were confirmed at the Virology Laboratory at the Faculty of Veterinary Science, Mahidol University.

Satellite telemetry technique and data analysis

Eight gulls negative for influenza antigen were chosen for the satellite telemetry study. The first gull was marked in March 2008 and the second lot of 7 gulls was marked during February and March 2009. Each bird was tagged with a ring band and fitted with a solar powered satellite platform transmitters (solar PTT-100, Microwave Telemetry, Inc., Columbia, MD) on its back by using Teflon harnesses (Bally Ribbon Mills, Bally, PA). A transmitter weighing 12 g was used for the first gull and transmitters weighing 9.5 g were used for remaining 7 gulls. On average, the transmitter packages weighed approximately 1.98 % of the bird's body weight. After being marked, birds were released, usually within 1 hour, to a place close to the capture sites.

The solar PTT-100 transmitter operates at frequency of 401.650 MHz; and flyway data from birds can be retrieved every 2 days. The standard duty cycle of the solar PTTs was set at 10 hours on and 48 off for recharging the batteries. Signals were processed and the data was provided by Argos CLS (Toulouse, France). Bird locations were analyzed and mapped with Google Earth Program version 5.1(Google, Mountain View, CA, USA) with a precision of <1500 m. When the transmitter signal from any tracked bird was lost, the observation was still going on for at least one more month before concluding its disappearance.

Detection of H5N1 HPAI infection in study birds

Prevalence of influenza virus in this flock of gulls as well as the confirmation of the negative results for influenza antigen detection was determined by real time reverse transcription-polymerase chain reaction (RT-PCR) for viral genome detection and the virus isolation from throat and cloacal swab samples. Serological techniques for detection of H5 antibody were performed by hemagglutination-inhibition (HI) assay and microneutralization (microNT) assay; and that for detection of pan-influenza antibody was performed by ELISA.

Real time RT-PCR: Protocols from the Organization des Epizooties (http://www.oie.int/fr /normes/mmanual/2008/pdf/2.03.04_AI.pdf) and/or those established by the U.S. Centers for Disease Control and Prevention (CDC) were used for viral genome detection and subtype identification. Throat and cloacal swabs from each bird were tested separately.

Virus isolation method: Cloacal and throat swab specimens were separately inoculated in duplicate in embryonated eggs and a Madin Darby canine kidney (MDCK) cell monolayer. Amniotic/allantoic fluid and culture supernatant were screened for presence of influenza virus by hemagglutination with 0.5% goose red blood cells before subjected to subtype identification by real time RT-PCR.

Hemagglutination-inhibition (HI) assay: The protocol for H5N1 antibody detection was based on the method given for avian influenza in the World Health Organization (WHO) manual on animal influenza diagnosis and surveillance [35] in which 0.5% goose erythrocytes were chosen as the indicator as previously described by Louisirirotchanakul et al. [36]. The assay was performed in micro-titer V plate in duplicate wells using replicating A/chicken/Thailand/ICRC-V143/07(H5N1) (accession No. EU233413-EU233420) at a final concentration of 4 hemagglutination units/25 μl as the test antigen. Titer was defined as the highest serum dilution that causes complete hemagglutination of the test erythrocytes.

Microneutralization assay: H5N1 antibody was detected by ELISA based MicroNT assay using the protocols as described in the WHO manual for avian influenza [35], and modified by Louisirirotchanakul et al. [36] in which MDCK cell suspension was replaced with MDCK cell monolayer. The assay was performed in micro-titer plates in duplicate. A/chicken/Thailand/ICRC-V143/07(H5N1) at a final concentration of 100 TCID50 was used as the test virus. Viral nucleoprotein synthesized was detected by a mouse monoclonal antibody (Chemicon International, Inc., Tecumala, CA) as the primary antibody together with horse radish peroxidase-conjugated rabbit anti-mouse Ig (Dako Cytomation, Denmark) as the secondary antibody. Titer was defined as the highest serum dilution that causes a 50% reduction in the amount of viral nucleoprotein synthesized.

ELISA: Sera were assayed for antibody to influenza A viruses (pan-influenza A subtypes), using the type A influenza multi species antibody test kit (AI MSp) (BioChek, London, UK) according to the manufacturer's instructions.

Virus challenge test

To study whether infected birds could survive the infection and still be able to carry on migratory activity, wild birds were trapped and kept in captivity for a week prior to viral inoculation. These gulls were negative for influenza virus infection. Each bird was intranasal inoculated with 100 μl of A/Brown-head gull/Thailand/vsmu-4/2008(H5N1) (accession No. EU676322-EU67329) at an inoculum dose of 10, 10^3 and 10^4 tissue culture infective dose 50

(TCID50). Inoculated birds were kept in an isolator in an animal biosafety laboratory level 3, Faculty of Veterinary Science, Mahidol University, and observed daily for signs, symptoms and death until one month after inoculation.

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Figure legends

Figure 1. Flock of Brown-headed gulls at the study site, and a gull fitted with satellite transmitter.

Figure 2. Migratory routes of all study gulls. (A) Gull I.D. 74795; (B) Gull I.D. 88215; (C) Gull I.D. 88216; (D) Gull I.D. 91416; and (E) Gull I.D. 91417. Data accessed on August 6, 2011, Co-ordinates (Main figure and A-E): 21.98°N 95.28°E.

Figure 3. Ecological characteristics of habitats in each country involving the inland lakes and coastal areas. Data accessed on August 6, 2011 Co-ordinates: (A) 36.97°N 89.23°E; (B) 35.23°N 91.11°E; (C) 31.10°N 90.85°E; (D) 13.58°N 100.45°E; (E) 12.95°N 103.92°E; and (F) 9.56°N 105.18°E.

Figure 4. Duration of stay of each gull in various countries.

Table 1. Demographic data of the tracked gulls.

Bird I.D.	Sex	Body weight (g)	Marking date	Time at signal loss from monitoring	Place at signal loss	Tracking period
74795	ND	475	Mar 25, 2008	Dec 2008	Cambodia	9 mos.
88215	Male	650	Mar 26, 2009	Mar 2011	Thailand	2 yrs.
88216	Female	600	Mar 13, 2009	May 2010	Tibet	1 yr, 2 mos.
88217	ND	450	Feb 17, 2009	Mar 2009	Thailand	<1 mo.
88218	Female	500	Mar 26, 2009	Apr 2009	Thailand	<1 mo.
91416	ND	440	Feb 17, 2009	Jan 2011	Cambodia	1 yr, 11 mos.
91417	ND	430	Feb 17, 2009	Nov 2010	Thailand	1 yr, 9 mos.
91418	ND	400	Feb 17, 2009	Mar 2009	Thailand	<1 mo.

ND = Not determined

Table 2. Flying distances of the tracked gulls.

		Total distance on migration (km)		Duration of migration (days)			Average distance at habitat per day (km)	
Bird I.D.	Year	Thailand to China	China to Thailand	Thailand to China	China to Thailand	Average flying distance on migration per day (km)	China	Thailand
74795	2008	2,419	2,747	7	9	326	26	28
88215	2009	3,167	2,372	12	16	201	2	7
	2010	2,223	2,404	9	21	174	9	6
	2011	Lost						6
88216	2009	2,343	2,014	5	15	310	19	6
	2010	2,074	Lost	7				2
91416	2009	2,255	2,067	12	12	177	1	2
	2010	1,954	1,968	23	12	137	3	3
91417	2009	2,924	2,917	39*	22	152	3	6
	2010	2,403	2,870	22	5	360	10	3
Average distance		2,418	2,420	12	14	230	9	7
95% CI		2,139- 2,698	2,143- 2,697	7-23	10-18	167 - 293	3-16	1-12

CI = Confidence interval

*The migratory route started from Thailand to Cambodia and Vietnam, and it took 39 days from Vietnam to China.

Table 3. Habitats of the tracked gulls.

		Length of stay (days)				
Bird I.D.	Location	2008	2009	2010	2011	Average
74795	Thailand: Samut Prakan, Samut Sakhon	44	-	-	-	-
	China: Qinghai, Xinjiang, Tibet	174	-	-	-	-
	Cambodia: Siem Reap	35	-	-	-	-
88215	Thailand: Samut Prakan, Samut Songkhram	-	90	119	61	90
	China: Xinjiang, Tibet	-	170	180	-	175
	Cambodia: Pursat, Siem Reap	-	-	40 (02/12/10 -11/01/11)		-
88216	Thailand : SamutPrakan, SamutSongkhram, Chachoengsao	-	90	98	-	94
	China: Tibet	-	183	-	-	-
91416	Thailand :SamutSongkhram	-	64	101	-	82
	China: Tibet	-	201	189	-	195
	Cambodia: Kampong Thom, Pursat, Siem Reap	-	-	42 (19/11/10 - 10/01/11)		-
91417	Thailand : Samut Prakan, Samut Songkhram	-	83	78	-	81
	China: Qinghai, Xinjiang	-	146	180	-	163
	Cambodia: Kampong Thom, Pursat, Siem Reap	-	32 (25/12/09- 25/01/10)		-	-

Table 4. Susceptibility of Brown-headed gulls in captivity to H5N1 virus challenge.

Inoculum dose (TCID50)	Gull No.	Day of shedding after virus inoculation from		Day at death	NT titer at day after inoculation					
		Trachea	Cloacae		0	5	10	15	20	25
10^4	1	No shedding	2, 3	4	<20					
	2	2, 3	2	4	<20					
	3	1, 2, 3, 4	3, 4	5	<20					
10^3	4	3, 4	No shedding	5	<20					
	5	1, 2, 3, 4	2, 3, 4	5	<20					
	6	2, 4	3	6	<20	20				
10	7	No shedding	No shedding	Alive	<20	<20	<20	<20	<20	<20
	8	No shedding	No shedding	Alive	<20	<20	160	1280		
	9	4	3	7	<20	<20				

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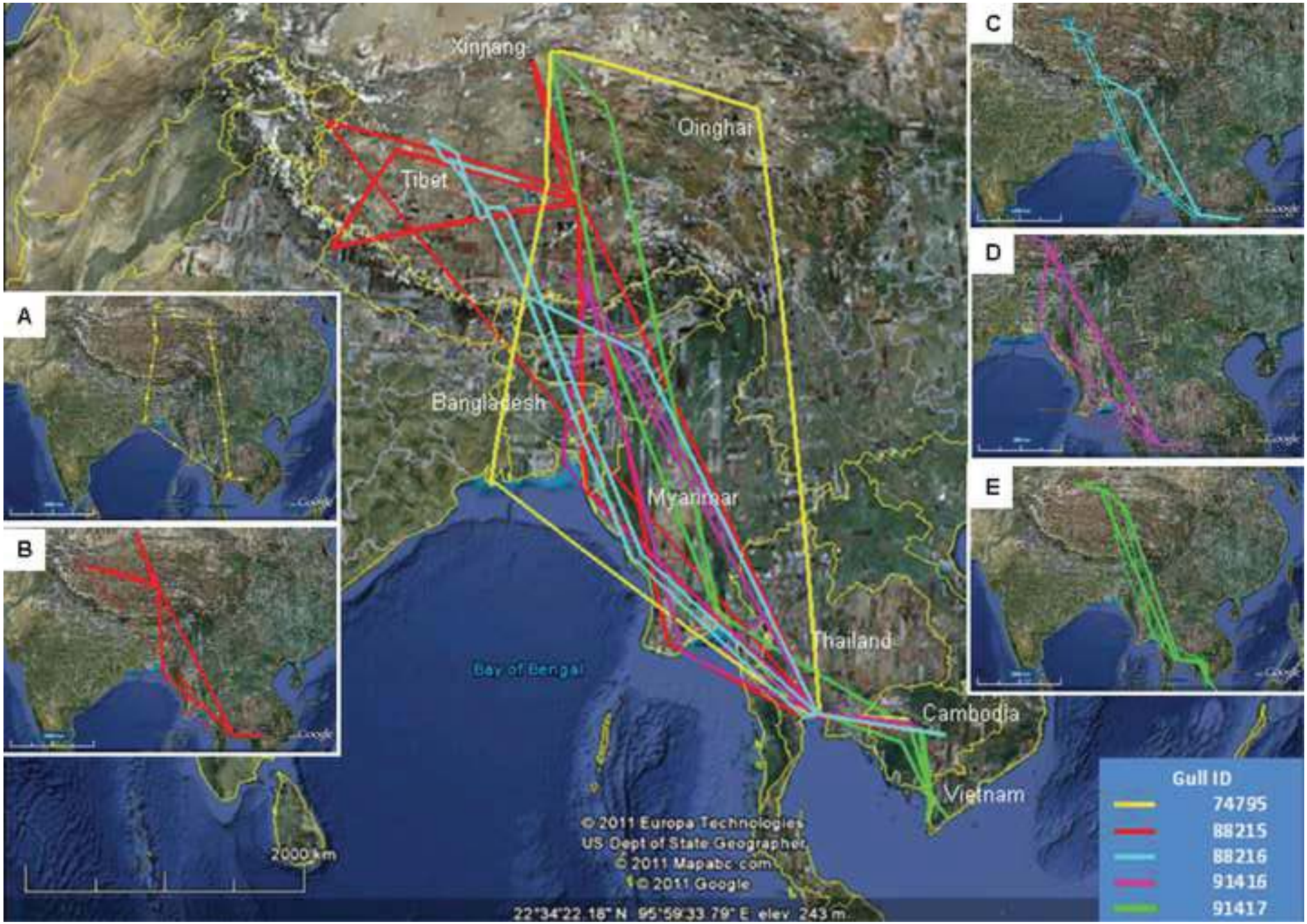

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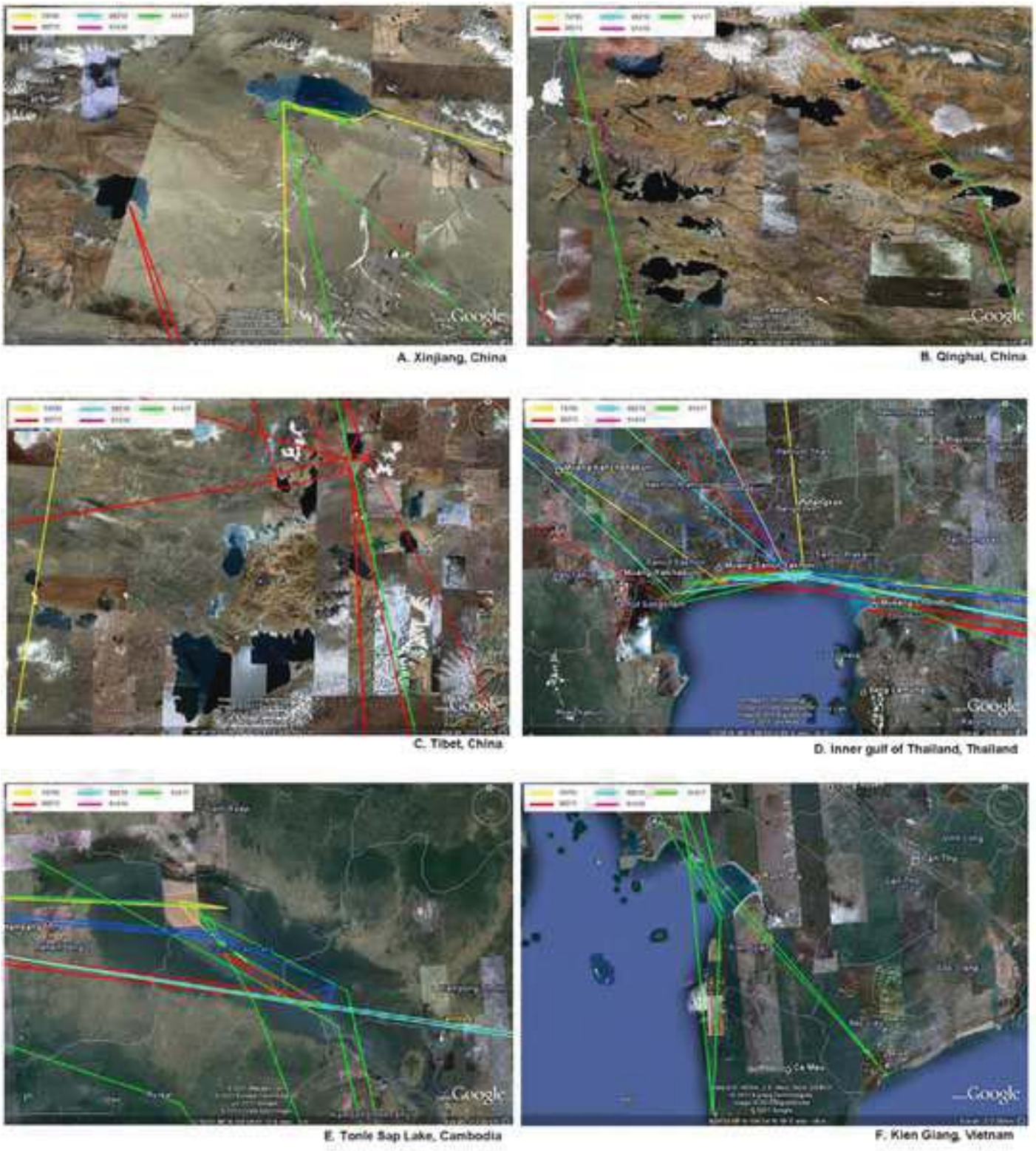

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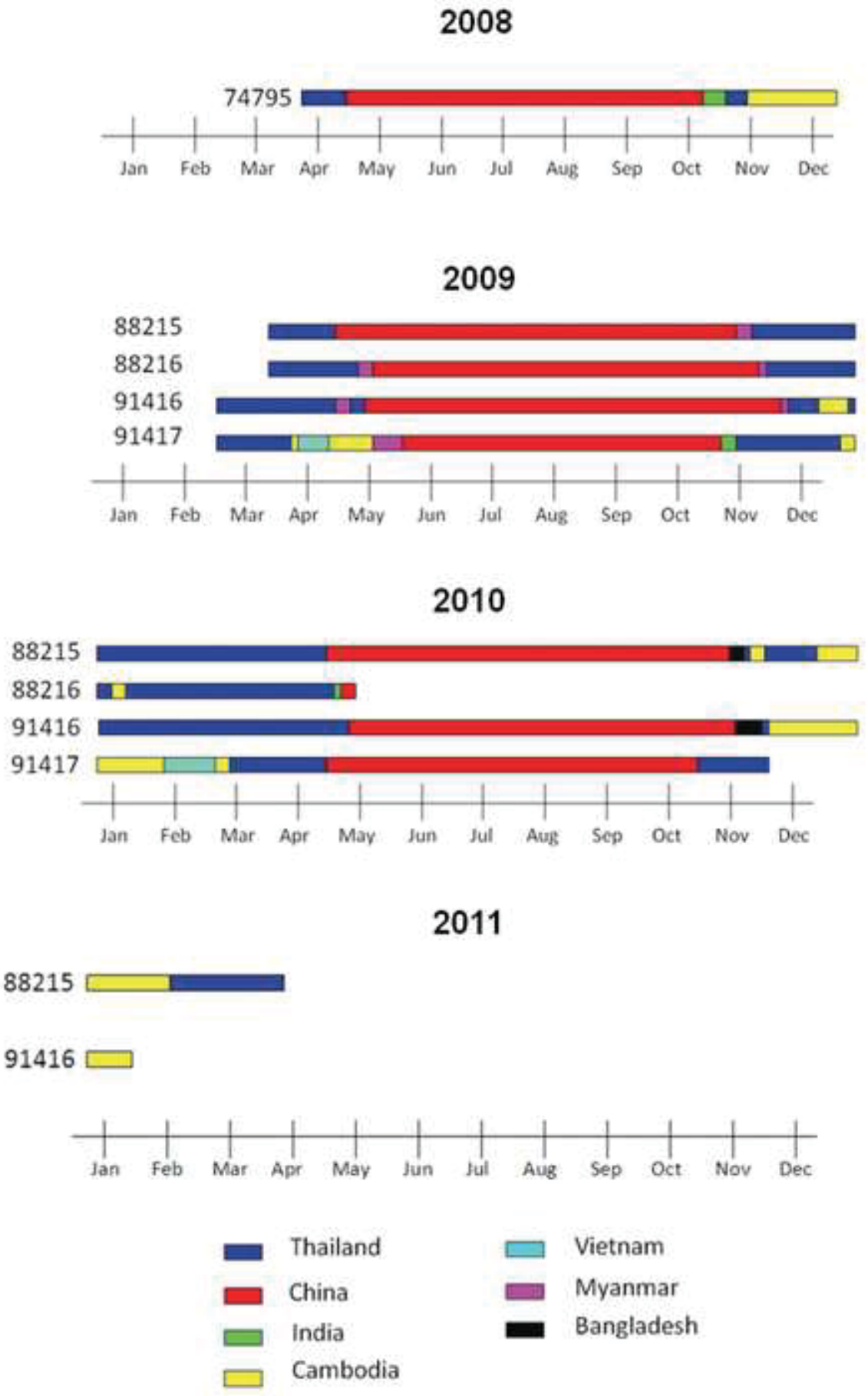

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สำนักระบาดวิทยา กรมควบคุมโรค กระทรวงสาธารณสุข / Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health

อัตราการติดเชื้อ ระยะเวลาของการขับเชื้อไวรัส และปริมาณเชื้อไวรัส ขณะมีการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในกลุ่มทหารเกณฑ์ ศูนย์ฝึกทหารใหม่แห่งหนึ่ง ประเทศไทย เดือนมิถุนายน พ.ศ. 2552

การสอบสวนทางระบาดวิทยา

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

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ความเป็นมา

ต้นเดือนเมษายน 2552 มีรายงานผู้ติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 (2009 pandemic influenza A (H1N1)) ในประเทศสหรัฐอเมริกา[1] และเม็กซิโก[2] ไวรัสได้แพร่กระจายไปยังภูมิภาคอื่นๆ ของโลกอย่างรวดเร็ว และตั้งแต่วันที่ 11 มิถุนายน 2552 องค์การอนามัยโลก (World Health Organization; WHO) ได้ประกาศยกระดับการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ขึ้นเป็นระดับ 6 ซึ่งเป็นระดับสูงสุด หมายถึงเกิดการระบาดไปทั่วโลก[3] ที่เกิดขึ้นภายในเวลาเพียง 6 สัปดาห์ ซึ่งรวดเร็วกว่าการระบาดใหญ่ในอดีตที่ต้องใช้เวลานานกว่า 6 เดือนก่อนแพร่กระจายไปทั่วโลก นับถึงวันที่ 15 มกราคม 2553 องค์การอนามัยโลกได้รับรายงานข้อมูลการระบาดของโรคไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ใน 208 ประเทศทั่วโลกที่มีผู้ป่วยยืนยันการติดเชื้อและมีผู้เสียชีวิตอย่างน้อย 13,554 ราย[4] สำหรับในประเทศไทยข้อมูลนับถึงวันที่ 13 มกราคม 2553 มีรายงานผู้ป่วยยืนยันไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 จำนวน 30,852 ราย คิดเป็นอัตราป่วยสะสมเท่ากับ 48.67 ต่อประชากรแสนคน เสียชีวิตจำนวน 196 ราย[5] การป้องกันการติดต่อที่สำคัญวิธีหนึ่งคือ การแยกผู้ป่วยออกจากกลุ่มคนเพื่อป้องกันการแพร่กระจายเชื้อไปสู่คนปกติ ดังนั้น ความรู้เกี่ยวกับระยะเวลาขับเชื้อไวรัสออกมาในทางเดินหายใจ (Viral shedding) และปริมาณไวรัส (Viral load) ในผู้ป่วยจึงมีความสำคัญในการกำหนดมาตรการป้องกันการแพร่ระบาดของโรคไข้หวัดใหญ่สายพันธุ์ใหม่

