 2005;162: 686–693.
3. Claas EC, Osterhaus AD, van Beek R, *et al.*: Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472–477.
4. Gioia C, Castilletti C, Tempestilli M, *et al.*: Cross-subtype immunity against avian influenza in persons recently vaccinated for influenza. Emerg Infect Dis 2008;14:121–128.
5. Gocnyk M, Fislova T, Mucha V, Sladkova T, Russ G, Kostolansky F, and Vareckova E: Antibodies induced by the HA2 glycopolypeptide of influenza virus haemagglutinin improve recovery from influenza A virus infection. J Gen Virol 2008;89:958–967.
6. Gocnyk M, Fislova T, Sladkova T, Mucha V, Kostolansky F, and Vareckova E: Antibodies specific to the HA2 glycopolypeptide of influenza A virus haemagglutinin with fusion-inhibition activity contribute to the protection of mice against lethal infection. J Gen Virol 2007;88: 951–955.
7. Katz JM, Lim W, Bridges CB, *et al.*: Antibody response in individuals infected with avian influenza A (H5N1) viruses and detection of anti-H5 antibody among household and social contacts. J Infect Dis 1999;180:1763–1770.
8. Kilbourne ED: Influenza pandemics of the 20th century. Emerg Infect Dis 2006;12:9–14.
9. Kitphati R, Pooruk P, Lerdsamran H, *et al.*: Kinetics and longevity of antibody response to influenza A H5N1 virus infection in humans. Clin Vaccine Immunol 2009;16: 978–981.
10. Kositanont U, Kanyok R, Wasi C, Wongsurakiat P, Suthamsmai T, and Maranetra N: Occurrence and protective level of influenza infections using serology in patients with COPD in vaccination study. J Med Assoc Thai 2004;87: 964–969.
11. Monto AS and Kendal AP: Effect of neuraminidase antibody on Hong Kong influenza. Lancet 1973;1:623–625.
12. Nguyen HH, Moldoveanu Z, Novak MJ, *et al.*: Heterosubtypic immunity to lethal influenza A virus infection is associated with virus specific CD8(+) cytotoxic T lymphocyte responses induced in mucosa-associated tissues. Virology 1999;254:50–60.
13. Nguyen HH, Ginkel van FW, Vu HL, McGhee JR, and Mestecky J: Heterosubtypic immunity to influenza A virus infection requires B cells but not CD8+ cytotoxic T lymphocytes. J Infect Dis 2001;183:368–376.
14. North Dakota Department of Health, Division of Disease Control: 2003–2004 Influenza Season Summary. http://www.ndhealth.gov/disease/Documents/pump%20handle/01–09.pdf
15. Okuno Y, Isegawa Y, Sasao F, and Ueda S: A common neutralizing epitope conserved between the hemagglutinin of influenza A virus H1 and H2 strains. J Virol 1993;67:2552–2558.
16. Remarque EJ, Bruijn de IA, Boersma WJ, Masurel N, and Ligthart GJ: Altered antibody response to influenza H1N1 vaccine in healthy elderly people as determined by HI, ELISA, and neutralization assay. J Med Virol 1998;55:82–87.
17. Rowe T, Abernathy RA, Hu-Primmer J, *et al.*: Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol 1999;37:937–943.
18. Sandbulte MR, Jimenez GS, Boon AC, Smith LR, Treanor JJ, and Webby RJ: Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. PLoS Med 2007;4:e59:265–272.
19. Tang JW, Karry LK, and Chan PKS: Lack of cross-immune reactivity against influenza H5N1 from seasonal influenza vaccine in humans. J Med Virol 2008;80:1992–1996.
20. Treanor JJ, Campbell JD, Zangwill KM, Rowe T, and Wolff M: Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med 2006;354:1343–1351.
21. Viboud C, Grais RF, Lafont BA, Miller MA, and Simonsen L: Multinational impact of the 1968 Hong Kong influenza pandemic: Evidence for a smoldering pandemic. J Infect Dis 2005;192:233–248.
22. Wongsurakiat P, Maranetra KN, Wasi C, Kositanont U, Dejsomritrutai W, and Charoenratanakul S: Acute respiratory illness in patients with COPD and the effectiveness of influenza vaccination: a randomized controlled study. Chest 2004;125:2011–2020.
23. World Health Organization: WHO manual on animal influenza diagnosis and surveillance. Geneva, World Health Organization, 2002 (document WHO/CDS/CSR/NCS/2002.5), pp 28–39.

24. World Health Organization: Cumulative number of confirmed human cases of avian influenza A/(H5N1), 2009. http://www.who.int/csr/disease/avian_influenza/country/cases _table _2009_04_08/en/index.html
25. World Health Organization: Influenza (seasonal) fact sheet No 211, 2009. http://www.who.int/mediacentre/factsheets/fs211/en/index.html
26. Yu H, Gao Z, Feng Z, *et al.*: Clinical characteristics of 26 human cases of highly pathogenic avian influenza A (H5N1) virus infection in China. PLoS ONE 2008;3: e2985.

Address correspondence to:
Prof. Pilaipan Puthavathana
Department of Microbiology
Faculty of Medicine Siriraj Hospital
Mahidol University
Bangkok 10700, Thailand

E-mail: siput@mahidol.ac.th

Received September 17, 2009; accepted January 17, 2010.

Ngaosuwankul *et al. Virology Journal* 2010, **7**:75
http://www.virologyj.com/content/7/1/75

VIROLOGY JOURNAL

RESEARCH Open Access

Influenza A viral loads in respiratory samples collected from patients infected with pandemic H1N1, seasonal H1N1 and H3N2 viruses

Nathamon Ngaosuwankul[1], Pirom Noisumdaeng[1], Pisut Komolsiri[1], Phisanu Pooruk[1], Kulkanya Chokephaibulkit[2], Tawee Chotpitayasunondh[3], Chariya Sangsajja[4], Charoen Chuchottaworn[5], Jeremy Farrar[6] and Pilaipan Puthavathana*[1]

Abstract

Background: Nasopharyngeal aspirate (NPA), nasal swab (NS), and throat swab (TS) are common specimens used for diagnosis of respiratory virus infections based on the detection of viral genomes, viral antigens and viral isolation. However, there is no documented data regarding the type of specimen that yields the best result of viral detection. In this study, quantitative real time RT-PCR specific for *M* gene was used to determine influenza A viral loads present in NS, NPA and TS samples collected from patients infected with the 2009 pandemic H1N1, seasonal H1N1 and H3N2 viruses. Various copy numbers of RNA transcripts derived from recombinant plasmids containing complete *M* gene insert of each virus strain were assayed by RT-PCR. A standard curve for viral RNA quantification was constructed by plotting each Ct value against the log quantity of each standard RNA copy number.

Results: Copy numbers of *M* gene were obtained through the extrapolation of Ct values of the test samples against the corresponding standard curve. Among a total of 29 patients with severe influenza enrolled in this study (12 cases of the 2009 pandemic influenza, 5 cases of seasonal H1N1 and 12 cases of seasonal H3N2 virus), NPA was found to contain significantly highest amount of viral loads and followed in order by NS and TS specimen. Viral loads among patients infected with those viruses were comparable regarding type of specimen analyzed.

Conclusion: Based on *M* gene copy numbers, we conclude that NPA is the best specimen for detection of influenza A viruses, and followed in order by NS and TS.

Background

Influenza A viruses are classified into 16 hemagglutinin (H) and 9 neuraminidase (N) subtypes [1]. Since the emergence of Russian influenza A (H1N1) in 1977 [2] to the emergence of pandemic influenza A (H1N1) in April 2009, only A/H1N1, A/H3N2 and influenza B viruses have been recognized as human or seasonal influenza. Influenza virus spreads via respiratory secretion. After an incubation period of about 1-3 days, the viruses are shed from various kinds of respiratory samples. Upper respiratory tract specimens, such as nasopharyngeal wash (NPW) or nasopharyngeal aspirate (NPA), nasal swab (NS), throat swab (TS), endothracheal swab, bronchoalveolar lavage and tissues, are recommended for virus detection in patients with respiratory tract infection. These specimens could be used for viral antigen detection, virus isolation and molecular methods for genome detection. Nevertheless, there is no documented data which addresses the type of specimen that gives the best yield for the disease diagnosis [3].

Genomes of influenza A and B viruses are composed of 8 negative sense, single-stranded RNA segments encoded for 10-11 proteins essential for infection and replication [1]. The genomic RNA has been used as targets for amplification by conventional and real time reverse transcription-polymerase chain reaction (RT-PCR). The highly conserved *M* gene-derived primers are usually utilized for diagnosis of all influenza A subtypes, whereas specific subtype identification targets *H* or *H* and *N* genes. In this

* Correspondence: siput@mahidol.ac.th
[1] Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Full list of author information is available at the end of the article

study, the protocol established by the U.S., Center for Disease Control (CDC) for detection of *M* gene [4] in adjunct with the standard curves of known copies of *M* RNA transcripts derived either from H1N1, H3N2 or the 2009 pandemic A (H1N1) viruses was used to quantify the viral loads in specimens collected from patients with severe influenza prior to receiving anti-viral drug. Our study provided the information on the clinical specimens that yielded the best diagnostic result; and the viral loads in patients infected with different influenza subtypes and strains were also compared.

Methods

Subjects and Specimen Collection

This study was approved by the Institutional Review Boards of the Committee on Ethics, Faculty of Medicine Siriraj Hospital, Mahidol University and the Ministry of Public Health, Thailand. NPA, NS and TS samples were collected in viral transport medium (MicroTest™ Multi-Microbe Media; Remel, Lenexa, KS) from patients with severe influenza. The collection of NPA was performed by flushing through a nasopharyngeal tube with 2 ml of sterile normal saline using a sterile NG-tube or sterile butterfly needle tube, inserted through the floor of nose. The NPA yield at approximately 0.5 ml volume was then added with VTM and the 3.5 ml final volume was obtained. The nose and throat swabbing were performed right after the NPA collection from nostrils and throat, respectively, using MicroTest™ kit with 3 ml of VTM.

Quantitative Real time Reverse Transcription-Polymerase Chain Reaction

Real time RT-PCR protocols established by CDC as well as viral antigen detection by QuickVue (Quidel Corporation, San Diego, CA), virus isolation in MDCK cell culture and serodiagnosis, were used to diagnose influenza virus infection in these patients. Positive results from at least two diagnostic tests were obtained for each case. A total of 29 patients enrolled in this study comprised 12 cases of pandemic influenza A/2009 (H1N1), 5 cases of A/Brisbane/59/2007(H1N1) like- and 12 cases of A/Brisbane/10/2007 (H3N2) like-virus infection. All respiratory specimens were kept at -70°C until tested.

In the preparation of standard *M*-RNA, viral RNA extracted from A/Nonthaburi/102/2009 (H1N1), A/Brisbane/59/2007-like (H1N1) and A/Brisbane/10/2007-like (H3N2) viruses were reverse transcribed into complementary DNA (cDNA) in a 20 μl reaction comprised 8 μl of viral RNA, 1× RT buffer, 5 mM $MgCl_2$, 10 mM DTT, 50 ng of random hexamers, 0.5 mM dNTPs, 40 units of RNaseOUT™ (Invitrogen Corporation, Carlsbad, CA) and 200 units of SuperScript™ III reverse transcriptase (Invitrogen) following the manufacturer's instruction. Thereafter, cDNA was subjected to PCR amplification in a 50 μl reaction mixture containing 5 μl of cDNA target, 5 μl of 10× High Fidelity PCR buffer, 1 mM dNTP mixture, 2 mM $MgSO_4$, 0.4 μM forward primer, 0.4 μM reverse primer (universal *M* primers, Bm-M-1 and Bm-M-1027R [5]; sequences as shown in Table 1) and 0.5 μl of High Fidelity Platinum®Taq DNA polymerase (Invitrogen). The PCR amplification cycle was set as 94°C for 2 min for initial denaturation, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 68°C for 90 sec, and followed by final extension at 68°C for 10 min. The PCR product of complete *M* segment of 1,056 base pairs in size was gel-purified and cloned into pGEM® T-Easy plasmid (Promega Corporation, Madison, WI). Thereafter, *M* RNA was *in vitro*-transcribed from the recombinant plasmid using Riboprobe® combination system-SP6/T7 (Promega), followed by step of RNase-free DNase (Promega) digestion in order to remove out the recombinant plasmid DNA

Table 1: Sequences of primers and probes for PCR and real time RT-PCR.

Primer and probe	Sequence (5'>3')	Reference
Bm-M-1	TAT TCG TCT CAG GGA GCA AAA GCA GGT AG	Hoffmann E et al.
Bm-M-1027R	ATA TCG TCT CGT ATT AGT AGA AAC AAG GTA GTT TTT	Hoffmann E et al.
FluA Forward	GAC CRA TCC TGT CAC CTC TGA C	CDC
FluA Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	CDC
FluA Probe[1]	TGC AGT CCT CGC TCA CTG GGC ACG	CDC
RnaseP Forward	AGA TTT GGA CCT GCG AGC G	CDC
RnaseP Reverse	GAG CGG CTG TCT CCA CAA GT	CDC
RnaseP Probe[1]	TTC TGA CCT GAA GGC TCT GCG CG	CDC

[1]TaqMan® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) at the 3'-end.

templates. *M* transcripts obtained were kept at -70°C until assayed.

To minimize the test variation, standard curves of *M* RNA transcripts were constructed in parallel with the detection of viral *M* RNA in clinical samples in the quantitative real time RT-PCR. The *M* RNA transcripts were measured by Quant-iT™ RNA Assay Kit (Invitrogen) and diluted to various copy numbers in a ten folded serial dilution manner; and each known *M* RNA copy number was assayed by real time RT-PCR according to that described by the 2009 CDC protocol [4]. The sequences of primer and probe sets used in this study are shown in Table 1. A 25 μl reaction mixture of real time RT-PCR comprised 5 μl of total RNA, 12.5 μl of 2× reaction mix, 0.5 μl of SuperScript™ III Platinum®Taq Mix (Invitrogen), each 0.8 μM of forward and reverse primers and 0.2 μM of labeled probe, and H_2O was added to bring up the final volume. The amplification was carried out in DNAEngine® Peltier Thermal Cycler with Chromo4™ Real-Time PCR Detector (Bio-Rad Laboratories, Inc., Hercules, CA) using the amplification cycles of 50°C for 30 min for reverse transcription, 95°C for 2 min for *Taq* polymerase activation, followed by 45 cycles of PCR amplification (95°C for 15 sec and 55°C for 30 sec). Fluorescence signal was obtained at 55°C. The results were analyzed by MJ OpticonMonitor™ Analysis Software version 3.1 (Bio-Rad). A standard curve was constructed by plotting each cycle threshold (Ct) value against the log quantity of standard RNA copy numbers. Total RNA was extracted from the NPA, NS and TS specimens by QIAamp® Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instruction. Real time RT-PCR for detection of influenza A *M* gene and the *RnaseP* (RNP) house keeping gene, was carried out. To obtain amount of viral load present in each clinical sample, the test Ct value was extrapolated against the standard curve derived from each virus subtype or strain (Fig. 1). The sensitivity of the assay for all 3 subtypes and strain was 100 copies of target *M* RNA/real time RT-PCR reaction when the cut-off for positive result was set at 40 cycles.

Data Analysis

Statistical analysis was performed with SPSS program. Pair *t*-test was used to compare the mean $\log_{10}$ viral loads among different types of specimens collected from the same subjects and at the same time. Student *t*-test was used to analyze the mean $\log_{10}$ viral copy numbers in contemporary specimens from patients infected with different virus subtypes and strain.

Results and Discussion

Real time RT-PCR protocol was analyzed for its applicability to amplify *M* genes derived from H1N1, H3N2 and

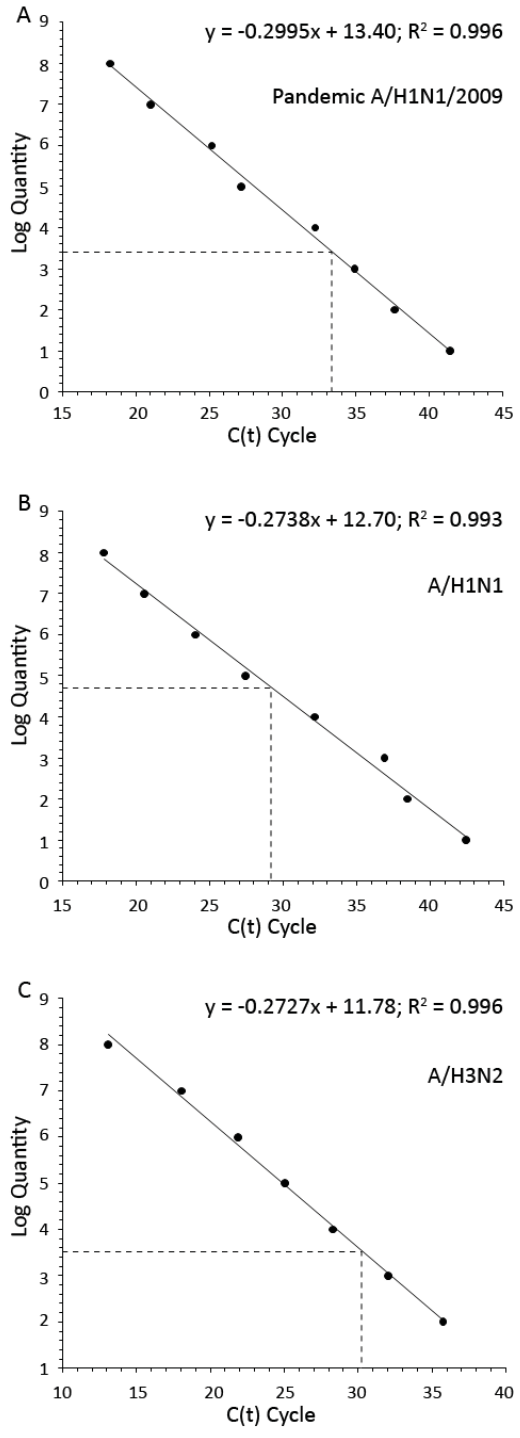


Figure 1 *M* transcript standard curve for quantitative detections of the pandemic A/H1N1 (A), seasonal A/H1N1 (B) and seasonal A/H3N2 viruses (C). The standard curve of *M* RNA copy numbers was generated by plotting the Ct value (X-axis) against $\log_{10}$ copy numbers of *M* transcripts (Y-axis). The amount of *M* copy number in clinical specimens was obtained by extrapolation of the Ct of the test sample against the standard curve.

the 2009 pandemic viruses by aligning the primers and probe nucleotide sequences against those *M* genes of various influenza subtypes and strains using BioEdit Sequence Alignment Editor (Fig. 2, Table 2). The forward and reverse primers bound to those *M* genes with higher than 90% identity, while the probe bound with 100% identity. This suggested that the CDC primers/probe set can be universally used for detection of *M* segments or viral loads of the novel influenza A/2009 (H1N1), seasonal H1N1 and seasonal H3N2 viruses.

Three standard curves of *M* RNA transcripts were constructed with the R^2 of 0.996, 0.993 and 0.996 for pandemic A/2009 (H1N1), seasonal H1N1 and seasonal H3N2, respectively (Fig 1). The *M* copy numbers per ml of VTM from patients infected with pandemic H1N1 or H3N2 viruses were significantly highest in NPA samples (pair *t*-test; $P \leq 0.05$) (Table 3). However, number of patients infected with seasonal H1N1 virus was too small for data analysis. Additionally, viral load levels in patients infected with either subtype or strain was comparable (student *t*-test, $P > 0.05$). *M* RNAs were detected in all NPA and NS, but not in all TS samples collected from patients infected with any one of the virus subtypes/strain. The detection rate was shown in Table 4.

RT-PCR for diagnosis of influenza viruses is generally more sensitive than viral isolation method. The technique detected the viral genome present in dead and alive viruses including excess viral RNA present in the infected cells; however, virus isolation detected only live virus particles. RT-PCR is a high through-put and less time consuming method. In addition, only RT-PCR can differentiate type, subtype and strain of influenza viruses. Sensitivity of RT-PCR to diagnose the disease not only depends on the protocol, but also the type of clinical sample used in the diagnosis. Our study has two advantages that are not commonly conducted in previous reports. Firstly, we had an opportunity to investigate 3 types of clinical specimens collected from the same individuals at the same time, e.g., NPA, NS and TS. Secondly, we had employed full length *M* RNA transcripts derived from A/H1N1, A/H3N2 and the 2009 pandemic viruses to construct 3 standard curves for quantifying viral RNA copy numbers of the contemporary subtype and strain present in the test specimens, with the assumption that the full length *in vitro M* RNA transcripts closely mimics the native structure of the viral *M* genomic segments. Regardless of viral subtypes and strains (H1N1, H3N2 and 2009 pandemic H1N1 virus), we found that all NPA and NS specimens were positive for viral genome detection, while the positive rate was lower in TS specimens.

Previous investigators reported that viral RNA concentration in respiratory samples and long duration of virus shedding were correlated with influenza disease severity [6]. Amount and duration of viral shedding are important in the disease treatment and control of virus spread. Different type of specimens contained different amount of viral RNA concentration; therefore, using different type of clinical specimens may yield different information. In addition, there is no reference method for viral load assay. Peiris et al. [7] reported that viral load in NPA samples of

Figure 2 Alignment of *M* gene fragment from the pandemic A/H1N1, seasonal A/H1N1 and seasonal A/H3N2 viruses against CDC real time RT-PCR primers and probe sequences. BioEdit Sequence Alignment Editor was used to locate the region of real time RT-PCR primers and probe binding site within *M* gene of various subtypes of influenza A viruses.

Table 2: Percentages of identity of primers and probe with the *M* sequences derived from different virus subtypes and strain.

Virus	% Identity with		
	Forward primer	Reverse primer	Probe
Pandemic A/H1N1/2009	95.45	91.67	100
A/H1N1	100	91.67	100
A/H3N2	95.45	91.67-95.83	100

H5N1 patients was lower than those of H3N2 patients. The finding was further extended by Ward et al. [8] that viral load in throat swab samples of H5N1 patients in 1997 and 2004 was 10-fold lower than that observed in H3N2 patients, i.e., 1.5×10^6 $TCID_{50}$/ml versus 1.6×10^5 $TCID_{50}$/ml (*t*-test, $P < 0.05$). On the other hand, de Jong et al. [9] found that viral load in TS from H5N1 patients was significantly higher than that from H3/H1 patients; and, additionally, TS contained significantly higher H5N1 viral load than nasal swab samples; meanwhile, viral load in TS and nasal swab samples from H1/H3 patients was not statistically different. The difference in results obtained from different groups of investigators might reflect process of specimen collection and also the different protocols for viral load measurement.

It has been reported that the 2009 pandemic virus preferentially binds sialic acid receptor with α 2, 6 linkage to galactose (SA α 2,6 Gal), the same as human influenza H1N1 and H3N2 viruses [10]. Fatality rate in patients infected with the novel virus is less than 1%, except in that which occurs in patients with underlying conditions, e.g., cardiovascular disease, hypertension, asthma and diabetes, etc. [11,12]. However, the study in a mammalian model demonstrated that the 2009 pandemic H1N1 virus was more pathogenic than the seasonal H1N1 virus [13]. Our study, therefore, explored the viral load in respiratory secretions collected prior to anti-viral treatment, and found that the level of viral RNA in cases infected with the 2009 pandemic H1N1 virus was not statistically different from those infected with seasonal H1N1 and

Table 3: Influenza viral loads in various types of clinical specimens collected from patients infected with different virus subtypes.

Virus	Number of cases	Statistics	Age	Days after onset	Log10 *M* RNA copy number in		
					NPA	NS	TS
Pandemic A/H1N1/2009	12	Mean	12	5	7.5[a, b]	6.5[a, c]	4.1[b, c]
		Median	8	5	7.5	6.9	4.9
		Range	3-53	2-10	5.8-8.9	2.7-8.7	Und.-6.9
A/H1N1	5	Mean	5	5	7.8	7.2	7.4
		Median	4	4	8.5	6.4	7.7
		Range	1-12	2-10	4.7-8.9	5.5-9.3	5.8-8.7
A/H3N2	12	Mean	17	5	8.0[a, b]	6.6[a]	5.6[b]
		Median	4	5	8.1	7.2	6.8
		Range	1-69	2-6	5.8-9.2	3.5-8.3	Und.-7.7

The viral loads are reported as $\log_{10}$ of *M* segment copy number/1 ml of VTM. Pair *t*-test was used to compare the mean $\log_{10}$ viral loads in different types of specimens collected from the same subjects and at the same time.

[a] indicates a significant difference of the viral loads in NPA and NS, [b] in NPA and TS and [c] in NS and TS (Pair *t*-test, $P < 0.05$).

Und., below detection limit; NPA, Nasopharyngeal aspirate; NS, Nasal swab; TS, Throat swab.

Table 4: Genome detection rate by type of clinical specimens.

Virus	Number of positive cases		
	NPA	NS	TS
Pandemic A/H1N/2009	12 (100%)	12 (100%)	9 (75%)
A/H1N1	5 (100%)	5 (100%)	5 (100%)
A/H3N2	12 (100%)	12 (100%)	11 (91.67%)

NPA, Nasopharyngeal aspirate; NS, Nasal swab; TS, Throat swab.

H3N2 viruses. Mean $\log_{10}$ copies/ml of viral RNA of 7.5-8.0 in NPA, 6.5-7.2 in NS and 4.1-7.4 in TS samples were found in our study. It is to be kept in mind that all of our patients had severe influenza at time of specimen collection, and most of them were pediatric patients (24 children and 5 adults). Duration of viral shedding of the seasonal influenza as reported by the other groups of investigators was 4-5 days in average [6,14]. A recent report by To et al. [15], showed that the level of the 2009 pandemic viral load of 8 $\log_{10}$ copies/ml was found in respiratory specimens collected before oseltamivir treatment; and the viral shedding peaked at the day of onset of symptom with median duration of 4 days [15]. On the other hand, when using plasmid containing amplification target to construct the standard curve together with using pool of throat and nasal swab as the test samples, the other study demonstrated that the H1/H3 viral loads of 5.06 ± 1.85 $\log_{10}$ copies/ml were found in patients with major co-morbidities and 3.62 ± 2.13 $\log_{10}$ copies/ml in patients without co-morbidities [6].

Conclusions

Our study suggested that when complete facilities are accessible, such as in clinics and hospitals, NPA will be the best specimen of choice; and in field investigation, NS will be the second choice, followed by TS specimen. Using the appropriate specimen will provide the highest diagnostic rate and the precise strategy for disease treatment and prevention control.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PP designed the research study; NN, PN, PK and PhP performed research; NN, PN and PK analyzed data; NN and PP wrote the manuscript. KK, TC, CS, CC and JF provided specimens. All authors read and approved the final manuscript.

Acknowledgements

This study is supported by the Thailand Research Fund for Senior Research Scholar and the South East Asia Infectious Disease Clinical Research Network (SEAICRN), supported by the US National Institute of Health. NN is supported by Postdoctoral Fellowship Scholarship, Mahidol University, Thailand. We thank Mrs. Caroline Fukuda and Dr. Steve Wignall, SEAICRN for their kind coordination.