วันที่ 23 มิถุนายน 2552 สำนักระบาดวิทยา ได้รับรายงานว่ามีทหารเกณฑ์ป่วยด้วยอาการโรคติดเชื้อทางเดินหายใจเฉียบพลันจำนวนมาก โดยส่วนใหญ่มีอาการไข้ ไอ เสมหะ น้ำมูก ปวดศีรษะ เกิดขึ้นภายในศูนย์ฝึกทหารใหม่แห่งหนึ่ง จังหวัดชลบุรี จากการสอบสวนโรคเบื้องต้นของทีมเฝ้าระวังสอบสวนเคลื่อนที่เร็วในพื้นที่และทีมระบาดวิทยาทหารเรือ สงสัยว่าเป็นการระบาดของโรคไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 เนื่องจากในอำเภอข้างเคียง คือ อำเภอบางละมุง จังหวัดชลบุรี มีรายงานการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่มาประมาณ 1 สัปดาห์ก่อนหน้านี้ สำนักระบาดวิทยาจึงร่วมกับทีมระบาดวิทยากองทัพเรือ ดำเนินการศึกษาระบาดวิทยาของโรคในศูนย์ฯ ตั้งแต่วันที่ 25 มิถุนายน 2552 โดยมีวัตถุประสงค์เพื่อยืนยันการระบาดของโรค หาอัตราการติดเชื้อที่มีและไม่มีอาการ

สารบัญ

วัตถุประสงค์ในการจัดทำ
รายงานการเฝ้าระวังทางระบาดวิทยาประจำสัปดาห์

1. เพื่อให้หน่วยงานเจ้าของข้อมูลรายงานเฝ้าระวังทางระบาดวิทยา ได้ตรวจสอบและแก้ไขให้ถูกต้อง ครบถ้วนสมบูรณ์ยิ่งขึ้น

2. เพื่อวิเคราะห์และรายงานสถานการณ์โรคที่เป็นปัจจุบัน ทั้งใน และต่างประเทศ

3. เพื่อเป็นสื่อกลางในการนำเสนอผลการสอบสวนโรค หรืองาน ศึกษาวิจัยที่สำคัญและเป็นปัจจุบัน

4. เพื่อเผยแพร่ความรู้ ตลอดจนแนวทางการดำเนินงานทางระบาดวิทยาและสาธารณสุข

ผู้เขียนบทความวิจัย

อดิสรณ์ วรรธนะศักดิ์[1] จักรรัฐ พิทยาวงศ์อานนท์[1]
วารินทร์ ปงกันคำ[2] รชต ปัญญารัตนศิลป์[3] วราพร วรรณา[1]
พิไลพันธ์ พุธวัฒนะ[4] ณฐมน เงาสุวรรณกุล[4] พิษณุ ภู่รักษ์[4]
หทัยรัตน์ เลิศสำราญ[4] สมคิด คงอยู่[1] โสภณ เอี่ยมศิริถาวร[1]
[1]สำนักระบาดวิทยา กรมควบคุมโรค กระทรวงสาธารณสุข
[2] กองเวชกรรมป้องกัน กรมแพทย์ทหารเรือ กองทัพเรือ
[3] โรงพยาบาลอาภากรเกียรติวงศ์ จังหวัดชลบุรี
[4]ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล
มหาวิทยาลัยมหิดล

ระยะเวลาของการขับเชื้อไวรัสและปริมาณของไวรัสในทางเดินหายใจในกลุ่มทหารเกณฑ์ที่มีผลตรวจยืนยันการติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009

วิธีการศึกษา

การศึกษาครั้งนี้ประกอบด้วย 2 ส่วน ได้แก่ 1) การสำรวจอัตราการติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ด้วย Serological survey และ 2) ศึกษาการขับไวรัส (Viral shedding) และ ปริมาณไวรัส (Viral load) ในผู้ป่วยรายใหม่ โดยในส่วนของการศึกษา Serological survey ได้สุ่มเลือกทหารเกณฑ์หนึ่งกองร้อยในศูนย์ฝึกทหารใหม่ จ.ชลบุรี เป็นตัวแทนในการศึกษา ทำการเจาะเลือดทหารเกณฑ์ 2 ครั้งห่างกัน 4 สัปดาห์ เป็นตัวอย่างซีรั่มคู่ (Paired sera) เพื่อส่งตรวจยืนยันการติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ด้วยวิธี Hemagglutination inhibition (HI) assay

ในส่วนของการศึกษาระยะเวลาของการขับเชื้อไวรัส (Duration of viral shedding) และปริมาณไวรัส (Viral load) คณะผู้ศึกษาได้คัดเลือกจากผู้ป่วยรายใหม่ที่กำลังมีอาการของโรคติดเชื้อทางเดินหายใจเฉียบพลัน (Acute Respiratory Infection; ARI) ด้วยการใช้แบบสอบถามเก็บข้อมูลกลุ่มทหารเกณฑ์กองประจำการ ครูฝึกและข้าราชการในศูนย์ฝึกทหารใหม่ จังหวัดชลบุรี โดยใช้นิยามผู้ป่วย ได้แก่ ผู้ที่มีอาการอย่างน้อย 2 อาการต่อไปนี้ ได้แก่ ไข้ (อุณหภูมิร่างกาย มากกว่า 37.8 องศาเซลเซียส) ไอ เจ็บคอ และน้ำมูกไหล ระหว่างวันที่ 26 พฤษภาคม - 26 มิถุนายน 2552 และเก็บสารคัดหลั่งจากคอหอยหลังโพรงจมูก (Nasopharyngeal swab) หรือสารคัดหลั่งจากคอหอย (Throat swab) นำไปตรวจหาสารพันธุกรรมของเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ทุกวันติดต่อกันเป็นเวลา 12 วัน โดยวิธี Real-time reverse transcriptase polymerase chain reaction (Real-time RT-PCR) ทั้งในเชิงคุณภาพและเชิงปริมาณ โดยส่งตัวอย่างตรวจที่ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

ผลการศึกษา

การระบาดของไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในทหารเกณฑ์ที่ศูนย์ฝึกทหารใหม่ จังวัดชลบุรี ที่มีหน่วยทหารจำนวน 4 กองพัน แต่ละกองพันประกอบด้วย 6 กองร้อย แต่ละกองร้อยมีทหารเกณฑ์ประมาณ 150 - 180 คน พบผู้ป่วยรายแรกเริ่มมีอาการตั้งแต่วันที่ 29 พฤษภาคม 2552 หลังจากนั้นพบจำนวนผู้ป่วยเพิ่มขึ้นเรื่อยๆ จนถึงวันที่ 26 มิถุนายน 2552 พบผู้ป่วยทั้งสิ้น 1,231 ราย คิดเป็นอัตราป่วย (Attack rate) ร้อยละ 29.3 ผู้ป่วยไปรับการรักษาที่แผนกแพทย์ศูนย์ฝึกทหารใหม่ โรงพยาบาลอาภากรเกียรติวงศ์ และโรงพยาบาลสมเด็จพระนางเจ้าสิริกิติ์ พบผู้ป่วยเสียชีวิต 1 ราย อัตราป่วยตาย (Case fatality rate) ร้อยละ 0.08 ทั้งหมดเป็นเพศชาย มีค่ามัธยฐานอายุ 21 ปี พบผู้ป่วยกระจายอยู่ในทุกกองร้อย พบอัตรา

ป่วยสูงสุดใน กองร้อย 6 กองพัน 1 และ กองร้อย 2 กองพัน 2 เท่ากับร้อยละ 57.9 และ 53.7 ตามลำดับ จำนวนผู้ป่วยรายใหม่เพิ่มมากขึ้นชัดเจนระหว่างวันที่ 7 - 18 มิถุนายน 2552 (รูปที่ 1) ผู้ป่วยมีอาการไอมากที่สุด (ร้อยละ 92.4) รองลง ได้แก่ ไข้ มีน้ำมูก ปวดศีรษะ ปวด เมื่อยกล้ามเนื้อ เจ็บคอ (รูปที่ 2)

ผลการศึกษาอัตราการติดเชื้อในทหารเกณฑ์ 90 นายที่ถูกสุ่มเลือกมาจากกองร้อยหนึ่ง เพื่อตรวจหาระดับภูมิคุ้มกันต่อเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 พบว่า ไตเตอร์ $\geq$ 1:40 ด้วยวิธี HI จำนวน 61 ราย เท่ากับร้อยละ 67.8 (ตารางที่ 1) ทั้งนี้ ในกลุ่มที่มีผลการตรวจยืนยันว่ามีการติดเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 พบว่าเป็นผู้ติดเชื้อที่ไม่มีอาการป่วย (asymptomatic infection) 18 ราย (ร้อยละ 29.5) และเป็นผู้ติดเชื้อที่มีอาการจำนวน 43 ราย (ร้อยละ 70.5)

การศึกษาเกี่ยวกับระยะเวลาของการขับเชื้อไวรัสและปริมาณไวรัส (viral shedding และ viral load) ในกลุ่มทหารเกณฑ์ที่ตรวจพบสารพันธุกรรมของเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในสิ่งส่งตรวจจากคอหอยหลังโพรงจมูกในผู้ป่วยจำนวน 25 ราย มีอายุ 18 - 26 ปี ค่ามัธยฐานอายุ (Median) เท่ากับ 21 ปี ทั้งหมดเป็นเพศชาย มี 1 รายมีน้ำหนักตัวมาก (102 กิโลกรัม) แต่ไม่มีโรคประจำตัวใดๆ และอีกหนึ่งรายกำลังป่วยเป็นวัณโรคปอดอยู่ระหว่างการรักษาด้วยยาต้านเชื้อวัณโรค ผู้ป่วยที่เหลือมีสุขภาพแข็งแรงและไม่มีโรคประจำตัว วันที่เก็บตัวอย่างสารคัดหลั่งจากคอหอยหลังโพรงจมูกในผู้ป่วยวันแรกห่างจากวันเริ่มป่วยตั้งแต่ 1 ถึง 9 วัน (ค่ามัธยฐาน 4 วัน) ระยะเวลาของการขับเชื้อไวรัสจากทางเดินหายใจในผู้ป่วยทั้ง 25 รายมีค่ามัธยฐาน 5 วัน โดยระยะเวลาของการขับเชื้อไวรัสออกจากทางเดินหายใจสั้นสุด 2 วันและนานสุด 11 วัน (รูปที่ 3) เมื่อพิจารณาข้อมูลรายบุคคล มีผู้ป่วย 3 รายที่สามารถเก็บสิ่งส่งตรวจได้ตั้งแต่วันแรกที่ผู้ป่วยเริ่มมีอาการ และพบเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่สามารถที่จะขับจากทางเดินหายใจได้ตั้งแต่ภายในวันแรกที่ผู้ป่วยเริ่มมีอาการป่วย

ปริมาณไวรัสของผู้ป่วยที่ตรวจพบในการศึกษาครั้งนี้มีจำนวน $2.6x10^2$ Copies/ml. ถึง $8.1X10^9$ Copies/ml. เฉลี่ย $2.4X10^4$ Copies/ml. ปริมาณเชื้อไวรัสที่พบในผู้ป่วยแต่ละราย ส่วนใหญ่จะมีปริมาณมากสุดในวันที่ 2 ถึงวันที่ 5 หลังจากเริ่มมีอาการป่วย อย่างไรก็ตาม ไม่พบว่ามีความสัมพันธ์ระว่างปริมาณเชื้อไวรัสกับระยะเวลาของการขับเชื้อไวรัสจากทางเดินหายใจในผู้ป่วยกลุ่มนี้ นอกจากนี้ พบว่าระยะเวลาการขับเชื้อไวรัสในผู้ป่วยที่มีน้ำหนักเกินและเสียชีวิตเท่ากับ 8 วัน และในผู้ป่วยที่กำลังป่วยเป็นวัณโรคปอดเท่ากับ 4 วัน

สรุปและวิจารณ์ผลการศึกษา

การระบาดของไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในทหารเกณฑ์ชายไทยอายุเฉลี่ย 21 ปี มีอัตราป่วยสูงถึงร้อยละ 29.3 และมีผู้ป่วยที่มีน้ำหนักเกินซึ่งจัดเป็นกลุ่มเสี่ยงเสียชีวิต 1 ราย อาการป่วยที่พบมากสุด ได้แก่ ไอ ไข้ น้ำมูก ปวดศีรษะ ปวดเมื่อยกล้ามเนื้อเจ็บคอ ซึ่งเป็นอาการของโรคติดเชื้อทางเดินหายใจเฉียบพลัน การระบาดเกิดขึ้นรวดเร็วเนื่องจากทหารเกณฑ์มีจำนวนมากและทำกิจกรรมร่วมกันตลอด ประกอบกับอยู่ในสถานที่แออัดจึงแพร่กระจายเชื้อต่อเนื่องได้ง่ายเมื่อไม่มีการแยกผู้ป่วยในระยะแรก ๆ ของการระบาดครั้งนี้ นอกจากนี้ยังพบว่าสัดส่วนผู้ติดเชื้อที่ไม่มีอาการป่วยมากถึงร้อยละ 29.5 ของผู้ติดเชื้อทั้งหมด อาจเป็นอีกสาเหตุหนึ่งที่ทำให้มีโอกาสแพร่ระบาดได้เพิ่มขึ้น เนื่องจากทหารกลุ่มนี้ไม่ได้ถูกแยกออกจากผู้ที่ไม่ป่วย ถึงแม้ว่าประชากรกลุ่มนี้จะไม่ไอ ไม่จาม ไม่มีน้ำมูก แต่กิจกรรมการใช้ชีวิตของทหารเกณฑ์มีโอกาสสัมผัสน้ำลายกันได้ง่าย เช่น การดื่มน้ำจากแก้วเดียวกัน หรือใช้ช้อนส้อมร่วมกันในขณะรับประทานอาหาร เป็นต้น

ในขณะมีการติดเชื้อไวรัสไข้หวัดใหญ่ ไวรัสจะถูกขับออกมาอยู่ในสารคัดหลั่งบริเวณจมูกและคอหอยหลังช่องปาก และแพร่กระจายเชื้อไวรัสนี้ผ่านทางการไอและจาม[6] นอกจากนี้มีรายงานการตรวจพบเชื้อไวรัสในอุจจาระ ปัสสาวะ และซีรั่ม[7] โดยทั่วไปในผู้ใหญ่ที่แข็งแรง ไม่มีโรคประจำตัว ไม่ได้รับยาต้านไวรัส ที่ป่วยด้วยไข้หวัดใหญ่ตามฤดูการจะมีระยะเวลาของการขับเชื้อไวรัสออกมาในทางเดินหายใจนาน 5-7 วันหลังจากเริ่มมีอาการ[8,9]

ในส่วนของไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 มีการศึกษาเรื่องระยะเวลาของการขับเชื้อไวรัสออกมาในทางเดินหายใจในต่างประเทศ พบว่าในขณะมีการระบาดของไข้หวัดใหญ่สายพันธุ์ใหม่ในนักเรียนเตรียมทหารของโรงเรียนนายเรืออากาศสหรัฐอเมริกาเดือนมิถุนายน พ.ศ. 2552 ระยะเวลาของการขับเชื้อไวรัสออกมาในทางเดินหายใจมีค่ามัธยฐาน 5 วัน (พิสัย 1 - 12 วัน) สูงสุดใน 24 - 72 ชั่วโมงหลังเริ่มมีอาการป่วย[10] สอดคล้องกับการศึกษาในประเทศไทยในการระบาดครั้งนี้ ที่พบว่าระยะเวลาการขับเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในทหารเกณฑ์มีระยะเวลานานเฉลี่ย 5 วัน หลังเริ่มแสดงอาการ แต่สามารถตรวจพบได้ตั้งแต่วันแรกที่เริ่มป่วยจนถึง 11 วัน ซึ่งนับว่าใกล้เคียงกันมาก ซึ่งน่าจะอธิบายได้จากการที่เป็นประชากรอายุใกล้เคียงกันและเป็นการติดเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ในระยะต้นของการระบาดใหญ่ ที่ยังมีสารพันธุกรรมใกล้เคียงกัน โดยปริมาณเชื้อไวรัสสูงสุดอยู่ระหว่างวันที่ 2 ถึงวันที่ 5 หลังจากเริ่มมีอาการ จึงเป็นระยะเวลาที่ต้องมีมาตรการป้องกันการแพร่เชื้อที่เข้มงวด และต้องป้องกันต่อเนื่องตลอดระยะเวลาที่มีการระบาดเพราะผู้ป่วยบางรายอาจจะขับเชื้อได้นานกว่า 10 วัน

ในอดีตมีหลายการศึกษา ที่รายงานประสิทธิผลของยาต้านไวรัสชนิด Neuraminidase inhibitor (NAI) เช่น oseltamivir และ zanamivir ว่าสามารถลดระยะเวลาที่มีการขับเชื้อไวรัสออกมาในทางเดินหายใจ

และลดปริมาณไวรัสในผู้ป่วยไข้หวัดใหญ่ตามฤดูกาล ถ้าหากได้รับยาภายใน 4 วันแรกนับจากเริ่มมีอาการ[11,12] ซึ่งในกรณีของไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่นี้ ผู้ป่วยที่ได้รับยา oseltamivir มีระยะเวลาของการขับเชื้อไวรัสที่สั้นลงเช่นกัน[13] อย่างไรก็ตามในการระบาดครั้งนี้มีทหารเกณฑ์ที่ป่วยเพียง 2 รายที่ได้รับยา oseltamivir จึงควรมีการศึกษาเพิ่มเติมถึงประสิทธิผลของยาต้านไวรัสในผู้ป่วยไทยต่อไป

การเก็บตัวอย่างสารคัดหลั่งจากคอหอยหลังโพรงจมูก (Nasopharyngeal swab) มีโอกาสได้ผลบวกสูง โดยการศึกษานี้พบปริมาณเชื้อไวรัสเฉลี่ย 2.4X10^4 Copies/ml. และปริมาณสูงสุดระหว่างวันที่ 2 - 5 หลังจากเริ่มมีอาการป่วย ดังนั้น การเก็บตัวอย่างวิธีนี้จึงมีเหมาะสมหากทำโดยบุคลากรที่มีประสบการณ์และใช้เทคนิกที่ถูกต้องเพราะเป็นการเก็บตัวอย่างจากจุดที่พบเชื้อไวรัสไข้หวัดใหญ่อยู่ในปริมาณมาก

ข้อจำกัดในการศึกษา

การศึกษานี้ ดำเนินการในศูนย์ฝึกทหารใหม่ ซึ่งผู้ป่วยเป็นทหารเกณฑ์ที่มีสุขภาพร่างกายแข็งแรงก่อนหน้าการป่วย ทั้งหมดมีอายุใกล้เคียงกันและมีกิจกรรมต่างๆ คล้ายกัน และเกือบทุกรายไม่ได้รับยาต้านไวรัส ทำให้ยากต่อการระบุปัจจัยที่มีผลทำให้ระยะเวลาของการขับเชื้อไวรัสออกมาจากทางเดินหายใจแตกต่างกัน ในส่วนของคุณภาพการเก็บและนำส่งตัวอย่างอาจจะมีความแตกต่างกันในแต่ละวัน เนื่องจากการเก็บตัวอย่างสารคัดหลั่งจากผู้ป่วยกระทำโดยพยาบาลในหอผู้ป่วย ซึ่งผลัดเปลี่ยนกันไปตามเวรของพยาบาล อาจเป็นสาเหตุทำให้ได้จำนวนเชื้อไวรัสที่ไม่แน่นอน เนื่องจากทักษะที่แตกต่างกันระหว่างผู้เก็บตัวอย่างหลายคน เพราะการป้ายเชื้อที่คอหอยหลังโพรงจมูกของผู้ป่วยขึ้นอยู่กับหลายปัจจัย เช่น ตำแหน่งที่ป้ายและความเที่ยงของมือผู้เก็บตัวอย่าง นอกจากนี้ การขนส่งตัวอย่างจากจังหวัดชลบุรีมาตรวจที่ห้องปฏิบัติการในกรุงเทพมหานครยังมีปัจจัยด้านระยะเวลา และคุณภาพการเก็บรักษาตัวอย่างที่ส่งผลต่อคุณภาพตัวอย่างและการตรวจพบปริมาณเชื้อไวรัสได้

กิตติกรรมประกาศ

ขอขอบคุณบุคคลและหน่วยงานที่ให้การสนับสนุนการศึกษาครั้งนี้ ได้แก่ กรมแพทย์ทหารเรือ ผู้อำนวยการโรงพยาบาลสมเด็จพระนางเจ้าสิริกิติต์ ผู้อำนวยการโรงพยาบาลอาภากรณ์เกียรติวงศ์ ผู้บัญชาการศูนย์ฝึกทหารใหม่ ฐานทัพเรือสัตหีบ สำนักงานสาธารณสุขจังหวัดชลบุรี สำนักงานป้องกันควบคุมโรคที่ 3 ชลบุรี ทีมเฝ้าระวังสอบสวนเคลื่อนที่เร็วอำเภอสัตหีบ จังหวัดชลบุรี ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล และสำนักงานกองทุนสนับสนุนการวิจัย (The Thailand Research Fund for Senior Research Scholar) โดยผ่านศาสตราจารย์ ดร.พิไลพันธ์ พุธวัฒนะ

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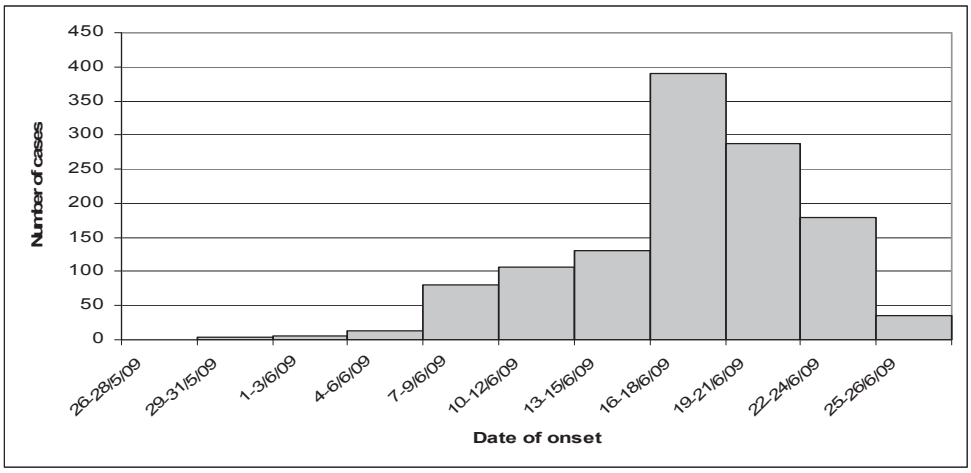
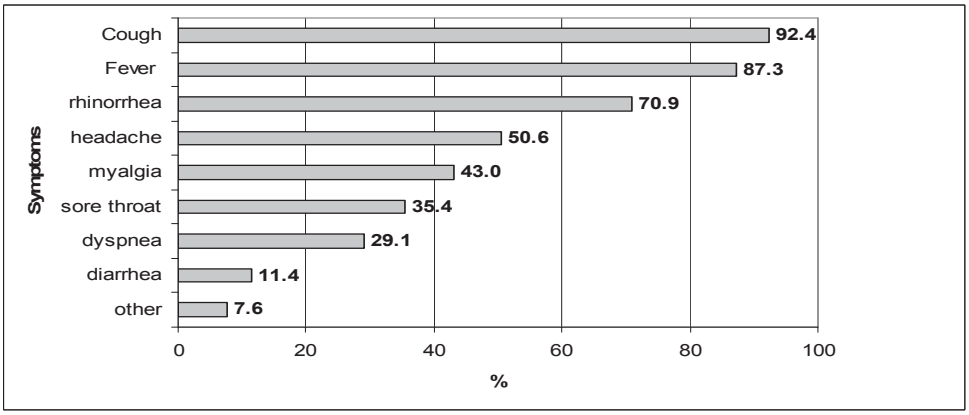
ตารางที่ 1 แสดงร้อยละของการติดเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 และร้อยละของผู้ติดเชื้อที่มีอาการและไม่มีอาการ ในทหารเกณฑ์ของศูนย์ฝึกทหารใหม่ ที่ได้รับการสุ่มตรวจ วันที่ 9 มิถุนายน 2552 (N = 90)

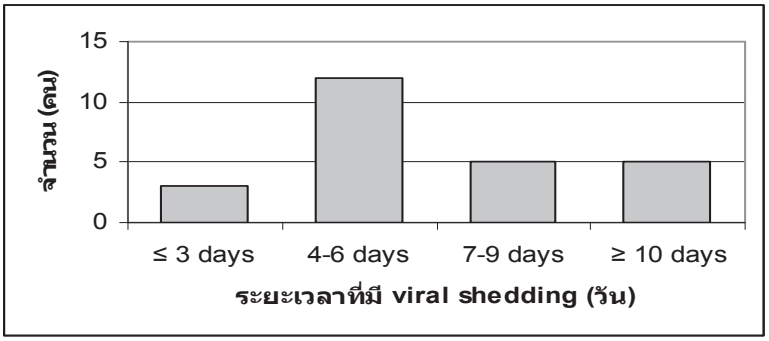
ผลการตรวจ	จำนวน	ร้อยละ	อาการป่วย	จำนวน	ร้อยละ
ติดเชื้อ	61	67.8	มีอาการ	43	70.5
			ไม่มีอาการ	18	29.5
ไม่ติดเชื้อ	29	32.2			
รวม	90	100.0			



รูปที่ 1 แสดงจำนวนผู้ป่วยสงสัยไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 จำแนกตามวันเริ่มป่วย ในทหารเกณฑ์ศูนย์ฝึกทหารใหม่แห่งหนึ่ง จังหวัดชลบุรี ระหว่างวันที่ 26 พฤษภาคม – 26 มิถุนายน 2552 (N = 1,231)



รูปที่ 2 แสดงร้อยละของอาการและและอาการแสดงในผู้ป่วยสงสัยไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 ในทหารเกณฑ์ศูนย์ฝึกทหารใหม่แห่งหนึ่ง ระหว่างวันที่ 1– 26 มิถุนายน 2552 (N = 77)



รูปที่ 3 แสดงจำนวนผู้ป่วยจำแนกตามระยะเวลา (วัน) ที่ตรวจพบไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ในทหารเกณฑ์ศูนย์ฝึกทหารใหม่ จังหวัดชลบุรี ระหว่างวันที่ 27 มิถุนายน - 8 กรกฎาคม 2552 (N=25 คน)

KEYSTONE SYMPOSIA

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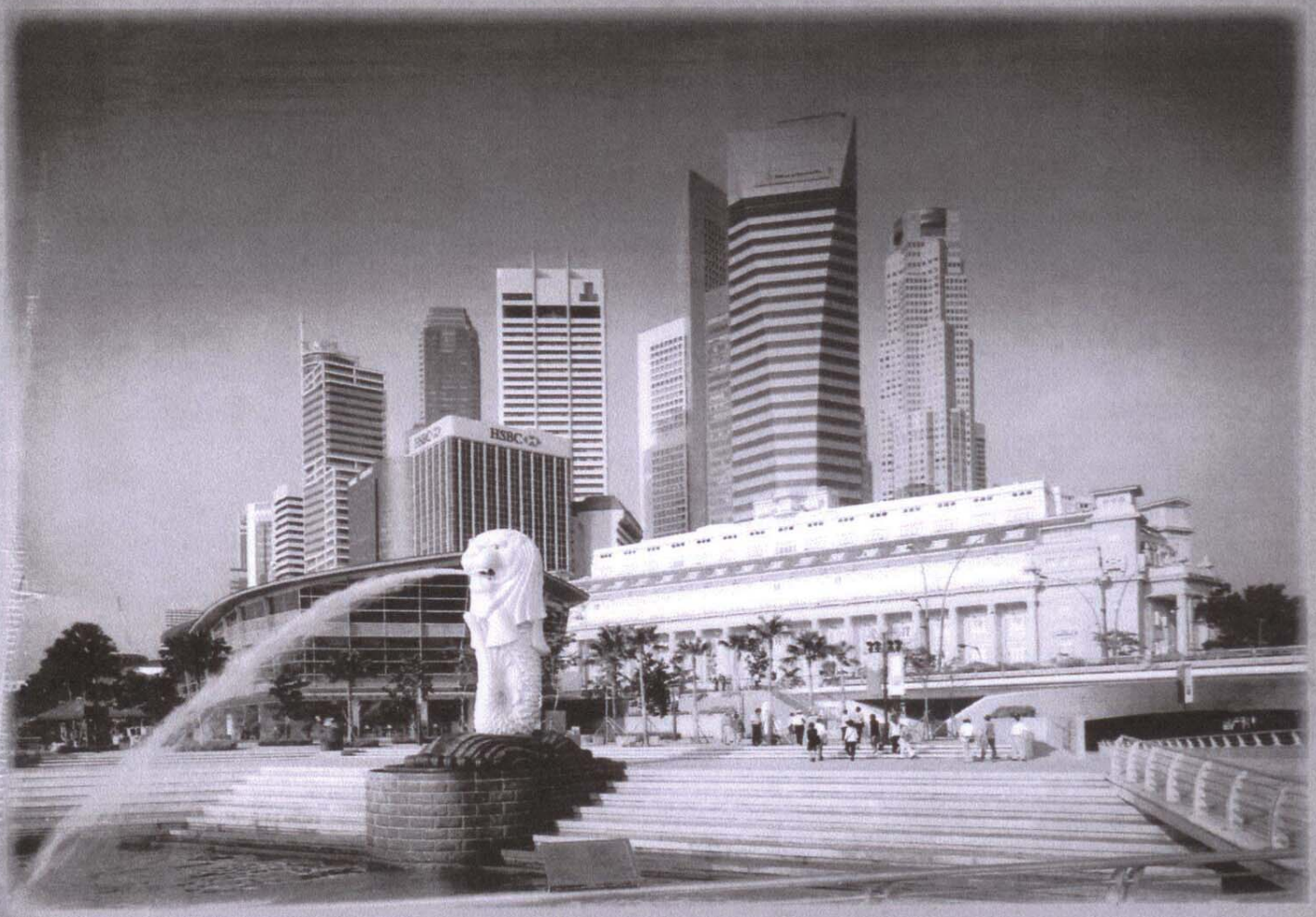
2006 ABSTRACT BOOK

Respiratory Viruses of Animals Causing Disease in Humans

Stanley A. Plotkin, Albert D.M.E. Osterhaus and Martin L. Hibberd

In collaboration with A*STAR (Agency for Science, Technology and Research), Singapore



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221 Pathogen Detection Microarray – from Lab to Bedside

Christopher W. Wong, Charlie Lee Wah Heng, Leong Wan Yee, Shirlena Soh, Martin L. Hibberd, Ken W-K Sung, Lance D. Miller. Microarray and Expression Genomics Laboratory, Genome Institute of Singapore, Republic of Singapore.

Background
Testing for infectious agents in clinical specimens has historically been a hypothesis driven process. While DNA microarrays have recently been used to identify pathogens in a more unbiased fashion, their routine use has been hampered by technical constraints that limit detection sensitivity and specificity.
Methods and Findings
To address these problems, we created an oligonucleotide microarray containing 390k probes spanning the genomes of 35 RNA viruses. By empirically determining signal detection thresholds related to probe-target sequence similarity, and studying the relationship between primer design and PCR-based amplification bias, we have defined novel criteria for selecting probes for the optimal detection of pathogens by microarray. With the *in silico* generation of optimized pathogen "recognition signatures" and the development of algorithms for predicting PCR bias and analyzing probe signal distributions, we were able to identify the presence of pathogens in clinical specimens at the species, genus, or family level. Using this approach, we tested 35 nasal wash samples from 35 children with lower respiratory infections that had been previously tested for respiratory syncytial virus, human metapneumovirus and rhinovirus by real-time PCR and identified pathogens with 94% diagnostic accuracy (75% sensitivity and 100% specificity).
Conclusion
Our findings show that microarrays can be used for the robust and accurate identification of pathogens in patient specimens, and further substantiate the use of microarray technology in clinical diagnostics.
This study was supported by funding from Singapore's Agency for Science, Technology and Research (A*STAR).

222 Genetic Characterization of an Avian Influenza Virus, subtype H5N2, isolated from ducks imported into Singapore

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The avian influenza viruses (AIVs) subtype H5N2 have been reported to mutate from low pathogenic (LPAI) to high pathogenic AIVs (HPAI) in Pennsylvania (1983), Mexico (1994), Italy (1997) and Texas (2004). In addition, avian to human transmission of influenza viruses of both subtypes, H5N1 and H5N2, have been documented.

In 2004, the Defence Medical & Environmental Research Institute received 2 AIVs, (F118 and F189), from the Agri-food and Veterinary Authority of Singapore for diagnostic testing. These samples were isolated from ducks imported into Singapore and suspected to be of the HPAI subtype 'H5N1'. We used real-time polymerase chain reaction (PCR) for diagnostic testing, targeting the hemagglutinin (HA) and neuraminidase (NA) genes. The PCR amplicons were subjected to nucleotide sequencing. Our preliminary results confirmed the AIV samples to be of the virus subtype H5N2, but of low pathogenicity. In this presentation, we fully sequenced the HA and NA genes, and the 6 internal gene segments of isolate F118. Analysis of the cleavage site of the F118-HA protein showed the absence of basic amino acid (aa) residues associated with HPAI. However, the phylogenetic analysis of the F118-HA gene showed a distinct clustering with the Asian HPAI of the subtype H5N1, rather than with the American LPAI of the subtype H5N2. The F118-NA gene showed no deletion in the stalk sequence, which was reported in American viruses of subtype H5N2 isolated in chickens. Phylogenetic analysis of the NA genes further clustered F118-NA closely with subtypes H9N2, H1N2, and H7N2. Examination of the internal proteins of F118 revealed avian-specific aa signatures in the M1, M2, PB1, PB2, PA and NP proteins. No residues associated with amantadine resistance (M2) nor virulence was observed (PB2 and NS1 proteins). Phylogenetic analyses further suggest that the internal genes of the F118 isolate were derived from different influenza A virus subtypes, originating from the Asia-Europe regions. Interestingly, the F118-PA gene clustered more closely with the Asian viruses subtype H5N1, than with any of the AIVs subtype H5N2.

The sequence and phylogenetic data collected in this work will be useful in future molecular and epidemiological studies of the dynamics and evolution of circulating avian influenza viruses, in particular in this region of South-East Asia.

223 Chinese herbs inhibited the entry of SARS-CoV in vitro

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The water extracts of seven Chinese herbs and five Kampo medicines which were used for the treatment of SARS patients, were examined to determine if they could inhibit the SARS viral replication. A human immunodeficiency virus (HIV)/SARS-CoV S pseudotyped virus, containing HIV lentiviral vectors encoding a reporter gene and S protein of SARS-CoV, was used for the screening.
Only Cinnamomi Cortex extract (CCE) and Caryophylli Flos extract (CFE), showed inhibitory activities against HIV/SARS-CoV S pseudovirus with a 50% effective concentration (EC_{50}) of 0.03 mg/ml for the former and 0.058 mg/ml for the latter. Both drugs also inhibited cell membrane fusion between 293T cells and Hela cells expressing either S protein or ACE2 protein almost completely at the concentrations of 0.3 mg/ml. Finally we confirmed that both fractions inhibited wild-type infection in the plaque reduction assay at the selective indices (SIs) of CCE and CFE were 9 and 3.6, respectively We further examined four fractionated samples from Cinnamomi Cortex (CC) by HIV/SARS-CoV S pseudovirus and found that two fractions (Ethanol extract and Butanol fraction) had inhibitory activities with SI of 2.38 and 5.0, respectively. In cell fusion assay both fractions completely inhibited below 0.3mg/ml. In wild type infection, again both fractions showed inhibitory activities with SI of 18 for Ethanol Fr. and 23 for Butanol Fr..
The results strongly indicated that CC and Caryophylli Flos (CF) contain a potent new inhibitor of SARS-CoV entry, and Butanol fraction of CC showed the strongest inhibitory activities.

This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan and the Scientific Research Expenses for Health and Welfare Program from the Ministry of Health and Welfare, Japan.

224 Hemagglutinin activity of avian influenza H5N1 viruses

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Horse hemagglutination inhibition (HI) test is recommended by WHO as an adjunct to micro-neutralization test for H5N1 antibody. As horse RBC can not be obtainable easily in most of Southeast Asian countries, the present study searched for an alternative species of RBC which provided as good result as that employing horse RBC in Hemagglutination (HA) and HI assays.
HA activity of 13 H5N1 viruses (5 human isolates and 8 animal isolates) was tested against 5 species of RBC: 1% horse, 0.5% goose, 0.5% chicken, 0.75% guinea pig and 0.75% human group O. Titration of a virus solution as using goose RBC yielded the highest titer, followed by chicken, guinea pig, human group O and horse RBC (Friedman test; $p<0.05$). The results were reproducible upon testing with 3 RBC donors from each species. HI antibody to two human H5N1 isolates was investigated in 15 sera from patients and survivors with the same 5 species of RBC donors. Using A/Thailand/1(KAN-1)/04 virus antigen, horse RBC gave significant higher antibody titer than the other 4 RBC species ($p<0.001$, Wilcoxon's signed-rank test, Bonferroni's adjustment). However, goose RBC yielded the highest titer when A/Thailand/676/05 virus was used as the antigen. These demonstrated that HI antibody activity was dependent on both the virus antigen and RBC species.
We also conducted complete HA genomic sequencing of all 15 H5N1 virus isolates. There were no difference in receptor binding site of these Thailand isolates as compared to Hong Kong 1997 and Vietnam 2005 viruses, except a mutational change of A138V of A/Thailand/676/05 virus. Our study proposes goose RBC as an alternative donor for HA assay and for virus recognition in the virus isolation method, and also as the indicator for HI assay.
This study was granted by BIOTEC and Thailand Research Fund.

225 Adenoviral vector-based pandemic influenza vaccine against antigenically distinct H5N1 strains

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Avian H5N1 influenza viruses currently circulating in Asia, Europe, the Middle East and Africa could potentially cause the next pandemic. However, currently licensed human vaccines are subtype-specific and do not protect against these H5N1 viruses. We aimed to develop an egg-independent strategy to combat the H5N1 influenza virus, because the virus is highly lethal to chickens and the maintenance of a constant supply of embryonated eggs could be difficult in a pandemic.

We constructed a replication-incompetent, human adenoviral vector (HAd-H5HA) that expressed subtype 5 hemagglutinin. Immunization of mice with HAd-H5HA induced both humoral and cell-mediated immune responses significantly better than that of a traditional subunit vaccine when tested against H5N1 viruses isolated from people. Vaccinated mice were effectively protected from H5N1 disease, death, and primary viral replication when challenged with antigenically distinct H5N1 strains. These findings highlight the potential of an adenoviral vector-based delivery system, which is both egg-independent and adjuvant-independent and offers stockpiling options for the development of a pandemic influenza vaccine.

Data from our ongoing pre-clinical studies with new vaccine strategies involving human or nonhuman adenoviral vectors aimed at providing broader protection against pandemic influenza will also be presented.

The work is supported by NIH-AI-059374 and NVPO grants.

226 Oseltamivir sensitivity of H5N1 influenza isolates, Thailand

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Surveillance for oseltamivir sensitivity was conducted in 17 H5N1 viruses (6 isolates from humans, 9 from domestic and wild birds and 2 from tigers) isolated in Thailand during 2004-2006. Plaque inhibition assay using virus at concentration of 50 pfu/well was firstly employed with good success; however it is a laborious and difficult technique to be performed on large sample size. Additionally, some influenza isolates do not form plaque which made the drug sensitivity assay by plaque inhibition impossible. Therefore, the present study established drug sensitivity assay by using ELISA nucleoprotein reduction based method.

H5N1 viruses at concentration of 100, 25 and 5 TCID50/100µl/well were tested with oseltamivir carboxylate at concentration of 800, 200, 50, 12.5, ... and 0.000003 µM in triplicate in MDCK cell monolayer. Inhibitory concentration 50 (IC50) of each virus concentration was established from the dose response curve. With the three virus concentrations tested, IC50 of the 6 human isolates varied from 0.091-36.1 (mean= 12.63), 0.006-1.68 (mean = 0.413), and 0.004-0.43 (mean= 0.134) µM; whereas those of the animal isolates varied from 0.19- >800 (mean= undetermined), 0.031- >800 (mean= undetermined), 0.0007-6.92 (mean= 0.978) µM, respectively. According to dose response curve, oseltamivir resistant virus has not been detected in our study, even though, there were at least 2 human viruses that were isolated from dead cases which had received full course of treatment before. Too high virus inoculum of 100 and 25 TCID50 in two animal isolates may explain the undetermined IC50 of the drug.

Sequencing of all viruses studied did not show mutational change at amino acid positions 119, 274, 292 and 294 in neuraminidase gene.

The present study demonstrated a wide range of IC50 of oseltamivir against human and animal H5N1 isolates. Nevertheless, oseltamivir is still the drug of choice for H5N1 patients. Failure of the treatment should be explained by other factors such as time at drug administration, the drug regimen including mixed infection with other pathogen.

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227 Use of conserved influenza antigens linked to immunostimulatory DNA (ISS) to generate broad immunity to divergent and potentially pandemic virus strains

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Standard trivalent inactivated influenza vaccines (TIVs) have several key limitations. Because TIVs induce immunity to strain-specific HA and NA antigens, they offer limited efficacy against drift virus strains and no expected efficacy against shift virus strains that can lead to pandemics. In addition, TIVs demonstrate only modest immunogenicity in high risk groups such as the elderly. To address these deficiencies, we have conjugated the conserved influenza antigens nucleoprotein (NP) and matrix protein 2, extracellular domain (M2e) to immunostimulatory DNA sequences (ISS) to generate highly immunogenic conserved antigens that can be administered alone or in combination with TIV to induce potent, broadly reactive immunity.

NP-ISS induces strong Th1 and CTL responses that reduce viral replication and provide protection against shift and drift strains in mouse challenge systems. Co-delivery of NP-ISS with TIV enhances the antibody responses to HA in both mouse and primate models. M2e-ISS induces strong IgG2a antibody responses in mice without requiring complex M2e formulations. Similar to NP-ISS, co-delivery of M2e-ISS with TIV enhances the antibody response to HA in mice.

NP-ISS and M2e-ISS represent unique vaccine components that can provide strong, cross-strain protective immunity and can be used with TIV to enhance immunogenicity and possibly provide dose sparing of the TIV components. The NP-ISS/M2e-ISS vaccine represents a promising approach to a broadly reactive, universal influenza vaccine.

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228 Acquisition and enhancement of fusogenicity by single amino acid substitutions in the spike protein are essential for adaptation and infectivity of coronavirus in cultured cells

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The cross-species transmission in coronavirus (CoV), such as SARS-CoV and human CoV-OC43, underlies the importance of studying the mechanisms of CoV host adaptation and interspecies transmission. Here we report studies on the adaptation of the Beaudette strain of infectious bronchitis virus (IBV) from chicken embryo to Vero cells and the underlying mechanisms. Following adaptation to Vero cells, a total of 49 amino acid mutations has taken place in the IBV genome, and 26 substitutions (53.06%) are located in the spike (S) protein.

Expression of S protein derived from the Vero cell-adapted strains (primary-adapted, p7; and secondary-adapted, p65) showed cell-cell fusion and syncytial formation. However, S protein obtained from the chicken embryo-adapted strain (EP3) could not cause cell-cell fusion. Construction of chimeric S genes and site-directed mutagenesis studies identified L857-F mutation in the heptad repeat 1 (HR1) is essential for the cell-cell fusion. In addition, G405-D mutation in the S1 domain enhances the fusogenicity of S protein and is responsible for the secondary adaptation. Introduction of F857-L into the S gene of the recombinant IBV virus based on the Vero cell-adapted IBV showed that the recovered virus do contain this mutation. However, only viruses with compensatory mutations at other positions of the S protein (Q523-L and I769-V; P327-S, Q523-L and I769-V) are infectious. Expression and mutagenesis studies confirmed that either Q523-L or I769-V mutation could compensate the F857-L mutation and restore the fusogenicity of the S protein. This study reveals that acquisition of fusogenicity by single amino acid substitutions in the S protein is essential for adaptation of a coronavirus from avian to mammalian species.

Hemagglutinin activity of avian influenza H5N1 viruses

Lerdsamran H, Louisirirotchanakul S, Wiriyarat W, Sangsiriwut K, Pooruk P, Kijphati R, Chaichoune K, Sawanpanyalert P, Pittayawonganon C, Ungchusak K, Auewarakul P, Puthavathana P.

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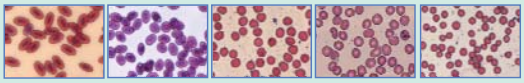
Horse hemagglutination inhibition (HI) antibody is one WHO notification for the H5N1 confirmed case. Unfortunately, horse erythrocytes can not be accessed easily in Asia countries.

Objective

To investigate the efficiency of avian influenza (H5N1) influenza viruses on erythrocytes receptor from five different species: horse, goose, chicken, guinea pig and human group O for using in both Hemagglutination (HA) and HI tests.