Author Details

[1]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand, [2]Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand, [3]Queen Sirikit National Institute of Child Health, Bangkok 10400, Thailand, [4]Bamrasnaradura Infectious Disease Institute, Nonthaburi 11000, Thailand, [5]Chest Disease Institute, Nonthaburi 11000, Thailand and [6]Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam

Received: 28 January 2010 Accepted: 20 April 2010
Published: 20 April 2010

References

1. Maines TR, Szretter KJ, Perrone L, Belser JA, Bright RA, Zeng H, Tumpey TM, Katz JM: **Pathogenesis of emerging avian influenza viruses in mammals and the host innate immune response.** *Immunol Rev* 2008, **225:**68-84.
2. Tognotti E: **Influenza pandemics: a historical retrospect.** *J Infect Dev Ctries* 2009, **3:**331-334.
3. **WHO information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans-update** [http://www.who.int/csr/resources/publications/swineflu/en/]
4. **CDC protocol of realtime RTPCR for influenza A (H1N1)** [http://www.who.int/csr/resources/publications/swineflu/en/]
5. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR: **Universal primer set for the full-length amplification of all influenza A viruses.** *Arch Virol* 2001, **146:**2275-2289.
6. Lee N, Chan PK, Hui DS, Rainer TH, Wong E, Choi KW, Lui GC, Wong BC, Wong RY, Lam WY, Chu IM, Lai RW, Cockram CS, Sung JJ: **Viral loads and duration of viral shedding in adult patients hospitalized with influenza.** *J Infect Dis* 2009, **200:**492-500.
7. Peiris JS, Yu WC, Leung CW, Cheung CY, Ng WF, Nicholls JM, Ng TK, Chan KH, Lai ST, Lim WL, Yuen KY, Guan Y: **Re-emergence of fatal human influenza A subtype H5N1 disease.** *Lancet* 2004, **363:**617-619.
8. Ward CL, Dempsey MH, Ring CJ, Kempson RE, Zhang L, Gor D, Snowden BW, Tisdale M: **Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement.** *J Clin Virol* 2004, **29:**179-188.
9. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha do Q, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J: **Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia.** *Nat Med* 2006, **12:**1203-1207.
10. Neumann G, Noda T, Kawaoka Y: **Emergence and pandemic potential of swine-origin H1N1 influenza virus.** *Nature* 2009, **459:**931-939.
11. **WHO, Pandemic (H1N1) 2009 - update 58, Laboratory-confirmed cases of pandemic (H1N1) 2009 as officially reported to WHO by States Parties to the International Health Regulations (2005)** [http://www.who.int/csr/don/2009_07_06/en/]
12. **WHO, Assessing the severity of an influenza pandemic** [http://www.who.int/csr/disease/swineflu/assess/disease_swineflu_assess_20090511/]
13. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, Muramoto Y, Tamura D, Sakai-Tagawa Y, Noda T, Sakabe S, Imai M, Hatta Y, Watanabe S, Li C, Yamada S, Fujii K, Murakami S, Imai H, Kakugawa S, Ito M, Takano R,

Iwatsuki-Horimoto K, Shimojima M, Horimoto T, Goto H, Takahashi K, Makino A, Ishigaki H, Nakayama M, Okamatsu M, Takahashi K, Warshauer D, Shult PA, Saito R, Suzuki H, Furuta Y, Yamashita M, Mitamura K, Nakano K, Nakamura M, Brockman-Schneider R, Mitamura H, Yamazaki M, Sugaya N, Suresh M, Ozawa M, Neumann G, Gern J, Kida H, Ogasawara K, Kawaoka Y: ***In vitro* and *in vivo* characterization of new swine-origin H1N1 influenza viruses.** *Nature* 2009, **460:**1021-1025.

14. Ison MG: **Influenza in hospitalized adults: gaining insight into a significant problem.** *J Infect Dis* 2009, **200:**485-488.
15. To KK, Chan KH, Li IW, Tsang TY, Tse H, Chan JF, Hung IF, Lai ST, Leung CW, Kwan YW, Lau YL, Ng TK, Cheng VC, Peiris JS, Yuen KY: **Viral load in patients infected with pandemic H1N1 2009 influenza A virus.** *J Med Virol* 2010, **82:**1-7.

doi: 10.1186/1743-422X-7-75
Cite this article as: Ngaosuwankul *et al.*, Influenza A viral loads in respiratory samples collected from patients infected with pandemic H1N1, seasonal H1N1 and H3N2 viruses *Virology Journal* 2010, **7**:75

CLINICAL AND VACCINE IMMUNOLOGY, July 2009, p. 978–981 Vol. 16, No. 7
1556-6811/09/$08.00+0 doi:10.1128/CVI.00062-09

Kinetics and Longevity of Antibody Response to Influenza A H5N1 Virus Infection in Humans▽

Rungrueng Kitphati,[1] Phisanu Pooruk,[2] Hatairat Lerdsamran,[2] Siriwan Poosuwan,[1] Suda Louisirirotchanakul,[2] Prasert Auewarakul,[2] Kulkanya Chokphaibulkit,[3] Pirom Noisumdaeng,[2] Pathom Sawanpanyalert,[1] and Pilaipan Puthavathana[2]*

National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000, Thailand,[1] and Department of Microbiology[2] and Department of Pediatrics,[3] Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Received 9 February 2009/Returned for modification 23 March 2009/Accepted 11 May 2009

Anti-H5N1 antibody was determined by microneutralization, hemagglutination inhibition, and Western blotting assays in serial blood samples collected from eight Thai patients, including four fatal cases and four survivors. The antibody was detected as early as 5 days and, typically, with an increase in titer in paired blood at about 15 days after disease onset. The anti-H5 antibody response was long-lasting, for almost 5 years in cases which can be followed that far. In addition, cross-neutralizing activity to related clade 1 viruses was observed.

During the first outbreak of human highly pathogenic avian influenza (HPAI) virus H5N1 disease in Hong Kong in 1997, the microneutralization (microNT) assay was used to detect acute antibody responses in virologically confirmed cases, but the durability of the response was not assessed (4). The microNT assay was more sensitive than the traditional hemagglutination inhibition (HI) assay, which used erythrocytes from avian species in detecting human antibodies induced by H5 viruses (7). The sensitivity and specificity of microNT could be improved when it was combined with a confirmatory Western blot (WB) assay (7).

Since late 2003, highly pathogenic avian influenza (HPAI) H5N1 viruses have caused unprecedented outbreaks in poultry in 61 countries (10). Human disease has accompanied the global spread of the virus, and as of 14 January 2009, 394 human H5N1 virus infections had been reported by the World Health Organization (WHO), with an overall fatality rate of over 60% (13). Despite these case numbers, information on the kinetics, and in particular the persistence of H5 virus-specific antibody in humans infected with H5N1 viruses, is lacking. This information could guide H5N1 vaccine studies and the optimal design of seroepidemiological investigations to better understand the extent of and risk factors associated with human H5N1 infection. In 2006, WHO included serodiagnosis as one among other criteria for an H5N1 confirmed case, i.e., (i) a fourfold or greater rise in neutralizing antibody titer in paired blood samples of which the acute blood has been collected within 7 days after symptom onset and the convalescent sample achieves a titer of ≥1:80, or (ii) presence of a neutralizing antibody titer of ≥1:80 in a single serum sample collected 14 or more days after symptom onset together with a positive result using a different serological assay, such as the horse erythrocyte HI assay or an H5-specific WB positive result (12).

Thailand reported the first human case of H5N1 disease on 23 January 2004. No human case has occurred after July 2006; in total, there have been 25 human cases with 17 deaths. The diagnosis of H5N1 infection in human cases in Thailand is primarily based on results of conventional and real-time reverse transcription-PCR (RT-PCR) and virus isolation methods. Serological tests have been introduced more recently. The present study reports the results of serological analyses conducted by using the microNT, HI, and WB assays to explore the kinetics and longevity of the antibody response to H5N1 virus infection in fatal cases and survivors.

MATERIALS AND METHODS

Ethical issues. This study has been approved by two Ethical Committee for Human Research panels: one from the Faculty of Medicine Siriraj Hospital, Mahidol University, and the second one from the Ministry of Public Health, Thailand. Subjects or parents gave consent to participate in the follow-up blood collection.

Subjects. Eight individuals, four fatal cases and four survivors, were included in this study. All of them were diagnosed with H5N1 virus infection by both RT-PCR and virus isolation methods. Nucleotide sequencing showed that all of the isolated viruses belonged to clade 1. The demographic data for these subjects are shown in Table 1. Serial blood samples from the four survivors were collected at approximately 6-month intervals. Serum or plasma samples were kept frozen at −20°C until tested.

Viruses. Two human H5N1 isolates belonging to genotype Z, clade 1, were used for serodiagnosis in this study. A/Thailand/1(KAN-1)/04 (KAN-1), the first human virus isolated in the country, was isolated in January 2004, and A/Thailand/676(NYK)/05 (NYK) was isolated in December 2005 during the third wave of the epidemic. The latter virus contains two mutational changes: A134V in the receptor binding site and R325K in the cleavage site of the hemagglutinin (HA) molecule (1, 6). Complete genomic sequences of the two virus isolates used in this study are available through GenBank. KAN-1 preferentially binds sialic acid α2,3-linked galactose (SAα2,3Gal), while NYK preferentially binds sialic acid α2,6-linked galactose (SAα2,6Gal) (1). The viruses were propagated in MDCK cell monolayers without serum supplement. Experiments related to infectious viruses were conducted in biosafety level 3 facilities.

Microneutralization assay. An enzyme-linked immunosorbent assay (ELISA)-based microNT assay was conducted for detection of NT antibody. The test protocol followed what is described in the WHO manual, with a small modifi-

* Corresponding author. Mailing address: Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. Phone: 662-4197059. Fax: 662-4182663. E-mail: siput@mahidol.ac.th.

▽ Published ahead of print on 20 May 2009.

TABLE 1. Demographic data for H5N1 patients

Subject no.	Age (yrs)	Gender	Estimated incubation period (days)	Disease outcome	Yr of infection
1	6	M		Death	2004
2	2	M	5	Survival	2004
3	29	M	3	Survival	2004
4	32	F		Survival	2004
5	48	M	12	Death	2005
6	7	M	8	Survival	2005
7	5	M	10	Death	2005
8	59	M		Death	2006

cation (11). The test serum was heat inactivated at 56°C for 30 min and then twofold diluted with maintenance medium starting from a dilution of 1:5 to 1:2,560. The assay was performed by mixing 60 μl of the diluted serum with 60 μl of the virus suspension at a concentration of 200 50% tissue culture infective doses ($TCID_{50}$) and incubated at 37°C for 2 hours. Then, 100 μl of the mixture was transferred onto an MDCK cell monolayer and further incubated at 37°C for 18 to 20 h. In order to verify the amount of virus inoculum, virus back-titrations at doses of 0.1, 1, 10, and 100 $TCID_{50}$ were included in every assay plate, together with the positive control serum and cell culture control. The test reaction was run in duplicate. Viral nucleoprotein produced in the infected MDCK cells was detected by indirect ELISA using mouse monoclonal antibody to influenza A virus nucleoprotein (Chemicon International, Inc., CA) as the primary antibody and goat anti-mouse immunoglobulin (Ig) conjugated with horseradish peroxidase (Southern Biotechnology Associates, Inc. Birmingham, AL) as the second antibody. The tetramethylbenzidine (TMB) peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, MD) was used as the chromogenic substrate. The color product was read for the optical density (OD) at dual wavelengths of 450 and 630 nm. The corrected OD value of the test serum was obtained after subtracting the cell control OD from the original OD value. The corrected OD value of the virus control at a working concentration of 100 $TCID_{50}$ was also obtained in the same manner. A positive microNT result was obtained when the test serum yielded a ≥50% reduction in the corrected OD value compared with that of the virus control. The antibody titer was defined as the reciprocal value of the highest serum dilution that gave ≥50% neutralization of 100 $TCID_{50}$ of the test virus. It should be noted that the antibody titer value as determined by our definition was twofold lower than that defined by WHO and CDC, such that our titer of 1:40 is equivalent to 80 as defined by WHO and CDC. In our assay protocol, the volume of the test virus in the reaction well was not taken into account in the calculation for the NT antibody titer; meanwhile, this volume is included in the WHO/CDC protocol (I. Stephenson, WHO manual on influenza microneutralization assay: CDC Influenza Training Course at Faculty of Medicine Siriraj Hospital, Mahidol University, November 2004). In addition, we found no significant difference in the NT titers, as the CDC reference serum samples were assayed in an MDCK cell monolayer or cell suspension such that under two types of cell conditions, a test serum showed the same antibody titer or the variation was within twofold of the difference.

Hemagglutination inhibition test. The two H5N1 isolates and horse and goose erythrocytes were used for antibody detection by an HI test. The protocol was as described previously (8, 11). A test serum was mixed with receptor-destroying enzyme (Denka Seiken, Japan) to obtain a dilution of 1:4, followed by incubation for 16 h at 37°C, heat inactivation at 56°C for 30 min, and adsorption with packed red blood cells (RBC) for 60 min at 4°C. The treated serum was serially diluted twofold to yield the starting dilutions of 1:20 to 1:2,560 and then added with 4 HA units of the test virus. One HA unit is defined as the highest virus dilution that yields complete hemagglutination. A reaction well containing 25 μl of the diluted serum and 25 μl of the virus suspension was incubated at room temperature for 30 min before adding 50 μl of the erythrocyte suspension and further incubated for 1 hour at 4°C. Either a 0.5% goose red blood cell (GRBC) or 1% horse red blood cell (HRBC) suspension was employed in our HI assay. Hemagglutination patterns of the test wells were examined. The HI antibody titer was defined as the reciprocal of the highest serum dilution that gave complete inhibition of hemagglutination.

Western blot assay. Based on WHO criteria, a human serum sample that possesses an NT antibody titer of ≥80 (or ≥40 by our assay) should be further confirmed for its specificity to H5 HA in a WB assay. The serum sample at the dilution of 1:100 was tested against baculovirus recombinant H5 HA antigen (Protein Sciences Corporation, CT). According to the product brochure, the HA1 and HA2 subunits possess molecular masses of 45 and 25 kDa, respectively. Goat antiserum against purified HA of A/Vietnam/1203/04(H5N1), kindly provided by Robert G. Webster and Richard Webby, St. Jude Children's Research Hospital, and a commercial monoclonal antibody to H5 HA (US Biological, Swampscott, MA) was used as the reference antibody control. The nitrocellulose membrane blotted with antigens was blocked with 5% skim milk in Tris-buffer saline plus 0.1% Tween 20 (TBS-T). The test sera were incubated with the blotted membrane overnight at 4°C before washing three times with TBS-T, followed by the secondary antibody, horseradish peroxidase enzyme conjugated with either rabbit anti-goat Ig (Dako Cytomation, Denmark), goat anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or goat anti-human IgG (Zymed Laboratories Inc., San Francisco, CA) for 2 hours at room temperature. Diaminobenzidine (Sigma-Aldrich, St. Louis, MO) mixed with $NiCl_2$ and H_2O_2 was used as the chromogenic substrate.

RESULTS AND DISCUSSION

A fourfold rise in antibody titer either by microNT or HI assay was noted in paired blood specimens, typically when the second specimen was collected 15 or more days after onset of disease (patient nos. 1, 4, and 6). The highest microNT and HI titers of 1:1,280 were detected in sera collected at 15 days (patient no. 6) or 21 days (patient nos. 1 and 4) after onset of disease (Table 2). In one surviving subject from whom repeated serial blood specimens were collected (patient no. 6), the early peak antibody level was followed by a marked decrease in titer about 5 months later, after which the antibody titer remained stable for at least another year. Similarly, all four survivors (patient nos. 2, 3, 4, and 6) also demonstrated microNT titers of ≥1:40 that persisted in all serial serum samples collected at over 3 or almost 5 years after disease onset, the longest periods that we could follow for an individual subject. Our titer of 1:40 was a critical value for indicating previous H5N1 infection, because it was not found in the general Thai population. When establishing the microNT assay, we had investigated more than 200 serum samples from healthy subjects. The results showed that 70% of the subjects had NT antibody to H1N1 virus as tested against A/New Caledonia/20/99 (H1N1) but none had antibody to KAN-1 virus (data not shown). We previously reported the result of an ELISA-based microNT assay in 901 residents of four villages with H5N1 human cases and found that 888 (98.6%) had no NT antibody titer to KAN-1 virus as screened at the serum dilution of 1:5. Among 13 positive cases, 11 had an NT titer of 5 and the other 2 had titers of 10 and 20 (2).

There were two patients (nos. 5 and 7) who died before convalescent blood samples were collected. No anti-H5 antibody was detected in patient no.7, whose acute blood was collected 9 days after onset of disease; even the NT assay was performed against NYK, which was the autologous virus. On the other hand, patient no. 5, who had a single serum sample collected 5 days after onset of disease, remarkably exhibited a serum antibody titer of about 1:320 by either microNT or HI assay. Finally, patient no. 8 had no detectable anti-H5 antibody in two blood samples collected at 24 and 27 days after onset of symptoms, beyond the time when a response was typically seen in other patients. Patient no. 8 was negative for anti-human immunodeficiency virus and anti-hepatitis C virus antibodies and was naturally immune to hepatitis B virus infection (positive anti-HBs, positive anti-HBc, and negative HBs antigen). However, he was an alcohol abuser, an underlying condition that might lead to the inability to develop high antibody re-

TABLE 2. Kinetics and longevity of antibody response to H5N1 virus infection

Subject no.	Blood sample No.	Specimen collection (time after disease onset)	Antibody titer to: A/Thailand/1(KAN-1)/04[a] as determined by: NT	HI with: GRBC	HI with: HRBC	A/Thailand/676(NYK)/05[b] as determined by: NT	HI with: GRBC	HI with: HRBC
1	1	10 days	<5	20	20		20	20
	2	21 days	1,280	640	1,280		1,280	640
2	1	2 yrs, 3 mos	80	80	160	160	160	80
	2	2 yrs, 9 mos	160	80	160	160	160	160
	3	3 yrs, 3 mos	80	80	80	160	160	160
	4	3 yrs, 11 mos	80	80		160	160	
	5	4 yrs, 5 mos	40	40		160	160	
	6	4 yrs, 11 mos	40	40		160	160	
3	1	2 yrs, 2 mos	160	80	160	80	160	80
	2	2 yrs, 8 mos	160	80	160	160	160	160
	3	3 yrs, 2 mos	160	80	160	160	160	160
	4	3 yrs, 10 mos	160	80		160	160	
	5	4 yrs, 3 mos	80	80		160	160	
	6	4 yrs, 10 mos	80	80		160	160	
4	1	10 days	<5	20	20			
	2	12 days	5	20	20		20	20
	3	21 days	1,280					
	4	1 yrs, 6 mos	160	80	80	320	160	160
	5	2 yrs	160	80	160	320	160	160
	6	2 yrs, 6 mos	160	80	160	160	80	160
	7	3 yrs, 3 mos	80	80		160	160	
	8	3 yrs, 8 mos	80	80		160	160	
	9	4 yrs, 2 mos	80	80		160	160	
5	1	5 days	320	80	320	160	640	640
6	1	4 days	5	<20	<20	<10	20	<20
	2	15 days	1,280	320	640	2,560	1,280	1,280
	3	20 days	640	160	320		640	640
	4	5 mos	80	80	160	80	160	160
	5	11 mos	80	80	160	80	160	160
	6	1 yrs, 5 mos	40	80	80	80	160	160
	7	2 yrs, 2 mos	40	40		80	80	
	8	2 yrs, 7 mos	40	40		80	80	
	9	3 yrs, 1 mos	40	40		80	80	
7	1	9 days	<5	<20	<20	<5	20	20
8	1	24 days	5	<20	<20		<20	<20
	2	27 days	5	<20	<20		<20	<20

[a] Autologous virus of patient no. 1.
[b] Autologous virus of patient no. 7.

sponse. He received standard oseltamivir treatment beginning 14 days after onset of symptoms but died 13 days later. We further tested these two blood samples against autologous virus (A/Thailand/1(NBL)/06 (H5N1), but still no NT antibody titer was detected (data not shown). Katz et al. (4) previously reported an adult H5N1 patient in whom no H5-specific antibody response was detected, despite the collection of appropriately timed paired blood specimens. The diagnosis of H5N1 virus infection in this case was similarly based on positive RT-PCR and virus isolation. However, in this case, the inability to mount an H5 antibody response was attributed to the immunocompromised status of the patient due to systemic lupus erythematosus.

Interestingly, we found that sera obtained from patients infected in 2004 could cross-neutralize the two virus isolates to comparable antibody titers. Similarly, sera collected in 2005 cross-neutralized the earlier H5N1 strain, despite the two viruses sharing 96% amino acid identity in the HA sequence (GenBank accession nos. AAS65615 and ABC72655) and 90% identity in NA sequence (GenBank accession nos. AAS65616 and ABC72646) and the difference in receptor site binding preference of SAα2,3Gal or SAα2,6Gal (1, 6). These results suggest that the two H5N1 viruses examined were antigenically closely related with respect to the human antibody response.

This study used infectious viruses as the test antigens in the HI assay. The protocol as described previously employed ei-

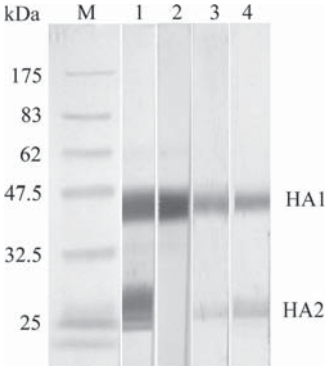


FIG. 1. Detection of anti-H5 HA antibody by WB assay using H5 HA recombinant protein as the test antigen. Lane M, protein molecular mass markers; lane 1, goat antiserum to purified H5 HA; lane 2, mouse monoclonal antibody to H5 HA; lane 3, serum from patient no. 4 at 1 year 6 months after disease onset; lane 4, serum from patient no. 6 at 11 months after disease onset.

ther 1% horse or 0.5% goose erythrocytes (5, 8). Even though horse erythrocytes have been recommended by previous investigators and WHO (8, 11) for detection of antibody to AI virus by HI assay, we did not see a marked difference in the antibody titers obtained when either kind of erythrocytes was used (Table 2). Our finding provides an alternative choice of erythrocytes that can be accessed more easily for Asian countries. We previously reported this finding in a smaller number of blood samples (5).

The result from the WB assay in 20 serum samples from healthy subjects was clearly negative, including the serum sample with anti-H5N1 NT antibody titers of 10 and 20 (data not shown). On the other hand, sera from all four survivors, including when using goat anti-H5 HA, reacted with both HA1 and HA2 domains of the H5 HA, while the monoclonal antibody reacted with the HA1 domain only. The WB assay confirmed the persistence of H5N1 antibody in serial serum samples collected at all time points in all survivors. Examples of serum specimens from patients 4 and 6 are shown in Fig. 1.

Our study was limited by the amount and number of blood specimens available for investigation for several reasons: diagnosis of H5N1 virus infection is generally performed on respiratory samples, not sera; criteria for serologic diagnosis have only been established more recently and are not useful for acute clinical care; based on early disease manifestations, clinicians were more likely to suspect other more common diseases, such as dengue, resulting in only small volumes of sera remaining after dengue serodiagnosis; most important is the high fatality rate of human H5N1 disease. Another limitation was the inability to obtain the exact time of exposure and infection of an individual. During a poultry die-off in a village, it was difficult to pinpoint the exact date of exposure, and some cases may have experienced multiple exposures. The incubation period for H5N1 virus infection in humans after exposure to infected poultry in many cases is 2 to 5 days and is generally 7 days or less. In clusters with probable human-to-human transmission, the incubation period appeared to be approximately 3 to 5 days but in one instance was estimated to be 8 to 9 days (3, 9, 14).

In summary, our study demonstrated high neutralizing antibody titers were achieved 2 to 3 weeks after disease onset in the majority of H5N1-infected symptomatic individuals tested. Furthermore, the anti-H5 neutralizing antibody response was long-lasting and cross-neutralizing for related clade 1 viruses. To what extent these features are implicated among asymptomatic individuals remains to be determined.

ACKNOWLEDGMENTS

This work was supported by the National Center of Genetic Engineering and Biotechnology, the Thailand Research Fund for Senior Research Scholars, and the Department of Medical Sciences, Ministry of Public Health.

We thank Jacqueline Katz, U.S. CDC, for her valuable comments and for editing part of the manuscript.

REFERENCES

1. **Auewarakul, P., O. Suptawiwat, A. Kongchanagul, C. Sangma, Y. Suzuki, K. Ungchusak, S. Louisirirotchanakul, H. Lerdsamran, P. Pooruk, A. Thititanyanont, C. Pittayawanganon, C.-T. Gao, H. Hiramatsu, W. Jampangern, S. Chunsutthiwat, and P. Puthavathana.** 2007. An avian influenza H5N1 virus that binds to a human-type receptor. J. Virol. **81:**9950–9955.
2. **Dejpichai, R., Y. Laosiritaworn, P. Puthavathana, T. M. Uyeki, M. O'Reilly, N. Yampikulsakul, S. Phurahong, P. Pooruk, J. Prasertsopon, R. Kularb, K. Nateerom, N. Sawanpanyalert, and C. Jiraphongsa.** 2009. Seroprevalence of antibodies to avian influenza virus A (H5N1) among residents of villages with human cases, Thailand, 2005. Emerg. Infect. Dis. **15:**756–760.
3. **Kandun, I. N., H. Wibisono, E. R. Sedyaningsih, Yusharmen, W. Hadisoedarsuno, W. Purba, H. Santoso, C. Septiawati, E. Tresnaningsih, B. Heriyanto, D. Yuwono, S. Harun, S. Soeroso, S. Giriputra, P. J. Blair, A. Jeremijenko, H. Kosasih, S. D. Putnam, G. Samaan, M. Silitonga, K. H. Chan, L. L. M. Poon, W. Lim, A. Klimov, S. Lindstrom, Y. Guan, R. Donis, J. Katz, N. Cox, M. Peiris, and T. M. Uyeki.** 2006. Three Indonesian clusters of H5N1 virus infection in 2005. N. Engl. J. Med. **355:**2186–2194.
4. **Katz, J. M., W. Lim, C. B. Bridges, T. Rowe, J. Hu-Primmer, X. Lu, R. A. Abernathy, M. Clarke, L. Conn, H. Kwong, M. Lee, G. Au, Y. Y. Ho, K. H. Mak, N. J. Cox, and K. Fukuda.** 1999. Antibody response in individuals infected with avian influenza A (H5N1) viruses and detection of anti-H5 antibody among household and social contacts. J. Infect. Dis. **180:**1763–1770.
5. **Louisirirotchanakul, S., H. Lerdsamran, W. Wiriyarat, K. Sangsiriwut, K. Chaichoun, T. Songserm, R. Kitphati, P. Sawanpanyalert, C. Komoltri, P. Auewarakul, and P. Puthavathana.** 2007. Erythrocyte binding preference of avian influenza H5N1 viruses. J. Clin. Microbiol. **45:**2284–2286.
6. **Puthavathana, P., P. Auewarakul, P. C. Charoenying, K. Sangsiriwut, P. Pooruk, K. Boonnak, R. Khanyok, P. Thawachsupa, R. Kitphati, and P. Sawanpanyalert.** 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J. Gen. Virol. **86:**423–433.
7. **Rowe, T., R. A. Abernathy, J. Hu-Primmer, W. W. Thompson, X. Lu, W. Lim, K. Fukuda, N. Cox, and J. M. Katz.** 1999. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J. Clin. Microbiol. **37:**937–943.
8. **Stephenson, I., J. M. Wood, K. G. Nicholson, and M. C. Zambon.** 2003. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza hemagglutinin. J. Med. Virol. **70:**391–398.
9. **Ungchusak, K., P. Auewarakul, S. F. Dowell, R. Kitphati, W. Auwanit, P. Puthavathana, M. Uiprasertkul, K. Boonnak, C. Pittayawonganon, N. J. Cox, S. R. Zaki, P. Thawatsupha, M. Chittaganpitch, R. Khongtong, J. M. Simmerman, and S. Chunsutthiwat.** 2005. Probable person-to-person transmission of avian influenza A (H5N1). N. Engl. J. Med. **352:**333–340.
10. **World Health Organization.** 2009. Countries report H5N1 avian influenza in domestic poultry/wildlife 2003–2009. http:// www.oie.int/eng/info_ev/en_AI_factoids_2.htm. Accessed 15 January 2009.
11. **World Health Organization.** 2002. WHO manual on animal influenza diagnosis and surveillance. WHO/CDS/CSR/NCS/2002.5. http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf. Accessed 24 November 2006.
12. **World Health Organization.** 2006. WHO case definitions for human infections with influenza A (H5N1) virus. 29 August 2006. http://www.who.int/csr/disease/avian_influenza/guidelines/case_definition2006_08_29/en/print.html. Accessed 22 October 2006.
13. **World Health Organization.** 2009. Cumulative number of confirmed human cases of avian influenza reported to WHO 14 January 2009. http:// www.who.int/csr/disease/avian_influenza/country/cases_table_2009_01_14/en/index.html. Accessed 15 January 2009.
14. **Writing Committee of the Second WHO Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Viruses.** 2008. Update on avian influenza A (H5N1) virus infection in humans. N. Engl. J. Med. **358:**261–273.

Virus Genes (2009) 38:404–407
DOI 10.1007/s11262-009-0349-y

Codon volatility of hemagglutinin genes of H5N1 avian influenza viruses from different clades

Prasert Auewarakul · Sunisa Chatsurachai · Alita Kongchanagul · Pumaree Kanrai · Sikarin Upala · Prapat Suriyaphol · Pilaipan Puthavathana

Received: 28 November 2008 / Accepted: 12 March 2009 / Published online: 24 March 2009

Abstract Codon volatility is a method recently developed to estimate selective pressures on proteins on the basis of their synonymous codon usage. Volatility of a codon was defined as the fraction of single nucleotide substitutions that would be nonsynonymous. Higher volatility may indicate that the gene has been under more positive selection in the recent past. We analyzed volatility of hemagglutinin genes of H5N1 viruses in the recent outbreaks and observed differences in the volatility among viruses of different clades. The codon volatility of subclade 2.1 viruses from Indonesia was the lowest among all H5N1 clades and subclades. Time series analyses since the beginning of the epidemic in 2004 showed that codon volatility of subclade 2.1 has gradually decreased, while those of other major clades have been increasing. This may reflect differences in the recent evolution of these viruses.

Keywords Avian influenza · H5N1 · Clade · Codon volatility · Selection · Evolution

Avian influenza H5N1 virus is a highly pathogenic influenza A virus that has emerged and caused wide-spread outbreaks in many countries [1]. The ability to infect and cause severe disease in human poses a serious threat that the virus may further evolve to the point that it can transmit efficiently from person to person [2]. This would potentially cause a catastrophic influenza pandemic, which could kill millions of people worldwide [3].

All influenza A viruses are believed to have a common reservoir source in water fowls, which are considered a natural host of the virus [4]. Avian influenza viruses have been transmitted to other avian and mammalian host species and have established several host-specific lineages [4]. The virus evolves differently in natural avian hosts than it does in non-reservoir species [5]. Immunological pressure and adaptation to new host species are probably the major driving forces of viral evolution [6]. All the highly pathogenic H5N1 viruses that are causing outbreaks in various countries have hemagglutinin (HA) genes that originated from the common ancestral strain that caused an outbreak in geese in Southern China in 1996, A/goose/Guangdong 1/1996 (H5N1) (GsGd) [7]. The virus has gone through many reassortment events giving rise to multiple genotypes [7]. The HA gene of H5 subtype has diverged into clades and subclades, some of which are different in the geographical distribution [8]. Viruses of clade 1 are responsible for the outbreaks in Thailand and Vietnam, whereas clade 2 viruses have a more wide-spread distribution and can be further classified into 5 subclades. Subclade 2.1 is responsible for the outbreak in Indonesia, while subclade 2.2 belongs to the Qinghai lineage that spread to Europe and Africa [8]. Clade 2.3.4 has recently replaced clade 1 viruses in northern Vietnam [9]. The ancestral GsGd and its closely related sequences are defined as clade 0. Other subclades of clade 2 (2.3–2.5) and clade 3–9 are confined to China and some neighboring countries [1, 10]. It is not clear whether the diversification among clades was merely a result of geographical separation or there is a more fundamental difference in the evolution of the clades. We sought to understand the evolutionary pattern of the clades and subclades by analyzing their codon bias. We used a

P. Auewarakul (✉) · S. Chatsurachai · A. Kongchanagul · P. Kanrai · S. Upala · P. Suriyaphol · P. Puthavathana
Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkok Noi, Bangkok 10700, Thailand
e-mail: sipaw@mahidol.ac.th

recently developed method called codon volatility, which assesses selective pressure by looking at codon usage of the gene [11–13]. We chose to analyze the HA1 portion of the hemagglutinin (HA) gene of the viruses. HA1 is a viral surface protein with high variability and has been previously shown to have high codon volatility, which indicates positive selection [14].

Codon volatility can be defined loosely as the proportion of a codon's point mutations that result in a nonsynonymous substitution [11]. Although there are several possible definitions of volatility, we used the formal definition described in Plotkin et al. [11] where the volatility of a gene is the summed volatility of its codons. Because codons with high volatility also have neighboring codons with high volatility, nonsynonymous substitutions are more likely to result in codons with high volatility. Therefore, after a positive selection has occurred, a site is more likely to be occupied by a codon of greater than average volatility. On the other hand, proteins containing a larger number of sites under negative selection will exhibit a statistical bias towards less volatile codons, after controlling for their amino acid content [13].

Whether codon volatility can be used to detect positive selection has been a controversial issue. Criticisms on the ability of codon volatility to detect positive selection include the fact that it is under influence from other factors, such as amino acid and nucleotide frequencies [15–19]. Nevertheless, the ability of codon volatility to detect relative positive selection has been shown in a number of models including influenza virus, *Mycobacterium tuberculosis*, and protozoan parasite *Trypanosoma cruzi* [11, 14, 20]. Although comparative method using the ratio of nonsynonymous/synonymous changes (dN/dS) is the most widely used approach for analysis of positive selection, it can be affected by time since divergences of the compared sequences and population size when closely related sequences are analyzed [21]. Therefore, dN/dS can be biased by how well the compared sequences represent the population structure in the timeline of evolution in each clade. In contract, codon volatility is a function of individual sequence, and not dependent on the selection of comparison pair.

Volatility calculation was performed using the Codon Volatility Computation Server (v1.0), Harvard University Bauer Center for Genomic Research (http://volatility.cgr.harvard.edu/cgi-bin/volatility.pl). Volatility of a codon is strongly influenced by the amino acid which it encodes. Therefore, in order to get rid of the bias caused by amino acid content of the gene, we used the ''volatility *P*-values'' that control for the amino acid sequence of each gene [13]. The volatility *P*-value for a gene is computed using a statistical test that evaluates whether or not its volatility is significantly elevated or depressed compared to the rest of the sequences in the set, controlling for its length and amino acid composition. The *P*-value can be calculated using a randomization procedure or by direct formulae based upon a normal approximation. This web-implementation uses the normal approximation, which produces *P*-values that are virtually identical to those obtained by the randomization procedure. *P*-values near zero indicate significantly elevated volatility where *P*-values near one indicate significantly depressed volatility.

HA1 sequences were retrieved from the NCBI Influenza Virus Resource. The sequences include all influenza A virus of subtype H5N1 of the year 2003–2007 with full-length HA1 coding region. In order to classify the retrieved sequences into clades and subclades, we performed a phylogenetic analysis using maximum likelihood method in PHYLIP 3.66 package. The retrieved HA1 sequences clustered into clades and subclades according to their geographical origins as previously described [1, 8, 10] (data not shown). Comparing codon volatility of HA1 from various clades and subclades revealed that the subclade 2.1 (2.1.1–2.1.3) from Indonesia had lower codon volatility (higher volatility *P*-value), while all other clades and subclades had comparable *P*-values (Fig. 1). The difference between the mean *P*-value of subclade 2.1 (0.710) and that of all other clades and subclades (0.457) was statistically significant ($P < 0.0001$, *t*-test). All the differences between the *P*-value of subclade 2.1 and that of each individual clade and subclade were also statistically significant at $P < 0.0001$ by Bonferroni test. Since all the H5N1 viruses of different clades are related and believed to diversify from a common group of ancestral viruses of GsGd lineage in the recent past, we asked whether the observed difference in codon volatility was present at the beginning of diversification as a result of founder effect or has developed over the years after the separation between clade and subclade founders. To address this issue, we separated codon volatilities by the year of viral isolation. The codon volatilities of clade 2.1 viruses decreased (with the codon volatility *P*-values increasing) gradually over the years since the beginning of the epidemic in 2003. In contrast, codon volatilities of closely related viruses of clade 2.2–2.5 increased (with the codon volatility *P*-values decreasing) steadily during the same period. There were significant correlations between year of viral isolation and the means of codon volatility *P*-values. The correlation coefficient (R^2) was 0.8959 and 0.8381 for clade 2.1 and clade 2.2–2.5, respectively (Fig. 2). For the other major clade (clade 1), we also observed a trend of increasing codon volatility over the years, but the correlation did not reach statistical significance ($R^2 = 0.3518$). Therefore, the difference in codon volatilities became more prominent in the more recent years. This suggested that the difference in codon volatility occurred as a result of different

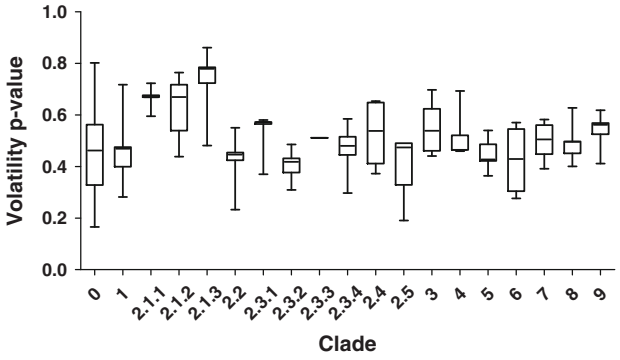


Fig. 1 HA1 volatility *P*-value of H5N1 viruses from different clades and subclades. Error bars, quadrangles, and horizontal lines inside the quadrangles represent ranges, 2nd and 3rd quartiles, and modes of codon volatility *P*-values of clades and subclades

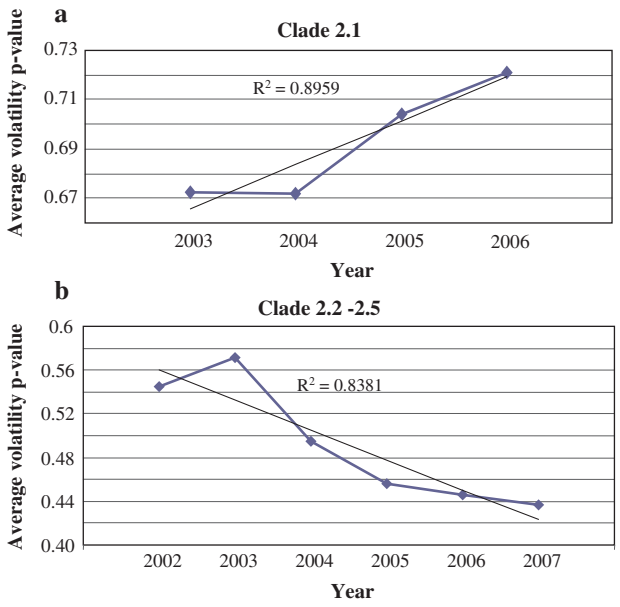


Fig. 2 Regression lines showing trends of decreasing codon volatility (increasing volatility p-value) over the recent years in HA1 of clades 2.1 (**a**) and increasing codon volatility of HA1 of clade 2.2–2.5 over the same period (**b**)

evolutionary pattern among the viruses as they separately evolved.

Our analyses indicate that H5N1 avian influenza viruses of clade 2.1 from Indonesia may be under a lower positive selective pressure or under a higher negative selection as compared to viruses of other clades. It is unclear what caused this differential selection. Certain environments of the transmission either intra-species or inter-species, especially avian-mammalian inter-species transmission, may impose a positive selective pressure on the virus in order to adapt to the new transmission environment. The environment and nature of transmission in Indonesia may be different from those in other countries and may require less viral adaptation. Because of the persistent outbreaks and continuous transmission in poultry in this country, the virus may not need to jump from species to species in order to maintain the transmission chain. This may provide the virus the ability to persist and spread without having to evolve rapidly. On the other hand, it is also possible that the HA1 of clade 2.1 viruses may be under a higher negative selection because of either its own structural constraint or any constraint required for effective spreading in specific environmental conditions in this country.

With the assumption that codon volatility reflected the level of positive selection in these HA1 sequences, the higher codon volatility of HA1 for all other clades and subclades as well as the continuously increasing codon volatility for clades 2.2–2.5 suggests that most of the current H5N1 viruses, except for the clade 2.1 from Indonesia, have been under positive selection since the beginning of the epidemic and still continue to evolve rapidly. This is in agreement with a recently published analysis showing rapid evolutionary dynamics in avian influenza viruses including H5N1 [22]. Another study also showed evidence of positive selection in HA genes of H5N1 viruses that might be different among viral lineages [23]. Since high codon volatility indicates viruses with high ability to evolve, it may pose a higher risk of successful viral adaptation to human host. Therefore, we have to maintain vigilance and continuous monitoring and control in order to prevent the emergence of a pandemic virus.

Acknowledgments This study was a part of the "Avian Influenza Surveillance in Thailand-Studies at Human-Animal Interface" project supported by Cooperative Agreement Number 1U19CI000399-01 from Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC. P.P. was supported by a Senior Research Scholar grant of the Thailand Research Fund. P.A. was supported by Chalermprakiat Fund of Faculty of Medicine Siriraj Hospital.

References

1. R.G. Webster, E.A. Govorkova, N. Engl. J. Med. **355**, 2174 (2006). doi:10.1056/NEJMp068205
2. K. Ungchusak et al., N. Engl. J. Med. **352**, 333 (2005)
3. N.M. Ferguson et al., Nature **442**, 448 (2006). doi:10.1038/nature04795
4. R.G. Webster, W.J. Bean, O.T. Gorman, T.M. Chambers, Y. Kawaoka, Microbiol. Rev. **56**, 152 (1992)
5. R.G. Webster, D.J. Hulse, Rev. Sci. Tech. **23**, 453 (2004)
6. M.I. Nelson, E.C. Holmes, Nat. Rev. Genet. **8**, 196 (2007). doi:10.1038/nrg2053
7. K.S. Li et al., Nature **430**, 209 (2004). doi:10.1038/nature02746
8. H. Chen et al., Proc. Natl. Acad. Sci. USA **103**, 2845 (2006). doi:10.1073/pnas.0511120103
9. M.T. Le et al., PLoS ONE **3**, e3339 (2008). doi:10.1371/journal.pone.0003339
10. A.N. Abdel-Ghafar et al., N. Engl. J. Med. **358**, 261 (2008)

11. J.B. Plotkin, J. Dushoff, H.B. Fraser, Nature **428**, 942 (2004). doi:10.1038/nature02458
12. J.B. Plotkin, J. Dushoff, M.M. Desai, H.B. Fraser, J. Mol. Evol. **63**, 635 (2006). doi:10.1007/s00239-005-0233-x
13. J.B. Plotkin, J. Dushoff, M.M. Desai, H.B. Fraser, Mol. Biol. Evol. **23**, 1457 (2006). doi:10.1093/molbev/msl021
14. J.B. Plotkin, J. Dushoff, Proc. Natl. Acad. Sci. USA **100**, 7152 (2003). doi:10.1073/pnas.1132114100
15. T. Dagan, D. Graur, Mol. Biol. Evol. **22**, 496 (2005). doi:10.1093/molbev/msi033
16. Y. Chen, J.J. Emerson, T.M. Martin, Nature **433**, E6 (2005). doi:10.1038/nature03223
17. R. Friedman, A.L. Hughes, Mol. Biol. Evol. **22**, 542 (2005). doi:10.1093/molbev/msi038
18. P.M. Sharp, Mol. Biol. Evol. **22**, 807 (2005). doi:10.1093/molbev/msi073
19. H. Tang, C.I. Wu, Mol. Biol. Evol. **23**, 372 (2006). doi:10.1093/molbev/msj043
20. F. Azuaje, J.L. Ramirez, J.F. Da Silveira, IEEE Trans. Nanobioscience **6**, 223 (2007). doi:10.1109/TNB.2007.903482
21. E.P. Rocha et al., J. Theor. Biol. **239**, 226 (2006). doi:10.1016/j.jtbi.2005.08.037
22. R. Chen, E.C. Holmes, Mol. Biol. Evol. **23**, 2336 (2006). doi:10.1093/molbev/msl102
23. W. Shi et al., Sci. China C Life Sci. **51**, 987 (2008). doi:10.1007/s11427-008-0131-8

Journal of General Virology (2009), **90**, 216–222 **DOI** 10.1099/vir.0.005660-0

Indigenous sources of 2007–2008 H5N1 avian influenza outbreaks in Thailand

Kridsada Chaichoune,[1] Witthawat Wiriyarat,[1] Arunee Thitithanyanont,[2] Rassameepen Phonarknguen,[1] Ladawan Sariya,[1] Sarin Suwanpakdee,[1] Thanom Noimor,[3] Sunisa Chatsurachai,[4] Prapat Suriyaphol,[4] Kumnuan Ungchusak,[3] Parntep Ratanakorn,[1] Robert G. Webster,[5] Mekkla Thompson,[6] Prasert Auewarakul[4] and Pilaipan Puthavathana[4]

Correspondence
Prasert Auewarakul
sipaw@mahidol.ac.th
or
Pilaipan Puthavathana
siput@mahidol.ac.th

[1]Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand

[2]Faculty of Science, Mahidol University, Bangkok 10400, Thailand

[3]Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Nonthaburi 11000, Thailand

[4]Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

[5]Department of Infectious Diseases, St Jude Children's Research Hospital, Memphis, TN 38105-2794, USA

[6]Westat Inc., Rockville, MD 20850-3195, USA

Outbreaks of H5N1 avian influenza show strong seasonality. It is not clear where the source of virus originates from in each new outbreak season. This study sought to understand the nature of viral resurgence in recent outbreak seasons in Thailand, where the epidemic is relatively well controlled. In such a situation, indigenous viruses surviving the inter-outbreak season would have to pass through a bottleneck. In order to look for evidence of the bottleneck effect, viral genome sequences from recent outbreaks in the country were analysed. H5N1 avian influenza viruses were isolated from six outbreaks in the rainy season and winter of 2007 through to early 2008. Most of the outbreaks were in the Yom–Nan River basin in the southern part of the northern region of the country. Sequences of these viral isolates were identified as clade 1, genotype Z, similar to viruses from previous years in the central region of the country. The sequences clustered into two groups, one of which was closely related to viruses isolated from the same area in July 2006. These analyses indicated that there was a strong bottleneck effect on the virus population and that only a few lineages remained in the area. In addition, evidence of reassortment among these viruses was found. These indicated re-emergence of viruses from a small pool of indigenous sources that had been silently perpetuated over the dry summer months. Therefore, an approach to eradicate H5N1 avian influenza from the area by eliminating these local reservoirs may be feasible and should be seriously considered.

Received 24 July 2008
Accepted 5 September 2008

INTRODUCTION

Thailand has suffered several rounds of H5N1 avian influenza (AI) outbreaks in poultry. The outbreaks in 2004–2005 were explosive and caused severe economic loss to the poultry industry. Poultry outbreaks, as well as human cases, in Thailand have a clear seasonal pattern. Each year, cases appear with the start of the rainy season in July, peak at the end of the rainy season and the start of winter in September–October, and disappear with the start of summer in February (Tiensin *et al.*, 2005). The outbreaks in 2004–2005 were widespread, covering all regions of the country (Buranathai *et al.*, 2007; Tiensin *et al.*, 2005). After massive campaigns and vigorous outbreak control by culling and movement restrictions, the epidemic subsided. Subsequent outbreaks in 2006 and later were limited to small clusters of backyard chickens and ducks. These outbreaks were concentrated in the central part (lower northern region) of the country and were caused by clade 1 viruses. Although a few limited outbreaks were reported in the north-eastern region of the

The GenBank/EMBL/DDBJ accession numbers for the avian influenza sequences are EU233413–EU233420, EU497919–EU497921, EU547798–EU547801, EU669187–EU669201, EU676306–EU676321 and EU875388–EU875397; details are available with the online version of this paper.

country bordering Laos in July 2006 and January 2007, they were caused by a different group of viruses, belong to clade 2 (Chutinimitkul *et al.*, 2007). Interaction or exchange of the clade 1 and clade 2 viruses between these two areas has never been detected. In this study, we confined our analyses to the outbreaks in the central part of the country, which has been suffering repeated H5N1 AI outbreaks since the beginning of the epidemic in 2003–2004. The provinces with repeated H5N1 outbreaks in poultry in 2006–2008 include Sukhothai, Phitsanulok, Pichit and Nakhonsawan in the lower northern region. In the rainy season and winter of 2007 through to early 2008, there were several reports of backyard chicken die-off in these provinces. Despite intensive control and the apparent complete disappearance of outbreak in the dry summer months, outbreaks reappeared in the rainy season of 2006 and 2007. This indicated either the existence of locally persistent viral reservoirs or the reintroduction of viruses from other countries. Although introduction of a new viral strain (clade 2.3.4) into the north-eastern region of the country was reported, the viral sequences from the north/central region remained clade 1, genotype Z (Chutinimitkul *et al.*, 2007; Keawcharoen *et al.*, 2005). This indicated that yearly re-emerging viruses in central Thailand belong to a similar lineage and suggested that they originate from a locally persistent reservoir (Amonsin *et al.*, 2006; Buranathai *et al.*, 2007). The disappearance of the virus in summer suggested that there might be only a small number of viruses surviving the inter-outbreak period, causing a bottleneck effect. Although previously published analyses of viral sequences in 2004–2005, when the epidemic was extensive and did not completely subside in summer, did not suggest any bottleneck effect (Amonsin *et al.*, 2006; Buranathai *et al.*, 2007), we questioned whether the situation would be different in 2006–2008, when the epidemic was better controlled. In order to understand the nature of viral perpetuation and resurgence, we analysed nucleotide sequences from viruses isolated between 2006 and 2008 in the lower northern provinces of Thailand, where outbreaks have taken place repeatedly in every outbreak season.

METHODS

Outbreaks. We investigated six episodes of poultry die-off. All but one occurred in backyard chicken flocks with limited spread. One outbreak in the Chumsaeng district of Nakhonsawan province was in a broiler chicken farm. These outbreaks were located in the Yom–Nan River basin. The Yom and Nan Rivers are branches of the Chaopraya River, the main river of the central plain of Thailand. The locations of the outbreaks are shown in Fig. 1, and characteristics of the outbreaks are given in Table 1.

Virus isolation and sequencing. Tracheal and cloacal swabs collected from backyard chickens and ducks were inoculated into Madin–Darby canine kidney cells. The subtype of viruses was identified by RT-PCR using H5- and N1-specific primers (Lee *et al.*, 2001). RT-PCR was performed on RNA samples extracted from haemagglutination-positive culture supernatants and the PCR

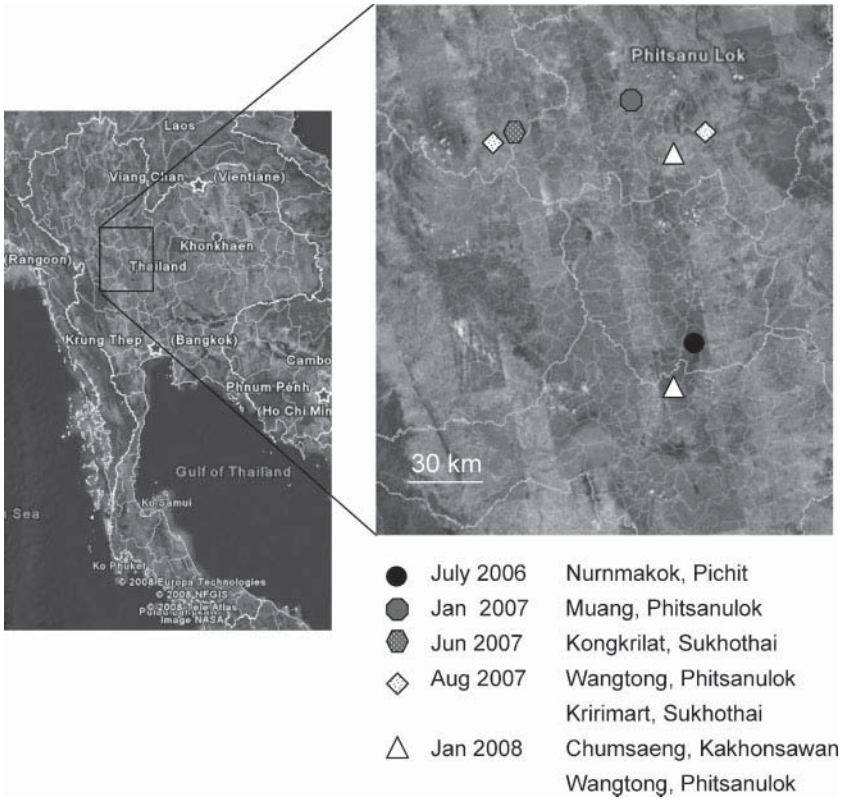


Fig. 1. Location of H5N1 outbreaks in poultry in 2007–2008, with the date of sample collection from the outbreaks.

Table 1. Characteristics of the poultry outbreaks in 2006–2008

Location (district, province)	Type of poultry	No. birds*	Time span	Virus name
Nummakok, Pichit	Backyard chickens	Unknown	24 July 2006	A/chicken/Thailand/PC-168/2006, A/chicken/Thailand/PC-170/2006
Muang, Phitsanulok	Ducks	207 died (30 backyard chickens, 177 ducks)	15 January 2007	A/duck/Phitsanulok/NIAH6-5-0001/2007
Kongkrilat, Sukhothai	Backyard chickens	Unknown	May–June, 2007	A/chicken/Thailand/ICRC-V143/2007
Wangtong, Phitsanulok	Backyard chickens	Eight died from 70 birds (11.4 %)	17–23 August 2007	A/chicken/Thailand/ICRC-V195/2007
Kririmart, Sukhothai	Backyard chickens	19 died from 70 birds (27.1 %)	21–23 August 2007	A/chicken/Thailand/ICRC-V213/2007
Chumsaeng, Nakhonsawan	Broiler	4085 died from 14 000 birds (29.2 %)	22–29 January 2008	A/chicken/Thailand/ICRC-V586/2008
Wangtong, Phitsanulok	Backyard chickens	127 died from 250 chickens (50.8 %)	24–29 January 2008	A/chicken/Thailand/ICRC-V618/2008
	Backyard ducks	A small flock of 10 apparently healthy ducks in the outbreak area		A/duck/Thailand/ICRC-V629/2008
Supanburi	Captive pheasant	One died from 500 captive wild birds (0.2 %)	17 January 2008	A/pheasant/Thailand/VSMU-1-SPB/2008

*Data from the Department of Livestock Development (http://www.dld.go.th/home/bird_flu/history.html).

products were directly sequenced as described previously (Puthavathana *et al.*, 2005). The sequences of A/chicken/Thailand/PC168/06, A/chicken/Thailand/PC170/06 and A/duck/Phitsanulok/NIAH6-5-0001/07 were obtained from GenBank. All sequences determined in this study were submitted to GenBank; the accession numbers are shown in Supplementary Table S1, available in JGV Online.