Materials and Methods

- Viruses : 14 human and animal H5N1 influenza viruses
- Serum samples : 15 human sera from 7 cases
- Erythrocytes from 5 species



Goose Chicken Guinea pig Human group O Horse

Table 1 : Condition of Hemagglutination test and Hemagglutination inhibition test

	Guinea pig	Human gr O	Horse	chicken	goose
Concentration (%)	0.75	0.75	1	0.5	0.5
Microtiter plate	U	U	U	V	V
Incubation time 4^0C, min	60	60	60	30	30

(Stephenson, 2003 and WHO,2002)

Results

Titration of a virus solution as using goose erythrocyte yielded the highest geometric mean titer, followed by chicken, guinea pig, human group O and horse erythrocyte (Friedman test; $p<0.05$)

Table 2. Hemagglutination titer of influenza A viruses as assayed with erythrocytes from different species

H5N1 Influenza A viruses	Passage history	* HA titer based on erythrocytes of				
		Goose	Chicken	Guinea pig	Human gr.O	Horse
A/Thailand/1(KAN-1)/04	MK2, MDCK8	512	512	128	128	128
A/Thailand/2(SP-33)/04	MDCK6	64	64	64	32	16
A/Thailand/3(SP-83)/04	MDCK5	64	64	16	32	16
A/Thailand/5(KK-494)/04	MDCK4	128	64	32	64	32
A/Thailand/676(NYK)/05	MDCK9	256	256	512	256	32
A/Tiger/Thailand/VSMU-11-SPB/2004	Egg 1	512	512	256	256	256
A/Clouded Leopard/Thailand/VSKU-6-CBI/2004	Egg 1	512	512	256	256	<2
A/Great Barbet/Thailand/VSMU-2-CBI/2005	MDCK2	128	128	64	64	32
A/Green Peafowl/Thailand/VSMU-3-CBI/2005	MDCK2	256	128	64	64	64
A/Crane/Thailand/VSMU-4-CBI/2005	MDCK2	512	256	128	128	128
A/Tree sparrow/Thailand/VSMU-16-RBR/2005	MDCK4	64	64	32	32	16
A/Golden Pheasant/Thailand/VSMU-21-SPB/2005	MDCK1	128	64	32	16	32
A/Pigeon/Thailand/VSMU-25-BKK/2005	MDCK3	64	64	32	16	16
A/Chicken/Thailand (SPB)/137/2005	MDCK4	512	512	64	64	128
Geometric Mean titer		190.21	156.03	74.25	67.25	33.62

* Only HA titer obtained from one out of three erythrocyte donors is presented

Table 3. H5N1 HI antibody titers as tested against different erythrocyte species

Serum Code (sequential blood)	A/Thailand/1(KAN-1)/04(H5N1) HI antibody titer					NT	A/Thailand/676(NYK)/05(H5N1) HI antibody titer					NT
	Goose	Chicken	Guinea pig	Human	Horse	Ab titer	Goose	Chicken	Guinea pig	Human	Horse	Ab titer
CB KAN-1 (1)	20	<20	20	<20	20	<5	20	<20	<20	<20	20	-
CB KAN-1 (2)	640	640	640	640	1280	1280	1280	1280	1280	1280	640	-
TN	20	<20	20	20	40	80	160	80	80	80	80	40
SJ	20	<20	20	20	40	160	160	80	80	80	80	80
PT (1)	20	<20	<20	20	20	<5	-	-	-	-	-	-
PT (2)	20	<20	20	<20	20	5	20	-	20	-	20	-
PT (3)	40	20	20	40	80	80	160	320	160	80	320	-
PT (4)	40	20	20	40	80	80	160	160	80	160	160	80
MY 676 (1)	<20	<20	<20	<20	<20	<5	20	20	<20	<20	<20	-
MY 676 (2)	<20	<20	<20	<20	<20	<5	20	<20	<20	<20	20	-
RB (1)	<20	<20	<20	<20	<20	5	<20	<20	<20	<20	<20	-
RB (2)	320	160	160	160	640	1280	1280	1280	1280	1280	1280	-
RB (3)	160	80	80	160	320	640	640	1280	640	640	640	-
RB (4)	20	20	20	20	40	80	160	80	80	40	40	80
BB	80	40	80	160	320	320	640	640	320	320	640	-
Geometric Mean antibody titer	36.47	22.97	28.95	33.25	57.89		118.9	116.19	80	84.38	92.81	

Table 4. Statistical values from the analyses on difference in HI antibody titers as assayed against different viruses

Viruses	Geometric mean antibody titer as using erythrocyte from:				
	Goose	Chicken	Guinea pig	Human	Horse
A/Thailand/1(KAN-1)/04	36.47	22.97	28.95	33.25	57.89
A/Thailand/676(NYK)/05	118.9	116.19	80	84.38	92.81
2-sided exact p-value (Wilcoxon's signed-rank test)	**0.001**	**0.002**	**0.004**	**0.004**	0.102

Conclusions

- Goose erythrocyte is the most sensitive for virus recognition for influenza A virus, whereas horse erythrocyte can recognize only avian influenza virus.
- Not all H5N1 isolate can agglutinate horse erythrocyte.
- Regarding to HI antibody detection, horse erythrocyte can not demonstrate in clear cut for its advantage over goose as it is depend upon the virus strain.
- Our study proposes that goose erythrocyte confers higher advantage than horse in the determination for both presence of H5N1 virus or HI influenza antibody

Acknowledgments

This study was granted by BIOTEC and Thailand Research Fund.

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Respiratory Viruses Associated with Severe Pneumonia

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Southeast Asia Influenza Clinical Research Network, Thailand

Abstract

Conventional multiplex RT-PCR using Seeplex kit and indirect immunofluorecence (IFA) assay had been used to investigate for viral agents associated with severe pneumonia. Seeplex can diagnose 12 viruses, while IFA can do so for 6 viruses. Seeplex was statistically compared with IFA by agreement over chance in the investigation of 101 patients with severe pneumonia; the index of > 0.9 was found when both tests were used to diagnose respiratory syncytial virus (RSV) infection only. When Seeplex was compared with the gold standard real time RT-PCR in the diagnosis of influenza in 110 patients, its sensitivity was 66.7% for influenza A virus (fluA) and 85.71% for influenza B virus (fluB), while its specificity was 100%. Validation of Seeplex in the diagnosis of other viral agents should be further explored. With combination of all tests, RSV was the most common virus found (19.8%), and followed in order by human metapneumovirus (13.9%), parainfluenza virus type 1 (PIV 1) (11.9%) and adenovirus (11.9%). Seeplex could detect mixed viral infections in 11 (10.9%) cases: of which adenovirus, the most common mixed agent, was found in 8 cases.

Introduction

The majority of acute upper and lower respiratory tract infections in human, especially pediatric patients, are caused by respiratory viruses such as RSV, PIV1, PIV3, adenoviruses, and influenza viruses. Laboratory assays including IFA, viral isolation, serodiagnosis, and molecular technique for viral genome detection were used for disease diagnosis. Recently, multiplex PCR using multiple primer pairs in single reaction is commercially available in order to investigate multiple agents simultaneously.

Objective

To investigate respiratory viruses associated with severe pneumonia by using multiplex RT-PCR (Seeplex) in comparison to real time RT-PCR and IFA

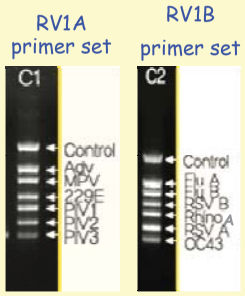
Materials and methods

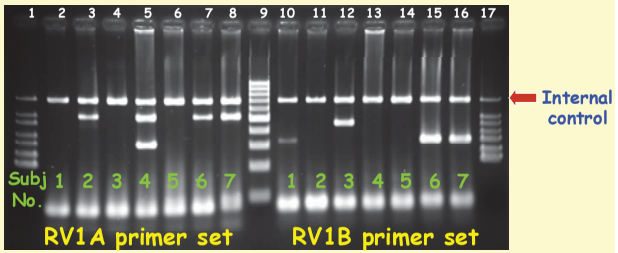
Real-time TaqMan RT-PCR: **This test** was used as the gold standard for diagnosis of fluA and B viruses.

IFA: Exfoliated cells present in NPA were used for detection of viral antigens by indirect IFA using Respiratory Panel IFA kit, Chemicon, USA. This method can diagnose the following viruses: RSV, PIV1, PIV2, PIV3, influenza A and B viruses, and adenovirus

Results

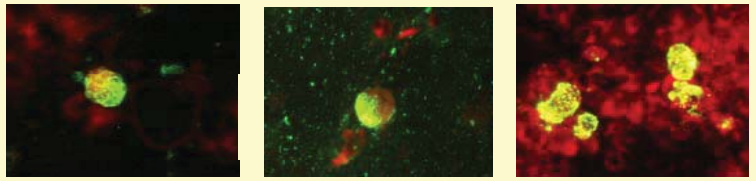
Multiplex RT-PCR for respiratory viruses (RV): RNA extracts (automated Biorobot EZ1 extraction, QIAGEN, USA) from NPA samples were reverse transcribed with random hexamer primers using Superscript III reverse transcriptase (Invitrogen, USA). Subsequently, cDNA was subjected to Seeplex RV12 ACE Detection (Seegene, Korea). This test kit includes 2 panels (RV1A and RV1B) of primer pairs specific to 12 RV: adenovirus, human metapneumovirus, coronavirus (229E), PIV1, PIV2, PIV3 in RV1A, and Flu A, Flu B, RSV B, Rhinovirus A, RSV A, and coronavirus (OC43) in RV1B.





PCR products were separated on 2.5% agarose gels

Fig 1. Immununofluorescence staining of virus infected cells in NPA. Positive cells exhibit an apple green color under fluorescence microscope.



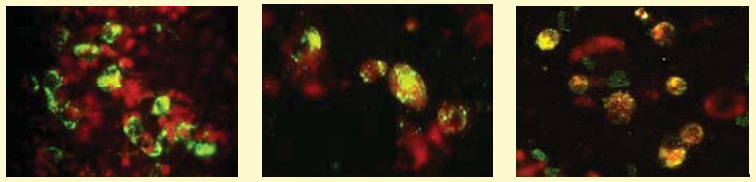


Table 1. Viral agents associated with severe pneumonia

Virus	No. of samples positive by	
	Seeplex method	IFA method
RSV A and B	17 (16.8%)	20 (19.8%)
Metapneumovirus	14 (13.9%)	Not Applicable
Parainfluenza type 1	12 (11.9%)	6 (5.9%)
Adenovirus	12 (11.9%)	5 (5.0%)
Rhino A virus	8 (7.9%)	Not Applicable
Influenza B	6 (5.9%)	5 (5.0%)
Influenza A	4 (4.0%)	5 (5.0%)
Coronavirus 229E and OC43	3 (3.0%)	Not Applicable
Parainfluenza type 3	2 (2.0%)	3 (2.97%)
Parainfluenza type 2	0 (0%)	0 (0%)
Total (n=101 cases)	78 viruses in 67 (66.3%)cases	44 viruses in 43(42.6%) cases

Table 2. Sensitivity and specificity of Seeplex and IFA for the detection of influenza virus *

Test	Virus	Sensitivity (%)	Specificity (%)	Predictive value	
				Pos	Neg
Seeplex	influenza A (n=4)	66.67	100	100	97.94
	influenza B (n=6)	85.71	100	100	98.94
IFA	influenza A (n=5)	71.43	100	100	98.10
	influenza B (n=5)	71.43	100	100	98.10

* real time RT-PCR was used as the gold standard

Among 101 cases investigated, 13 flu cases were diagnosed by real time RT PCR, while Seeplex can diagnose 10 cases

Among 110 cases investigated, 14 flu cases were diagnosed by real time RT PCR, while IFA can diagnose 10 cases

Table 3. Determination for agreement over chance index between Seeplex and IFA on the detection of respiratory viral agents

Virus	Agreement over chance index
RSV	0.96
Influenza A	0.88
Influenza B	0.71
Parainfluenza type 1	0.63
Parainfluenza type 3	0.38
Adenovirus	0.55

Index value between 0.9-1.0 indicates real agreement between two assays

Conclusion

An acute viral respiratory disease can be caused by several respiratory viruses leading to need of tools that can diagnose multiple agents simultaneously, and at the same time, require small amount of respiratory specimens. IFA, with limited supply of specific antibodies, can diagnose 7 viruses; while Seeplex can diagnose 12 viruses. Therefore, Seeplex should be superior to IFA in term of number of agents it can diagnose; and it is also less subjective and requires less experience to run multiplex PCR. However, accuracy of Seeplex has never been demonstrated. When Seeplex and IFA were statistically compared by agreement over chance, good agreement of >0.9 was obtained with RSV diagnosis only. Moreover, when Seeplex was compared with the gold standard RT-PCR for influenza diagnosis, Seeplex could detect only 10 of 13 of cases. Other tools to validate Seeplex on its capability to diagnose other viral agents are needed.

Acknowledgements

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Respiratory Viruses Associated with Severe Pneumonia

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Abstract

Conventional multiplex RT-PCR using Seeplex kit and indirect immunofluorecence (IFA) assay were used to investigate for viral agents associated with severe pneumonia in Thai patients. Our result demonstrated that > 80% of pediatric cases were associated with viral infection; and 60% of them were two years old. RSV was the most common virus found (23.6%), and followed in order by adenovirus (14.3%), Flu A (8.7%), and HuMV (8.2%). Viral infections were found only in 30% of adult cases. Seeplex could detect mixed viral infections in 14 (7.2%) cases: of which adenovirus was the most common mixed agent. Seeplex was statistically compared with IFA by agreement over chance and it was found that the agreement over 0. 80 was found with RSV, flu A and flu B only.

Objective

To investigate respiratory viruses associated with severe pneumonia by using multiplex RT-PCR (Seeplex RV detection kit, Seegene, Korea) and IFA.

Materials and Methods

Subjects: This study comprised 195 severe pneumonia patients: 163 children and 32 adults from 4 hospitals in Thailand. 99 (60.7%) pediatric cases were under two years old. Adult cases had age-range between 27 and 81, of which (71.9%) were older than 50.

Immunofluorescence assay (IFA): Exfoliated cells present in NPA were used for detection of viral antigens by indirect IFA using Respiratory Panel IFA kit, Chemicon, USA.

Multiplex RT-PCR for respiratory viruses (RV): RNA extracts from NPA samples were reverse transcribed with random hexamer primers Subsequently, cDNA was subjected to Seeplex RV Detection (Seegene, Korea). This test kit includes 2 panels (RV1A and RV1B) of primer pairs specific to 12 RV: adenovirus, human metapneumovirus, coronavirus (229E), PIV1, PIV2, PIV3 in RV1A, and Flu A, Flu B, RSV B, Rhinovirus A, RSV A, and coronavirus (OC43) in RV1B.

Results

Fig 1. Immunofluorescence staining of virus infected cells in NPA. Positive cells exhibit an apple green color under fluorescence microscope.

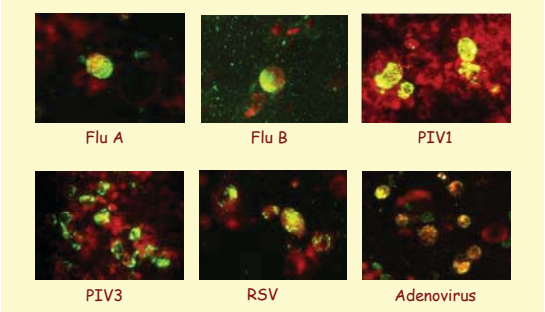
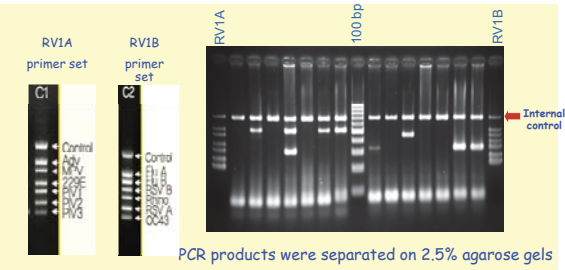


Fig 2. Seeplex® RV detection of respiratory viruses in NPA specimens.



PCR products were separated on 2.5% agarose gels

Table 1. Viral agents associated with severe pneumonia

Virus	Positive by						
	IFA			Seeplex			Grand
	Adult	Pediatric	Total	Adult	Pediatric	Total	total
Flu A	1	15	16	1	14	15	17 (8.7%)
Flu B	0	8	8	1	7	8	9 (4.6%)
PIV 1	1	6	7	1	11	12	12 (6.1%)
PIV 2	0	1	1	0	1	1	1 (0.5%)
PIV 3	1	4	5	1	4	5	6 (3.0%)
RSV	0	43	43	0	41	41	46 (23.6%)
Adenovirus	1	12	13	1	26	27	28 (14.3%)
HuMV	NA	NA	NA	2	14	16	16 (8.2%)
Rhino A virus	NA	NA	NA	1	15	16	16 (8.2%)
Coronavirus	NA	NA	NA	1	2	3	3 (1.5%)
Total viruses	4	89	93	9	135	144	154 (78.9%)
Total positive pediatric cases = 130/163 (79.7%) Total positive adult cases = 10/32 (31.2%)							

Table 2. Investigation for viral agents by IFA and Seeplex

		Seeplex (+)	Seeplex (-)	Total
IFA	(+) Flu A	14	2	16
	(-) Flu A	1	178	179
Total		15	180	195

		Seeplex (+)	Seeplex (-)	Total
IFA	(+) Flu B	7	1	8
	(-) Flu B	1	186	187
Total		8	187	195

		Seeplex (+)	Seeplex (-)	Total
IFA	(+) RSV	38	5	43
	(-) RSV	3	149	152
Total		41	154	195

		Seeplex (+)	Seeplex (-)	Total
IFA	(+) Adeno	12	1	13
	(-) Adeno	15	167	182
Total		27	168	195

		Seeplex (+)	Seeplex (-)	Total
IFA	(+) PIV 1	7	0	7
	(-) PIV 1	5	183	188
Total		12	183	195

		Seeplex (+)	Seeplex (-)	Total
IFA	(+) PIV 2	1	0	1
	(-) PIV 2	0	194	194
Total		1	194	195

		Seeplex (+)	Seeplex (-)	Total
IFA	(+) PIV 3	4	1	5
	(-) PIV 3	1	189	190
Total		5	190	195

Table 3. Determination for agreement over chance index between Seeplex and IFA on the detection of respiratory viral agents

Virus	Agreement over chance index
PIV 2	1.00
Flu A	0.89
RSV	0.88
Flu B	0.87
PIV 3	0.79
PIV 1	0.72
Adenovirus	0.56

Index value between 0.9-1.0 indicates real agreement between two assays

Conclusions

An acute viral respiratory disease can be caused by several respiratory viruses leading to need of tools that can diagnose multiple agents simultaneously, and at the same time, require small amount of respiratory specimens. Seeplex should be superior to IFA in term of number of agents it can diagnose; and it is also less subjective and requires less experience to run multiplex PCR. However, accuracy of Seeplex has never been demonstrated.

Acknowledgements

This study was supported by Thailand Research Fund for Senior Research Scholar, Mahidol University Research Grant, and South East Asia Infectious Disease Clinical Research Network (SEAICRN)

Human Influenza Virus-Specific T Cells Mediated Cross Reactive Immune Response to Nucleoprotein (NP) Derived from Avian Influenza H5N1 Virus

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[1]Department of Microbiology; [2]Medical Molecular Biology Unit, Office for Research; and [3]Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Rd., Bangkok-noi, Bangkok 10700, THAILAND.

Background:

Protection against influenza A virus by specific antibody is relatively strain specific; meanwhile, broader immunity may be conferred by cell mediated immune response (CMIR) (1). Therefore, development of universal influenza vaccine that can confront seasonal influenza viruses as well as avian influenza H5N1 viruses of multiple clades, is desirable. Previous studies had shown that immunity induced by old influenza isolates could alleviate the severity of disease caused by novel strains or subtypes. It is suggested that this cross immunity is mediated by the heterosubtypic T-cell response. In this context, induction of cross-reactive $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes (CTL) that recognize conserved epitopes in internal protein of influenza virus are of interest (2,3). This prompted us to identify and characterize a set of T cell conserved epitopes that would contribute to broad immune response across influenza virus subtypes. This study measured cross cell-mediated immunity to H5N1 virus in healthy subjects by ELISpot and flow cytometry assays using overlapping peptides derived from nucleoprotein (NP) as the test antigen.

Materials and Methods:

A total of 30 subjects included 23 healthy individuals in which 18 had history of receiving seasonal influenza vaccine at least once, and 7 subjects who had history of influenza infection one month ago. Peripheral blood mononuclear cells (PBMCs) from these subjects were kept frozen until used. A panel of 20 mer peptides with 10 mers overlapping, were synthesized based on amino acid sequence derived from NP of A/Thailand/1(KAN-1)/2004 (H5N1) virus (KAN-1 virus) (accession number AAV 35112). Totally, this panel comprised 49 peptides. Memory T cells that cross-reacted to these NP overlapping peptides were measured by the *ex vivo* IFN-γ enzyme-linked immunospot (ELISpot) assay. Flow cytometry employing intracellular cytokine staining (ICS) was used for immunophenotyping of the peptide restricted T cells. Microneutralization (MicroNT) assay to KAN-1 virus was performed to determine status of previous H5N1 virus infection.

A)

```
>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
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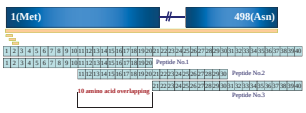
B)



C)

Nucleoprotein H5N1 peptide pools

Pool	A1	A2	A3	A4	A5	A6	A7
B1	NP5-1	NP5-2	NP5-3	NP5-4	NP5-5	NP5-6	NP5-7
B2	NP5-8	NP5-9	NP5-10	NP5-11	NP5-12	NP5-13	NP5-14
B3	NP5-15	NP5-16	NP5-17	NP5-18	NP5-19	NP5-20	NP5-21
B4	NP5-22	NP5-23	NP5-24	NP5-25	NP5-26	NP5-27	NP5-28
B5	NP5-29	NP5-30	NP5-31	NP5-32	NP5-33	NP5-34	NP5-35
B6	NP5-36	NP5-37	NP5-38	NP5-39	NP5-40	NP5-41	NP5-42
B7	NP5-43	NP5-44	NP5-45	NP5-46	NP5-47	NP5-48	NP5-49

Fig. 1 Amino acid sequence of NP of KAN-1 virus (A); NP overlapping peptides were synthesized by Sigma-Genosys (Sigma-Aldrich, Singapore) in the PEPscreen® custom peptide libraries format. A total of 49 peptides are overlapped by 10 amino acid residues (B); Peptides were pooled in a 2-dimensional matrix system (C).

Results:

All 30 subjects had no antibody to H5N1 virus as tested by microNT assay. However, 13 (43%) subjects exhibited cross-reactive specific T cell response by ELISpot. Altogether, these subjects recognized only 4 of 49 peptides tested (Fig. 3A). The average magnitudes of *ex vivo* ELISpot IFN-γ were 63 SFU/10^6 PBMCs, and the frequency of responsiveness was 1 to 2 peptides/subject (Fig. 3B). $CD4^+$ T cells were mainly responsible for peptide recognition as they were present in all 13 responders; while $CD8^+$ T cells were found only in one responder. Examples are shown in Fig. 4. There was only one responder who had both peptide specific $CD4^+$ and $CD8^+$ T cells, but both types of cells recognized different epitopes. In addition, there was one peptide that can stimulate both $CD4^+$ and $CD8^+$ T cell response (Table 1).

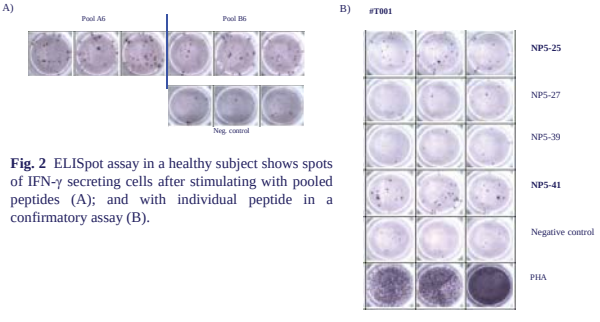


Fig. 2 ELISpot assay in a healthy subject shows spots of IFN-γ secreting cells after stimulating with pooled peptides (A); and with individual peptide in a confirmatory assay (B).

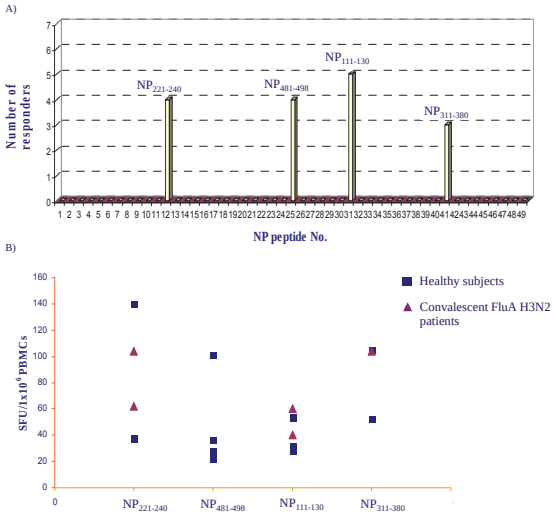


Fig. 3 Peptide recognition and frequency of responsiveness, $NP_{221-240}$ (RMCNILKGKFQTAAQRAMMD) denotes NP5-12, $NP_{418-498}$ (MNNEGSYFFGDNAEEYDN) denotes NP5-25, $NP_{111-130}$ (YDKEEIRRIWRQANNGEDAT) denotes NP5-31, and $NP_{311-380}$ (QVFSLIRPNENPAHKSQLVW) denotes NP5-41 in the 2 dimentional matrix system (A); magnitudes of ELISpot response in 13 responders (B).

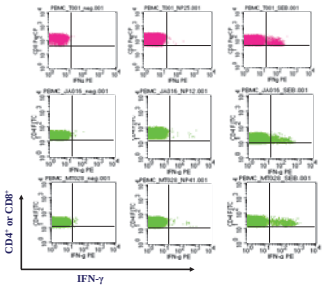


Fig. 4 Immnophenotyping of peptide-restricted T cells by ICS technique. Staphylococcal enterotoxin B (SEB) is used as the positive control.

Table 1. Frequency of NP peptides recognition by $CD4^+$ or $CD8^+$ T cells from 13 responders

Responders	NP peptide recognized by T cells							
	$NP5\text{-}12_{(NP\ 221\text{-}240)}$		$NP5\text{-}25_{(NP\ 481\text{-}498)}$		$NP5\text{-}31_{(NP\ 111\text{-}130)}$		$NP5\text{-}41_{(NP\ 311\text{-}330)}$	
	$CD4^+$ T cells	$CD8^+$ T cells	$CD4^+$ T cells	$CD8^+$ T cells	$CD4^+$ T cells	$CD8^+$ T cells	$CD4^+$ T cells	$CD8^+$ T cells
Healthy (n=9)	2	-	3	1	3	-	2	-
Convalescent FluA patients (n=4)	2	-	-	-	2	-	1	-

Conclusions:

Healthy individuals who had never been infected with H5N1 virus may exhibit cross cellular immunity against this virus as assayed by ELISpot and flow cytometry using overlapping peptides derived from H5N1 NP. Four NP immunodominants were identified. Our study may be useful in the vaccine design and understanding of natural T cell immunity.

Acknowledgement:

This study was supported by the Thailand Research Fund for Senior Research Scholar, and the National Science and Technology Development Agency.

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Electron micrographs of H5N1 virus and its replication in MDCK cells

Pornparn Rojanasang[1], Suda Louisirirotchanakul [1], Kleophant Thakergpol [2], Naree Choosrichom[2], Witthawat Wiriyarat[3], Kridsada Chaichoune[3], Robert Webster[4], Pilaipan Puthavathana [1, 5]

[1]Departments of Microbiology and [2]Pathology; [5]Siriraj Influenza Cooperative Research Center (ICRC), Faculty of Medicine Siriraj Hospital and [3]Faculty of Veterinary Science, Mahidol University, Bangkok, Thailand; [4]Department of Infectious Diseases, St. Jude Children's Research Hospital, Tennessee, USA.

Background: Since 2003, re-emergence of highly pathogenic avian influenza (HPAI) H5N1 virus has swept across continents and causes poultry die-off including severe pneumonia in humans. Morphology of this influenza virus subtype has never been characterized. In this study, we demonstrated electron micrographs of HPAI H5N1 particles together with low pathogenic avian influenza (LPAI) viruses. In addition, localization of H5N1 viruses in MDCK infected cells was shown under a transmission electron microscope (TEM).

Materials and methods: The viruses employed in this study included 9 HPAI H5N1 (4 from human and 5 from avian); 4 LPAI virus subtypes H1N1, H3N2, H5N3 and H7N1; and 2 human isolates of H1N1 and H3N2 subtypes. Human viruses were isolated and propagated in cell culture, whereas avian viruses were isolated and propagated in embryonated eggs. These viruses, except one LPAI virus with uncertain passage history, were sub-cultivation for less than 10 passages. The virus particles were visualized by negative staining with 2% phosphotungstic acid. Meanwhile, thin sections of MDCK cells infected with A/Thailand/1(KAN-1)/04 (H5N1) were positively double stained with uranyl acetate and lead citrate. The grids were examined under a transmission electron microscope (JEOL 1230, Japan).

Results: Influenza virus particles were pleomorphic. Three forms of morphology of different sizes were demonstrated: spherical (diameter 80-120 nm), rod (length 121-300 nm) and filamentous (length >300 nm) (Fig. 1). Regarding virus replication in MDCK cells, we demonstrated ultrastructure of virus inclusions in the cell cytoplasm, including virus particles at entry and the budding out particles at exit (Fig. 2).

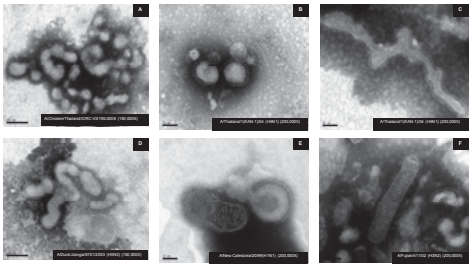

Fig 1. Pleomorphic morphology of influenza A virus from human and avian isolates in culture fluid with negative staining 1) H5N1 HPAI of A/Chicken/Thailand/ICRC-VS195/08 [A] and A/Thailand/1(KAN-1)/04 [B and C];
2) LPAI of A/Duck/Jiangxi/6151/03 (H5N3) [D]; human influenza A/New Caledonia/20/99 (H1N1) [E] and A/Fujian/411/02 (H3N2) [F] strains.

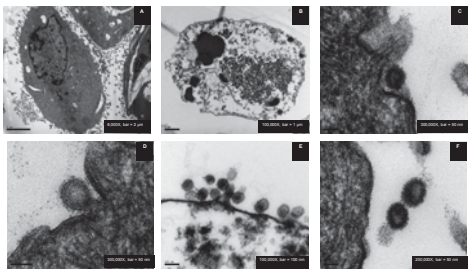

Fig 2. Electron micrograph of H5N1 A/Thailand/1(KAN-1)/04 in MDCK cell culture with positive staining.
Negative control [A] and the infected cell [B]. Attachment of the virus onto the cell [C] and budding out from the infected cell [D] and a large number of viruses releasing from the infected cells [E and F].

Conclusions: Electron micrographs of influenza virus particles demonstrated that spherical form was the predominate population among all virus subtypes studied, regardless of host of origin, virus virulence or passage history. Positive staining of uninfected MDCK cells as observed at low magnification showed surface pilli which might be miss interpreted as the filamentous form of virus particles. Nevertheless, this appearance could be identified by higher resolution and magnification of the modern electron microscope. Herein, we showed lines of spherical particles budding from the cell surface. Our finding was not different from what had been previously reported with human viruses.

Acknowledgements:
We thank the skillful technical assistance of the personnel from Virology Laboratory of Department microbiology and Veterinary Science, Mahidol University, for providing human and animal influenza viruses collection. And electron microscope team, Department of pathology, Faculty of Medicine Siriraj Hospital for assistance with photographs

This study was supported by Thailand Research fund for Senior Research Scholar through Prof. Pilaipan Puthavatana

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Surveillance for Sensitivity of Influenza A and B Viruses to Neuraminidase Inhibitors

Hatairat Lerdsamran[1], Phisanu Pooruk[1], Achareeya Korkusol[1], Kantima Sangsiriwut[1, 2], Pirom Noisumdaeng[1], Pisut Komolsiri[1], Kannika Nateerom[1], Charoen Chuchottaworn[3], Chariya Sangsajja[4], Tawee Chotpitayasunondh[5], Kulkanya Chokephaibulkit[6], Pilaipan Puthavathana[1]

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Background

Oseltamivir and zanamivir are neuraminidase inhibitors against both influenza A and B viruses. Monitoring for neuraminidase inhibitor resistant viruses can be accomplished by phenotypic and genotypic based assays. Our study employed neuraminidase inhibition (NAI) assay together with nucleotide sequence analysis for amino acid substitution in *NA* gene to determine sensitivity of influenza A and B viruses to oseltamivir and zanamivir.

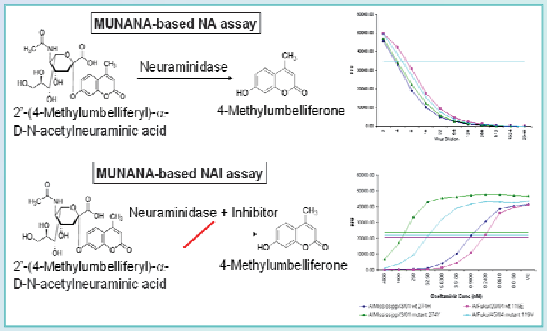
Materials and Methods

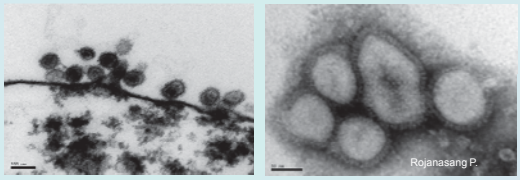
Test viruses:

38 influenza virus isolates from 2007-2009
- 8 pandemic influenza A/H1N1 isolates
- 6 human influenza A/H1N1 isolates
- 15 human influenza A/H3N2 isolates
- 9 influenza B isolates

Reference viruses:
- A/Mississippi/03/01(H1N1)wild type-274H and its resistant mutant-274Y
- A/Fukui/20/04 (H3N2) wild type (119E)
- A/Fukui/45/04 (H3N2) mutant (119V)

MUNANA-based NAI assay | NA gene sequencing





Results

Table. Drug sensitivity and NA molecular marker of influenza A/H1N1, A/H3N2, pandemic influenza A/H1N1/2009 and influenza B viruses

Viruses	IC_{50} values (nM)		Resistant markers						
			Oseltamivir				Zanamivir		
	Oseltamivir	Zanamivir	E119V	D198N	H274Y	R292K	E119G/A/D	R152K	R292K
Reference viruses									
A/Mississippi/03/01(H1N1) wildtype-274H	1.53	1.18			H				
A/Mississippi/03/01(H1N1) mutant-274Y	591.66	2.26			Y				
A/Fukui/20/04(H3N2) wildtype-119E	0.31	0.67	E				E		
A/Fukui/45/04(H3N2) mutant-119V	72.63	5.74	V				V		
H1N1 viruses (N=6)									
3043	875.25	3.89	E	D	Y	R	E	R	R
3422	1.27	3.54	E	D	H	R	E	R	R
3461	2.16	2.15	E	D	H	R	E	R	R
3479	391.16	1.53	E	D	Y	R	E	R	R
3509	584.91	2.33	E	D	Y	R	E	R	R
3619	646.77	1.02	E	D	Y	R	E	R	R
Range	1.27- 875.25	1.02-3.89							
Median	488.04	2.24							
Mean	416.92	2.41							
H3N2 viruses (N=15)			E	D	H	R	E	R	R
Range	0.29-1.04	0.25-2.64	for all isolates						
Median	0.5	0.62							
Mean	0.54	1.05							
Pandemic H1N1 viruses (N=8)									
Range	0.5-1.43	0.27-1.03	Not done						
Median	1.08	0.69							
Mean	1.10	0.68							
Influenza B viruses (N=9)									
Range	10.97- 45.83	5.26-23.34	Not done						
Median	23.93	6.58							
Mean	25.24	10.87							

- Both seasonal and pandemic influenza A/H1N1 together with A/H3N2 isolates were zanamivir sensitive.
- All pandemic A/H1N1 and A/H3N2 isolates were oseltamivir sensitive; whereas, 4 of 6 (66.7%) seasonal A/H1N1 viruses were oseltamivir resistant viruses
- All influenza B isolates showed a decrease in sensitivity to both oseltamivir and zanamivir
- NA sequencing data showed the molecular marker H274Y in seasonal A/H1N1 oseltamivir resistant viruses

Acknowledgements

This study was supported by the Thailand Research Fund for Senior Research Scholar, and the South East Asia Infectious Disease Clinical Research Network.

Conclusion

Oseltamivir resistant seasonal H1N1 virus is a growing problem worldwide. Nevertheless, influenza B viruses should be closely monitored for emerging of neuraminidase inhibitor resistance as it has been shown to require higher dose for the disease treatment.

Hemagglutinin-Inhibition and Microneutralization Assays for Antibodies to Pandemic Influenza A (H1N1) 2009 Virus

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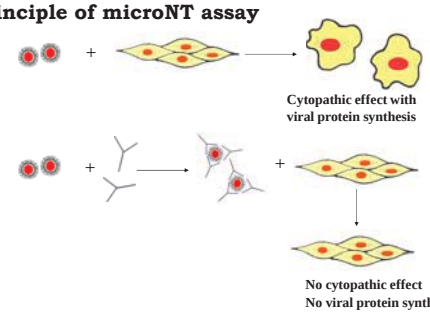
Introduction

Hemagglutinin-inhibition (HI) assay has been established for decades for human influenza diagnosis and evaluation of vaccine immunogenicity. Additionally, on the basis of antigenic drift, HI employing a panel of reference antisera is used for vaccine strain selection by the World Health Organization annually. In contrary, microneutralization (microNT) assay is recommended for detection of antibody to H5N1 and pandemic influenza A (H1N1) 2009 viruses. The present study applied those two assays to determine the cut-off titer for diagnosis of the pandemic influenza A(H1N1) 2009.

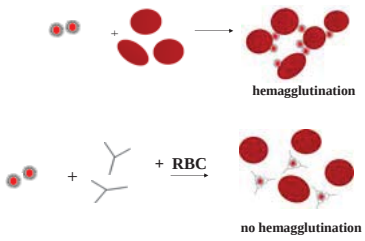
Material and Method

A total of 37 patients (9 children and 28 adults) who were diagnosed pandemic influenza A(H1N1) 2009 by RT-PCR, were enrolled. There were 18 subjects whose first blood samples were collected at ≤ 7 days after disease onset, and later for the remaining cases. Serum samples were assayed for specific antibody by goose erythrocyte HI and ELISA based microNT using A/Thailand 104/09 (H1N1) as the test virus.

Principle of microNT assay



Principle of HI assay



Result:

Rising in antibody titer could be demonstrated mostly in patients whose first blood samples were collected within 7 days and convalescent blood samples were collected between 3 to 5 weeks after onset of disease. Nevertheless, frequency of a four folded rise in HI or microNT titer was low, even in subjects whose first blood samples were collected earlier. This is according to presence of high antibody level in the first blood samples (Table 1 and Figs 1A and 1B).

Table 1. Serodiagnosis of pandemic influenza 2009

Patient	No. cases	Number with a 4-folded rise in antibody titer	
		MicroNT	HI
Adult	28	3	6
Children	9	5	4
Total	37	8 (21.6%)	10 (27.0%)

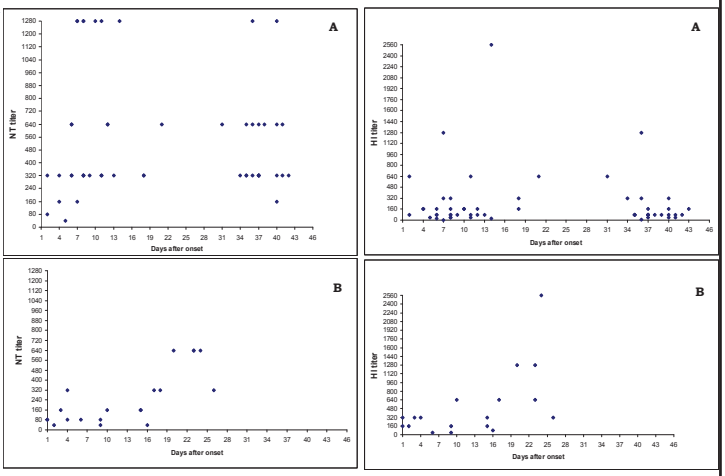


Fig.1 Antibody response in paired blood from adult (A), and pediatric cases (B)

Table 2. Cut-off titers for disease diagnosis

Criteria	Percentages of sensitivity
MicroNT titer ≥ 320	91.9
HI titer ≥ 40	97.3
MicroNT titer ≥ 320 and HI titer ≥ 40	89.2

Conclusion:

Previous seasonal H1N1 infection might boost an anamnestic response, and then, results in a rapid rise in antibody level in acute blood sample such that a marked increase in antibody titer in convalescent blood samples cannot be shown in most of the patients. Therefore, serodiagnosis does not help much in disease diagnosis, but it will be useful for epidemiological study. Our preliminary result proposed the cut-off titers for the diagnosis of pandemic influenza 2009 as shown in Table 2. More data is needed before this criteria is firmly established.

Acknowledgement: This study is supported by the Thailand Research Fund for Senior Research Scholar and the South East Asia Infectious Disease Clinical Research Network (SEAICRN), Thailand.

Viral Load of Pandemic Influenza A (H1N1) 2009 Virus in Various Types of Respiratory Specimens

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Background: Nasopharyngeal aspirate (NPA), nasal swab (NS), and throat swab (TS) are common specimens used for respiratory virus diagnosis by PCR/RT-PCR, antigen detection and viral isolation. However, there is no documented data regarding a type of specimen that yields the best result for viral detection. In this study, real time RT-PCR specific for *M* gene of influenza A virus, was used to determine viral load present in NPA, NS and TS samples collected from the same patient with pandemic A/2009 (H1N1) infection. Copy numbers of *M* genomic segments present in each type of specimen were compared. The results, then, can guide clinicians in making choice of clinical samples for the H1N1 2009 diagnosis.

Materials and methods: A total of 12 patients: 2 adults and 10 pediatric cases, with severe pneumonia caused by pandemic influenza A (H1N1) 2009 virus were enrolled in the study. From each patient, NPA, NS and TS swabs were collected and put into each tube containing 2 ml of viral transport media (VTM). Viral load present in each kind of clinical sample was determined based on copy number of *M* genomic segments. Briefly, known copy numbers of *in vitro M* RNA transcripts derived from pandemic influenza A (H1N1) 2009 virus were used as standard RNA, and amplified in parallel with the test sample by real time RT-PCR using CDC protocol version 2009. Alignment of primers/probe against the pandemic 2009 viruses is shown in Fig.1. Standard curve of *M* copy numbers was constructed from the C(t) values of standard RNA transcripts; and then, copy number of *M* segments in the test sample was obtained by extrapolation of its C(t) value against this standard curve (Fig.2).

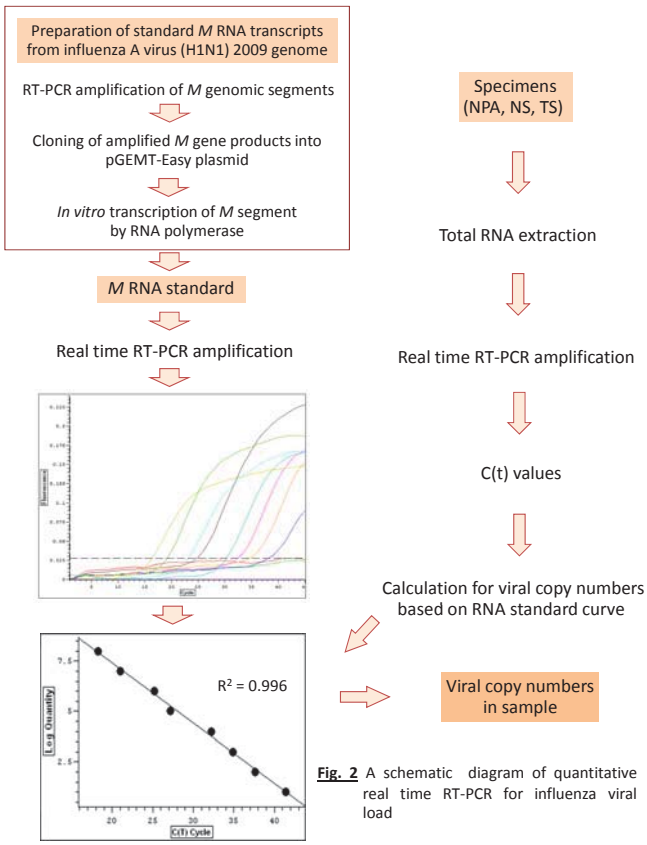

Fig. 2 A schematic diagram of quantitative real time RT-PCR for influenza viral load

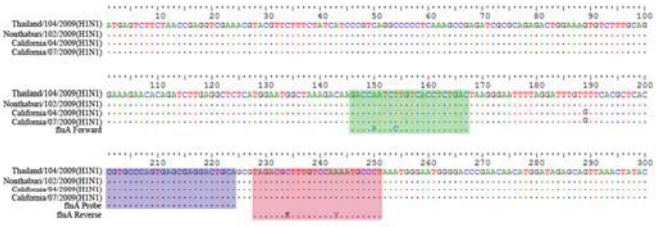

Fig. 1 Sequence alignment of primers/probe set against *M* gene of influenza A (H1N1) 2009 viruses

Results: Of 12 subjects, *M* RNA segments were detected in 12 NPA, 12 NS and 9 TS samples (Table 1). NPA specimens were found to contain the highest amount of influenza viral load, and followed in order by NS and TS samples. Mean copy numbers of viral load in NPA specimens was 1.54×10^8 (range 6.54×10^5 - 8.10×10^8) copies/ml of VTM; and it was 6.53×10^7 (range 5.62×10^2 - 4.93×10^8) copies/ml for NS and 1.05×10^6 (range 0 - 7.38×10^6) copies/ml for TS (Table 2). Examples of real time RT-PCR results are shown in Fig.3.

Table 1 Detection rate of *M* gene in each kind of specimen by real time RT-PCR

Number of subjects	Number of positive cases		
	NPA	NS	TS
12	12 (100%)	12 (100%)	9 (75%)

Table 2 Mean, median and range of copy numbers of viral load in each kind of sample

Virus	Statistics	NPA	NS	TS
H1N1/2009	Mean	1.54×10^8	6.53×10^7	1.05×10^6
	Median	4.02×10^7	8.49×10^6	7.56×10^4
	Range	6.54×10^5 - 8.10×10^8	5.62×10^2 - 4.93×10^8	0 - 7.38×10^6

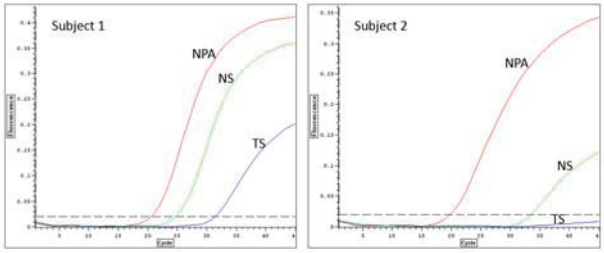

Fig. 3 Examples of real time RT-PCR results

Conclusion: Based on *M* gene copy numbers, we conclude that NPA is the best specimen for detection of pandemic influenza A (H1N1) 2009 virus , and followed in order by NS and TS.

Acknowledgement: This study is supported by Thailand Research Fund for Senior Research Scholar. We thank South East Asia Infectious Disease Clinical Research Network (SEAICRN), Thailand for providing clinical specimens.

Seroprevalence to HPAI H5N1 virus in healthy backyards chickens living in repeated outbreak areas of Thailand

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Background:
Thailand has been affected by several waves of H5N1 HPAI outbreaks since 2004. AI control with biosecurity is strictly implemented in industrial poultry farms. However, this type of control is costly and difficult to implement in villages where flocks of backyard poultry are raised for family consumption. Even though, there has been no human case since August 2006, AI is occasionally reported in free ranging birds in remote areas. Thai native chickens and free ranging ducks are relatively resistant to HPAI H5N1 virus. Some may be infected without developing clinical signs and death. Viruses shed from these infected poultry may contaminate the environment and become sources of viral spread in nature. Therefore, this study conducted serosurveillance to determine H5N1 virus infection rate in backyard, Thai native chickens resided in AI repeated outbreak areas.

Materials and methods:
This study was approved by the Ethical Committee for Animal Health and Welfare, Faculty of Veterinary Science and the Animal Assurance PHS from Office of Laboratory Animal Welfare, USA (OLAW No. A5731-01). The study was carried out during January 2007 to August 2009 in 6 provinces: *Nakhonsawan, Lopburi,* Sukhothai, *Phitsanulok,* Kamphaeng Phet and Uttradit. Blood samples were collected from backyards chickens with consent from the owners. ELISA based microneutralization assay in MDCK cell monolayer were conducted in biosafety level-3 containment facilities. The reciprocal antibody titer of 40 was the cut-off level for positive result.

Results:
Among 707 healthy backyards chickens, 12 (1.7%) had H5N1 neutralizing antibody. By location, 6 chickens were *in Phitsanulok*, 5 in Sukhothai and one in Uttradit provinces.

Table 1. Seroprevalence to HPAI H5N1 virus in healthy backyards chickens

Provinces	No. of positive/ no. of chickens	Seropositive (%)