Phylogenetic reconstruction. Sequence alignment was carried out using MUSCLE version 3.6 and adjusted manually using BioEdit version 7.0.9. The maximum-likelihood (ML) method from the PhyML package was applied to generate a phylogenetic tree of the aligned sequences. Bootstrap analyses were performed on the ML trees for 1000 pseudo-replicates.

RESULTS

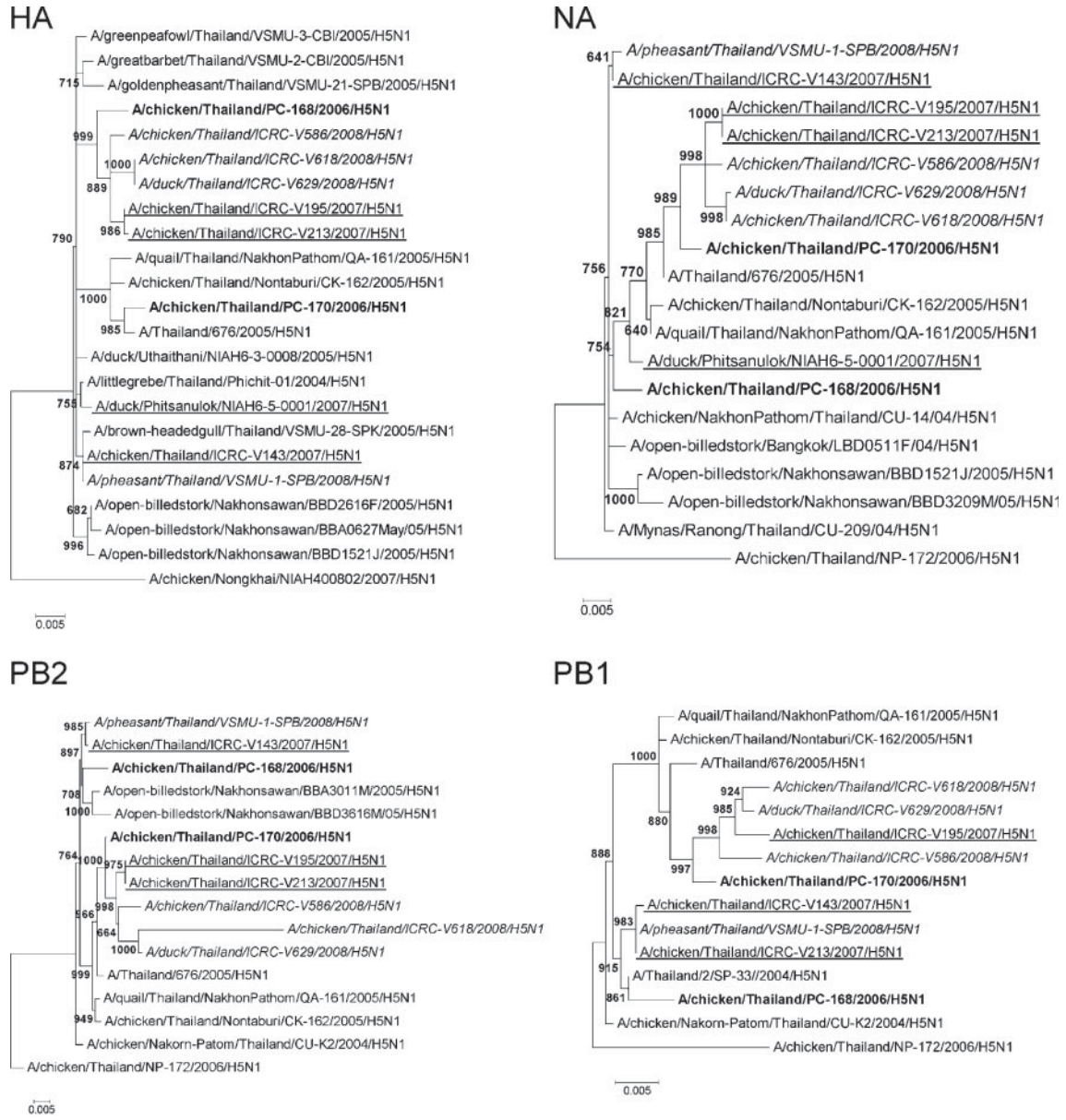
We sequenced all of the genomic fragments of the isolated viruses. The sequences were inspected for unusual changes. We found no mutations at key determinant residues, including receptor-binding preference in the haemagglutinin (HA) gene at positions 138, 186, 196 and 226–228 (H3 numbering system) (Auewarakul *et al.*, 2007; Stevens *et al.*, 2006; Yamada *et al.*, 2006); adaptation to mammalian hosts and growth at lower temperatures in the polymerase basic gene (PB2) at position 627 (Hatta *et al.*, 2007); innate immunity escape and cytokine induction in the non-structural gene (NS1) at position 92 (Lipatov *et al.*, 2005; Seo *et al.*, 2004); and oseltamivir resistance in the neuraminidase (NA) gene at position 274 (Le *et al.*, 2005).

We performed phylogenetic analyses of all of the genomic segments using the ML algorithm. All of the sequences clustered with clade 1, genotype Z viruses isolated previously in the central region of Thailand, and could be clearly separated from the previously reported clade 2.3.4 from north-eastern Thailand (Chutinimitkul *et al.*, 2007) (Fig. 2). When we looked at the relationship among the newly isolated viruses, the HA sequences of ICRC-V143 and ICRC-V213 did not appear to be more related to each other than to other Thailand sequences (Fig. 2). This was unexpected, as the two viruses were isolated from neighbouring areas over consecutive time periods. In contrast, sequences with clear epidemiological linkage clustered closely and formed separated branches, e.g. sequences from open-billed storks in Nakhonsawan in 2005, which indicated a common origin of viruses within the same outbreak cluster. On the other hand, the HA sequence of ICRC-V213 was closely related to that of ICRC-V195 (Fig. 2). The two viruses were isolated in the same week of August 2007 but from areas about 60 km apart. Surprisingly, the patterns of relationship among these three viruses were not consistent for some other genomic segments. The polymerase subunits PB1 and PA of ICRC-V213 were closely related to those of ICRC-V143 rather than ICRC-V195. This suggested that ICRC-V213 could be a reassortant between ICRC-V143- and ICRC-V195-like viruses. Interestingly, the HA, matrix (M),

nucleoprotein (NP) and PA genes of ICRC-V195 were closely related to those of A/chicken/Thailand/PC-168/2006 (PC-168), a virus isolated in a neighbouring province, Pichit, 1 year earlier. Moreover, the NA, NS, PB1 and PB2 genes of ICRC-V195 were closely related to those of A/chicken/Thailand/PC-170/06 (PC-170), a virus isolated in the same area and time period as PC-168 (Fig. 2). Although PC-168 and PC-170 were from the same area in the same time period, these two viruses were not more related to each other than to other clade 1 Thailand viruses. This suggested a reassortment event between the PC-168 and PC-170, which might have given rise to ICRC-V195. Another 2007 virus, A/duck/Phitsanulok/NIAH6-5-0001/2007, was identified in January 2007, but its HA and NA sequences did not show any closer relationship to other recent isolates than to other clade 1 isolates, suggesting that this HA/NA lineage may have been extinct after January 2007. However, as sequences of other genomic segments of this virus are not available, we do not know whether the virus passed on some of its other genomic segments to other viruses via reassortment. In January 2008, outbreaks reappeared in Nakhonsawan, Phitsanulok and Supanburi. The viruses A/chicken/Thailand/ICRC-V586/2008, A/chicken/Thailand/ICRC-V618/2008 and A/duck/Thailand/ICRC-V629/2008, and A/pheasant/Thailand/VSMU-1-SPB/2008 were isolated from Nakhonsawan, Phitsanulok and Supanburi, respectively. The virus A/pheasant/Thailand/VSMU-1-SPB/2008 was isolated from a zoo in an area where poultry die-off had occurred. All of the genomic segments of this virus clustered closely with those of ICRC-V143, whereas sequences of the other viruses isolated in January 2008 were closely related to those of ICRC-V195. In summary, viruses from a cluster of outbreaks in January 2008 (ICRC-V586, ICRC-V618 and ICRC-V629) appeared to be direct descendents of a virus similar to an isolate from August 2007 (ICRC-V195), which was probably a reassortant of two viruses similar to those identified in July 2006 (PC-168 and PC-170). Another isolate from January 2008 (A/pheasant/VSMU-1-SPB/2008) seemed to be a descendant of a virus similar to a chicken isolate from June 2007 (ICRC-V143) (Fig. 3). These data indicated that at least two varieties of H5N1 virus persisted silently over the dry summer months and gave rise to outbreaks in the 2007–2008 season, and that reassortment among these viruses may have occurred. The two varieties were probably responsible for all of the outbreaks in season 2007–2008. Other strains from 2004–2005 were not detected after 2006, suggesting that they were extinct, resulting in a strong bottleneck effect on the virus population.

DISCUSSION

A bottleneck effect is an evolutionary event in which a substantial fraction of a population is eliminated, leaving the surviving minority to repopulate the milieu. Population bottlenecks can increase genetic drift. The re-emergence of H5N1 viruses of similar lineage every year in the rainy season after a silent period in the summer suggests that either there is an unrecognized infected reservoir or there is a low-level transmission chain during the summer months. Either way, if the reservoir or the level of the transmission chain during the summer is small enough, there would be a bottleneck effect on the virus population after re-emergence, as only a few viruses would re-emerge each year and give rise to a few branches, leaving other branches extinct. In this case, phylogenetic trees would show subclusters with some chronological and spatial correlations. However, if the reservoir or the transmission chain during summer was large, the emerging virus population structure would be similar to that of the previous season. In this case, phylogenetic trees would show no specific pattern. Previously published phylogenetic analyses of H5N1 sequences from central Thailand in 2004–2005 did not show any specific sublineage within the virus population (Amonsin *et al.*, 2006; Buranathai *et al.*, 2007; Keawcharoen *et al.*, 2005). This suggested that the virus was endemic in the area throughout the year without interruption, despite the brief periods of undetectable transmission during the summer months. In contrast, the outbreaks in 2006–2008 were much smaller in their geographical extent and the number of birds affected. This reduced the size of the virus population in the outbreak season and also in the inter-outbreak period. The virus populations in the summers of 2006 and 2007 were probably small enough to cause a bottleneck effect. Our data support this model. Despite the evidence supporting the existence of a bottleneck, our analyses did not indicate where this bottleneck might be. Mild or asymptomatic infection with limited virus shedding is ideal to render transmission chains undetectable. One of the recent isolates in this study was from an apparently healthy duck, and ducks have been shown to be infected with mild disease by some H5N1 strains (Sturm-Ramirez *et al.*, 2005). Free-grazing duck raising is still common practice in the Yom–Nan River basin, and previous risk analyses have shown that populations of free-grazing ducks correlate well with the risk of AI outbreak (Gilbert *et al.*, 2006, 2007, 2008). It is therefore likely that free-grazing ducks served as the viral reservoir and that limitation of transmission chains within duck populations in inter-outbreak periods caused the observed bottleneck effect. Infection of vaccinated animals can also make the infection asymptomatic and render the transmission undetectable. Although poultry vaccination is prohibited in Thailand, illegal vaccination is believed to be widespread in the area, especially in fighting cocks. However, accurate information on poultry vaccination in Thailand is not available. The extent and role of poultry vaccination in this country needs to be clearly elucidated. Accurate identification of the reservoir and bottleneck requires more detailed surveillance, especially in the inter-outbreak periods. Such intensified surveillance would



greatly benefit our understanding and future control of H5N1 AI epidemics.

The similarity between ICRC-V143 and A/pheasant/Thailand/VSMU-1-SPB/2008 was intriguing, as the locations of the two outbreaks were 280 km apart. This suggests that a long-range carrier, such as birds or the poultry trade, might be involved in the transmission. Understanding how the virus spreads over long distances is crucial to the successful control of the epidemic and deserves further investigation.

H5N1 AI outbreaks have been occurring repeatedly in the Yom–Nan River basin in recent years. The area has a good irrigation system and the water supply is abundant all year round. Rice cultivation is also carried out in the summer in this area, whilst it is limited to the rainy season in most other areas of the country. The largest freshwater lake in Thailand, Bung Borapet, with an area of over 200 km^2, is also located in this area. Whether these geographical characteristics contribute to the repeated AI outbreaks requires further investigation. The limited geographical area of the sporadic outbreaks in 2006–2008 to the Yom–Nan River basin suggests that future attempts at controlling the virus reservoirs and outbreaks should be focused in this area.

The small pool of viruses surviving inter-outbreak periods may be amplified periodically in the outbreak seasons. It is

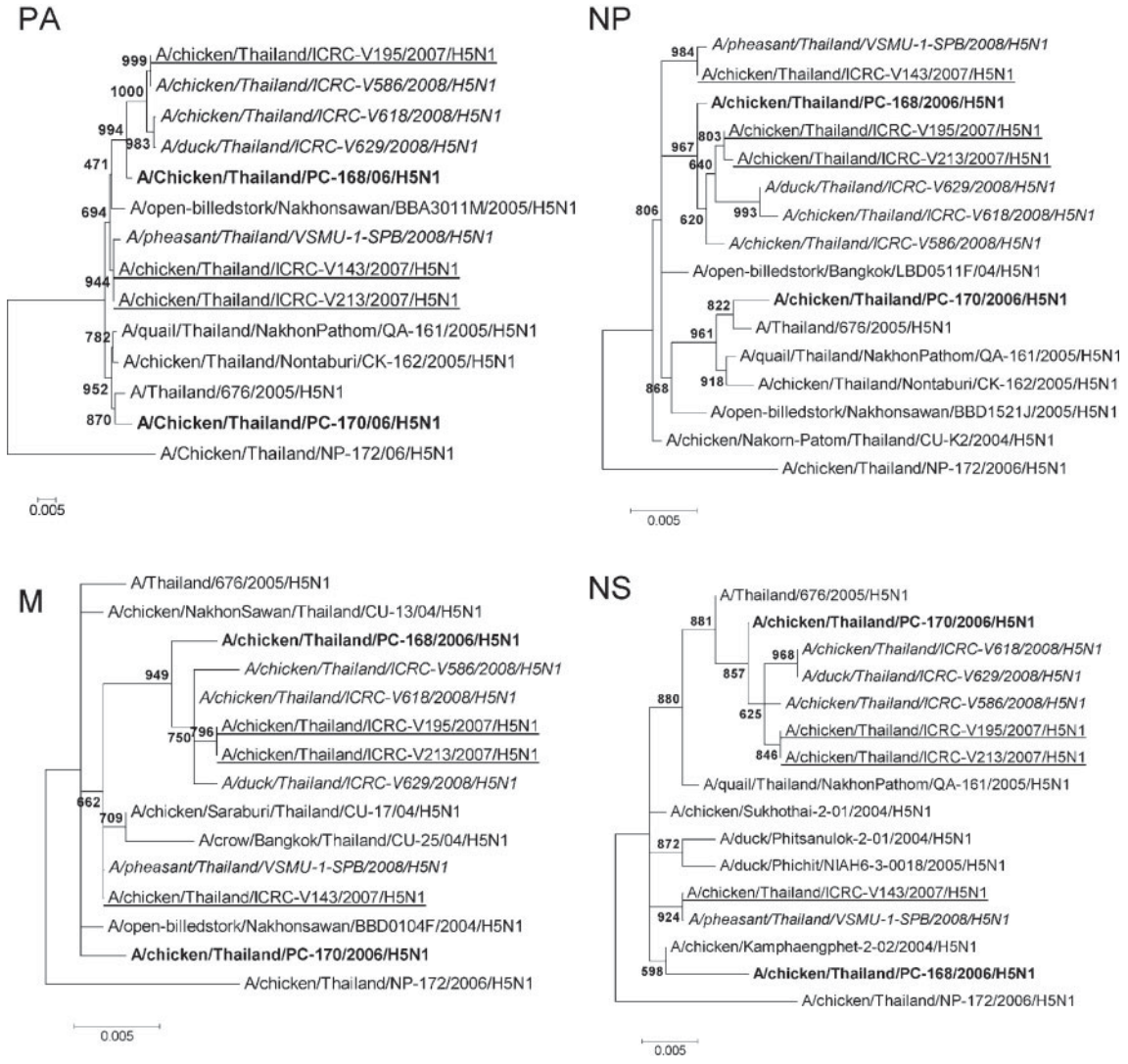


Fig. 2. ML trees with bootstrap values of genes of recent H5N1 viruses from Thailand. The viral sequences isolated in 2006, 2007 and 2008 are bold, underlined and italicized, respectively.

plausible that the cycle of low-level perpetuation in the summer and amplification in the rainy season and winter may together play crucial roles in persistence of the virus in this area. Our analyses suggest that the virus population during the inter-outbreak period may be quite small and could be the weak link in the cycle. Targeting this weak link by both extensive surveillance for virus reservoirs in summer and disrupting transmission chains at the end of the outbreak season to prevent the establishment of virus reservoirs may be the most appropriate strategy in the current situation in Thailand. Such a strategy could potentially lead to the eradication of the virus from this country.

ACKNOWLEDGEMENTS

This study was a part of the 'Avian Influenza Surveillance in Thailand – Studies at Human–Animal Interface' project supported by Cooperative Agreement Number 1U19CI000399-01 from the Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the CDC. Viral strain collection and characterization were facilitated by the Thailand Avian Influenza Monitoring Network (TAIM Net) established by the National Center for Genetic Engineering and Biotechnology. A. T. is supported by the Ellison Foundation and P. P., the grant principal investigator, is supported by a Senior Research Scholar grant of the Thailand Research Fund.

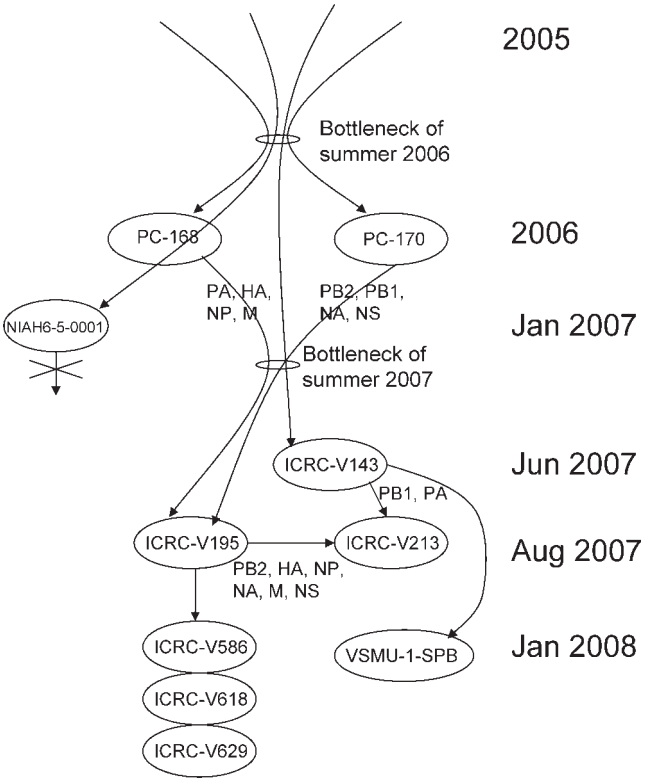


Fig. 3. Schematic diagram showing the predicted evolutionary paths of recent H5N1 isolates in Thailand and the proposed bottlenecks. The linear relationships among viruses were derived from the clustering of their genomic segment sequences in the phylogenetic trees in Fig. 2.

REFERENCES

Amonsin, A., Chutinimitkul, S., Pariyothorn, N., Songserm, T., Damrongwantanapokin, S., Puranaveja, S., Jam-On, R., Sae-Heng, N., Payungporn, S. & other authors (2006). Genetic characterization of influenza A viruses (H5N1) isolated from 3rd wave of Thailand AI outbreaks. *Virus Res* **122**, 194–199.

Auewarakul, P., Suptawiwat, O., Kongchanagul, A., Sangma, C., Suzuki, Y., Ungchusak, K., Louisirirotchanakul, S., Lerdsamran, H., Pooruk, P. & other authors (2007). An avian influenza H5N1 virus that binds to a human-type receptor. *J Virol* **81**, 9950–9955.

Buranathai, C., Amonsin, A., Chaisigh, A., Theamboonlers, A., Pariyothorn, N. & Poovorawan, Y. (2007). Surveillance activities and molecular analysis of H5N1 highly pathogenic avian influenza viruses from Thailand, 2004–2005. *Avian Dis* **51**, 194–200.

Chutinimitkul, S., Songserm, T., Amonsin, A., Payungporn, S., Suwannakarn, K., Damrongwatanapokin, S., Chaisingh, A., Nuansrichay, B., Chieochansin, T. & other authors (2007). New strain of influenza A virus (H5N1), Thailand. *Emerg Infect Dis* **13**, 506–507.

Gilbert, M., Chaitaweesub, P., Parakamawongsa, T., Premashthira, S., Tiensin, T., Kalpravidh, W., Wagner, H. & Slingenbergh, J. (2006). Free-grazing ducks and highly pathogenic avian influenza, Thailand. *Emerg Infect Dis* **12**, 227–234.

Gilbert, M., Xiao, X., Chaitaweesub, P., Kalpravidh, W., Premashthira, S., Boles, S. & Slingenbergh, J. (2007). Avian influenza, domestic ducks and rice agriculture in Thailand. *Agric Ecosyst Environ* **119**, 409–415.

Gilbert, M., Xiao, X., Pfeiffer, D. U., Epprecht, M., Boles, S., Czarnecki, C., Chaitaweesub, P., Kalpravidh, W., Minh, P. Q. & other authors (2008). Mapping H5N1 highly pathogenic avian influenza risk in Southeast Asia. *Proc Natl Acad Sci U S A* **105**, 4769–4774.

Hatta, M., Hatta, Y., Kim, J. H., Watanabe, S., Shinya, K., Nguyen, T., Lien, P. S., Le, Q. M. & Kawaoka, Y. (2007). Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathog* **3**, 1374–1379.

Keawcharoen, J., Amonsin, A., Oraveerakul, K., Wattanodorn, S., Papravasit, T., Karnda, S., Lekakul, K., Pattanarangsan, R., Noppornpanth, S. & other authors (2005). Characterization of the hemagglutinin and neuraminidase genes of recent influenza virus isolates from different avian species in Thailand. *Acta Virol* **49**, 277–280.

Le, Q. M., Kiso, M., Someya, K., Sakai, Y. T., Nguyen, T. H., Nguyen, K. H., Pham, N. D., Ngyen, H. H., Yamada, S. & other authors (2005). Avian flu: isolation of drug-resistant H5N1 virus. *Nature* **437**, 1108.

Lee, M. S., Chang, P. C., Shien, J. H., Cheng, M. C. & Shieh, H. K. (2001). Identification and subtyping of avian influenza viruses by reverse transcription-PCR. *J Virol Methods* **97**, 13–22.

Lipatov, A. S., Andreansky, S., Webby, R. J., Hulse, D. J., Rehg, J. E., Krauss, S., Perez, D. R., Doherty, P. C., Webster, R. G. & Sangster, M. Y. (2005). Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses. *J Gen Virol* **86**, 1121–1130.

Puthavathana, P., Auewarakul, P., Charoenying, P. C., Sangsiriwut, K., Pooruk, P., Boonnak, K., Khanyok, R., Thawachsupa, P., Kijphati, R. & Sawanpanyalert, P. (2005). Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. *J Gen Virol* **86**, 423–433.

Seo, S. H., Hoffmann, E. & Webster, R. G. (2004). The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. *Virus Res* **103**, 107–113.

Stevens, J., Blixt, O., Tumpey, T. M., Taubenberger, J. K., Paulson, J. C. & Wilson, I. A. (2006). Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science* **312**, 404–410.

Sturm-Ramirez, K. M., Hulse-Post, D. J., Govorkova, E. A., Humberd, J., Seiler, P., Puthavathana, P., Buranathai, C., Nguyen, T. D., Chaisingh, A. & other authors (2005). Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? *J Virol* **79**, 11269–11279.

Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., Parakamawongsa, T., Premashthira, S., Amonsin, A. & other authors (2005). Highly pathogenic avian influenza H5N1, Thailand, 2004. *Emerg Infect Dis* **11**, 1664–1672.

Yamada, S., Suzuki, Y., Suzuki, T., Le, M. Q., Nidom, C. A., Sakai-Tagawa, Y., Muramoto, Y., Ito, M., Kiso, M. & other authors (2006). Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. *Nature* **444**, 378–382.

Supplementary Table S1. List of GenBank accession numbers of the viral sequences

Virus name	GenBank accession number for gene							
	PB2	**PB1**	**PA**	**HA**	**NP**	**NA**	**M**	**NS**
A/chicken/Thailand/PC-168/2006	DQ999884	DQ999886	DQ999885	DQ999880	DQ999883	DQ999881	DQ999882	DQ999879
A/chicken/Thailand/PC-170/2006	DQ999893.1	DQ999892	DQ999894	DQ999887	DQ999891	DQ999888	DQ999889	DQ999890
A/duck/Phitsanulok/NIAH6-5-0001/2007	–	–	–	EF501997	–	EF501996	–	–
A/chicken/Thailand/ICRC-V143/2007(H5N1)	EU233413	EU233414	EU233415	EU233416	EU233417	EU233418	EU233419	EU233420
A/chicken/Thailand/ICRC-V195/2007(H5N1)	EU669192	EU669191	EU669190	EU497920	EU669189	EU669188	EU669193	EU669194

A/chicken/Thailand/ICRC-V213/2007(H5N1)	EU875391	EU875392	EU875393	EU497921	EU875394	EU875395	EU875396	EU875397
A/chicken/Thailand/ICRC-V586/2008(H5N1)	EU669195	EU669196	EU669197	EU497919	EU669198	EU669199	EU669201	EU669200
A/chicken/Thailand/ICRC-V618/2008(H5N1)	EU875390	EU875389	EU547800	EU547798	EU547799	EU875388	EU669187	EU547801
A/duck/Thailand/ICRC-V629/2008 (H5N1)	EU676312	EU676313	EU676306	EU676307	EU676308	EU676311	EU676309	EU676310
A/pheasant/Thailand/VSMU-1-SPB/2008 (H5N1)	EU676314	EU676315	EU676316	EU676317	EU676320	EU676318	EU676319	EU676321

Chaichoune, K., Wiriyarat, W., Thitithanyanont, A., Phonarknguen, R., Sariya, L., Suwanpakdee, S., Noimor, T., Chatsurachai, S., Suriyaphol, P., Ungchusak, K., Ratanakorn, P., Webster, R. G., Thompson, M., Auewarakul, P. and Puthavathana, P. (2009). Indigenous sources of 2007–2008 H5N1 avian influenza outbreaks in Thailand. *J Gen Virol* **90**, 216–222.

P. jirovecii independent of environmental hazards.

Isolation of pathogens from an aborted fetus does not necessarily mean that they have caused the death of the fetus because many agents appear to pass through the fetal-placental unit and cause little damage. However, fungal infection is a major worldwide cause of abortion in cattle (*10*), and the surprising high prevalence of *P. jirovecii* infection found in dead fetuses in our study emphasizes the need to study the possible role of this fungal organism in human abortion.

Our findings could be of potential clinical importance and could open a new field of research, which should be explored. Further research should assess the scope of the problem and design rational preventive strategies, if necessary.

This study is part of the project "*Pneumocystis* Pathogenomics: Unravelling the Colonization-to-Disease Shift," a Coordination Action supported by the European Commission (ERANET PathoGenoMics). This study was partially supported by the Spanish Ministry of Health (FIS 03/1743). M.A.M.-C. and C.d.l.H. were supported by the Spanish Ministry of Health (FIS CP-04/217 and FIS CM-04/146).