Nakhonsawan	0/4	0
Kamphaeng Phet	0/6	0
Phitsanulok	6/40	15
Lopburi	0/6	0
Sukhothai	5/588	0.85
Phichit	0/1	0
Uttaradit	1/62	1.61
Total	**12/707**	**1.70**

Conclusion:
The study demonstrated that backyards chickens resided in repeated outbreak areas, could survive H5N1 infection. Asymptomatically infected chickens may play role as silent spreaders and maintain the virus in nature. Outbreak may occur when the virus is spread to population of susceptible animals. Our finding lead to the suggestion that HPAI H5N1 virus might has become an endogenous pathogen of Thailand.

Acknowledgement:
This study supported by US. CDC (Cooperative Agreement Number 1U19CI00399-01). The study was also partial supported by Thailand Research Fund for Senior Research Scholar through P.P., the grant PI.

Seroprevalence of HPAI H5N1 Virus in Domestic Dogs and Cats Living in Repeated Outbreak Areas in Thailand

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[1] Faculty of Veterinary Science, [2] Faculty of Science, [3] Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand, [4]Bureau of Epidemiology, Thai Ministry of Public Health

Background: Highly pathogenic avian influenza (HPAI) H5N1 virus had been documented for its infectivity in several kinds of mammals including tigers and domestic dogs and cats which are the most popular pets of humans. HPAI has been well controlled; and no human cases occurred in human since August 2006. However, small outbreaks occasionally occur in backyard poultry, together with the infection in wild birds and mammal species. Thus, there is a possibility that dogs and cats, especially those living in H5N1 repeated outbreak areas or suspected outbreak areas where abnormal death of poultry were found, have high risk of exposure to H5N1 virus from eating the infected animal carcasses or closed contact with the infected animals. To demonstrate this, we performed serological surveillance in dogs and cats living in 5 provinces where H5N1 HPAI outbreaks or suspected outbreaks were found, by using microneutralization (microNT) assay.

Materials and methods: This study was carried out during January 2007 to August 2009 in dogs and cats living in 5 provinces where H5N1 HPAI outbreaks or suspected outbreaks repeatedly occurred, i.e., Sukhothai, Phichit, Phitsanulok, Uttaradit and Kamphaeng Phet. The study protocol was approved by the Ethical Committee for Animal Health and Welfare, Faculty of Veterinary Science and Animal Assurance PHS from Office of Laboratory Animal Welfare, USA (OLAW No. A5731-01). Blood sample collection was employed under consent from the animal owners. ELISA based microNT assay in MDCK monolayer was performed in biosafety level-3 containment facilities. The reciprocal antibody titer 40 was established as the cut-off level for positive result.

Results: Totally, serum samples from 157 dogs and 42 cats were tested. H5N1 NT antibody was detected in 25 (15.9%) dogs and 2 (4.7%) cats. Highest prevalence was found in animals from Phitsanulok province, and followed in order by Phichit and Sukhothai provinces.

Table 1. Seroprevalence of HPAI H5N1 virus in domestic dogs and cats

Animal	Provinces	No. of positive/ no. of animals	Seropositive (%)
Dog	Nakhonsawan	0/1	0
	Kamphaeng Phet	0/2	0
	Uttaradit	0/3	0
	Phichit	1/3	33.33
	Phitsanulok	8/17	47.06
	Sukhothai	16/131	12.21
	Total	**25/157**	**15.92**
Cat	Nakhonsawan	0/0	0
	Kamphaeng Phet	0/1	0
	Uttaradit	0/3	0
	Phichit	0/5	0
	Phitsanulok	2/5	40
	Sukhothai	0/28	0
	Total	**2/42**	**4.76**

Conclusion: We demonstrated that dogs and cats living in the H5N1 outbreak areas could be infected asymptomatically or developed only mild symptom. However, time at infection is not known. Dogs and cats can serve as a potential source of HPAI spread in addition to avian species.

Acknowledgement: This study supported by US. CDC (Cooperative Agreement Number 1U19CI00399-01). The study was also partial supported by Thailand Research Fund for Senior Research Scholar through P.P., the grant PI.

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Satellite Telemetry Reveals Migratory Routes of Brown-headed Gulls (*Larus brunnicephalus*) Captured in Thailand.

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Background

A flock of brown headed-gulls (*Larus brunnicephalus*) is seen along the muddy beach at Bang Pu, Samut Prakarn every year during November to March. However, its long distant migratory route has never been reported. Our group could isolate HPAI H5N1 virus from 4 (2.6 %) of 153 birds of this flock in 2005 and 2008. Thus, this flock might play a role in the spread of HPAI virus along its fly ways. The present study conducted satellite telemetry technique to demonstrate the migratory routes of this bird species, including an attempt to isolate H5N1 virus and detect specific antibody by ELISA and microneutralization (microNT) assay.

Methods

Gulls negative for influenza A antigen were tagged with solar powered satellite transmitters (Microwave); and their movements were monitored by Argos satellite tracking system. Most locations, used for analysis and mapping with Google Earth Programme version 4.3, had a precision of <1500 m. Tracheal and cloacal swabs as well as serum samples from birds were collected at convenience, while the flock stayed in Thailand.

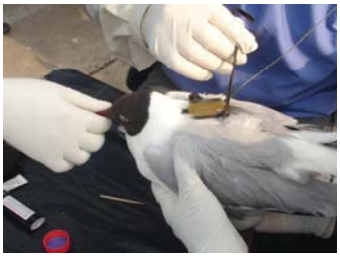

Figure 1 : Brown headed-gull (*Larus brunnicephalus*) was being tagged with solar powered satellite transmitters.

Results

We started tracking the first gull in March 2008 and the second group of 7 gulls between February and March 2009. Collective data from the two experiments confirmed that gulls migrated out of Thailand in April. Five of them flew to Xinjiang, Qinhai and Tibet, places of their long stay. The first gull moved from Tibet to West Bengal, India in October before coming back to Thailand. Nevertheless, this bird stayed in Thailand only shortly; then, it flew to Siem Reap, Cambodia in late October and inhabited there until the signals lost in December 2008. Birds could fly at a distance of 330.07 km/day in average. MicroNT assay was performed in 85 birds, and the results were all negative; meanwhile 3.5 % of samples were positive by ELISA for pan-influenza A antibody.

Figure 2 : Migratory route of a Brown headed-gull during March – December 2008.

Conclusions

Brown-headed gulls had migrated across several countries. However, it needs to be further explored that gulls can be infected asymptomatically and the infected gulls can migrate that far. Then, role of this species in the spread of H5N1 virus can be concluded.

Acknowledgement

This study supported by US. CDC (Cooperative Agreement Number 1U19CI00399-01). The study was also partial supported by Thailand Research Fund for Senior Research Scholar through P.P. (the grant PI).

Neuraminidase Inhibitor Resistant Seasonal Influenza H1N1 Viruses in Thailand During 2006 to 2009

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Introduction:

Two neuraminidase inhibitors (NAI), oseltamivir and zanamivir, have been used for treatment and prophylaxis of influenza A and B viruses. At present, almost 100% of seasonal influenza H1N1 viruses are resistant to oseltamivir owing to mutational change from histidine to tyrosine at amino acid position 274 (H274Y in N2 numbering) in NA molecule . Nevertheless, the H274Y mutant viruses are still sensitive to zanamivir. The H274Y mutation was seen in N1 in both cell culture passaged viruses and clinical specimens from immunocompetent or immunocompromised patients treated with oseltamivir. Our study conducted a surveillance of oseltamivir and zanamivir resistant seasonal H1N1 viruses prevalent in Bangkok and rural area of Thailand between 2006 and 2009 by using the fluorometric based NA inhibition assay as well as nucleotide sequencing for mutational change in N gene. Our study provided additional data from different geographical sites for both drugs as well as the earlier time period of investigation.

Materials and Methods:

Seasonal influenza H1N1 viruses from 3 provinces of Thailand during 2006 to 2009 : 28 virus isolates and 41 respiratory samples.

Neuraminidase (NA) inhibition assay using MUNANA substrate was used to determine 50% inhibitory concentration (IC50) of the virus isolates

Direct nucleotide sequencing of PCR products derived from amplification of *N* genomic segments present in clinical samples was performed. The nucleotide sequences were analyzed for amino acid mutation position indicating of drug resistance.

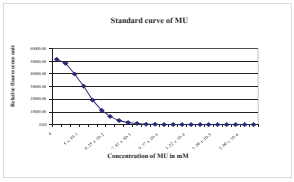
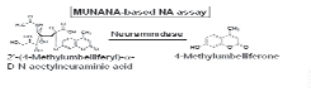


Fig 1. Standard curve of 4-methylumbelliferone: The MU solution was serially two fold diluted (4 to 10^{-6} mM), then the RFU (relative fluorescence unit) values obtained were plotted against MU concentrations to generate a standard curve. Therefore, the amount of viruses that yielded RFU value between 20,000 and 40,000 was selected as the standard dose. In general, all tested viruses in our study were assayed at the RFU value about 30,000.



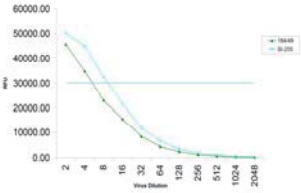
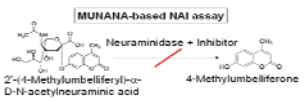


Fig 2. NA activity assay of SI-255 and U184/49 virus isolates. These two viruses will be used at the dilutions of 1: 8 and 1: 4, respectively in the NA inhibition assay



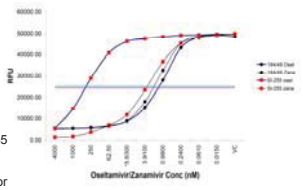


Fig 3. NA inhibition assay of U184/49 and SI-255 against oseltamivir and zanamivir.
U184/49 is sensitive to both drugs (IC50 = 1.46 nM for oseltamivir, and 2.51 nM for zanamivir), while SI-255 is sensitive to zanamivir (IC50=3.31 nM), but resistant to oseltamivir (IC50 = 372.18 nM)

Fig 4. Chromatogram of N1 gene of mutant show changing of amino acid position at position 274

Table 1. Mutation positions indicating NA inhibitor resistance

NA subtype	NA mutation positions (N2 numbering)	
	Oseltamivir	Zanamivir
A/H1N1	H274Y, N294S	Q136K
A/H3N2	E119V, D151E, I222V , R224K , R292K, N294S, R371K , E276D	E119A/D/G , R224K , R292K, R371K , E276D
B	E119A/D/G/V , R152K, D198N, R292K	E119A/D/G , R152K, D198N, R292K

Result:

All 22 influenza isolates investigated in 2006 and 2007 were drug susceptible with mean IC50 of 0.93 and 1.40 nM, respectively for oseltamivir.

The isolates in 2008 and 2009 were oseltamivir resistant with mean IC50 of 437.12 and 709.05 nM, respectively.

All of the 28 isolates tested were zanamivir sensitive, i.e., mean IC50 of 0.42 to 4.89 nM

The resistant mutation H274Y in N gene (N2 numbering) was found in all 6 clinical samples collected in 2008, but none of the 35 samples collected between 2006 and 2007.

All of the 28 isolates tested were zanamivir sensitive, i.e., mean IC50 of 0.42 to 4.89 nM; and no mutational change indicating zanamivir resistance was observed in all clinical samples tested. all of the 28 isolates tested were zanamivir sensitive, i.e., mean IC50 of 0.42 to 4.89 nM; and no mutational change indicating zanamivir resistance was observed in all clinical samples tested.

Table 2. Susceptibility of seasonal influenza H1N1 virus isolates to NA inhibitors

Year	Number tested	Mean, median and range of IC50 in nM	
		Oseltamivir	Zanamivir
2006	18	0.93, 0.94, (0.50-1.42)	1.22, 1.02, (0.42-2.11)
2007	4	1.40, 1.32, (1.02-1.94)	2.04, 1.91, (0.63-3.71)
2008	5	437.12, 428.03, (370.73-495.54)	2.19, 2.13, (1.45-3.30)
2009	1	709.05	4.89

Table 3. Nucleotide sequencing for drug resistant markers in clinical samples from patients with seasonal H1N1 virus infection

Year	Number tested	Amino acid positions on NA gene (N2 numbering)			
		H274	H274Y	N294	N294S
2006	32	32	-	32	-
2007	3	3	-	3	-
2008	6	-	6	6	-

Discussion:

Based on phenotypic and genotypic based assay, our study could not detect any drug resistant seasonal influenza H1N1 virus between 2006 and 2007; meanwhile the high percentages of drug resistance have been discovered in 2008 and 2009.

Regarding zanamivir phenotypic-based sensitivity assay, all of the viruses tested were drug sensitive. Additionally, mutation indicating zanamivir resistant was not found in all clinical samples investigated. Nevertheless, it is interesting to see that the viruses have a trend towards an increasing IC50 values by years; and this point is to be further explored.

Acknowledgement:
This study was supported by the Thailand Research Fund for Senior Research Scholar; and the National Science for Technology Development Agency

Susceptibility of influenza A viruses to neuraminidase inhibitors as determined by phenotypic and genotypic-based assays

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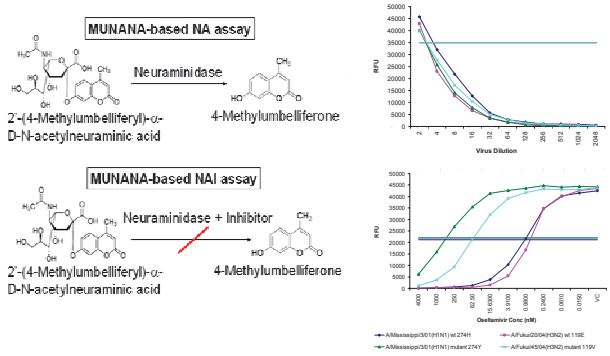
Introduction

Susceptibility of influenza viruses against anti-viral drugs can be determined by genotypic based assay (such as nucleotide sequencing and RT-PCR) or phenotypic-based assays (such as NAI assay, plaque reduction assay and viral protein reduction assay). The present study proposed that no single assay is adequate for anti-influenza drug resistance surveillance; and at least two assays of different principles should be performed in order to compensate for the disadvantage of another one. Herein, all four assays mentioned above were employed in the detection of oseltamivir and zanamivir resistant influenza viruses.

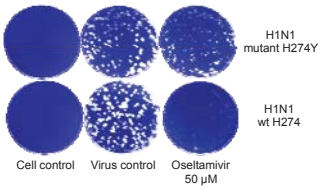
Phenotypic based assays

MUNANA-based NAI assay



Plaque reduction assay

Virus + NA inhibitor
↓ 37C, 1 hr
Add to MDCK monolayer
↓ 37C, 1 hr
Wash out
↓
Add agarose gel with various concentration of NA inhibitor
↓ 37C, 48 hr
Stain with crystal violet and determine for IC_{50}

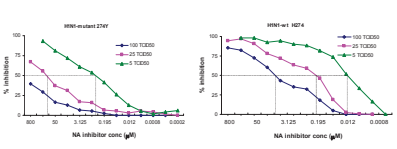


Results

Viruses	Oseltamivir carboxylate			Zanamivir		
	MUNANA-NAI assay (nM)	Plaque reduction assay (μM)	NP reduction assay (μM)	MUNANA-NAI assay (nM)	Plaque reduction assay (μM)	NP reduction assay (μM)
Reference strains						
A/Mississippi/03/01(H1N1) wt (274H)	1.799	0.27	0.050	1.28	0.50	0.11
A/Mississippi/03/01(H1N1) mutant (274Y)	**477.76**	**43.64**	**131.12**	**1.24**	**0.52**	**0.331**
Seasonal viruses						
A/Brisbrane/59/07(H1N1)-like SEA_3043	**875.25**	**104.44**	**781.49**	**3.89**	**0.39**	**1.659**
A/Brisbane/10/07(H1N1)-like SEA_3026	1.21	0.01	0.833	0.64	0.045	2.205
A/Brisbrane/10/07(H3N2)-like SEA_3613	0.85	0.055	0.166	1.82	0.50	1.331
2009 H1N1 virus						
A/Thailand/104/09(H1N1)	1.02	0.39	1.94	0.60	1.52	4.671
H5N1 viruses						
A/Thailand/1(KAN-1)/04	**0.15**	**1.084**	**5.68**	**1.82**	**14.32**	**50.282**
A/Thailand/3(SP-83)/04	0.19	0.003	0.008	1.02	0.017	0.231
A/Thailand/5(KK-494)/04	0.26	0.031	0.223	2.21	0.885	0.186
A/Thailand/676(NYK)/05	0.17	0.035	0.012	3.12	0.479	0.932
A/Laos/Nong Khai 1/07	**8.05**	**0.528**	**3.165**	**1.13**	**1.104**	**3.163**
A/Thailand/276(NBL)/06	0.86	0.006	0.004	2.04	0.009	0.053
A/Tiger/Thailand/BF154/04	0.13	0.238	0.4395	0.40	0.042	0.099
A/Golden Pheasant/Thailand/BF2743/05	**0.18**	**No plaque formation**	**>800**	**0.24**	**No plaque formation**	**>800**
A/Clouded Leopard/Thailand/VSKU-6/04	0.14	0.003	3.266	0.16	0.038	1.982
A/chicken/Thailand/VS143/07	1.88	0.002	0.007	7.68	0.013	0.14
A/chicken/Thailand/VS195/07	**0.53**	**No plaque formation**	**560.73**	**4.9**	**No plaque formation**	**259.45**

Nucleoprotein reduction assay

Virus + NA inhibitor
↓ 37C, 1 hr
Add to MDCK monolayer
↓ 37C, 1 hr
Wash out
↓
Add media with various concentration of NA inhibitor
↓ 37C, 24 hr
ELISA based nucleoprotein assay and determine IC_{50}



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Conclusion

There is no amino acid substitution indicating drug resistance was detected in *N* gene among all of the study isolates. However, based on phenotypic assays, few resistant mutants were detected by infectivity reduction assays but not NAI assay. Our result suggested the novel mutational change that might confer the resistance to NA inhibitors.

20 ปี สมาคมไวรัสวิทยา (ประเทศไทย)

การประชุมวิชาการประจำปี ครั้งที่ ๒๐

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Normal guinea pig serum mediated inhibitory effects on influenza virus replication

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Abstract

The present study employed normal guinea pig serum as the model to investigate innate immunity against seasonal influenza virus subtypes A(H1N1) and A(H3N2), 2009 pandemic A(H1N1) (H1N1pdm) and highly pathogenic avian influenza A(HPAI) H5N1 viruses. Native guinea pig serum was incubated with 1000TCID50 of influenza viruses: then, the mixtures were transferred onto MDCK cell monolayers to assay for virus replication. In presence of the serum inhibitors, the viruses were neutralized and resulted in a reduction of the amount of viral nucleoprotein produced in the inoculated cell cultures. The results demonstrated that seasonal influenza A (H3N2) virus was the most sensitive to normal guinea pig serum; meanwhile, seasonal influenza A (H1N1) and H1N1pdm viruses were less sensitive. On the other hand, HPAI H5N1 virus was resistant to guinea pig serum. When guinea pig serum was heat inactivated, the inhibitory effect did not diminish, but decreased by 1.4 to 5.9 folds as compared to those employed the native serum. These results suggested that both heat labile and heat stable inhibitory factors were present in guinea pig serum; and H3N2 subtypes were more sensitive to heat labile factor than H1N1 subtype. The study

also analyzed for correlation between susceptibility to guinea pig serum and number of glycosylation sites present in hemagglutinin (HA) and neuraminidase (NA) molecules. It was found that H3N2 viruses contained highest number of glycosylation sites and followed in order by H1N1 and HPAI H5N1 viruses. Thus, inversely correlation between sensitivity to guinea pig serum and number of glycosylation sites had been demonstrated. This finding may explain a mechanism to pathogenesis of the virulent H5N1 virus.

Introduction

Host defense against influenza viruses could be mediated by both adaptive and innate immunity. However, the function of specific immunity may be compromised in genetic variants; but function of innate immunity is not affected. In addition to cellular effectors, there are several soluble factors in alveolar secretion and serum of human and animals that possess anti-influenza activity, such as SP-D and SP-A, collectin, mannose-binding lectin (MBL), human mannose binding protein (MBP) and complement (1). 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 (2). α-inhibitor is not destroyed by inactivation at 56 C for 30 minutes. It is a sialylated glycoprotein which inhibits hemagglutination of influenza type A and B viruses by competitive binding with the cell receptor. However, it can not inhibit infection; and its activity was destroyed by receptor destroying enzyme (RDE). β-inhibitor type is heat labile, Ca^{2+} dependent and not sialylated. β-inhibitor in guinea pig serum had been shown to be MBL which was serologically cross reacted with human MBP (3). β-inhibitors inhibited influenza hemagglutination; and also shown to inhibit

influenza subtype H1 and H3 infections. It is not receptor analogue; therefore, it is not destroyed by RDE. Therefore, hemagglutination inbiting activity of β-inhibitors was probably mediated through stearic hindrance occurring as the result of binding between lectin to carbohydrate side chain on hemagglutinin (HA) molecule, and thus, prevent the viruses from binding with the cell receptor. γ-inhibitor had many properties which were similar to α- inhibitor, but it was alike β-inhibitors on possessing both hemagglutination inhibiting and virus neutralizing activities (4). While β-inhibitors inhibited H1 and H3 subtypes; γ-inhibitor inbited H2 and H3, but not H1 subtype (5, 6). γ-inhibitor was heat stable, sialylated glycoprotein and inhibited influenza hemagglutination by the mechanism of competitor analogue. Nevertheless, γ-inhibitor was not destroyed by RDE due to unusual sialic acid which was resistant to RDE hydrolysis. In horse and guinea pig serum, γ-inhibitor had been identified as α_2- macroglobulin (7).