Marco A. Montes-Cano, Magali Chabe, Maria Fontillon-Alberdi, Carmen de la Horra, Nieves Respaldiza, Francisco J. Medrano, Jose M. Varela, Eduardo Dei-Cas, and Enrique J. Calderon

Author affiliations: Virgen del Rocío University Hospital, Seville, Spain (M.A. Montes-Cano, M. Fontillon-Alberdi, C. de la Horra, N. Respaldiza, F.J. Medrano, J.M. Varela, E.J. Calderon); Center for Biomedical Research in Epidemiology and Public Health, Seville (M.A. Montes-Cano, M. Fontillon-Alberdi, C. de la Horra, N. Respaldiza, F.J. Medrano, J.M. Varela, E.J. Calderon); and Lille Pasteur Institute, Lille, France (M.A. Montes-Cano, M. Chabe, E. Dei-Cas)

DOI: 10.3201/eid1501.080242

References

1. Dei-Cas E. *Pneumocystis* infections: the iceberg? Med Mycol. 2000;38(Suppl.1):23–32.
2. Sanchez CA, Chabé M, Aliouat el M, Durand-Joly I, Gantois N, Conseil V, et al. Exploring transplacental transmission of *Pneumocystis oryctolagi* in first-time pregnant and multiparous rabbit does. Med Mycol. 2007;45:701–7. DOI: 10.1080/13693780701531156
3. Cere N, Drouet-Viard F, Dei-Cas E, Chanteloup N, Coudert P. In utero transmission of *Pneumocystis carinii* sp. f. *oryctolagi*. Parasite. 1997;4:325–30.
4. Pavlica F. The first observation of congenital pneumocystic pneumonia in a fully developed stillborn child. Ann Paediatr. 1962;198:177–84.
5. Mortier E, Pouchot J, Bossi P, Molinie V. Maternal-fetal transmission of *Pneumocystis carinii* in human immunodeficiency virus infection. N Engl J Med. 1995;332:825. DOI: 10.1056/NEJM199503233321219
6. Hughes WT. Pneumocystis in infants and children. N Engl J Med. 1995;333:320–1. DOI: 10.1056/NEJM199508033330515
7. Montes-Cano MA, de la Horra C, Martin-Juan J, Varela JM, Torronteras R, Respaldiza N, et al. *Pneumocystis jiroveci* genotypes in the Spanish population. Clin Infect Dis. 2004;39:123–8. DOI: 10.1086/421778
8. Ng VL, Yajko DM, Hadley WK. Extrapulmonary pneumocystosis. Clin Microbiol Rev. 1997;10:401–18.
9. Vargas SL, Ponce CA, Sanchez CA, Ulloa AV, Bustamante R, Juarez G. Pregnancy and asymptomatic carriage of *Pneumocystis jiroveci*. Emerg Infect Dis. 2003;9:605–6.
10. Kirkbride CA. Etiologic agents detected in a 10-year study of bovine abortions and stillbirths. J Vet Diagn Invest. 1992;4:175–80.

Address for correspondence: Marco A. Montes-Cano, Servicio de Inmunología, Hospital Universitario Virgen del Rocío, Avda Manuel Siurot s/n, 41013 Seville, Spain; email: mmontescano@hotmail.com

Avian Influenza Virus (H5N1) in Human, Laos

To the Editor: The first avian influenza (H5N1) outbreak in poultry in Laos occurred in 2003 and subsided in March 2004 after massive killing of poultry to contain the disease. Extensive surveillance from July 2005 through January 2006 did not detect any influenza virus subtypes in chicken, ducks, quails, and pigs in live bird markets in the Vientiane, Champasak, and Savannakhet Provinces (*1*). Avian influenza virus (H5N1) was reintroduced into Laos in February 2006 but showed a lower incidence. Viruses isolated in this country in 2004 belonged to genotype Z, clade 1, and 2006 isolates belonged to clade 2.3.4 (online Appendix Figure, panel A, available from www.cdc.gov/EID/content/15/1/127-appF.htm) (*1*).

Avian influenza (H5N1) had not been reported in humans in Laos until February 27, 2007 (*2*). Our patient was a 15-year-old adolescent girl who lived in a suburb of Vientiane where an outbreak of influenza (H5N1) in poultry had been confirmed on February 7, 2007. Influenza-like symptoms developed in the patient on February 10. She was hospitalized in Vientiane with fever and respiratory symptoms on February 15. On February 17, her parents brought her to a private hospital in Nong Khai Province, Thailand. Oseltamivir was prescribed on February 19. On February 20, she was transferred to the Nong Khai Provincial Hospital because of rapid, progressive, severe pneumonia with acute respiratory distress syndrome. When we suspected avian influenza in this patient, clinical specimens were tested.

A diagnosis of infection with avian influenza (H5N1) was based on positive results obtained by reverse transcription–PCR (RT-PCR), viral isolation in MDCK cells inoculated with an endotracheal suction specimen

collected on February 22, and a 4-fold increase in neutralizing antibody titers from 80 to 320 in paired blood specimens collected on February 25 and March 1, as assayed against autologous virus. This virus isolate was named A/Laos/Nong Khai 1/07(H5N1). Subsequent samples were collected on February 25 and March 7 (day of death). Results of RT-PCR were positive for the sample collected on February 25 only; virus isolation results were negative for both samples.

The virus was screened for a novel reassorted gene by a multiplex RT-PCR and 8 primer pairs specific for each genomic segment of genotype Z, clade 1 virus (*3*). All segments except the polymerase A (PA) segment were amplified, which indicated that the new virus was different from genotype Z viruses. The viral genome was sequenced and submitted to GenBank (accession nos. EU499372–EU499379 for hemagglutinin, nonstructural protein, matrix protein, nucleoprotein, PB1, PB2, neuraminidase, and PA genes, respectively). Phylogenetic analysis showed that this virus belonged to genotype V (online Appendix Figure, panel B) (*4*); phylogenetic analysis of the hemagglutinin gene (www.who.int/csr/disease/avian_influenza/smaltree.pdf) showed that it belonged to clade 2.3.4 (online Appendix Figure, panel A).

Protein sequence at the hemagglutinin cleavage site harbored many basic amino acids (RERR_RKR). One amino acid deletion and 1 amino acid change were found when compared with RERRRKKR, which is present in most avian influenza viruses (H5N1). There was no change in receptor binding site. This virus had glutamic acid at aa 627 in the PB2 protein, aspartic acid at aa position 92 in nonstructural protein 1, and 5 aa deletions at positions 80–84 in the nonstructural protein 1. Analysis of the neuraminidase gene showed a 20-aa deletion in the stalk protein; there was no mutation of histidine to tyrosine at aa position 274, a position shown to be the oseltamivir resistance marker in the neuraminidase 1 viral genome (*5*). Mutations in the matrix 2 gene showed that amantadine resistance was not present in our virus (*6*). Our in vitro assay (*7*) showed that this virus was sensitive to oseltamivir and amantadine.

Since 2003, genotype V influenza viruses (H5N1) have been reported in some East Asian countries. Genetic diversity in the hemagglutinin gene has classified those genotype V viruses into distinct clades. Viruses from avian species in South Korea in 2003 and Japan in 2004 (*8*,*9*) belong to clade 2.5. A/chicken/Shanxi/2/2006 isolate belonged to clade 7. Human cases in People's Republic of China, i.e., A/China/GD01/06, A/Shenzhen/406H/06, A/Jiangsu/1/2007, and A/Jiangsu/2/2007, belong to clade 2.3.4, the same clade as A/chicken/Thailand/NP-172/2006 and the virus from our study.

Highly pathogenic avian influenza viruses (H5N1) that caused outbreaks in Thailand since 2004 belong to genotype Z, clade 1. Introduction of genotype V clade 2.3.4 virus, A/chicken/Thailand/NP-172/2006, to Nakhon Phanom Province occurred in November 2006 (*10*), the same year that clade 2.3.4 virus was introduced into Laos (online Appendix Figure, panel A). On the basis of hemagglutinin gene phylogeny, A/Laos/Nong Khai 1/2007 is closely related to A/chicken/Nongkhai/NIAH 400802/2007 and A/chicken/Thailand/NP-172/2006. Phylogenetic analysis suggested that viruses from these 2 countries shared the same origin. There was extensive movement across the Mekong River even before the bridge linking Nong Khai from Vientiane was opened. However, the route of transmission of genotype V viruses from east Asian to Southeast Asian countries could not be elucidated.

Acknowledgments

We thank the Lao Public Health Department, the Vientiane Municipal and Nong Khai Provincial Health Office, and Nong Khai Provincial Hospital for collaborative support of this study. The oseltamivir used in drug sensitivity assay was kindly provided by Roche, Ardenne, Belgium.

This study was supported by the National Biotechnology for Genetic Engineering, the Thailand Research Fund for Senior Research Scholar, and a Mahidol University research grant.

Pilaipan Puthavathana, Kantima Sangsiriwut, Achareeya Korkusol, Phisanu Pooruk, Prasert Auewarakul, Chakrarat Pittayawanganon, Derek Sutdan, Rungrueng Kitphati, Pathom Sawanpanyalert, Bounlay Phommasack, Khanthong Bounlu, and Kumnuan Ungchusak

Author affiliations: Mahidol University, Bangkok, Thailand (P. Puthavathana, K. Sangsiriwut, A. Korkusol, P. Pooruk, P. Auewarakul); Ministry of Public Health, Nonthaburi, Thailand (C. Pittayawanganon, D. Sutdan, R. Kitphati, P. Sawanpanyalert, K. Ungchusak); and Ministry of Health, Vientiane, Laos (B. Phommasack, K. Bounlu)

DOI: 10.3201/eid1501.080524

References

1. Boltz DA, Douangngeun B, Sinthasak S, Phommachanh P, Rolston S, Chen H, et al. H5N1 influenza viruses in Lao People's Democratic Republic. Emerg Infect Dis. 2006;12:1593–5.
2. World Health Organization. First human case of avian influenza in the Lao People's Democratic Republic. 2007 [cited 2008 Oct 6]. Available from http://www.wpro.who.int/media_centre/press_releases/pr_20070228.htm
3. Auewarakul P, Sangsiriwut K, Chaichoune K, Thitithanyanont A, Wiriyarat W, Songserm T, et al. Surveillance for reassortant virus by multiplex reverse transcription-PCR specific for eight genomic segments of avian influenza A H5N1 viruses. J Clin Microbiol. 2007;45:1637–9. DOI: 10.1128/JCM.00382-07

4. Li KS, Guan Y, Wang J, Smith GJD, Xu KM, Duan L, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature. 2004;430:209–13. DOI: 10.1038/nature02746
5. Wang MZ, Tai CY, Mendel DB. Mechanism by which mutations at His274 alter sensitivity of influenza A virus N1 neuraminidase to oseltamivir carboxylate and zanamivir. Antimicrob Agents Chemother. 2002;46:3809–16. DOI: 10.1128/AAC.46.12.3809-3816.2002
6. Suzuki H, Saito R, Matsuda H, Oshitani H, Sato M, Sato I. Emergence of amantadine-resistant influenza A viruses: epidemiological study. J Infect Chemother. 2003;9:195–200. DOI: 10.1007/s10156-003-0262-6
7. Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pooruk P, Boonnak K, et al. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J Gen Virol. 2005;86:423–33. DOI: 10.1099/vir.0.80368-0
8. Mase M, Tsukamoto K, Imada T, Imai K, Tanimura N, Nakamura K, et al. Characterization of H5N1 influenza A viruses isolated during the 2003–2004 influenza outbreaks in Japan. Virology. 2005;332:167–76. DOI: 10.1016/j.virol.2004.11.016
9. Mase M, Kim J-H, Lee Y-J, Tsukamoto K, Imada T, Imai K, et al. Genetic comparison of H5N1 influenza A viruses isolated from chickens in Japan and Korea. Microbiol Immunol. 2005;49:871–4.
10. Chutinimitkul S, Thaweesak S, Amonsin A, Payungporn S, Suwannakarn K, Damrongwatanapokin S, et al. New strain of influenza A virus (H5N1), Thailand. Emerg Infect Dis. 2007;13:506–7.

Address for correspondence: Pilaipan Puthavathana, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand; email: siput@mahidol.ac.th

Fatal HIV Encephalitis in HIV-Seronegative Patients

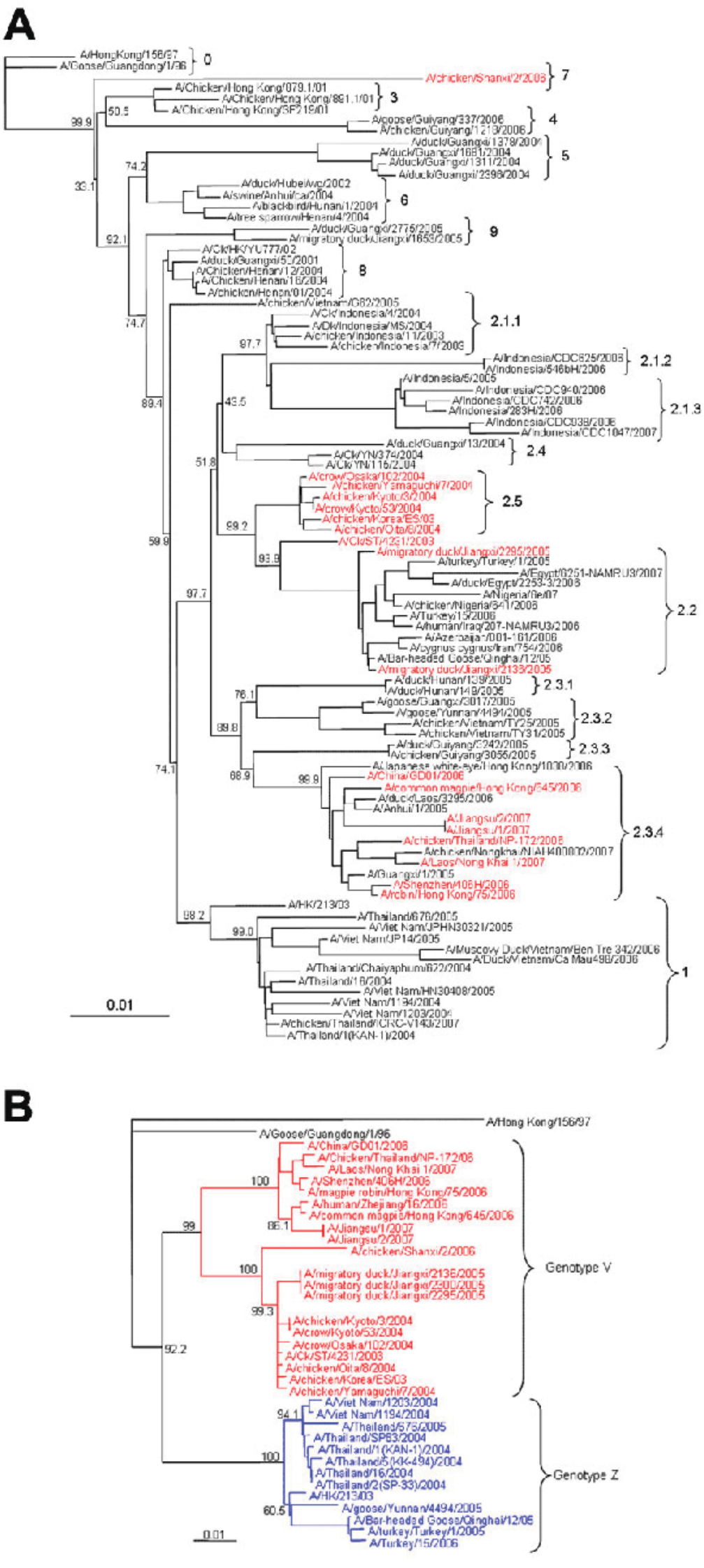
To the Editor: Acute encephalitis is rarely seen in patients infected with HIV (*1*). In addition, HIV in patients who are seronegative is extremely rare, particularly in the setting of current screening ELISAs (*2*). We report a case of encephalitis and HIV in the same patient, which resulted in death.

A 44-year-old Caucasian woman sought treatment at our hospital with a 1-week history of fever, unsteady gait, and progressive confusion. Her medical history included hypothyroidism, depression, and chronic alcohol abuse. The patient's first tests for HIV were negative at 19 and 12 months prior to admission during routine intake screening for jail inmates (Abbott HIV AB HIV-1/HIV-2 [rDNA] enzyme immunoassay [EIA] kit; Abbott Laboratories, Abbott Park, IL, USA). Six months before admission, the patient had a viral exanthem of blistering rash on her lips, palate, and chest. Two weeks later, she had oral thrush and a leukocyte count of 1,700 cells/μL. An HIV ELISA result was negative. Three months before admission, she was admitted to a different hospital for weakness, abdominal pain, intermittent fever, diarrhea, persistent oral candidiasis, and ethanol withdrawal. She had leukopenia and thrombocytopenia. A fourth HIV ELISA result was negative. The patient had been admitted to our hospital one week before the current admission with symptoms of fever, confusion, and urinary tract infection. Lumbar puncture showed an elevated protein level (106 mg/dL). A fifth HIV test result 6 days before most recent admission was negative. Five days before admission, she had been discharged to a rehabilitation facility.

On this hospitalization, she had fatigue, headache, disequilibrium, dysarthria, and blurred vision. Initial examination showed fever of 101.3°F, poor word recall, and a wide-based gait. Laboratory tests showed mild anemia and a leukocyte count of 2×10^3 cells/μL.

Over the next few days the patient's fever persisted and her mental status fluctuated. Tests on hospital day 2 showed a CD4 count of 101/mL (16.9%). Magnetic resonance imaging (MRI) of the brain showed diffuse symmetric white matter disease (Figure, panel A). Samples sent on hospital day 9 eventually showed wild-type HIV with a viral load >500,000 copies/mL. Repeat cerebrospinal fluid (CSF) test results were negative for cryptoccocus antigen, and PCR results were negative for cytomegalovirus, herpes simplex virus (HSV), and JC polyoma virus. The next day, a sixth HIV ELISA result was negative. The serum level of HIV p24 antigen was 202 pg/mL.

On hospital day 13, the patient began treatment with zidovidine, lamuvidine, didanosine, and nevirapine. Within 24 hours, seizures and catatonia developed in the patient. An electroencephalogram showed diffuse wave form slowing. A repeat MRI showed worsened white matter disease (Figure, panel B). The result of a seventh HIV screening ELISA performed on hospital day 15 was negative. Two days later, the HIV viral load was 241,789 copies/mL. On hospital day 19, her serum levels were within normal limits: immunoglobulin (Ig) M level (164 mg/dL), IgG level (1,440 mg/dL), a 3× normal IgA level (1,060 mg/dL), and no oligoproteins. The CSF had an IgG level >10× normal (72 mg/dL), elevated IgG levels for HSV1 (1:160) and HSV2 (1:40), was negative for virus culture, and showed a negative PCR result for JC polyoma virus. On hospital day 23, the eighth HIV ELISA result was negative. The Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA was used throughout the hospitalization. On hospital day 24, supportive care was withdrawn and the patient


A
A/HongKong/156/97
A/Goose/Guangdong/1/96
0
A/chicken/Shanxi/2/2006
7
A/Chicken/Hong Kong/079.1/01
A/Chicken/Hong Kong/881.1/01
A/Chicken/Hong Kong/SF219/01
3
A/goose/Guiyang/337/2006
A/chicken/Guiyang/1213/2006
4
A/duck/Guangxi/1378/2006
A/duck/Guangxi/1681/2004
A/duck/Guangxi/1311/2004
A/duck/Guangxi/2998/2004
5
A/duck/Hubei/wc/2002
A/swine/Anhui/ca/2004
A/blackbird/Hunan/1/2004
A/tree sparrow/Henan/4/2004
6
A/duck/Guangxi/2776/2005
A/migratory duck/Jiangxi/1653/2005
9
A/Ck/HK/YU777/02
A/duck/Guangxi/52/2001
A/Chicken/Henan/12/2004
A/Chicken/Henan/16/2004
A/chicken/Henan/01/2004
8
A/chicken/Vietnam/C82/2005
A/Ck/Indonesia/4/2004
A/Dk/Indonesia/MS/2004
A/chicken/Indonesia/11/2003
A/chicken/Indonesia/7/2003
2.1.1
A/Indonesia/CDC525/2005
A/Indonesia/546bH/2006
2.1.2
A/Indonesia/5/2005
A/Indonesia/CDC340/2006
A/Indonesia/CDC742/2006
A/Indonesia/283H/2006
A/Indonesia/CDC038/2006
A/Indonesia/CDC1047/2007
2.1.3
A/duck/Guangxi/13/2004
A/Ck/YN/374/2004
A/Ck/YN/116/2004
2.4
A/crow/Osaka/102/2004
A/chicken/Yamaguchi/7/2004
A/chicken/Kyoto/3/2004
A/crow/Kyoto/53/2004
A/chicken/Korea/ES/03
A/chicken/Oita/8/2004
2.5
A/Ck/ST/4231/2003
A/migratory duck/Jiangxi/2295/2005
A/turkey/Turkey/1/2005
A/Egypt/0251-NAMRU3/2007
A/duck/Egypt/2253-3/2006
A/Nigeria/flei07
A/chicken/Nigeria/3-1/2006
A/Turkey/15/2006
A/human/Iraq/207-NAMRU3/2006
A/Azerbaijan/001-161/2006
A/Cygnus cygnus/Iran/754/2006
A/Bar-headed Goose/Qinghai/12/05
A/migratory duck/Jiangxi/2133/2005
2.2
A/duck/Hunan/139/2005
A/duck/Hunan/148/2005
2.3.1
A/goose/Guangxi/3017/2005
A/goose/Yunnan/1491/2005
A/chicken/Vietnam/TY25/2005
A/chicken/Vietnam/TY31/2005
2.3.2
A/duck/Guiyang/3242/2005
A/chicken/Guiyang/3056/2005
2.3.3
A/Japanese white-eye/Hong Kong/1038/2006
A/China/GD01/2006
A/common magpie/Hong Kong/645/2006
A/duck/Laos/3295/2006
A/Anhui/1/2005
A/Jiangsu/2/2007
A/Jiangsu/1/2007
A/chicken/Thailand/NP-172/2006
A/chicken/Nongkhai/NAH400002/2007
A/Laos/Nong Khai 1/2007
A/Guangxi/1/2005
A/Shenzhen/406H/2006
A/robin/Hong Kong/75/2006
2.3.4
A/HK/213/03
A/Thailand/676/2005
A/Viet Nam/JPHN30321/2005
A/Viet Nam/JP14/2005
A/Muscovy Duck/Vietnam/Ben Tre 342/2006
A/Duck/Vietnam/Ca Mau498/2006
A/Thailand/Chaiyaphum/672/2004
A/Thailand/16/2004
A/Viet Nam/HN30408/2005
A/Viet Nam/1194/2004
A/Viet Nam/1203/2004
A/chicken/Thailand/CRC-V140/2007
A/Thailand/1(KAN-1)/2004
1
0.01
B
A/Hong Kong/156/97
A/Goose/Guangdong/1/96
A/China/GD01/2006
A/Chicken/Thailand/NP-172/06
A/Laos/Nong Khai 1/2007
A/Shenzhen/406H/2006
A/magpie robin/Hong Kong/75/2006
A/human/Zhejiang/16/2006
A/common magpie/Hong Kong/645/2006
A/Jiangsu/1/2007
A/Jiangsu/2/2007
A/chicken/Shanxi/2/2006
A/migratory duck/Jiangxi/2136/2005
A/migratory duck/Jiangxi/2400/2005
A/migratory duck/Jiangxi/2295/2005
A/chicken/Kyoto/3/2004
A/crow/Kyoto/53/2004
A/crow/Osaka/102/2004
A/Ck/ST/4231/2003
A/chicken/Oita/8/2004
A/chicken/Korea/ES/03
A/chicken/Yamaguchi/7/2004
Genotype V
A/Viet Nam/1203/2004
A/Viet Nam/1194/2004
A/Thailand/676/2005
A/Thailand/SP83/2004
A/Thailand/1(KAN-1)/2004
A/Thailand/3(KK-494)/2004
A/Thailand/16/2004
A/Thailand/2(SP-33)/2004
A/HK/213/03
A/goose/Yunnan/4494/2005
A/Bar-headed Goose/Qinghai/12/05
A/turkey/Turkey/1/2005
A/Turkey/15/2006
Genotype Z
0.01

Erythrocyte Binding Preference of Avian Influenza H5N1 Viruses

Suda Louisirirotchanakul, Hatairat Lerdsamran, Witthawat Wiriyarat, Kantima Sangsiriwut, Kridsda Chaichoune, Phisanu Pooruk, Taweesak Songserm, Rungrueng Kitphati, Pathom Sawanpanyalert, Chulaluk Komoltri, Prasert Auewarakul and Pilaipan Puthavathana

J. Clin. Microbiol. **2007, 45(7):2284. DOI: 10.1128/JCM.00921-07.**
Published Ahead of Print 23 May 2007.

JOURNAL OF CLINICAL MICROBIOLOGY, July 2007, p. 2284–2286 Vol. 45, No. 7
0095-1137/07/$08.00+0 doi:10.1128/JCM.00921-07

Erythrocyte Binding Preference of Avian Influenza H5N1 Viruses▽†

Suda Louisirirotchanakul,[1] Hatairat Lerdsamran,[1] Witthawat Wiriyarat,[2] Kantima Sangsiriwut,[1] Kridsda Chaichoune,[2] Phisanu Pooruk,[1] Taweesak Songserm,[3] Rungrueng Kitphati,[4] Pathom Sawanpanyalert,[4] Chulaluk Komoltri,[5] Prasert Auewarakul,[1] and Pilaipan Puthavathana[1]*

Department of Microbiology[1] and Division of Research Development,[5] Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand; Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand[2]; Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom, Thailand[3]; and Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand[4]

Received 3 May 2007/Accepted 9 May 2007

Five erythrocyte species (horse, goose, chicken, guinea pig, and human) were used to agglutinate avian influenza H5N1 viruses by hemagglutination assay and to detect specific antibody by hemagglutination inhibition test. We found that goose erythrocytes confer a greater advantage over other erythrocyte species in both assays.

Endemicity of H5N1 avian influenza in Southeast Asian countries since late 2003 has led to the application of various techniques to diagnose the disease. For the isolation technique, influenza virus progenies released from the infected cells are primarily recognized by hemagglutination (HA) test. In addition, HA is employed to quantify the amount of hemagglutinin antigen used in HA inhibition (HI) assay (7). Importantly, the World Health Organization (WHO) has specified the presence of a horse erythrocyte HI titer of ≥160 in adjunct with a microneutralization (microNT) antibody titer of ≥80 in a single serum collected at day 14 or later as one among other criteria for a confirmed case of H5N1 infection (8).

Agglutination of erythrocytes by influenza viruses is mediated by the interaction between the receptor binding site (RBS) in hemagglutinin molecule and the sialyl receptor. Human influenza H1N1 and H3N2 viruses preferentially bind to a sialic acid receptor, the oligosaccharide side chain of which is linked with α2,6-galactose linkage (SAα2,6Gal), while avian and equine influenza viruses prefer an α2,3-galactose linkage (SAα2,3Gal). Horse and cow erythrocytes contain mainly an SAα2,3Gal linkage but no SAα2,6Gal (1). Chicken and goose erythrocytes contain more SAα2,3Gal linkage than SAα2,6Gal, while this is reversed with human O cells and pig, guinea pig, and turkey erythrocytes (1, 2).