It is difficult to investigate serum factors that exert innate immunity against influenza viruses in human serum due to interfering effect from specific antibody arose from past infection or vaccination. Guinea pig serum should be a good model to study the serum factors that mediated innate immunity because it is a rich source of complement; moreover, its anti-influenza activity had been well characterized. Guinea pig erythrocytes treated with influenza virus were lysed during incubation in autologous serum at 37 C via an activation of the classical complement pathway (8). Nevertheless, influenza viruses were also reported to activate complement via alternative pathway (9). Guinea-pig serum lacking detectable antiviral antibody efficiently neutralized the infectivity of influenza B virus grown in chick embryos or MDCK cells (10). MBL as well as complement in guinea pig serum exerted lytic activity on influenza

infected BHK-21 cells *via* classical pathway of complement activation (11). In the present study, seasonal influenza virus subtypes A/H1N1 and A/H3N2, H1N1pdm and HPAI H5N1 viruses were investigated for their susceptibility to inhibitory factors present in guinea pig serum. We postulated that degree of sensitivity to guinea pig serum may be inversely correlated with pathogenicity of the viruses.

Materials and Methods

Cell lines

MDCK (Madin Darby Canine Kidney) cells were grown in Eagle's Minimal Essential Medium (1X EMEM, GIBCO, N.Y.) supplemented with 10% fetal bovine serum (FBS, GIBCO) plus penicillin, gentamycin and fungizone.

Viruses

A total of 9 influenza virus isolates including seasonal A (H1N1) and A (H3N2), H1N1pdm and HPAI H5N1 viruses of human origins were employed in this study (Table 1). The study viruses were propagated in MDCK cell monolayers maintained in EMEM medium supplemented with 2 µg/ml trypsin TPCK (Tosyl phenylalanyl chloromethyl ketone, Sigma-Aldrich, MO.) and without FBS supplement. Supernatants of the infected cell cultures were centrifuged, aliquoted and stored at -80 C until used.

Guinea pig serum

Guinea pig serum collected from clotted blood was aliquoted and kept at -80 C.

Complement titration

In order to standardize the amount of guinea pig serum used in each experiment, guinea pig serum was titrated for amount of complement by using hemolysin sensitized sheep red blood cells as the

indicator. Titration was performed prior to its use in every experiment. One hemolytic unit of complement is defined as the highest serum dilution which yields complete hemolysis of the sensitized sheep red blood cell.

Virus titration

Influenza viruses were titrated in MDCK cell monolayer in quadruplicate in a 96 well micro-culture plate using ELISA based viral nucleoprotein assay. Mouse monoclonal antibody to viral nucleoprotein (Chemicon International, Inc., CA.) and goat anti-mouse Ig conjugated horse reddish immunoperoxidase (Southern Biotech) were used as the detector system. The viral titer was calculated by Reed and Munch method and expressed in term of 50% tissue culture infective dose (TCID50).

Influenza virus neutralization by serum factors

Fifty µl of 1000TCID50 of influenza virus was mixed with 25 µl of guinea pig serum at concentration 2 hemolytic units and 50 µl of viral growth media in quadruplicate wells of the micro-titer U-plate. The reaction plate was incubated at 37 C for 45 minutes. Then, the virus-serum mixture was transferred onto MDCK cell monolayers and incubated overnight prior to virus titration. The viruses from the reaction plate was titrated by ELISA based nucleoprotein (NP) production in MDCK cells. High O.D. indicated high viral titer and *vice versa*. Then, the inhibiting activity of guinea pig serum against the test virus was calculated in compared with the virus control without guinea pig serum.

The assay involved native guinea pig serum was run in parallel with the heat inactivated serum in order to compare their inhibiting activity against the tested influenza strain/subtype.

Result

Influenza nucleoprotein produced in MDCK cell monolayers inoculated with the mixtures of the test viruses and guinea pig serum was assayed by ELISA using mouse specific monoclonal antibody. HPAI H5N1 virus infected cultures yielded highest amount of viral nucleoprotein and followed in order by H1N1 and H3N2 viruses. The percentages of viral NP production as compared to the virus control cultures are as shown in Table 1. The result demonstrated that guinea pig serum contained inhibitory factors that could neutralize influenza virus infectivity, and the degree of inhibition was dependent on the virus subtypes. H3N2 subtype was the most sensitive and H1N1 subtype was intermediately sensitive; meanwhile, H5N1 isolates of both clades were resistant to guinea pig serum. The inhibiting activity was 1.4 to 5.9 folds increased when the native guinea pig serum was heat inactivated (Table 1). Comparison between native serum and heat inactivated serum on the inhibiting activity against H1N1 and H3N2 subtypes are shown in Figs. 1 and 2. Meanwhile, no difference between native serum and heat inactivated serum on inhibiting activity against HPAI H5N1 viruses (data not shown).

Table 1 Viral NP production in MDCK infected cells

Influenza subtypes	Virus name	% production of viral NP in presence of		Fold increase
		Native serum	Heat inactivated serum	
H1N1	A/New caledonia/20/99	57.6	78.7	1.4
	A/Brisbane/59/07	38.9	64.9	1.7
H3N2	A/Fujian/411/02	8.8	52.1	5.9
	A/Moscow/10/99	12.8	74.0	5.8
	A/Sydney/05/97	15.9	61.2	3.8
H1N1 pdm	A/Thailand/104/09	46.8	66.4	1.4
H5N1 clade 1	A/Thailand/1(KAN-1)/04	94.4	99.2	1.1
	A/Thailand/676(NYK)/05	93.9	92.9	1.0
H5N1 clade 2.3.4	A/Laos/Nong Khai 1/07	99.2	94.7	0.9

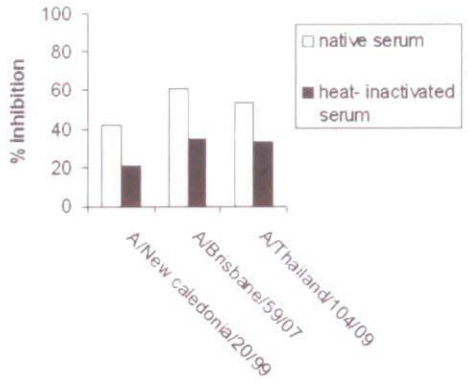

Fig. 1 Comparison between inhibiting activity of native guinea pig serum and heat inactivated serum against seasonal H1N1 viruses: A/New Caledonia/20/99 and A/Brisbane/59/07 and the 2009 pandemic virus: A/Thailand/104/09

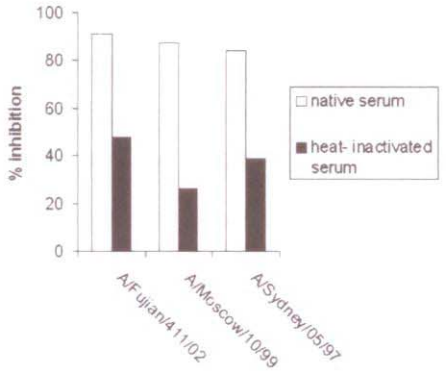

Fig. 2 Comparison between inhibiting activity of native guinea pig serum and heat inactivated serum against seasonal H3N2 viruses: A/Fujian/411/02, A/Moscow/10/99, and A/Sydney/05/97

Glycosylation sites on HA and NA molecules of the study viruses

Amino acid sequences of the HA and NA molecules of the study viruses were retrieved from GenBank; and glycosylation sites present on each molecule were predicted by CountGS application in BioEdit program

version 7.0.4.1. The results in Table 2 shows that H5N1 subtype contained least number of glycosylation sites on both HA and NA proteins.

Table 2 Glycosylation site on HA and NA of influenza viruses

Virus subtype	Number of glycosylation sites on	
	HA	NA
A/New caledonia/20/99 (H1N1)	10	9
A/Brisbane/59/07 (H1N1)	10	9
A/Moscow/10/99 like-virus (H3N2)	11	9
A/Thailand/104/09 (H1N1 pdm)	8	8
A/Thailand/1(KAN-1)/04 (H5N1)	8	3
A/Thailand/676(NYK)/05 (H5N1)	8	4
A/Laos/Nong Khai 1/07 (H5N1)	8	4

Discussion

Serum inhibitory factors which were able to inhibit influenza virus activities had long been demonstrated in different animal species. The present study employed guinea pig serum as the model to study innate immunity against different subtypes and strains of influenza viruses. It was demonstrated that degree of neutralizing activity of guinea pig serum was inversely correlated to number of glycosylation sites present in HA and NA molecules. Nature of the inhibitory factors present in the guinea pig serum was not identified in this study. However, it was demonstrated that there were consisted of at least two components: heat labile and heat stable factors. Based on findings of previous investigators, the heat

stable components could be either α- or β-inhibitors or both; and the heat labile component could be both complement and MBL, whose activity was correlated with glycosylation of the influenza viruses.

Activation of the complement system results in virus aggregation, virolysis or opsonization. It can lead to increased vascular permeability and recruitment of phagocytic cells to destroy the pathogens. There are three pathways of complement activation: the classical pathway that occurs when C1q binds to antibody, the alternative pathway in which the pathogen bypasses C1, C4 and C2 binding and initiates the activation pathway by binding with C3, and the lectin binding pathway in which the activation is mediated by binding of MBL with the oligosaccharide present on gram negative bacterial cell wall (12) or glycoprotein on the HIV envelope (13) or mannose glycan on HA of influenza virus (4, 14, 15). Mutations that lead to loss of specific glycosylation gave rise to influenza strains that were resistant to MBL as compared to their MBL sensitive parents (4). MBL is a C-type lectin member in the collectin family, whose molecule contains a collagen like sequence and carbohydrate recognition domain (16). MBL could activate classical pathway of the complement system without antibody binding as well as activate the alternative pathway. In addition, MBL could exert its anti-influenza activity with and without complement involvement (3, 16).

In conclusion, our study suggested that resistance to innate immunity may be an explanation for mechanism to virulence of HPAI H5N1 viruses.

Acknowledgements

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Immune Complex of HBsAg and anti-HBs in Occult HBV Infection

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Abstract

Since Nucleic acid testing (NAT) was developed to detect low level of hepatitis B virus (HBV) DNA, more information has been revealed on occult hepatitis B virus infection (OBI) with positive HBV DNA but undetectable hepatitis B surface antigen (HBsAg). One of the reasons for the absence of detectable HBsAg may be formation of immune complex with specific antibody. In order to determine the circulating immune complex in occult HBV infection, we investigated 29 plasma samples with occult HBV. HBsAg and immune complex in the samples were precipitated with polyethylene glycol (PEG) method. Subsequently, HBsAg was compared before and after acid dissociation with or without PEG. During the method validation, we found an increase in HBsAg after acid dissociation (p-value<0.05). HBsAg signal became positive in all the occult hepatitis cases after acid dissociation with PEG (p-value<0.05). Immune complex formation might be one of the explanations in the lack of detectable HBsAg in occult hepatitis B infection.

20 ปี สมาคมไวรัสวิทยา (ประเทศไทย)

การประชุมวิชาการประจำปี ครั้งที่ ๒๐

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The 2009 Pandemic Influenza A (H1N1) Virus Infection and Induction of Cytokines and Chemokines in Human Umbilical Vein Endothelial Cells

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Abstract

Three 2009 pandemic A (H1N1) virus isolates: A/Nonthaburi/102/09, A/Thailand/104/09 and A/Thailand/ICRC-1/09, were investigated for their capability to infect and induce cytokine/chemokine production in human umbilical vein endothelial cells (HUVECs). The infected HUVEC cultures showed degenerative cytopathic effect suggesting of direct cellular damage. However, in term of virus inoculum dose of 1TCID50 per cell with 36 hours incubation, the efficiency of infection was low. Only few percentages of infected cells were detected as determined by immunofluorescence assay using monoclonal antibody specific to influenza A nucleoprotein. Supernatants from the infected cultures were collected at 12, 24 and 36 hours post-infection and titrated for virus titers in MDCK cells as well as assayed for cytokine/chemokine production by ELISA. The result demonstrated successful productive infection with all virus isolates tested. It was noted that the isolates obtained from dead patient (ICRC-1) yielded the highest virus titers and followed in order by the other two isolates obtained from mild cases, Thailand/104 and Nonthaburi/102. IP-10 and IL-8, but not TNF-α and IL-1β, were detected in the supernatants of cultures infected by any of the test viruses. Our study suggested that ability to infect endothelial cells and induce cytokine/chemokine production may contribute to mechanism of pathogenesis in severe cases infected with the 2009 pandemic virus.

Introduction

The first pandemic influenza of this century was caused by the 2009 pandemic influenza A (H1N1) virus (H1N1pdm), a reassortant derived from influenza viruses of 4 origins: classical swine, European swine, avian and human influenza viruses (1). There were few differences between H1N1pdm and seasonal H1N1 virus, such as the patients infected with H1N1pdm elicited higher degree of gastrointestinal symptoms (2) the H1N1pdm had higher propensity to infect lower respiratory tract as demonstrated in ferret model, an occurrence which is consistent with the gastrointestinal symptom in patients (3) and the H1N1pdm could infect mouse without prior adaptation, meanwhile, it is needed to adapt seasonal H1N1 virus before mouse infection (4). This finding is suggestive of broader receptor binding activity of the H1N1pdm.

The disease produced by H1N1pdm is mild with fatality rate of 1.2% (5). Deaths are usually associated with underlying complications such as cardiopulmonary disease, diabetes, and pregnancy (6,7). Nevertheless, approximately 25-50% of deaths or severe cases occurred in people without predisposing risk factors. Role of cytokine storm as the major pathogenic mechanism of the H1N1pdm is not clearly elucidated. Macrophage cultures infected with H1N1pdm produced lower level of cytokines and chemokines as compared to seasonal H1N1, H3N2 and highly pathogenic avian influenza (HPAI) H5N1 virus (8). On the other hand, increased level of cytokines and chemokines was reported in two severe cases infected with the H1N1pdm (9).

It is interesting that pneumonia hemorrhage was the common pathological finding among the 1918 Spanish influenza A (H1N1), HPAI H5N1 virus and the H1N1pdm infected cases (7, 10, 11). Endothelial cells play an important role in regulating vessel permeability and maintaining hemostasis (12). Hemorrhage and edema are the characteristics suggestive of activation or infection of the endothelial cells which consequently lead to loss of tight junction constituents and vascular hyper-permeability, and probably virus dissemination in the body (13). H1N1pdm could infect various cell types such as macrophages (8, 14) and conjunctival cells *in vitro* (15). Moreover, the viruses could be detected in stools and urine of the patients (16). Therefore, it is possible that the H1N1pdm can infect many cell types and disseminate beyond the respiratory tract in the human body. Nevertheless, H1N1pdm infections of endothelial cells have never been reported. The present study explored mechanism of pathogenesis involving H1N1pdm dissemination through investigation on their ability to infect, replicate and induce cytokine and chemokine production in primary endothelial cells derived from human umbilical veins (HUVECs).

Materials and Methods

Cell cultures

Human umbilical vein endothelial cells

Primary culture of HUVECs prepared from pool of 8-10 umbilical cords were grown in Medium 199 (M199, GIBCO, N.Y.) plus human endothelial-SFM basal growth medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO).

Madin Darby Canine Kidney cells (MDCK)

MDCK cells were grown in Eagle's Minimal Essential Medium supplemented with 10% FBS plus penicillin, gentamycin and fungizone.

Influenza A viruses

The 2009 pandemic influenza A (H1N1) viruses (H1N1pdm) employed in this study were: A/Thailand/102/09, A/Thailand/104/09 and A/Thailand/ICRC-1/09. The first two strains were isolated from mild cases and the third strain was isolated from a dead case with underlying condition of obesity. These viruses were isolated and propagated in MDCK cell cultures in presence of 2μg/ml trypsin TPCK (Tosyl phenylalanyl chloromethyl ketone, Sigma-Aldrich, MO.) without FBS supplement. The virus inoculated culture was incubated at 37 C in a CO_2 incubator and daily observed for cytopathic effect (CPE). The supernatants from the virus infected cultures were harvested, aliquoted and kept at -70 C before titration in MDCK cell monolayers.

Virus titration

The test viruses were titrated in MDCK cell monolayers in quadruplicate using ELISA based nucleoprotein assay as the end point measurement for virus infection. The virus titer was calculated by Reed and Muench method and presented as 50% tissue culture infectious dose ($TCID_{50}$).

Virus infection in HUVECs

Confluent HUVEC cultures at the 3^{rd} – 4^{th} passage were trypsinized and adjusted to the concentration of 2×10^5 cells/500 μl/well of a 24 well culture plate. The plates were incubated at 37 C overnight before replacing with maintenance medium prior to virus inoculation. The cultures were absorbed with the test virus at inoculum dose of 1 $TCID_{50}$/cell for one hour, then washed once and added with the

maintenance medium supplemented with trypsin TPCK, the culture supernatants were harvested at 12, 24 and 36 hours post infection and titrated for infectious viral titers by $TCID_{50}$ assay in MDCK cell monolayer as well as quantified for cytokines/chemokines (TNF-α, IL-1β, IP-10 and IL-8) by ELISA (R&D system, Minneapolis, MN.). The infected HUVEC cells were scraped out of the culture plate for detection of viral antigen by immunoflorescence (IF) assay using mouse monoclonal antibody to influenza A nucleoprotein (Chemicon International, Inc., CA.).

Results

H1N1pdm infection in HUVECs

Cytopathic effect (CPE) in HUVECs infected with the test viruses could be clearly recognized after 24 hours of infection. Round and reflection cells were found discretely in the infected cultures. Approximately 2% of HUVECs were positive for viral antigen in nucleus and cytoplasm as measured by IF assay. Successful infection of influenza A virus in HUVECs could be demonstrated by the release of viral progenies in the culture supernatants collected at 12, 24 and 36 hours post infection (hpi.). A/Thailand/ICRC-1/09 yielded the highest virus titers and followed in order by A/Thailand/104/09 and A/Nonthaburi/102/09 (Fig.1).

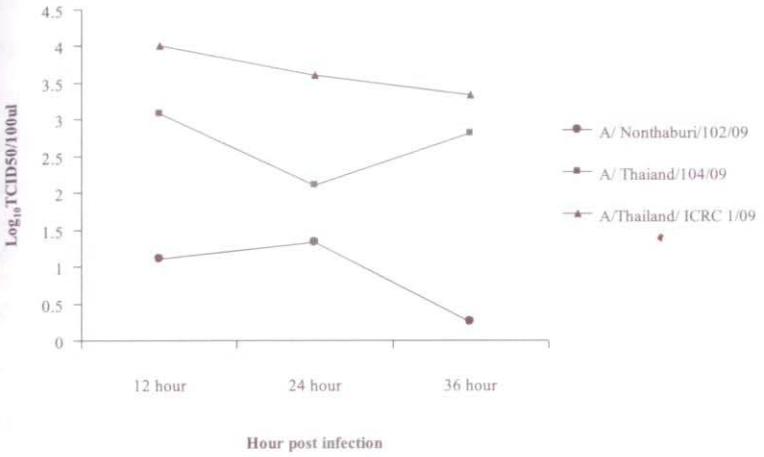

Fig. 1 Titers of H1N1pdm in supernatants of the infected HUVEC culture harvested at different intervals after infection

Cytokines and chemokines produced from viral infected HUVECs

Supernatants collected from the infected HUVECs cultures at 12, 24 and 36 hpi as well as the uninfected cell controls, were investigated for presence of IL-8, IP-10, TNF-α and IL-1β by ELISA in duplicate wells. Amount of cytokines/chemokines released from the uninfected cells as the background control were subtracted from the test values of the infected cultures to obtain the corrected concentrations. IP-10 and IL-8 were detected in HUVEC cultures infected with any of the test viruses with comparable concentration. On the other hand, TNF-α and IL-1β were below the detectable level (Table 1).

Table 1. Cytokine/chemokine production from HUVECs infected with H1N1pdm

Cytokine/chemokine	Virus	pg/ml of cytokine/chemokine at		
		12 hpi.	24 hpi.	36 hpi.
IP-10	A/Nonthaburi/102/09	526	1058	1108
	A/Thailand/104/09	502	683	1104
	A/Thailand/ ICRC-1/09	489	1725	1601
IL-8	A/Nonthaburi/102/09	0	112	394
	A/Thailand/104/09	69	156	638
	A/Thailand/ ICRC-1/09	0	143	1219

Note: hpi = hours post-infection

Discussion

Endothelial cells are abundant in all organs, particularly the lung. They are target and source of cytokines/chemokines (12). Previous investigators reported that human microvascular endothelial cells could be infected at limited extent by seasonal influenza H1N1, but the infection was more pronounced with HPAI H5N1 viruses (17). HPAI H5N1 virus produces severe pneumonia in more than half of the infected cases. Cytokine storm, virus dissemination and multi-organ failure are likely to be the key role to the virus virulence. However, cytokine storm was also linked with human influenza associated neurological complication and encephalopathy (18). In addition, it was reported that HUVEC cells infected with human H1N1 and H3N2 viruses induced the production of a variety of cytokines and chemokines such as IL-6 and TNF-α (19,20). The present study demonstrated that H1N1pdm was able to infect and