Herein, five erythrocyte species (horse, goose, chicken, guinea pig and human O cells) were tested by HA assay against 14 H5N1 clade 1 isolates from Thailand, including five from humans, seven from wild and domestic birds, one from a tiger and, one from a clouded leopard, together with one human H1N1 isolate and five H3N2 isolates. Final concentrations of 0.5% goose, 0.5% chicken, 0.75% guinea pig, 0.75% human group O, and 1% horse erythrocytes were used. Except for those from the horse, erythrocytes were suspended in phosphate-buffered saline, pH 7.2. Horse erythrocytes were suspended in phosphate-buffered saline plus 0.5% bovine serum albumin. These protocols were followed as described previously (6, 7). A reaction well, consisting of 50 μl of diluted virus and 50 μl of erythrocyte suspensions, was incubated for 1 h at 4°C before the agglutination pattern was read. One HA unit is defined as the highest virus dilution that yields complete HA.

The experiments demonstrated that 13 of 14 H5N1 isolates could agglutinate erythrocytes from all five species with a statistical difference in the extent of titer (Friedman test, $P < 0.05$). Interestingly, an isolate from the clouded leopard could not agglutinate horse erythrocytes (Table 1). The result was consistent, as repeatedly tested with erythrocytes from three donors within one species. Goose erythrocytes yielded the highest HA titer, followed in order of sensitivity by chicken, guinea pig, human, and horse erythrocytes (Wilcoxon's signed-rank test, $P < 0.005$).

Hemagglutinin amino acid sequences of our H5N1 isolates were compared with those of A/Goose/Guangdong/1/96 (the ancestor) and with Hong Kong virus 1997 (5) (see Fig. S1 in the supplemental material). No change in RBS was found, except for one isolate, A/Thailand/676(NYK)/05, which contained a mutational change A134V in RBS. However, this mutational change did not relate to erythrocyte binding preference. It remains to be elucidated why the isolate from the clouded leopard could not agglutinate horse erythrocytes while there were no change in RBS and no difference in the deduced amino sequence of hemagglutinin. Receptor specificity of influenza viruses is influenced by both the galactose linkage and species of sialic acid: *N*-acetylneuraminic acid (NeuAc) or *N*-glycolylneuramic acid (NeuGc). Horse erythrocytes contained only NeuGcα2,3Gal (1, 3). Therefore, loss of the ability to agglutinate horse erythrocytes may be related to loss of the ability to recognize either NeuGc or galactose linkage (1, 3).

* Corresponding author. Mailing address: Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. Phone: 662-419-7059 or 662-418-2663. Fax: 662-418-4148 or 662-418-2663. E-mail: siput@mahidol.ac.th.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 23 May 2007.

TABLE 1. HA titers of influenza A viruses as assayed with erythrocytes from different species

Influenza A virus and HA GMT	Passage history	Accession no.	HA titer by erythrocyte source[a]				
			Goose	Chicken	Guinea pig	Human	Horse
H1N1 human influenza virus							
A/New Caledonia/20/99-like virus (Siriraj 07/00)	MDCK7	EF568930	128	64	128	64	<2
H3N2 human influenza viruses							
A/Sydney/05/97-like virus (Siriraj 08/98)	MDCK4	EF568929	64	64	128	64	<2
A/Fujian/411/02-like virus (Siriraj 03/04)	MDCK6	EF568924	32	<2	64	64	<2
A/Fujian/411/02-like virus (Siriraj 01/03)	MDCK3	EF568925	32	<2	32	32	<2
A/Fujian/411/02-like virus (Siriraj 02/03)	MDCK2	EF568926	4	<2	32	32	<2
A/California/07/04-like virus (Siriraj 12/04)	MDCK4	EF568927	4	<2	16	32	<2
H5N1 influenza viruses							
A/Thailand/1(KAN-1)/04	LLC-MK2, MDCK8	AY555150	512	512	128	128	128
A/Thailand/2(SP-33)/04	MDCK6	AY555153	64	64	64	32	16
A/Thailand/3(SP-83)/04	MDCK5	AY577314	64	64	16	32	16
A/Thailand/5(KK-494)/04	MDCK4	AY627885	128	64	32	64	32
A/Thailand/676(NYK)/05	MDCK9	DQ360835	256	256	512	256	32
A/Great Barbet/Thailand/VSMU-2-CBI/2005	MDCK2	EF206697	128	128	64	64	32
A/Green Peafowl/Thailand/VSMU-3-CBI/2005	MDCK2	EF206700	256	128	64	64	64
A/Gray-Crowed Crane/Thailand/VSMU-4-CBI/2005	MDCK2	EF206696	512	256	128	128	128
A/Tree Sparrow/Thailand/VSMU-16-RBR/2005	MDCK4	EF178506	64	64	32	32	16
A/Golden Pheasant/Thailand/VSMU-21-SPB/2005	MDCK1	EF178517	128	64	32	16	32
A/Pigeon/Thailand/VSMU-25-BKK/2005	MDCK3	EF206698	64	64	32	16	16
A/Chicken/Thailand (Suphanburi)/137/05	MDCK4	EF568922	512	512	64	64	128
A/Tiger/Thailand/VSMU-11-SPB/2004	Egg 1	EF178531	512	512	256	256	256
A/Clouded Leopard/Thailand (Chonburi)/AI-1216A/2004	Egg 1	EF568923	512	512	256	256	<2
HA GMT of H5N1 viruses			190.21	156.03	74.25	67.25	33.62

[a] The most frequent titer obtained from three erythrocyte donors within the same species is presented.

The study of human influenza H1N1 and H3N2 viruses showed that all six isolates could not agglutinate horse erythrocytes (Table 1). An A/Sydney/05/97(H3N2)-like isolate could agglutinate chicken erythrocytes, while all three A/Fujian/411/02(H3N2)-like isolates and one A/California/07/04(H3N2)-like isolate could not. This result supported previous findings that current H3N2 isolates have lost their agglutinating activity with chicken erythrocytes (2, 4). However, all of the data, including ours, were discrete and could not conclude that there was a correlation between certain mutational positions and loss of the agglutinating activity (data not shown).

Fourteen serum samples from seven H5N1 patients, including the survivors, were assayed for H5N1 antibody. Two H5N1 strains, A/Thailand/1(KAN-1)/04 which was the first human isolate from the country (5), and A/Thailand/676(NYK)/05, as described above, were selected as the test antigens. This study was approved by the Institution Ethical Committee for Human Research.

In the HI test, serum was pretreated with a receptor-destroying enzyme (Denka Seiken, Japan) at final dilution of 1:4 for 16 h at 37°C followed by heat inactivation for 30 min at 56°C and absorbed with a 50% erythrocyte suspension for 60 min at 4°C. A mixture of 25 μl of the diluted serum and 25 μl of the test virus at a concentration of 4 HA units was incubated for 30 min at room temperature before addition of 50 μl of erythrocyte suspension. The end result was read after incubation for 1 h at 4°C. HI antibody titer is defined as the final serum dilution that completely inhibits HA. Again, three donors from each of the five erythrocyte species were tested in separate runs with consistent results.

The results demonstrated that horse erythrocytes, which were the least sensitive in HA, gave the highest geometric mean titer (GMT) of antibody when A/Thailand/1(KAN-1)/04 was used as the test antigen (Wilcoxon's signed-rank test, $P < 0.005$). Goose erythrocytes were ranked second, followed by human, guinea pig, and chicken erythrocytes. In contrast, when A/Thailand/676(NYK)/05 was used as the test antigen, goose erythrocytes yielded the highest GMT, followed in order by chicken, horse, human, and guinea pig erythrocytes. However, a statistically significant difference was not found (Wilcoxon's signed-rank test, $P > 0.005$) (Table 2). Collectively, the level of HI antibody titer was dependent on both the erythrocyte species and the test antigen used. We also showed that HI is more sensitive for strain differentiation than microNT.

Our study proposes that goose erythrocytes confer a greater advantage for recognition of H5N1 viruses and HI antibody

TABLE 2. H5N1 HI antibody titers as tested by different erythrocyte species

Patient, sequential sample no., and HI antibody GMT	Time after onset of illness	A/Thailand/1(KAN-1)/04(H5N1)[a]						A/Thailand/676(NYK)/05(H5N1)[b]					
		HI antibody titer by erythrocyte source					NT antibody titer	HI antibody titer by erythrocyte source					NT antibody titer
		Goose	Chicken	Guinea pig	Human	Horse		Goose	Chicken	Guinea pig	Human	Horse	
Patient 1													
1	8 days	20	<20	20	<20	20	<5	20	<20	<20	<20	20	
2	17 days	640	640	640	640	1,280	1,280	1,280	1,280	1,280	1,280	640	
Patient 2	2 yr, 2 mo	20	<20	20	20	40	80	160	80	80	80	80	40
Patient 3	2 yr, 1 mo	20	<20	20	20	40	160	160	80	80	80	80	80
Patient 4													
1	10 days	20	<20	<20	20	20	<5						
2	12 days	20	<20	20	<20	20	5	20		20		20	
3	6 mo	40	20	20	40	80	160	160	320	160	80	320	
4	1 year	40	20	20	40	80	160	160	160	80	160	160	80
Patient 5	7 days	<20	<20	<20	<20	<20	<5	20	<20	<20	<20	20	
Patient 6													
1	4 days	<20	<20	<20	<20	<20	5	<20	<20	<20	<20	<20	
2	15 days	320	160	160	160	640	1,280	1,280	1,280	1,280	1,280	1,280	
3	21 days	160	80	80	160	320	640	640	1280	640	640	640	
4	5 mo, 9 days	20	20	20	20	40	80	160	80	80	40	40	80
Patient 7	5 days	80	40	80	160	320	320	640	640	320	320	640	
GMT of HI antibody		40.00	24.38	31.23	36.26	65.63		136.35	134.54	93.88	100.79	110.16	

[a] A/Thailand/1(KAN-1)/04 was isolated from patient 1.
[b] A/Thailand/676(NYK)/05 was isolated from patient 5.

assay. Whether this finding is also generalized for H5N1 clade 2 viruses need to be investigated.

We would like to thank the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, and the Thailand Research Fund for Advanced Research Scholar for support.

We also thank Stephen Durako, Westat, for manuscript review and Jarunee Prasertsophon, Kannika Nateerom, and Rasameepen Pongakern for laboratory support.

REFERENCES

1. **Ito, T., Y. Suzuki, L. Mitnaul, A. Vines, H. Kida, and Y. Kawaoka.** 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology **227:**493–499.
2. **Medeiros, R., N. Escriou, N. Naffakh, J.-C. Manuguerra, and S. van der Werf.** 2001. Hemagglutinin residues of recent human A (H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology **289:**74–85.
3. **Neumann, G., and Y. Kawaoka.** 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerg. Infect. Dis. **12:**881–886.
4. **Nobusawa, E., H. Ishihara, T. Morishita, K. Sato, and K. Nakajima.** 2000. Change in receptor-binding specificity of recent human influenza A viruses (H3N2): a single amino acid change in hemagglutinin altered its recognition of sialyloligosaccharides. Virology **278:**587–596.
5. **Puthavathana, P., P. Auewarakul, P. C. Charoenying, K. Sangsiriwut, P. Pooruk, K. Boonnak, R. Khanyok, P. Thawachsupa, R. Kijphati, and P. Sawanpanyalert.** 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J. Gen. Virol. **86:**423–433.
6. **Stephenson, I., J. M. Wood, K. G. Nicholson, and M. C. Zambon.** 2003. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J. Med. Virol. **70:**391–398.
7. **World Health Organization.** 2002. WHO manual on animal influenza diagnosis and surveillance. WHO/CDS/CSR/NCS/2002.5. http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf. Accessed 24 November 2006.
8. **World Health Organization.** 2006. WHO case definitions for human infections with influenza A (H5N1) virus, 29 August 2006. http://www.who.int/csr/disease/avian_influenza/guidelines/case_definition2006_08_29/en/print.html. Accessed 24 November 2006.

Journal of Clinical Microbiology

Surveillance for Reassortant Virus by Multiplex Reverse Transcription-PCR Specific for Eight Genomic Segments of Avian Influenza A H5N1 Viruses

Prasert Auewarakul, Kantima Sangsiriwut, Kridsda Chaichoune, Arunee Thitithanyanont, Witthawat Wiriyarat, Taweesak Songserm, Rasameepen Ponak-nguen, Jarunee Prasertsopon, Phisanu Pooruk, Pathom Sawanpanyalert, Parntep Ratanakorn and Pilaipan Puthavathana

J. Clin. Microbiol. **2007, 45(5):1637. DOI: 10.1128/JCM.00382-07.**
Published Ahead of Print 21 March 2007.

JOURNAL OF CLINICAL MICROBIOLOGY, May 2007, p. 1637–1639 Vol. 45, No. 5
0095-1137/07/$08.00+0 doi:10.1128/JCM.00382-07

Surveillance for Reassortant Virus by Multiplex Reverse Transcription-PCR Specific for Eight Genomic Segments of Avian Influenza A H5N1 Viruses†▽

Prasert Auewarakul,[1] Kantima Sangsiriwut,[1] Kridsda Chaichoune,[2] Arunee Thitithanyanont,[3] Witthawat Wiriyarat,[2] Taweesak Songserm,[4] Rasameepen Ponak-nguen,[2] Jarunee Prasertsopon,[1] Phisanu Pooruk,[1] Pathom Sawanpanyalert,[5] Parntep Ratanakorn,[2] and Pilaipan Puthavathana[1]*

Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Bangkok,[1] Faculty of Veterinary Science, Nakhon Pathom,[2] Faculty of Science, Bangkok,[3] Mahidol University, Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom,[4] and Department of Medical Sciences, Ministry of Public Health, Nonthaburi,[5] Thailand

Received 19 February 2007/Returned for modification 23 February 2007/Accepted 14 March 2007

Avian influenza H5N1 virus is a global threat. An emergence of a reassortant virus with a pandemic potential is a major concern. Here we describe a multiplex reverse transcription-PCR assay that is specific for the eight genomic segments of the currently circulating H5N1 viruses to facilitate surveillance for a virus resulting from reassortment between human influenza virus and the H5N1 virus.

The genome of influenza A viruses is composed of eight gene segments, which encode polymerase (PB1, PB2, and PA), nucleocapsid (NP), hemagglutinin (HA), neuraminidase (NA), matrix (M) protein, and nonstructural (NS) protein. Since influenza viruses have segmented genomes, they are able to evolve through reassortment (1). Both the 1957 and 1968 pandemic strains are believed to be reassortants in which HA and PB1 genes, with or without the NA gene, were replaced by the corresponding genomic segments of avian influenza virus strains (5). The reassortments provided the viruses with new HA and NA antigenic profiles and the ability to spread efficiently from human to human. The ongoing outbreak of the highly virulent avian influenza virus H5N1 is concerning because of the possibility of a reassortant virus emerging with pandemic potential (3). Monitoring for a reassortant with mixed genomic segments from human and avian viruses is therefore very important. However, detection of a reassortant requires sequencing of all eight genomic segments, a process which is laborious and time consuming. Therefore, we developed a simple and high-throughput screening method using multiplex reverse transcription-PCR (RT-PCR) specific for eight genomic segments of recent H5N1 viruses to detect the probable reassortant viruses worthy of further characterization.

For the primer design, 100 full-length sequences arbitrarily selected from the Influenza Virus Resource Database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/genomes/FLU/) were used to search for the sequence(s) that was conserved in H5N1 and that differed from those of other subtypes. The viruses used in this study comprised 28 human influenza virus isolates and 103 H5N1 isolates from various animal species (94 isolates from poultry and other birds, 6 isolates from tigers, 2 isolates from leopards, and 1 isolate from a cat). These viruses have been collected since the beginning of the epidemic in the country. In 2003, 2004, 2005, and 2006, 7, 34, 60, and 2 animal H5N1 virus isolates were collected, respectively. The human viruses comprised 6 H5N1, 3 H1N1, 8 H3N2, and 11 influenza B viruses. These viruses were propagated in MDCK cells or embryonated eggs. The H5N1 viruses were originally subtyped by RT-PCR. The subtyping was confirmed by sequencing for all human H5 and some animal H5 viruses (2). Available accession numbers of some of the H5N1 virus isolates used in this study are shown in Table S1 in the supplemental material and also in a report by Puthavathana et al. (4). The human H1, H3, and influenza B viruses were characterized and confirmed with respect to their subtypes by hemagglutination inhibition assay at the WHO Reference Laboratories, Melbourne, Australia, or the Centers for Disease Control and Prevention of the United States via the National Reference Laboratory on Influenza, NIH, Thailand.

RNA was extracted by using a QIAamp viral RNA Mini kit (QIAGEN, Valencia, CA). A 140-μl volume of virus suspension from the infected culture or embryonated egg yielded a volume of 60 μl of extracted RNA. Multiplex RT-PCR was carried out in three reaction tubes with combinations of the following primer sets: NA plus PB1 plus NP, HA plus PA plus PB2, or NS plus M. Sequences of these primers are shown in Table 1. The thermocycling was performed in a GeneAmp PCR system 2400 thermal cycler (Perkin Elmer) using a QIAGEN OneStep RT-PCR kit (QIAGEN, Valencia, CA) with standard reaction conditions (a 50 μl volume containing 1× PCR buffer, 5 to 10 μl of extracted RNA, 400 μM (each) deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 2 μl enzyme mix, and 2 U RNase inhibitor). The thermocycling profile was 50°C for 30 min, 95°C for 15 min, 35 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C

* Corresponding author. Mailing address: Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkok Noi, Bangkok 10700, Thailand. Phone: 662 419 7059. Fax: 662 418 4148. E-mail: siput@mahidol.ac.th.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 21 March 2007.

TABLE 1. Sequences, concentrations used in the reaction, nucleotide positions, and product sizes of PCR primers for the eight gene segments

Gene	Primer name	Sequence	Concn in PCR (μM)	Nucleotide positions	Product size (bp)
PB2	SPB2F	GAA TGA ACA AGG GCA GAC G	0.3	237–255	117
	SPB2R	ACT GCA CTT GTC GCC GGC	0.3	336–353	
PB1	SPB1F	AAT GAG AAT CAG AAT CCT AGG	0.4	952–972	377
	SPB1R	GTG GTT TTG GTG TAC CTT TTC	0.4	1308–1328	
PA	SPAF	AAT TGA AAG CAT GAT TGA AGC C	0.3	1797–1818	167
	SPAR	TGA ACA CAG ACT TCG CCA GC	0.3	1944–1963	
HA	HA-H5f	ACT CCA ATG GGG GCG ATA AA	0.6	897–916	352
	HA-H5r	CAA CGG CCT CAA ACT GAG TGT	0.6	1228–1248	
NP	SNPF	GAC AGC TAT CCA CCA GAG GG	0.3	1112–1131	174
	SNPR	CCG AGA AAG TGG GCT GAA CG	0.3	1266–1285	
NA	NA-N1f	CTC ATG CTC CCA CTT GGA	0.6	332–349	515
	TNAr5	CCC TGC ACA CAC ATG TG	0.6	830–846	
M	SMF	TCG CAC TCA GCT ACT CAA CC	0.3	369–388	142
	SMR	TGA GAC CGA TGC TGT GAA TCT	0.3	490–510	
NS	SNSF	GAT AAG GCA CTT AAA ATG CCG	0.4	250–270	253
	SNSR	ACG GTG AGA TTT CTC CCA CG	0.4	483–502	

for 45 s, and a final extension of 72°C for 10 min. The PCR products were visualized by ethidium bromide staining after electrophoresis in a 2% agarose gel.

In our multiplex RT-PCR assay, potential reassortants would show the lack of one or more of the eight amplicons in the case of H5N1 viruses and the presence of one or more amplicons in the case of H1N1 and H3N2 viruses. With the designed primer set, we could initially amplify all eight genomic segments from 105 out of 109 H5N1 viruses. Four viruses initially gave negative amplification results for NA. However, these four viruses yielded positive NA band results in separated single-gene amplifications, and multiplex reamplification using 1.5- to 2-fold-higher RNA input gave positive amplification results for all genes. Representative results obtained with the multiplex RT-PCR products are shown in Fig. 1. None of the human H3N2, H1N1, or influenza B virus isolates yielded any amplification products by this method (data not shown). In order to verify the lack of reassortment, all human H5N1 viruses and a subset of animal viruses (62 out of 103) were subjected to a full-genome sequencing protocol as previously described (4). Phylogenetic analyses of the sequence data revealed that all viruses contained their respective genomic segments and that no evidence of reassortment was found (data not shown). In addition to detecting human-avian virus reassortants, our assay may also be able to detect some reassortants that acquire genomic segments from some other unrelated avian viruses. However, the assay was not designed to detect reassortment among avian viruses, which are unlikely to play a role in viral adaptation to the human host. The absence of a band in the amplification of H5N1 viruses is not always due to reassortment. Full-genome sequencing is needed for result confirmation. Because the performance of this assay is directly affected by variation in the sequences of circulating H5N1 strains, the primer sequences should be frequently reevaluated and updated accordingly.

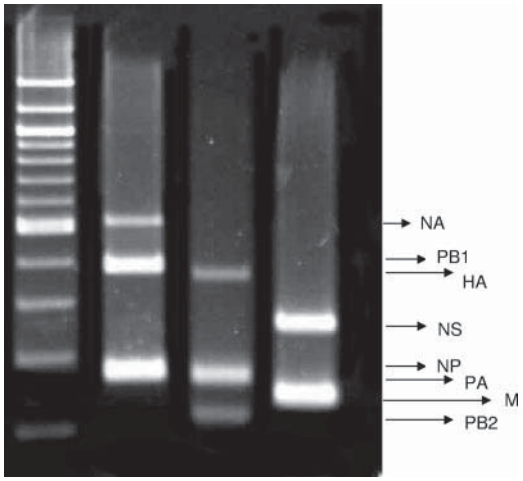


FIG. 1. A representative photograph of multiplex RT-PCR products from an H5N1 virus after electrophoresis in 2% agarose and ethidium bromide staining. Each lane shows products from each tube. The first lane is a 100-bp DNA ladder, and the target gene of each amplification product is labeled on the right.

Our multiplex RT-PCR was designed for egg- or MDCK-propagated viruses. Although the assay may be able to detect some of the genes in some clinical specimens, the level of sensitivity is probably not sufficient to reliably detect all the genomic segments simultaneously. This makes our assay less suitable for direct screening using clinical specimens. Nevertheless, the possibility of using a more sensitive version of this assay with clinical specimens should be further explored.

This study was supported by research grants from the National Center for Genetic Engineering and Biotechnology and the Thailand Research Fund for Advanced Research Scholar.

We thank Darat Lauhakirti and Sirawat Srichatrapimuk for their collaboration in the preparation of the manuscript.

REFERENCES

1. **Guan, Y., J. S. Peiris, A. S. Lipatov, T. M. Ellis, K. C. Dyrting, S. Krauss, L. J. Zhang, R. G. Webster, and K. F. Shortridge.** 2002. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proc. Natl. Acad. Sci. USA **99:**8950–8955.
2. **Hoffmann, E., J. Stech, Y. Guan, and R. G. Webster.** 2001. Universal primer set for the full-length amplification of all influenza viruses. Arch. Virol. **146:** 2275–2289.
3. **Li, K. S., Y. Guan, J. Wang, G. J. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. Estoepangestie, A. Chaisingh, P. Auewarakul, H. T. Long, N. T. Hanh, R. J. Webby, L. L. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster, and J. S. Peiris.** 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature **430:**209–213.
4. **Puthavathana, P., P. Auewarakul, P. C. Charoenying, K. Sangsiriwut, P. Pooruk, K. Boonnak, R. Khanyok, P. Thawachsupa, R. Kijphati, and P. Sawanpanyalert.** 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J. Gen. Virol. **86:**423–433.
5. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. **56:**152–179.

IRV 12016 B
Journal Name Manuscript No.
Dispatch: 6.9.12 Journal: IRV CE: Liyagat Ali
Author Received: No. of pages: 5 PE: Vigneswari

DOI:10.1111/irv.12016
www.influenzajournal.com

Short Article

Seroprevalence of 2009 H1N1 Virus Infection and Self-Reported Infection Control Practices Among Healthcare Professionals Following the First Outbreak in Bangkok, Thailand

Kulkanya Chokephaibulkit,[a] Susan Asanasaen,[b] Anucha Apisarnthanarak,[c] Yong Rongrungruang,[b] Kanchana Kachintorn,[d] Yuwadee Tuntiwattanapibul,[d] Tepnimitr Judaeng,[d] Pilaipan Puthavathana[e]

[a]Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. [b]Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. [c]Division of Infectious Diseases, Faculty of Medicine, Thammasat University Hospital, Bangkok, Thailand. [d]Division of Center for Nosocomial Infection Control, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. [e]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
Correspondence: Kulkanya Chokephaibulkit, Division of Infectious Diseases, Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.
E-mail: kulkanya.cho@mahidol.ac.th

Accepted 10 August 2012. Published online Xx Xxxx 20XX.

A serologic study with simultaneous self-administered questionnaire regarding infection control (IC) practices and other risks of influenza A (H1N1) pdm09 (2009 H1N1) infection was performed approximately 1 month after the first outbreak among frontline healthcare professionals (HCPs). Of 256 HCPs, 33 (13%) were infected. Self-reported adherence to IC practices in >90% of exposure events was 82·1%, 73·8%, and 53·5% for use of hand hygiene, masks, and gloves, respectively. Visiting crowded public places during the outbreak was associated with acquiring infection (OR 3·1, $P = 0·019$). Amongst nurses, exposure to HCPs with influenza-like illness during the outbreak without wearing a mask was the only identified risk factor for infection (OR = 2·3, $P = 0·039$).

Keywords 2009 H1N1 pandemic, Thailand, healthcare workers, seroprevalence, hemagglutination assay

Please cite this paper as: Chokephaibulkit *et al.* (2009) Seroprevalence of 2009 H1N1 Virus Infection and Self-Reported Infection Control Practices Among Healthcare Professionals Following the First Outbreak in Bangkok, Thailand. Influenza and Other Respiratory Viruses DOI: 10.1111/irv.12016.

Introduction

The outbreak of influenza A (H1N1) pdm09 (2009 H1N1) pandemic in Thailand started in early June, peaked around July to August, and waned in September.[1] Healthcare professionals (HCPs) are at risk of exposure to infection from the community as well as in the workplace. However, they also have the greatest access to personal protective equipment (PPE) and are likely to receive the annual seasonal vaccine that may provide some protection against 2009 H1N1 infection.[2] The 2009 H1N1outbreak was an opportunity to evaluate the effectiveness of infection control (IC) practices in HCPs.

We conducted a serologic study of 2009 H1N1infection among frontline HCPs who were involved in the care of 2009 H1N1 patients during the first outbreak in two large public hospitals in Bangkok, Thailand. The aim of the study was to understand the magnitude of acquisition of infections among HCPs in relation to self-reported IC practices and the factors associated with infection during the outbreak. This information may be useful for future outbreak control and vaccination strategies.

Methods

HCPs who worked during the peak of the 2009 H1N1 outbreak (June–August, 2009) on the wards that cared for patients with influenza and at emergency rooms of Siriraj Hospital and Thammasat Hospital, two large public tertiary care centers in Bangkok, were randomly invited to participate in the study. These two hospitals served adults and children patients. The wards at which the HCPs in this study were working were 3 ERs, four pediatric wards, three adult wards, and three intensive care units. Immunocompromised patients were being cared for in some of the wards participating the study. Approximately, a third of HCPs in each of these wards were invited and 97% accepted. The study was conducted from October 1 to 19,

2009, approximately 1 month after the end of the outbreak. An anonymous self-administered questionnaire was administered prior to a single blood draw for assessment of hemagglutination inhibition (HI) titer. The questionnaire consisted of demographic information, medical history, and factors that may be associated with community-acquired and occupational-acquired influenza. Adherence to hand hygiene (alcohol-based hand rub and/or hand washing with water) and using mask and gloves when in contact (defined as having activities or procedures that potentially resulted in contact or droplet transmission) with patients with suspected 2009 H1N1 were categorized as: every time or >90–100%, mostly or 70–90%, and <60%, respectively. The wards were classified as isolation wards (1–three patients in a room, PPE practice for airborne and contact precautions when entering the patients' room), semi-open ward (share up to 12 patients in a room, PPE practice for airborne and contact precaution when entering patients' area), and open ward or emergency room (large ward hold up to 24 beds or walk-in patients, PPE practice for contact and droplet precaution as needed). There was no verification of the accuracy of responses in the questionnaire.

HI assay was performed using the protocol previously described.[3,4] We defined an HI titer ≥40 as seropositive and a marker of acquiring recent infection assuming that none of the HCPs had been infected with the 2009 H1N1 virus prior to the outbreak and that pre-existing HI antibody to 2009 H1N1 was uncommon.

Statistical analysis

Descriptive analysis was performed on the demographic and other variables associated with risk of influenza infection. Univariate analysis was performed using a binomial test. Multiple logistic regression analysis was used for multivariate analysis of self-reported factors including the IC practices associated with an HI titer ≥40. Stata (Version 9.2) was used for the data analysis.

Results

There were 256 HCPs, 93% female, who participated in this study. The median (range) age was 34 (20–61) years. The majority (81·3%) were nurses and nurse assistants. Of the 198 HCPs working in specific wards, 72 (36·4%) were working in isolation wards, 57 (28·8%) in semi-open wards, and 69 (34·8%) in open wards or emergency rooms. The majority (82%) had received seasonal influenza vaccines (Southern Hemisphere strain 2008) between April 2008 and May 2009, at least 1–2 months before the outbreak started. Adherence to IC practices in >90% of the exposure events when in contact with patients with suspected 2009 H1N1 infection was 82·1%, 73·8%, and 53·5% with hand hygiene, using a mask, and using gloves, respectively. One hundred and twenty (47·1%) HCPs reported a history of respiratory tract infection (RTI) during the outbreak.

Thirty-three (13%) HCPs had a serum HI titer ≥40 suggesting acquisition of 2009 H1N1 infection during the outbreak. The proportions seropositive in HCPs aged <25, 25–49, and ≥50 years were 20·9%, 12·9%, and 2·8%, respectively. HCPs with a history of RTI during the outbreak tended to have a higher proportion seropositive than those who had no history of RTI (16·7% versus 8·9%, $P = 0·06$). The proportions seropositive among nurses and nurse assistants in different patient care areas were not different: 12·5% versus 19·3% versus 11·6% ($P = 0·411$) in isolation, semi-open, and open wards, respectively.

The univariate analysis revealed that a younger age (<25 years) and visiting crowded public places during the outbreak were associated with acquiring infection. In multivariate analysis, the only risk factor was visiting crowded public places during the outbreak (odds ratio = 3·1, 95% CI = 1·2-8·1, $P = 0·019$, Table 1). The rates of adherence to IC practices were not associated with acquisition of infection. The HCPs with adherence to hand hygiene and mask use at 70–90% of exposure events had a similar rate of infection to those with adherence >90–100%. In a subgroup analysis of 198 nurses and nurse assistants, there was a weak association between acquisition of 2009 H1N1 infection and close contact with HCPs with influenza-like illness (ILI) during the outbreak without wearing a mask (odds ratio = 2·3, 95% CI 0·9–5·6, $P = 0·039$).

Discussion

This study revealed a rate of 2009 H1N1 infection among frontline HCPs of 13%, indicated by a serum HI titer ≥40, after the first outbreak in Bangkok. This result was consistent with a previous report of 18% of HCPs in a large hospital in Bangkok who became sick with the 2009 H1N1 infection during the same outbreak,[5] and higher than the 7% reported among healthy blood donors in Bangkok around the same period of this study,[6] suggesting that HCPs had a higher risk of getting 2009 H1N1 infection than the general population.

A report of the 2009 H1N1 outbreak in England found that 42%, 20%, and 6% of the general population age 5–14, 15–24, and 25–44 years in London and West Midlands had an HI titer against 2009 H1N1 of ≥32.[7] This evidence of higher prevalence in a younger age group correlated well with a large study of the 2009 H1N1 outbreak in the US in which 40% and 35% of the patients were in the age groups 10–18 and 19–50 years, respectively, and only 5% occurred at age >50 years.[8] This age bias was probably due to the increased number of social or institutional gathering

Table 1. Factors associated with serologic evidence of recent infection defined by hemagglutination inhibition (HI) titer ≥40

Characteristics	No. (%)	No. (%) Cases with HI ≥ 40	No. (%)Cases with HI <40	Crude odds ratio (95% CI)	*P*-value	Adjusted odds ratio (95% CI)	*P*-value
Age (years)	(*n* = 256)	(*n* = 33)	(*n* = 223)				
<25	43 (16·8)	9 (27·3)	34 (15·2)	9·0 (1·1–405·8)	0·0198*	5·7 (0·7-49·0)	0·111
25–49	178 (69·5)	23 (69·7)	155 (69·5)	5·0 (0·8–213·8)	0·14*	4·3 (0·6-33·6)	0·163
50–64	35 (13·7)	1 (3)	34 (15·3)	1			
Body Mass Index	(*n* = 254)	(*n* = 33)	(*n* = 221)				
≥25	44 (17·3)	4 (12·1)	40 (18·1)	0·6 (0·2–1·9)	0·47*		
<25	210 (82·7)	29 (87·9)	181 (81·9)	1			
Career	(*n* = 256)	(*n* = 33)	(*n* = 223)				
Physician	30 (11·7)	3 (9·1)	27 (12·1)	1			
Nurse or nurse assistant	208 (81·3)	28 (84·8)	180 (80·7)	1·4 (0·4–7·7)	0·78*		
Others	18 (7)	2 (6·1)	16 (7·2)	1·1 (0·1–10·9)	1·00*		
Received the seasonal influenza vaccine between April 2008 and May 2009	(*n* = 250)	(*n* = 32)	(*n* = 218)				
Yes	205 (82)	27 (84·4)	178 (81·7)	1·2 (0·4–4·3)	0·71		
No	45 (18)	5 (15·6)	40 (18·3)	1			
Living with children younger than 5 years	(*n* = 255)	(*n* = 33)	(*n* = 222)				
Yes	52 (20·4)	8 (24·2)	44 (19·8)	1·3 (0·5–3·2)	0·56		
No	203 (79·6)	25 (75·8)	178 (80·2)	1			
Living with children age 5–15 years	(*n* = 253)	(*n* = 33)	(*n* = 220)				
Yes	59 (23·3)	4 (12·1)	55 (25·0)	0·4 (0·1–1·3)	0·12*		
No	194 (76·7)	29 (87·9)	165 (75·0)	1			
Having household member sick with respiratory tract infection during the outbreak	(*n* = 253)	(*n* = 33)	(*n* = 220)				
Yes	117 (46·2)	12 (36·4)	105 (47·7)	0·6 (0·3–1·4)	0·22		
No	136 (53·8)	21 (63·6)	115 (52·3)	1			
Visiting crowded public places during the outbreak	(*n* = 256)	(*n* = 33)	(*n* = 223)				
Yes	149 (58·2)	27 (81·8)	122 (54·7)	3·7 (1·4–11·4)	0·0032	3·1 (1·2–8·1)	0·019
No	107 (41·8)	6 (18·2)	101 (45·3)	1			
Mask type used when caring for patients with suspected/confirmed 2009 H1N1	(*n* = 239)	(*n* = 30)	(*n* = 209)				
N95 respirator	142 (59·4)	16 (53·3)	126 (60·3)	1			
Surgical Mask	78 (32·6)	10 (33·3)	68 (32·5)	1·2 (0·4–2·9)	0·73		
Use either mask	19 (8)	4 (13·4)	15 (7·2)	2·1 (0·4–7·7)	0·26*		
Frequency of hand hygiene before and after caring for patients with suspected/confirmed 2009 H1N1	(*n* = 246)	(*n* = 32)	(*n* = 214)				
All the time (>90–100%)	202 (82·1)	27 (84·4)	175 (81·8)	1·2 (0·4–4·1)	0·76		
Most of the time (70–90%)	43 (17·5)	5 (15·6)	38 (17·8)	1			
Sometimes (<60%)	1 (0·4)	0	1 (0·4)	–			
Frequency of mask use when caring for patients with suspected/confirmed 2009 H1N1	(*n* = 244)	(*n* = 32)	(*n* = 212)				
All the time (>90–100%)	180 (73·8)	24 (75·0)	156 (73·6)	0·9 (0·4–2·5)	0·86		
Most of the time (70–90%)	56 (23)	8 (25)	48 (22·6)	1			
Sometimes (<60%)	8	0	8 (3·8)	–			
Frequency of glove use when caring for patients with suspected/confirmed 2009 H1N1	(*n* = 245)	(*n* = 32)	(*n* = 213)				
All the time (>90–100%)	131 (53·5)	19 (59·4)	112 (52·6)	1·4 (0·4–4·9)	0·57		
Most of the time (70–90%)	69 (28·2)	8 (25)	61 (28·6)	1·0 (0·3–4·4)	0·94		
Sometimes (<60%)	45 (18·3)	5 (15·6)	40 (18·8)	1			
Having respiratory tract infection during the outbreak	(*n* = 255)	(*n* = 32)	(*n* = 223)				
Yes	120 (47·1)	20 (62·5)	100 (44·8)	2·1 (0·9–4·8)	0·06	1·8 (0·8-4·0)	0·132
No	135 (52·9)	12 (37·5)	123 (55·2)	1			

*Fisher's exact test.

activities and less pre-existing cross-protective antibody in the younger compared with the older participants.[9] In our study, we also found that having an HI titer ≥40 was associated with younger age and visiting crowded public places, suggesting that the outbreak spread more widely in a younger population, probably from gathering in public places.

Conducting a serologic study immediately after the first outbreak of 2009 H1N1 infection was a unique opportunity to look at the effectiveness of IC practices. Assuming from a previous study that approximately 11% of the HCPs had an HI antibody titer to 2009 H1N1 ≥ 40 before the outbreak,[6] we can expect that the serologic evidence reported is primarily due to the acquisition of infection during the outbreak. The HCPs who participated in this study were the frontline personnel at highest risk of exposure to 2009 H1N1-infected patients and were well trained in using PPE and IC practices. Ideally, these HCPs should not acquire 2009 H1N1 infection from patient care. However, our setup was not ideal. Aerosol-generating procedures were performed in many of the patient care areas and none of the wards had the perfect negative pressure needed to limit aerosol spread. Moreover, adherence to IC practices was not perfect in real life.

A previous report revealed that frontline HCPs were actually less likely to contract 2009 H1N1 than other HCPs, probably because of good adherence to IC practices and use of PPE.[10] However, we found that only about half of our HCPs reported perfect adherence (>90% of exposure events) to wearing of gloves, and around 70–80% perfectly adhered to mask use and hand hygiene. Despite this imperfect adherence, it seemed that the HCPs in our study acquired infection from the community rather than from patient care. A report from the US also found acquisition from the community as a major route of infection in HCPs.[11] We did not find the levels of adherence to hand hygiene and mask use (70–90% versus 90–100%) to be associated with infection. This is in line with a previous study that revealed a protective effect of >75% adherence to hand hygiene.[10]

The hospital environment is the setting where HCPs spend much of their time and may be the source of infection, in addition to patient care activities. Environmental contamination in household settings of patients with influenza was well documented.[12] A study in Singapore reported that contact with H1N1-infected colleagues was associated with 2009 H1N1infection in hospital staff.[13] We found that exposure to HCPs with ILI was associated with 2009 H1N1 infection in a subgroup analysis of nurses and nurse assistants, but not in the whole cohort analysis. Of note, almost half of the HCPs in this study had an RTI during the outbreak but only 16·7% of these RTI episodes appeared to be associated with HI seroconversion to 2009 H1N1 suggesting that other respiratory viruses co-circulated during the outbreak, and this may have masked the effect of ILI contact in the whole cohort. Hospital policies to monitor ill HCPs and prevent transmission of infection from ill HCPs are needed.

There are several limitations of the study. First of all, our sample size was not large. Inherent self-report survey-based research compared with observer-based research, in particular when reporting hand hygiene and PPE habits, is that over-reporting of the habit cannot be excluded. We do not have verification of the accuracy of the answers to the questionnaire and we did not monitor for adherence to IC practices in our study.

In conclusion, we found that of 2009 H1N1 infection among frontline HCPs was somewhat higher than in the general population. The risk of infection was found to be associated with community exposure risk, particularly in the young, as well as exposure to other HCPs with ILI without protection.

Acknowledgements

We thank Varaporn Pumsuwan, M.Sc., Natcha Viruchkul, M.N.S, Wanatpreeya Phongsamart, MD, Orasri Wittawatmongkol, MD, Alan Maleesatharn, MBA, Amy Downing, MD, and Hatairat Lerdsamran, M.Sc., and Tim R Cressey, Ph.D for their contribution to the study. We thank all the participants for their volunteerism to join the study and response to the questionnaire. This study was supported by the National Science and Technology Development Agency and the Thailand Research Fund for Senior Research Scholar.

References

1 WHO. Pandemic H1N1 2009- Thailand 31st March 2010. Available at http://www.searo.who.int/EN/Section10/Section2562_15043.htm.; Accessed 22 February 2011.
2 Garcia-Garcia L, Valdespino-Gomez JL, Lazcano-Ponce E *et al.* Partial protection of seasonal trivalent inactivated vaccine against novel pandemic influenza A/H1N1 2009: case-control study in Mexico City. BMJ 2009; 339:b3928.
3 Louisirirotchanakul S, Lerdsamran H, Wiriyarat W *et al.* Erythrocyte binding preference of avian influenza H5N1 viruses. J Clin Microbiol 2007; 45:2284–2286.
4 Kitphati R, Pooruk P, Lerdsamran H *et al.* Kinetics and longevity of antibody response to influenza A H5N1 virus infection in humans. Clin Vaccine Immunol 2009; 16:978–981.
5 Kiertiburanakul S, Apivanich S, Muntajit T *et al.* H1N1 2009 influenza among healthcare workers in a tertiary care hospital in Thailand. J Hosp Infect 2010; 74:300–302.
6 Lerdsamran H, Pittayawonganon C, Pooruk P *et al.* Serological Response to the 2009 Pandemic Influenza A (H1N1) Virus for Disease Diagnosis and Estimating the Infection Rate in Thai Population. PLoS ONE 2011; 6:e16164.
7 Miller E, Hoschler K, Hardelid P, Stanford E, Andrews N, Zambon M. Incidence of 2009 pandemic influenza A H1N1 infection in

England: a cross-sectional serological study. Lancet 2010; 375:1100–1108.
8 Dawood FS, Jain S, Finelli L *et al.* Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 2009; 360:2605–2615.
9 Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. MMWR Morb Mortal Wkly Rep 2009;58:521–524.
10 Apisarnthanarak A, Mundy LM. Factors associated with health care-associated 2009 influenza a (H1N1) virus infection among Thai health care workers. Clin Infect Dis 2010; 51:368–369.
11 Novel influenza A (H1N1) virus infections among health-care personnel - United States, April-May 2009. MMWR Morb Mortal Wkly Rep 2009;58:641–645.
12 Simmerman JM, Suntarattiwong P, Levy J *et al.* Influenza virus contamination of common household surfaces during the 2009 influenza A (H1N1) pandemic in Bangkok, Thailand: implications for contact transmission. Clin Infect Dis 2010; 51:1053–1061.
13 Chen MI, Lee VJ, Barr I *et al.* Risk factors for pandemic (H1N1) 2009 virus seroconversion among hospital staff, Singapore. Emerg Infect Dis 2010; 16:1554–1561.

Author Query Form

Journal: IRV

Article: 12016

Dear Author,
During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.

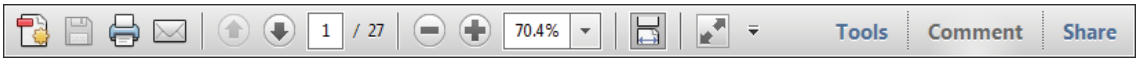
Many thanks for your assistance.

Query reference	Query	Remarks
Q1	**WILEY-BLACKWELL: Please supply date of Published Online.**	
Q2	**AUTHOR: Please check author and affiliations.**	
Q3	**AUTHOR: Please give address information for Stata: town, state (if applicable), and country.**	

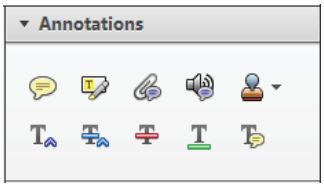
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

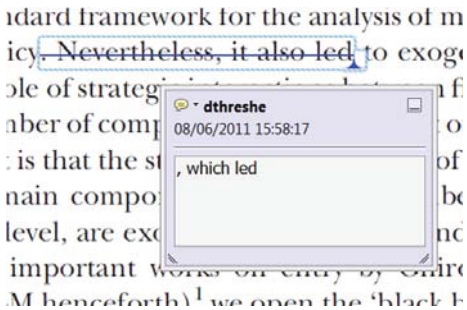


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

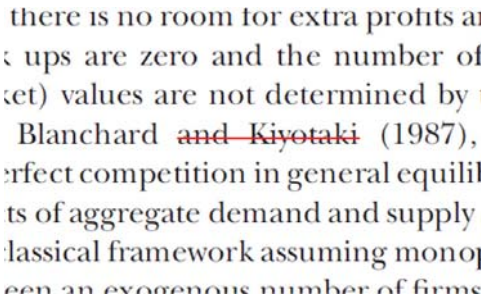


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

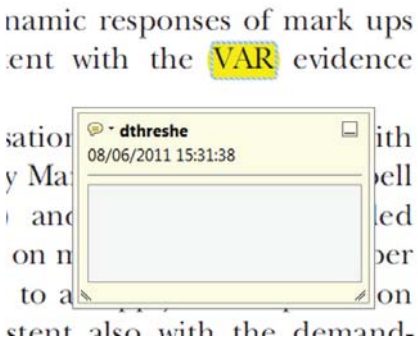


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

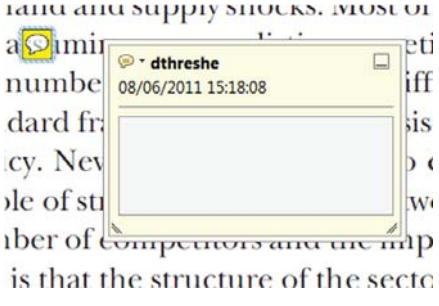


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



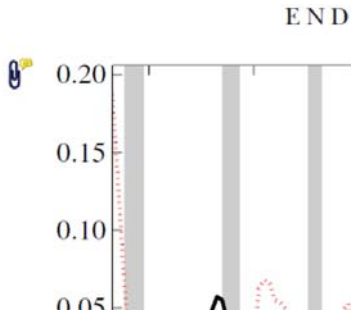
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

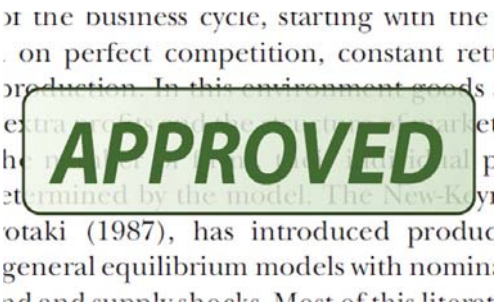


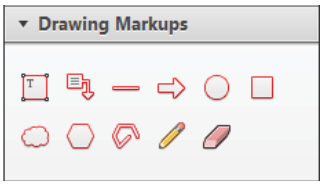
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

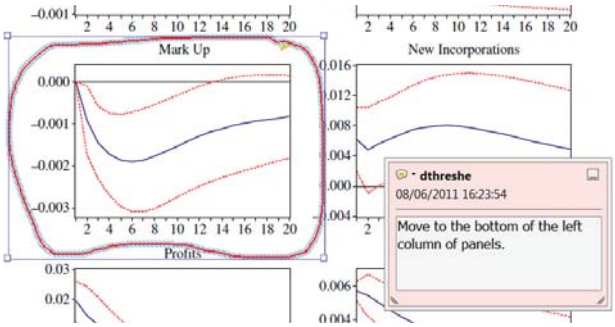
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).





7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

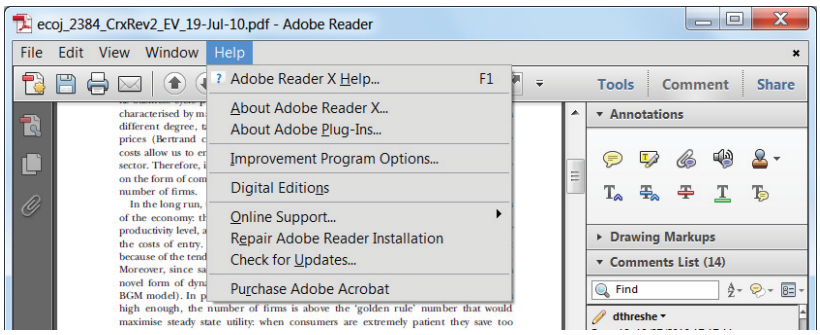
Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:



Viral Immunology: http://mc.manuscriptcentral.com/viral

Biological properties of H5 hemagglutinin expressed by vaccinia virus vector and its immunological reactivity with human sera

Journal:	*Viral Immunology*
Manuscript ID:	VIM-2012-0055.R1
Manuscript Type:	Articles
Date Submitted by the Author:	n/a
Complete List of Authors:	Noisumdaeng, Pirom; Mahidol University, Microbiology Pooruk, Phisanu; Mahidol University, Microbiology Kongchanagul, Alita; Mahidol University, Microbiology Assanasen, Susan; Mahidol University, Medicine Kitphati, Rungrueng; Ministry of Public Health, Disease Control Auewarakul, Prasert; Mahidol University, Microbiology Puthavathana, Pilaipan; Mahidol University, Microbiology
Keyword:	Influenza, Immunity, Antibodies, Animal model

Biological properties of H5 hemagglutinin expressed by vaccinia virus vector and its immunological reactivity with human sera

Pirom Noisumdaeng,[1,3] Phisanu Pooruk,[1,3] Alita Kongchanagul,[1,4] Susan Assanasen,[2] Rungrueng Kitphati,[5] Prasert Auewarakul,[1,3] and Pilaipan Puthavathana[1,3]

[1]Department of Microbiology and [2]Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok-noi, Bangkok 10700, Thailand

[3]Center for Emerging and Neglected Infectious Disease, Mahidol University, Thailand

[4]Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand

[5]Department of Disease Control, Ministry of Public Health, Nonthaburi 11000, Thailand

Corresponding author: Prof. Pilaipan Puthavathana

Department of Microbiology

Faculty of Medicine Siriraj Hospital, Mahidol University

Bangkok 10700, THAILAND

Tel: 662 419 7059

Fax: 662 418 2663

E-mail: siput@mahidol.ac.th

Abstract

A recombinant vaccinia virus harboring the full length hemagglutinin (*HA*) gene derived from a highly pathogenic avian influenza A/Thailand/1(KAN-1)/2004 (H5N1) virus (rVac-H5 HA virus) was constructed. The immunogenicity of the expressed HA protein was characterized using goat antiserum, mouse monoclonal antibody and human sera. The expressed HA protein localized both in the cytoplasm and on the cytoplasmic membrane of the thymidine kinase negative cells infected with the rVac-H5 HA virus, as determined by immunofluorescence assay. Western blot analysis demonstrated that the rVac-H5 HA protein was post-translationally processed by proteolytic cleavage of the HA0 precursor into HA1 and HA2 domains; and all of these HA forms were immunogenic in BALB/c mice. The molecular weight (MW) of each HA domain was the same as the wild-type H5 HA produced in Madin-Darby canine kidney cells infected with the H5N1 virus, but was higher than that expressed by a baculovirus-insect cell system. Sera from all H5N1 survivors reacted to HA0, HA1 and HA2 domains; whereas sera from H5N1-uninfected subjects reacted to the rVac-H5 HA2 domain only, but not to HA0 or HA1, indicating that some cross-subtypic immunity exists in the general population. There was a lot-to-lot variation of the recombinant HA produced in the baculovirus-insect cell system that might affect the detection rate of antibody directed against certain HA domains.

Introduction

Based on hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are classified into 17 HA (H1-17) and 9 NA (N1-9) subtypes, respectively. While influenza A virus subtypes H1-16 are found in aquatic birds (1), H17 was recently discovered in bats in Guatemala by using nucleotide sequence analysis (2). The major influenza A subtypes currently circulating in the human population include H3N2 viruses and H1N1 viruses derived from the 2009 pandemic H1N1 virus (H1N1pdm). The presence of H1N2 viruses are also sporadically reported in many countries such as United Kingdom, Germany, France, Egypt, Singapore, Canada and United States (3-5). Occasionally, avian influenza viruses have crossed species barriers to infect humans (6-7). Among the various subtypes reported, the highly pathogenic avian influenza (HPAI) H5N1 virus is the most virulent. This subtype has spread globally throughout the world by migrating birds and poultry shipments, especially in Asian countries; and its transmission to humans posts a pandemic threat (7).

HA is a type I integral membrane glycoprotein and is a major component of the viral envelope where it exists as noncovalent homotrimers. Initially, it is synthesized as a precursor polypeptide (HA0) that further undergoes post-translational modifications including glycosylation, proteolytic cleavage and signaling peptide removal (8-10). SDS-polyacrylamide gel electrophoresis of purified virus particles has demonstrated that the monomeric HA0 has a molecular weight (MW) of approximately 80 kilodaltons (kDa) and is cleaved into two glycosylated functional subunits, HA1 (MW 56-58 kDa) and HA2 (MW 25-26 kDa) which are connected together by disulfide linkages (11-13). The cleavage of the HA0 precursor into HA1 and HA2 is necessary for viral infectivity (9, 14). The HA1 is a hypervariable domain, which shares only 34-59% amino acid sequence identity between influenza virus subtypes (15). This domain is responsible for binding to host cellular receptors and the antibody against HA1 is relatively strain or subtype specific (13). On the

other hand, HA2 mediates fusion between the viral envelope and the endosomal membrane during the uncoating step of the viral replication cycle (13). It is more conserved than HA1, sharing 51-80% amino acid sequence identity between subtypes and inducing heterosubtypic immunity (15, 16). Neutralizing antibody against HA is the most important correlate of protection against influenza virus infection and is used for assessing vaccine immunogenicity. In addition, Western blot assay for antibody to H5 HA has been recommended by the World Health Organization (WHO) as the confirmatory test for serodiagnosis of patients infected with HPAI H5N1 virus (17).

Several protein expression systems have been employed to express the recombinant H5 HA for use as vaccine candidates, in studying host immune responses, and for disease diagnosis. These expression systems include: the infection of mammalian or avian cells with recombinant vaccinia viruses (18-22) or pseudotype lentiviral viruses (23), the infection of insect cells with recombinant baculoviruses (24-26), and the infection of plant cells with recombinant bacteria (27-28). In addition, the expression of H5 HA in yeast or bacterial cells transformed with recombinant plasmids have also been reported (29-30). Among these expression systems, the vaccinia virus vector provides a powerful tool for production of antigenically and biologically active proteins. Several seasonal influenza proteins including HA, NA, NP, M, NS, PB1, PB2 and PA have been expressed in the recombinant vaccinia viruses (31-34) and used to demonstrate influenza subtype-specific and cross-reactive immune responses (35-36).

Even though the rVac-H5 HA has been expressed by several groups of investigators (18-22), their immunobiological activities have only been investigated in animal models and never with human samples. Herein, we constructed a recombinant vaccinia virus harbouring the complete influenza *HA* gene derived from an HPAI H5N1 virus. Localization of the rVac-H5 HA in the infected cells was visualized by immunofluorescence microscopy; and its

antigenicity was determined by Western blot assay using serum samples from H5N1 survivors and H5N1-uninfected subjects. The use of rVac-H5 HA as the test antigen was compared with the H5 HA produced from Madin-Darby canine kidney cells (MDCK) infected with wild-type H5N1 virus, and with the recombinant H5 HA expressed in a baculovirus-insect cell system.

Materials and Methods

Ethical issues

This study has been approved by the Institutional Review Boards of the Faculty of Medicine Siriraj Hospital, Mahidol University.

Human subjects and blood specimen

A total of 20 serum samples employed in this study comprised 11 anonymously archived samples, 3 from HPAI H5N1 survivors, 8 from influenza A/H3N2-infected patients, and 9 serum samples from healthy individuals with informed consent. All patients were diagnosed by real time reverse transcription polymerase chain reaction (real time RT-PCR) using the protocols established by the U.S. Centers for Disease Control and Prevention. Virus isolation and/or serodiagnosis were also performed in most of the patients. The acute and convalescent blood samples were collected from H3N2 patients at approximately 3 weeks apart while single blood samples were collected from healthy individuals. Serum and plasma samples were kept frozen at -20ºC until used.

Cell lines and viruses

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (EMEM) (Gibco, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco,

NY) and antibiotics. Influenza A viruses: A/Thailand/Siriraj-Rama-TT/2004 [A/New Caledonia/20/1999 (H1N1)-like virus], A/Siriraj ICRC/SI-154/2008 [A/Brisbane/10/2007 (H3N2)-like virus] and A/Thailand/1(KAN-1)/2004 (H5N1) clade 1 virus (KAN-1 virus) were propagated in MDCK cell monolayers maintained in EMEM in presence of trypsin-tosylphenylalanyl chloromethyl ketone (trypsin-TPCK) (Sigma-Aldrich, MO) and antibiotics, and without FBS supplement. The culture supernatants were harvested, centrifuged, aliquoted and kept as virus stocks.

TK$^-$ (thymidine kinase negative) cells were grown in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% FBS and antibiotics. The culture media was replaced with DMEM supplemented with 2% FBS for virus propagation and titration. Vaccinia virus vaccine strain Lister, kindly provided by the Thai Government Pharmaceutical Organization, was used as the gene vector for construction of the recombinant virus harboring H5 *HA* gene insert. The virus infected cell cultures were harvested, frozen and thawed three times, followed by treatment with 0.1 volume of 0.25% trypsin (Gibco) for 15 minutes at 37ºC in order to break the aggregates of the viral progenies and facilitating the viral release. Subsequently, a 0.1 volume of FBS was added to terminate the trypsin activity. The cell lysates were centrifuged, aliquoted and kept as the virus stocks.

Construction of recombinant vaccinia virus carrying H5 *HA* gene insert

Total RNA was extracted from MDCK cells infected with the KAN-1 virus using a QIAamp® viral RNA mini kit (Qiagen GmbH, Hilden, Germany). The complete HA genomic segment was amplified by One-step RT-PCR kit (Qiagen) using universal primers: Bm-HA-1 (5'-TATTCGTCTCAGGGAGCAAAAGCAGGGG-3') and Bm-NS-890R (5'-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3') (37). The PCR product of 1,807 base pairs (bp) in length was purified by using QIAquick® gel extraction kit (Qiagen)

and cloned into pGEM®-T Easy vector (Promega Corporation, Madison, WI) by using T4 DNA ligase (Promega) before transforming into *E. coli* JM109 cells. The *Not*I fragment containing complete *HA* gene with sticky ends derived from the cutting product of the recombinant plasmid was repaired by using klenow DNA polymerase (New England Biolabs Inc., Ipswich, MA) in order to generate blunt end DNA strands. The DNA product was subcloned into a pSC11 expression vector kindly provided by Prof. Bernard Moss, National Institute of Allergy and Infectious Disease, Maryland, USA. This vector contains the *Sma*I insertion site located downstream of vaccinia virus p7.5 promoter together with the *E. coli lacZ* gene which encodes for β-galactosidase under a p11 promoter, and is flanked with thymidine kinase sequences (TK_R and TK_L). *E. coli* JM109 cells were transformed with the recombinant pSC11 containing *HA* gene insert (pSC11/*HA*) and plated on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Promega) plus ampicillin as the selective marker. The cloned bacterial colonies were cultured in LB broth and the plasmids were extracted by a QIAprep® Spin mini kit (Qiagen). The purified plasmids were cut by the enzyme *Pst*I in combination with *Xho*I (New England Biolabs) in order to check for the presence of the *HA* gene insert with correct orientation. DNA sequencing was performed in order to determine the in-frame translation of the *HA* insert using a set of 4 sequencing primers comprising pSC11 P7.5F (5'-GCACGGTAAGGAAGTAGAATC-3'), HAH5F (5'-ACTCCAATGGGGGCGATAAA-3'), HAH5R (5'-CAACGGCCTCAAACTGAGTGT-3') and pSC11R (5'-CATCGAGTGCGGCTACTATAAC-3'). A mixture of pSC11/*HA* recombinant plasmids and DMRIE-C transfection reagent (Invitrogen, Carlsbad, CA) in DMEM was transfected into the TK^- cells that had been pre-infected with vaccinia vaccine virus at the multiplicity of infection of 0.01 plaque forming unit (pfu)/ml for 2 hours. The HA sequence flanked with TK_R and TK_L was inserted into the parental vaccinia viral genome by homologous recombination with the *tk* gene. As a result, the recombinant virus harboring

the *HA* gene insert lost the ability to produce thymidine kinase enzyme (TK^- phenotype). The transfected culture was further incubated for 2 days to allow virus replication. The recombinant vaccinia virus was distinguished from the parental TK^+ vaccinia virus by plaque selection on the TK^- cell monolayer in the presence of 5-bromo-2'-deoxyuridine (BrdU) (Sigma Aldrich, St. Louis, MO) and X-gal in which the plaques produced by cells infected with the recombinant vaccinia virus appeared blue. Plaque purification of the recombinant virus was performed three times using low melting point agarose containing BrdU and X-gal. In parallel, the recombinant vaccinia virus containing pSC11 vector (rVac-pSC11) was constructed for use as the vaccinia virus control.

Immunofluorescence assay

TK^- cells infected with recombinant vaccinia viruses containing the *HA* gene insert were investigated for expression and localization of HA protein by immunofluorescence assay (IFA). Goat antiserum against HA from A/Vietnam/1203/04 (H5N1) (VN1203) kindly provided by Prof. Robert G. Webster and Dr. Richard Webby, St. Jude Children's Research Hospital, USA and a mouse monoclonal antibody raised against purified VN1203 HA (US Biological, Swampscott, MA) were used as the primary antibodies. Fluorescein isothiocyanate (FITC) conjugated-rabbit anti-goat Ig (Dako Cytomation, Glostrup, Denmark) or FITC conjugated-goat anti-mouse Ig (Light Diagnostics™, Temecula, CA) was used as the secondary antibody. The slides were counterstained with trihydrochloride trihydrate (Hoechst 33342- Invitrogen, Eugene, Oregon) together with 5% Evan blue's dye and examined for the presence of fluorescent cells under laser scanning confocal microscopes (LSM 510 Meta, Zeiss, Jena, Germany).

Western blot (WB) assay for detection of anti-H5 HA antibody

The WB assay was performed for 2 purposes in this study. Firstly, to investigate the expression, MW, and post-translational proteolytic cleavage of HA protein in TK$^-$ cells infected with either rVac-H5 HA or rVac-pSC11 virus in comparison with the KAN-1 virus infected MDCK cells and recombinant HA expressed in a baculovirus-insect cell system. Secondly, the assay was used to detect specific and cross-reactive antibodies to HA antigen in H5N1 survivors and H5N1-uninfected subjects.

The H5 HA antigens used in the WB assay were derived from 3 sources: the lysates of TK$^-$ cells infected with rVac-H5 HA virus; the lysates of MDCK cells infected with KAN-1 virus; and the recombinant H5 HA expressed in baculovirus-insect cell system (rBV-H5 HA), either purchased from Protein Sciences Corporation, Meriden, CT (lot numbers 45-05034RA-2 [designated lot no. 1] and 0880-125 [designated lot no. 2]) or kindly donated by BEI Resources through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH (catalog number NR-660, lot number 59137402 [designated lot no.3]). Briefly, infected cell lysates or recombinant antigens were mixed with 4X reducing sample buffer (8% SDS, 250 mM Tris Cl pH 6.8, 8% β-mercaptoethanol, 0.4% bromophenol blue, 40% glycerol), boiled for 5 minutes and subjected to 10% denaturing (SDS) discontinuous polyacrylamide gel electrophoresis (SDS-PAGE- Laemmli method) (38). The proteins present in gel were blotted onto a nitrocellulose membrane (Protran®, Whatman, GmbH, Germany) by using Trans-Blot® SD semidry transfer cell (Bio-Rad). The blotted membrane was blocked with 5% skim milk in Tris-buffer saline plus 0.1% tween-20 (TBS-T). Characterization of the rVac-H5 HA protein employed specific antibodies of 3 origins: goat antiserum to VN1203 HA (St. Jude Children's Research Hospital), mouse monoclonal antibody to VN1203 HA (US Biological), and pooled mouse sera collected from BALB/c mice immunized with the recombinant vaccinia virus carrying the *HA* gene of KAN-1 virus

(kindly provided by Dr. Molvibha Vongsakul, Faculty of Science, Mahidol University). The anti-species specific immunoglobulin: horseradish peroxidase enzyme (HRP) conjugated-rabbit anti-goat Ig (Dako Cytomation, Glostrup, Denmark) or goat anti-mouse Ig (Dako) was used as the secondary antibody. The antigen blotted on nitrocellulose membrane was incubated with the primary test serum overnight at 4ºC before washing with TBS-T and followed by incubation with the corresponding secondary antibody for 2 hours at room temperature. The mixture of 3, 3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO), 8% $NiCl_2$ and H_2O_2 was used as the chromogenic substrate. The detection of specific or cross reactive antibody to H5 HA in H5N1 survivors and H5N1-uninfected subjects employed sera at dilution of 1: 100. The HRP conjugated-goat anti-human IgG (Invitrogen) at dilution of 1: 1000 was used as the secondary antibody.

Microneutralization (microNT) assay

The protocol of ELISA-based microNT assay for detection of neutralizing (NT) antibodies was conducted according to that described in the WHO manual (39) and Lerdsamran, *et al* (40). The test serum was heat inactivated at 56ºC for 30 minutes before making a serial two-fold dilution starting from the dilution of 1: 5 and proceeding to 1: 2560. The assay was performed by mixing 60 microliters (µl) of the diluted serum with 60 µl of the virus suspension at a concentration of 200 tissue culture infective dose 50 (TCID50) and incubated at 37ºC for 2 hours. One hundred microliters of the serum-virus mixture were transferred onto an MDCK cell monolayer and further incubated for 24 hours. In order to verify the amount of virus inoculum, virus back-titration at concentrations of 0.1, 1, 10 and 100 TCID50 was included in every reaction plate in duplicate. The relative amount of influenza viral nucleoprotein in the reaction plates was determined by an ELISA using a mouse-specific monoclonal antibody (Chemicon, Temecula, CA) as the primary antibody,

goat anti-mouse Ig conjugated with HRP (Southern Biotechnology, Birmingham, AL) as the secondary antibody, and TMB (KPL Inc., Gaithersburg, MD), as the chromogenic substrate. The NT antibody titer was defined as the reciprocal of the highest serum dilution that reduces ≥50% of the amount of viral nucleoprotein in the reaction wells as compared to the virus control wells.

Results

Characterization of rVac-H5 HA

Recombinant baculovirus H5 HA protein (rBV-H5 HA) of known molecular weights (72 kDa for HA0, 45 kDa for HA1 and 25 kDa for HA2 domains as indicated in the product brochure) was used to characterize specificity of a goat antiserum and mouse monoclonal antibody against H5 HA by WB assay. Collective results from 3 lots of rBV-H5 HA demonstrated that the goat antiserum was reactive against HA0, HA1 and HA2 domains; whereas mouse monoclonal antibody was reactive only to HA0 and HA1 domains (Figure 1). The goat antiserum and mouse monoclonal antibody were further used to characterize our rVac-H5 HA. Lysates of TK^- cells infected with rVac-H5 HA virus were analyzed on their mobility in SDS-PAGE and WB assay in parallel with those of TK^- cells infected with rVac-pSC11 as the negative control. Three bands of proteins at MW of approximately 75, 55 and 27 kDa corresponding to the uncleaved HA0 precursor and its HA1 and HA2 cleavage products, respectively were observed as analyzed with goat anti-H5 HA antiserum (Figure 1A). On the other hand, 2 bands corresponding to HA0 and HA1 polypeptides were observed when anti-H5 HA monoclonal antibody was employed (Figure 1B). Notably, the MWs of HA1 and HA2 derived from the recombinant baculovirus virus were clearly lower than those derived from the recombinant vaccinia virus. The discrepancy in MW between our system

and the baculovirus system may be due to differences in the glycosylation pattern of HA produced in insect cells compared to vertebrate cells, as discussed below.

Expression and localization of rVac-H5 HA protein expressed in the infected TK$^-$ cells

Localization of rVac-H5 HA in the infected TK$^-$ cells was determined by indirect immunofluorescence assay using a goat anti-H5 HA antiserum and mouse anti-H5 HA monoclonal antibody. The rVac-pSC11 virus infected TK$^-$ cells were used as the negative control. The result showed that the fluorescent rVac-H5 HA protein localized both in the cytoplasm and on the cytoplasmic membrane of the infected cells; no fluorescent signal was observed in the rVac-pSC11 infected TK$^-$ cell control as visualized under a confocal fluorescence microscope (Figure 2). It was noted that our recombinant H5 HA could be recognized by antibodies raised against wild-type HA derived from strain VN1203 which belongs to the same clade.

Comparison between rVac-H5 HA and wild-type H5 HA

The recombinant H5 HA protein expressed in TK$^-$ cells infected with rVac-H5 HA virus was compared with the wild-type HA protein synthesized in MDCK cells infected with KAN-1 virus. The results showed that HA proteins produced in both virus-cell systems were similar in size and number of the cleavage products (Figure 3).

Immunogenicity and antigenicity of rVac-H5 HA

The immunogenicity of our rVac-H5 HA in the induction of antibody response was investigated in a mouse model. Serum samples collected from BALB/c mice immunized with rVac-H5 HA virus (gift from Dr. Molvibha Vongsakul) were reactive with rVac-H5 HA protein and demonstrated reactivity against HA0, HA1 and HA2 domains (Figure 4), while

serum samples from non-immunized mice, or the mice immunized with rVac-pSC11 virus were non-reactive by WB assay (data not shown). Furthermore, the mouse anti-H5 HA antiserum recognized the HA0, HA1 and HA2 domains present either in the recombinant H5 HA expressed in the baculovirus-insect cell system or in the wild-type H5 HA synthesized in MDCK cells infected KAN-1 virus.

Reactivity of human sera to rVac-H5 HA protein

Human sera from various groups of subjects were assayed for the presence of anti-H5 antibody by WB assay using crude cell lysates containing rVac-H5 HA and the recombinant H5 HA expressed in insect cells (rBV-H5 HA lot nos. 1 and 3) as the test antigens. The result showed that sera from all 3 H5N1 survivors contained antibodies to HA0, HA1 and HA2 domains, while the H5N1-uninfected subjects contained antibody to HA2 domain only (Figure 5 and Table 1). We further investigated these sera for presence of NT antibody against various influenza virus subtypes. All 3 H5N1 survivors contained high titers of anti-H5N1 NT antibody, whereas none of the H5N1-uninfected subjects including 8 H3N2-infected patients and 9 healthy individuals did so. Interestingly, these H5N1-uninfected subjects had NT antibody to seasonal H1N1 and H3N2 viruses. Therefore, it is plausible that the heterosubtypic antibodies cross-reacted with the H5 HA2 domain in the WB assay. Nevertheless, the frequency of antibody against the HA2 domain in H5N1-uninfected subjects varied according to sources of the recombinant protein antigen used. The recombinant H5 HA antigen from BEI Resources yielded the strongest reactivity with antibody to HA2, whereas our rVac-H5 HA virus expressed a lower amount of HA2 protein as demonstrated by a weaker reaction with antibody to HA2 antigen.

Discussion

In the present study, the rVac-H5 HA virus was constructed and its immunogenicity was characterized in comparison to wild-type H5 HA produced in MDCK cells infected with KAN-1 virus and to recombinant H5 HA produced in a baculovirus-insect cell system using a goat antiserum and a mouse monoclonal antibody against H5 HA protein. The MW of approximately 75, 55 and 27 kDa of HA0, HA1 and HA2 as expressed by our rVac-H5 HA virus were comparable to those derived from the KAN-1 virus infected MDCK cells. The results demonstrated that the expressed rVac-H5 HA protein was localized in the cytoplasm and on the cytoplasmic membrane of the TK^- cells infected with rVac-H5 HA virus; and this expression was stable after serial propagation of the recombinant virus in TK^- cells (data not shown). This is the first documentation of H5 HA expression in TK^- cells which is a human cell line in origin, however, our recombinant virus had been previously shown to infect and express the H5 HA protein in U937 cells, a human monocyte cell line (41).

The TK^- cells infected with our rVac-H5 HA virus expressed 3 forms of HA: the HA0 polyprotein precursor and its HA1 and HA2 cleavage products, suggesting post-translational proteolytic cleavage of the precursor protein. All these forms of HA were immunogenic, as sera from mice immunized with crude lysates of rVac-H5 HA virus infected cells revealed 3 bands of the appropriate size for HA0, HA1 and HA2 against the recombinant H5 HA expressed in insect cells or wild-type H5 HA produced in MDCK cells infected with KAN-1 virus in a WB assay. Additionally, we have also investigated for the presence of specific neutralizing antibody in sera from BALB/c mice immunized with our rVac-H5 HA virus by the CPE-based NT assay using the highly pathogenic H5N1 (KAN-1) parental virus as the test virus. We found that the mouse sera contained the neutralizing antibody at titers between 80 and 640.

The proteolyic cleavage of HA0 precursor into HA1 and HA2 domains is required for the viral infectivity (9, 14). The HA protein of HPAI viruses which contain multiple basic amino acids at cleavage site will be cleaved intracellularly by ubiquitous subtilisin-like proteases, while the HA that contains single arginine at the cleavage site (as found in nonpathogenic viruses) is cleaved extracellularly by trypsin-like proteases (9, 14). Our rVac-H5 HA virus carries the full length HA coding sequence with multiple basic amino acids PQRERRRKKRG at cleavage site (GenBank accession number AY555150). This explains why the expressed HA was found in the cleaved forms (HA1 and HA2) together with residue of the uncleaved form (HA0). Other investigators who work on the expression of HA of influenza viruses that contain a monobasic amino acid at the cleavage site had incorporated trypsin into the virus growth medium in order to achieve post-translational proteolytic cleavage of the HA product (42).

The MW of approximately 75, 55 and 27 kDa for HA0, HA1 and HA2, respectively as expressed by our rVac-H5 HA virus were comparable not only to the wild-type H5 HA derived from the KAN-1 virus infected MDCK cells, but also to that reported by other investigators using DF-1 cell line infected with modified vaccinia Ankara containing H5 HA from the VN1203 virus (18). However, the MW of these domains were higher than those of the recombinant H5 HA expressed by the recombinant baculovirus, i.e., 72, 45 and 25 kDa for HA0, HA1 and HA2, respectively as shown in this study and also indicated in the product brochure. However, the MW of 70 kDa for HA0 and 50 kDa for HA1 were previously reported by the other group of investigators (43). Several published data demonstrated that the glycosylation of HA in insect cells was significantly retarded and less efficient than in vertebrate cells; the reduction in carbohydrate contents and truncated oligosaccharides was also observed (45-46). Moreover, the HA produced in different types of insect cells

(*Spodoptera frugiperda* cells and *Estigmene acrea* cells) was different in glycosylation patterns (47).

The MW of deglycosylated forms of HA0, HA1 and HA2 is 64.5, 37.6 and 25.1 kDa, respectively according to our prediction by using 2 web-based tools: Compute pI/Mw tool (http://web.expasy.org/compute_pi/) and protein molecular weight calculator (http://www.sciencegateway.org/tools/proteinmw.htm). We have also determined the potential sites of glycosylation in the *HA* sequences of H5N1 (KAN-1) and VN1203 viruses using CountGS application in BioEdit program. Both viruses have 8 potential glycosylation sites, 2 within the HA1 globular domain and 6 in the HA2 fusion domain. Therefore, it is conceivable that the recombinant H5 HA expressed in vertebrates (TK^- cells from human; MDCK from dog; and DF1 from chicken) had a higher MW than those expressed in insect cells due to the difference in glycosylated moieties of the HA molecule which varies among different influenza strains and subtypes and host of origin (18, 44).

Our rVac-H5 HA virus at the concentration of $6x10^6$ pfu/50 µl reaction did not agglutinate goose erythrocytes and the infected TK^- cells did not exhibit a hemadsorption property (data not shown). This is in contrast to recombinant vaccinia viruses expressing the HA protein of H1N1 or H3N2 viruses, constructed by Itamura *et al* (34), which had hemagglutination, hemadsorption and cell fusion activities. We have found out that the H5 HA sequence of the recombinant vaccinia virus in this study is different from its parental HA sequence originally reported by our group for the wild type KAN-1 virus (GenBank accession number AY555150) by 2 positions (R139G and K218E, H5 numbering). These 2 positions were responsible for recognizing the sialic acid receptor present on the host cell membrane according to those previously reported for the HPAI H5N1 virus (48-50). It might be possible that the inability to recognize the cell receptor caused the loss of hemagglutinating activity of our recombinant virus. There are 5 antigenic sites on HA1

domains for seasonal and 2009 pandemic A(H1N1) (sites Sa, Sb, Ca1, Ca2, Cb) and H3N2 viruses (sites A-E), which are not related to the receptor binding site (51-52). The antigenicity-associated sites corresponding to the sites A-E have been reported for H5N1 virus (52). This might be an explanation why our recombinant virus could induce the production of neutralizing antibody in BALB/c mice despite its inability to agglutinate the goose red blood cells.

Our previous work confirmed the presence of antibody directed against different H5 HA domains by WB assay using recombinant VN1203 HA expressed in a baculovirus-insect cell system (Protein Sciences) as the test antigen. HA1 and HA2 specific antibodies were detected in all of 4 H5N1 survivors and lasted for years (53). Unfortunately, that lot of recombinant HA did not contain HA0 as characterized by the reference antiserum (53), while the second and the third lots in this study did. Our WB assay demonstrated that H5N1-uninfected subjects contained the antibodies directed to the HA2 domain, but not the HA0 or HA1 domains. However, the number of individuals with HA2 antibodies varied according to the lot of the recombinant proteins used. These H5N1-uninfected subjects had no NT antibody to H5N1 virus, while all had NT antibody to seasonal H1N1 and H3N2 viruses. We can conclude that the subjects who have no experiences of H5N1 infection contained cross-reactive antibodies against H5 HA2 as the result of previous or recent infection with seasonal influenza viruses. Therefore, our study can strengthen the WHO criteria for serodiagnosis of HPAI H5N1 in that the true H5N1 positive case should contain antibodies which directed against both the HA1 domain and the HA2 domain in a WB assay.

In terms of vaccine development for preparedness against pandemic influenza, it is difficult to predict which subtype of vaccine should be prepared in advance. Collective information suggests that HA2 peptide might be a good candidate (54). While the antibodies to HA1 is strain specific, the antibodies direct against HA2 are conserved and can cross-

neutralize various influenza subtypes including H1, H2, H5, H6, H18, H9, H11, H12, H13 and H16 (16, 55-56). In addition, it has been shown that B lymphocytes from healthy individuals without experience of H5N1 HPAI could generate antibodies to H5 HA2 domain that exhibited neutralizing activity across influenza subtypes including the HPAI H5N1 virus (55). However, those works have generated the clone of monoclonal antibodies with cross reactive neutralizing activity against influenza virus subtypes by phage display technique. These monoclonal antibodies should be derived from the selected clones and assayed in purified and concentrated form for to exhibit the neutralizing activity. On the other hand, our assay for neutralizing antibody against H5N1 virus in the non-H5N1 subjects employed the native sera. Even though those native sera could bind H5 HA2 protein as demonstrated by WB assay, it is likely that those sera contained undetectable level of the neutralizing antibody but higher level of binding antibody against the non-neutralizing epitope. An additional advantage is a potential robust booster effect in vaccinees most of whom have experienced natural influenza virus infection before. Thus, a high level of anti-HA2 antibodies which broadly neutralize across heterologous influenza subtypes might be expected. However, the antigenicity and stability of the protein produced is of concern, and especially, the host cell species for HA2 protein production.

Acknowledgements

This study was supported by the Thailand Research Fund for Senior Research Scholar, the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, and the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative. The authors would like to thank all participants who enrolled in the study and Dr. Andrew Broadbent for editing manuscript. We used the laser scanning confocal microscope at the Division of Medical Molecular Biology,

Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University.

Author Disclosure Statement

No competing financial interests exist.

References

1. Forrest HL and Webster RG: Perspectives on influenza evolution and role of research. Anim Health Res Rev 2010;11:3-18.
2. Tong S, Li Y, Rivailler P, *et al.*: A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci USA 2012;109:4269-4274.
3. World Health Organization: Influenza update. http://www.who.int/influenza/surveillance_monitoring/updates/latest_update_GIP_surveillance/en/index.html.
4. World Health Organization: Influenza A(H1N2) viruses (update). Wkly Epidemiol Rec 2002;77:77-80.
5. Gregory V, Bennett M, Orkhan MH, *et al.*: Emergence of influenza A H1N2 reassortant viruses in the human population during 2001. Virology 2002;300:1-7.
6. Center for Disease Control and Prevention: Avian influenza A virus infections of humans. http://www.cdc.gov/flu/avain/gen-info/avain-flu-humans.htm
7. Peiris JSM, de Jong MD and Guan Y: Avian influenza virus (H5N1): a threat to human health. Clin Microbiol Rev 2007;20:243-267.
8. Gallagher PJ, Henneberry JM, Sambrook JF and Gething MH: Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus. J Virol 1992;66:7136-7145.

9. Steinhauer DA: Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology 1999;258:1-20.
10. Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y and Nakajimai K: Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 1991;182:475-485.
11. Skehel JJ and Waterfield MD: Studies on the primary structure of the influenza virus hemagglutinin. Proc Natl Acad Sci USA 1975;72:93-97.
12. Privalsky ML and Penhoet EE: Influenza virus proteins: identity, synthesis, and modification analyzed by two-dimensional gel electrophoresis. Proc Natl Acad Sci USA 1978;75:3625-3629.
13. Skehel JJ and Wiley DC: Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 2000;69:531-569.
14. Zhirnov OP, Ikizler MR and Wright PF: Cleavage of influenza A virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases. J Virol 2002;76:8682-8689.
15. Gerhard W, Mozdzanowska K and Zharikova D: Prospects for universal influenza virus vaccine. Emerg Infect Dis 2006;12:569-574.
16. Sui J, Hwang WC, Perez S, *et al.*: Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 2009;16:265-273.
17. World Health Organization: Recommendations and laboratory procedures for detection of avian influenza A (H5N1) virus in specimens from suspected human cases. http://www.who.int/influenza/resources/documents/RecAIlabtestsAug07.pdf