induced CPE in HUVECs, suggesting that this virus can cause direct cellular damage even though the extent of infection was low as shown by low number of infected cells by IF assay. In addition, the infected HUVECs secreted IP-10 and IL-8 production. However, we could not detect TNF-α and IL-1β in our system, meanwhile, these two cytokines could be detected in sera of the H1N1pdm infected patients in the other studies (9, 21). Moreover, one of those two studies also showed elevated serum levels of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 and MCP-1 in the patients infected with H1N1pdm (21). Thus, the negative finding in *in vitro* system may not reflect the real situation occurring *in vivo*. In contrast to our result in HUVECs, the others reported low level of cytokine production in macrophages and dendritic cells infected with the H1N1pdm (14). We postulated that different cell types release different kind of cytokines and chemokines.

Nevertheless, in our study, we could detect (unpublished data) IL-8, but not TNF-α, IL-1 β, and IP-10 in culture supernatants of HUVECs infection with seasonal H1N1 and H3N2 viruses. Therefore, we demonstrated that IP-10 synthesis could be induced by H1N1pdm, but not seasonal H1N1 and H3N2 virus; meanwhile, TNF-α, IL-1β were not found in all.

Collective data demonstrated that H1N1pdm infection could lead to pulmonary hemorrhage in the dead cases (22) and the virus was able to disseminate beyond respiratory tract (16). Thus, our finding suggested that the ability of H1N1pdm to infect HUVECs and induced cytokine/chemokine production may contribute to the pathogenesis and severity of respiratory complications in the patients.

Acknowledgements

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Normal guinea pig serum mediated inhibitory effects on influenza virus replication

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Abstract

The present study employed normal guinea pig serum as the model to investigate innate immunity against seasonal influenza virus subtypes A(H1N1) and A(H3N2), 2009 pandemic A(H1N1) (H1N1pdm) and highly pathogenic avian influenza A(HPAI) H5N1 viruses. Native guinea pig serum was incubated with 1000TCID50 of influenza viruses: then, the mixtures were transferred onto MDCK cell monolayers to assay for virus replication. In presence of the serum inhibitors, the viruses were neutralized and resulted in a reduction of the amount of viral nucleoprotein produced in the inoculated cell cultures. The results demonstrated that seasonal influenza A (H3N2) virus was the most sensitive to normal guinea pig serum; meanwhile, seasonal influenza A (H1N1) and H1N1pdm viruses were less sensitive. On the other hand, HPAI H5N1 virus was resistant to guinea pig serum. When guinea pig serum was heat inactivated, the inhibitory effect did not diminish, but decreased by 1.4 to 5.9 folds as compared to those employed the native serum. These results suggested that both heat labile and heat stable inhibitory factors were present in guinea pig serum; and H3N2 subtypes were more sensitive to heat labile factor than H1N1 subtype. The study

Influenza A virus infection and induction of cytokines and chemokines in human umbilical vein endothelial cells

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Abstract

Infection of human endothelial cells may be an explanation for influenza virus dissemination beyond the respiratory tract. Herein, seasonal influenza A(H1N1) and A(H3N2), and H5N1 highly pathogenic avian influenza (HPAI) viruses were determined for their capability to infect and induce cytokine/chemokine production in human umbilical vein endothelial cells (HUVECs). The influenza infected HUVEC cultures showed cytopathic effect (CPE) and expressed viral nucleoprotein as determined by immunofluorescence assay (IFA). Supernatants from the infected cultures were collected at intervals and titrated for virus titers in MDCK cells as well as assayed for cytokine/chemokine production by ELISA. The result showed that most of H_5N_1 isolates yielded high or moderately high titers of virus progenies; while, only one isolate poorly replicated. On the other hand, seasonal influenza viruses could also replicate in HUVECs, but to a lesser extent. Using commercial ELISA, IL-8 could be detected in supernatants of HUVEC cultures infected with all influenza subtypes; but TNF-α and IL-1β could not be detected at all. Interestingly, H5N1 HPAI dramatically induced IP-10 production; while H3N2 viruses induced very low level and seasonal H1N1 viruses could not. Our study suggested that an ability to infect and induce cytokine/chemokine production in HUVECs may contribute to mechanism of virulence of H5N1 HPAI viruses in humans.

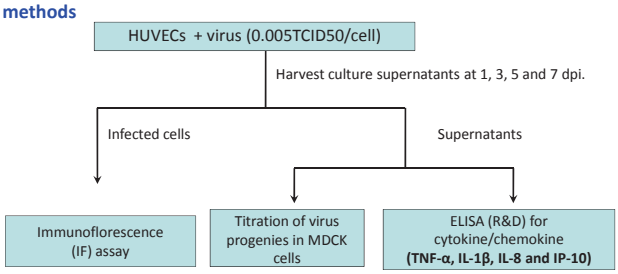
Introduction

With very few exception, an illness produced by human influenza viruses usually confines within the respiratory tract and subsides without sequel in most of the cases. On the other hand,H5N1 HPAI virus is the most virulent influenza subtypes ever reported in human infections. A virulent factor of H5N1 HPAI virus is its ability to disseminate beyond the respiratory tract to distal organs and cause multi-organ failure in most of the patients. Moreover, pneumonia hemorrhage was the common pathological finding in HPAI. Hemorrhage and edema are the characteristics suggestive of activation or infection of the endothelial cells which consequently lead to loss of tight junction constituents and vascular hyper-permeability, and probably virus dissemination in the body. The present study explored mechanism of pathogenesis involving influenza A virus dissemination through investigation on their ability to infect, replicate and induce cytokine and chemokine production in primary endothelial cells derived from pooled HUVECs.

Objectives

- To explore the ability of 15 influenza virus isolates including 9 H5N1 HPAI, 3 seasonal H1N1 and 3 H3N2 viruses to infect HUVECs. Kinetics of their replication was also determined at 1, 3, 5 and 7 days post infection (dpi.).
- To study the kinetics of cytokine and chemokine (TNF-α,IL-1β, IL-8 and IP-10) release in supernatants of the infected HUVEC cultures

Materials and methods



Result

Virus infection in HUVECs

All influenza A subtypes could produce CPE in HUVEC cultures. Nevertheless, CPE produced by H5N1 isolates could be recognized as early as 1 day post infection (Fig.1). The viral antigen was found in nucleus and/or cytoplasm of the infected HUVECs as shown by IFA (Fig.2). Successful productive infection of influenza virus in HUVECs was demonstrated by the release of viral progenies in the culture supernatants collected at 1, 3, 5 and 7 dpi. Among 9 H5N1 isolates, KAN-1, Nong Khai 1 and NBL 1 (feces) viruses replicated efficiently and yielded high virus titers; SP-33, SP-83, 676 NYK, NBL 1 (lung) and NBL 1 (trachea) replicated at moderate efficiency; while, KK-494 poorly replicated in HUVEC cultures. H3N2 viruses could fairly replicate in HUVECs, but least efficiency of replication was noted with Moscow virus. On the other hand, all of the 3 seasonal H1N1 viruses investigated poorly replicated in HUVECs (Fig.3).

Cytokine and chemokine production

All influenza viruses investigated could induce IL-8 in the infected HUVECs as assayed in the culture supernatants collected at different dpi., while none of them induce TNF-α and IL-1β. Interestingly, H5N1 HPAI viruses was the only subtype that could induce IP-10 production (Fig.4).

Acknowledgements

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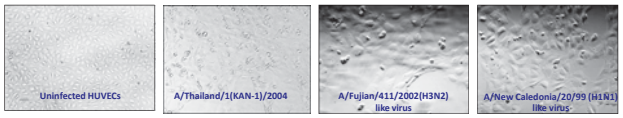

Fig.1 Cytopathic characteristics of HUVECs infected with influenza virus

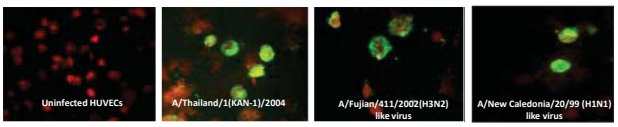

Fig.2 Influenza nucleocapsid antigen in nucleus and/or cytoplasm of the infected HUVECs

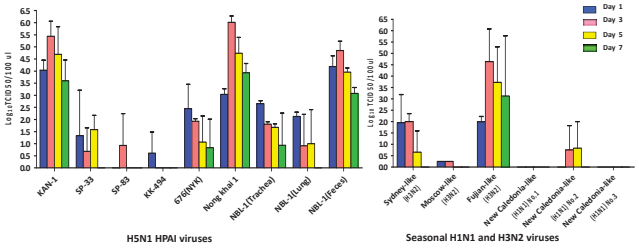

Fig.3 Titers of influenza viruses in supernatants of the infected HUVEC cultures

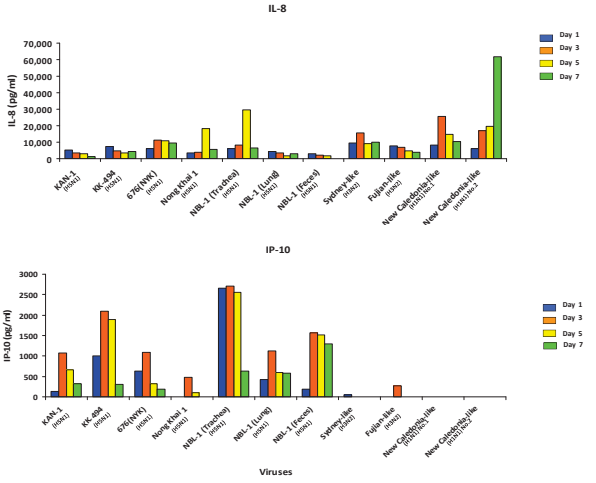

Fig.4 Levels of cytokines and chemokines in supernatants of the infected HUVEC cultures

Conclusion

H5N1 HPAI, seasonal H1N1 and H3N2 viruses could elicit productive infection in HUVECs; and highest efficiency of replication was noted with H5N1 HPAI viruses. IL-8 could be detected in supernatants of HUVEC cultures infected with all influenza subtypes; while TNF-α and IL-1β could not be detected at all. Moreover, IP-10 induction was typical for H5N1 viruses. Our study suggests that direct cellular damage and induction of high level of IP-10 in the infected HUVECs may contribute to mechanism of pathogenesis in severe H5N1 HPAI patients.

Normal guinea pig serum mediates inhibitory effects on influenza virus infection

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Abstract

Normal guinea pig sera (GPS) were used as the tool to study serum innate immunity against seasonal influenza A(H1N1) and A(H3N2), 2009 pandemic A(H1N1) (H1N1pdm) and H5N1 highly pathogenic avian influenza (HPAI) viruses. GPS was incubated with influenza viruses; and then, the mixtures were transferred onto MDCK cell monolayers to assay for virus infectivity. The results demonstrated that H3N2 virus was the most sensitive to GPS, while seasonal influenza A(H1N1) and H1N1pdm viruses were less sensitive. When GPS was heat inactivated or treated with receptor destroying enzyme (RDE) followed by heat inactivation, the serum inhibitory effect dramatically decreased. Interestingly, all of the H5N1 strains tested were highly resistant to GPS. It was suggested that H or both H and N of H5 viruses may confer this resistance. Two reverse genetic viruses, PR8-H5HA and PR8-H5HN, were tested against GPS in parallel with the reassorted PR8 wild type virus. The result showed that both reverse genetic viruses were resistant, while the reassoted PR-8 wild type was sensitive to GPS. It was found that H3N2 viruses contained highest number of glycosylation sites and followed in order by H1N1 and HPAI H5N1 viruses. Thus, inverse correlation between sensitivity to GPS and number of glycosylation sites had been demonstrated. This finding may explain a mechanism to pathogenesis of H5N1 HPAI virus on its ability to spread beyond the respiratory tract.

Introduction

Several soluble factors as well as non-specific inhibitors in sera of various animal species possess blocking activity against influenza virus infection. In presence of the serum inhibitors, the viruses were neutralized and resulted in the reduction of the amount of viral nucleoprotein produced in the inoculated cell cultures. It is difficult to study innate immunity against influenza viruses by using human sera due to interference effect from pre-existing specific antibodies arose during past infections or vaccination. Therefore, guinea pigs were chosen as the model in this study.

Objective

- To study seasonal influenza virus subtypes A/H1N1 and A/H3N2, H1N1pdm and HPAI H5N1 viruses on their susceptibility to guinea pig serum inhibitors
- To characterize biological properties of the serum inhibitors
- To determine correlation between glycosylation site on HA and NA and susceptibility to serum inhibitors
- To determine role of HA/NA on susceptibility/resistance to serum inhibitors

Materials and methods

1000TCID50 of virus + 2 hemolytic units of GPS
(native, heat inactivated , RDE treated followed by heat inactivation)

↓ 37 C for 45 minutes

MDCK cell monolayers

↓ 37 C, overnight

Assay for the amount of viral nucleoprotein (NP) produced in the inoculated MDCK cells by ELISA

Results

Table 1. Viral NP production in MDCK infected cells

Virus subtype	Virus name	% of NP produced in presence of		
		Native serum	Heat inactivated serum	RDE treated plus heat inactivated serum
H1N1	Reassorted A/PR/8/34	50.1	96.8	96.6
	A/New Caledonia/20/99-like virus	79.1	86.8	98.8
	A/Brisbane/59/07-like virus	69.3	85.1	88.3
H1N1 pdm	A/Thailand/104/09	65.8	85	94.7
	A/Nonthaburi/102/09	16.4	19.4	58.4
	A/California/07/09	11.3	25.5	91.9
H3N2	A/Sydney/05/97-like virus	19.2	95.3	100
	A/Moscow/10/99-like virus	0	79.7	100
	A/ Fujian/411/02-like virus No.1	3.1	77.8	88
	A/Fujian/411/02-like virus No.2	3.8	51.2	97
H5N1	A/Thailand/1(KAN-1)/04	92.2	-	-
	A/Thailand/5(KK-494)/04	89.2	-	-
	A/Thailand/676(NYK)/05	94	-	-
	A/Thailand/NBL-1/06 feces	96.5	-	-
	A/Thailand/NBL-1/06 lung	97.8	-	-
	A/Laos/Nong Khai 1/07	99.2	-	-
PR8-H5HA	rgPR8 H5HA (KAN-1)	97.7	98.2	97.8
PR8-H5HN	rgPR8 H5HN (KAN-1)	93.3	94.6	94.5

Note: % of NP produced as compared to that of the virus control

Table 2 Numbers of glycosylation site on HA and NA of the study viruses

Virus subtype	Virus name	Number of glycosylation sites on	
		HA	NA
H1N1	A/PR/8/34	7	4
	A/New Caledonia/20/99-like virus	10	9
	A/Brisbane/59/07-like virus	10	9
H1N1 pdm	A/Thailand/104/09	8	8
	A/Nonthaburi/102/09	8	8
	A/California/07/09	8	8
H3N2	A/Sydney/05/97-like virus	11	-
	A/Moscow/10/99-like virus	11	9
	A/Fujian/411/02-like virus No.1	12	-
	A/Fujian/411/02-like virus No.2	12	-
H5N1	A/Thailand/1(KAN-1)/04	8	3
	A/Thailand/5(KK-494)/04	8	3
	A/Thailand/676(NYK)/05	8	4
	A/Thailand/NBL-1/06	8	3
	A/Laos/Nong Khai 1/07	8	4
PR8-H5HA	rgPR8 H5HA (KAN-1)	8	4
PR8-HN	rgPR8 H5HN (KAN-1)	8	3

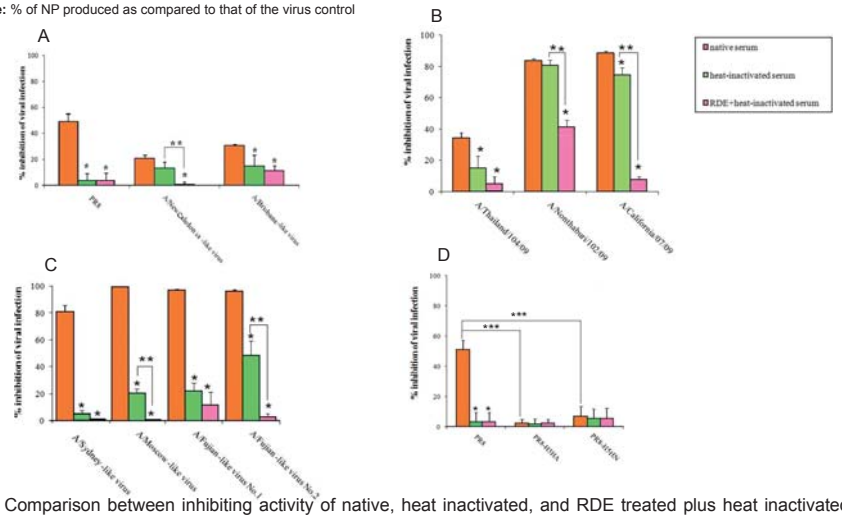

Figure Comparison between inhibiting activity of native, heat inactivated, and RDE treated plus heat inactivated serum against : A. Seasonal H1N1; B. H1N1pdm; C. H3N2 viruses; and D. Reverse genetic viruses

. * There was statistically significant difference between the inhibitory activity of native serum and the heat inactivated serum or the RDE + heat treated sera (*t*-test: $p \leq 0.05$).
** There was statistically significant difference between the inhibitory activities of the heat inactivated serum and the serum treated with RDE + heat inactivation (*t*-test: $p \leq 0.05$).
*** There was statistically significant difference between the inhibitory activity against rgPR8H5HA or rgPR8H5HN and the PR8 wild type virus (*t*-test: $p \leq 0.05$).

Conclusion

GPS contained inhibitory factors that could neutralize influenza virus infectivity; and the degree of inhibition was dependent on the virus subtypes and inversely correlated to number of glycosylation sites present in HA or HA and NA molecules. These inhibitory factors were consisted of heat labile, heat stable, RDE resistant, and RDE sensitive factors. Among all virus isolates tested, the inhibitor sensitive H3N2 viruses contained highest number of glycosylation sites. However, the inhibitor sensitive reassorted PR8 and the inhibitor resistant HPAI H5N1 viruses contained the similar least number. Our study has demonstrated a novel mechanism to virulence of H5N1 HPAI viruses, i.e., the resistance to innate serum inhibitors which makes the viruses readily to disseminate beyond the respiratory tract through viremia.

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Serological response to the 2009 influenza A (H1N1) virus strains derived from different epidemic waves in Thailand

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Abstract

Up to the end of 2010, Thailand has encountered 3 epidemic waves of the 2009 pandemic influenza A (H1N1) virus. In order to determine the viral antigenic drift that might occur during these epidemic waves, a total of 105 archival serum samples collected from patients and non-patients in July 2009 were assayed by hemagglutination inhibition (HI) assay using two isolates of the 2009 pandemic viruses derived from each pandemic wave as the test antigens. The result obtained from the two viruses derived from the same epidemic wave (intra-epidemic viruses) showed no significant difference in level of HI titers, i.e., GMT 46.9 and 44.2 for viruses from the first epidemic wave, GMT 55.6 and 51.1 for viruses from the second epidemic wave; and GMT 28.6 and 23.3 for viruses from the third epidemic wave (*t*-test; $p > 0.05$). On the other hand, HI antibody titers obtained from the viruses of the first or second epidemic waves were significantly higher when compared with those titers obtained from viruses of the third epidemic wave (*t*-test; $p < 0.05$). However, HI titers obtained from viruses belonged to the first and the second epidemic waves were not significantly different. Nucleotide sequences derived from *HA* gene of the six viruses were analyzed; and the result showed that these viruses were grouped into three separate clusters of the phylogenetic tree. The viruses isolated from the same epidemic wave were belonging to the same cluster. Taken together, the results suggested an antigenic change of the 2009 H1N1 viruses to the point that the antibody arose from the first exposure might not be able to protect from the viruses derived from the subsequent epidemic waves. The virus strain incorporated as a component of vaccine in use at present, therefore, might not match with the current strains circulating in the community.

Methodology

- A total of 105 archival serum samples collected in July 2009 from patients and non patients were investigated by HI assay (Figure 1) as described in the WHO manual on animal influenza diagnosis and surveillance. Six virus isolates derived from 3 epidemic waves were used as the test antigens (Table 1).
- Nucleotide sequences derived from complete *HA* gene of the six viruses were genetically analyzed using BioEdit and MEGA 5 software.

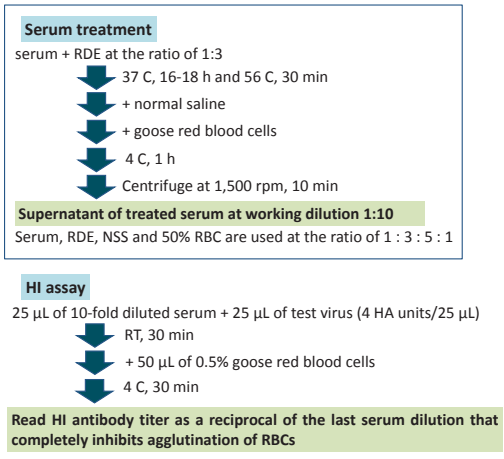

Figure 1. Hemagglutination inhibition (HI) assay

Background

Thailand had encountered 3 epidemic waves of the 2009 pandemic influenza A (H1N1) between 2009 and 2010. The first wave lasted between May and October 2009; and followed by the second wave between November 2009 and April 2010, and the third wave between May and October 2010. Based on the RNA nature of influenza virus, genetic drift commonly occurs and subsequently leads to viral antigenic variation such that antibody arose against the virus originated during the first epidemic wave may be not able to protect against those arose during the subsequent epidemic waves.

Objectives

- To determine GMT of antibody in serum samples collected during the first epidemic wave against the 2009 pandemic A (H1N1) (H1N1pdm) viruses derived from the three epidemic waves by hemagglutination inhibition (HI) assay
- To characterize *HA* gene of the viruses derived from the three epidemics

Results

The test sera harbored various HI antibody titers against each of the test viruses as shown in Table 2. GMT obtained from each of the two viruses derived from the same epidemic wave (intra-epidemic viruses) were not significant different (*t*-test; $p > 0.05$) as shown in Figure 2. And also, GMT obtained from viruses belonged to the first and the second epidemic waves were not significantly different. On the other hand, GMT of HI antibody against the viruses of the third epidemic waves were significantly lower than those against viruses of the first and second waves (*t*-test; $p < 0.05$, *). Nucleotide sequences derived from *HA* gene of the six viruses were analyzed; and phylogenetic tree demonstrated that viruses derived from the three epidemic waves belonged to different clusters as shown in Figure 3.

Table 2. HI antibody titers against the test viruses

Virus	No. of subjects with HI antibody titer of									GMT
	<10	10	20	40	80	160	320	640	1280	
A/Thailand/104/2009	11	8	6	26	34	16	3	1	-	46.9
A/Thailand/ICRC_CBI_10/2009	13	8	15	19	27	15	5	2	1	44.2
A/Thailand/SEA 34002/2010	11	6	6	24	25	25	6	2	-	55.6
A/Thailand/SEA 34004/2010	10	5	11	26	29	15	7	2	-	51.1
A/Thailand/ICRC_BKK_1/2010	18	11	24	32	16	3	1	-	-	28.6
A/Thailand/ICRC_NSN_1/2010	22	12	21	29	14	6	1	-	-	23.3

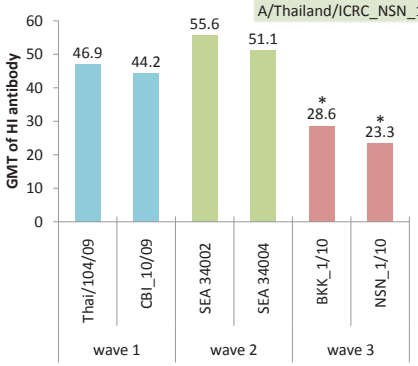

Figure 2. GMT of HI antibodies against each of the six test viruses. * represents statistic different with $p < 0.05$.

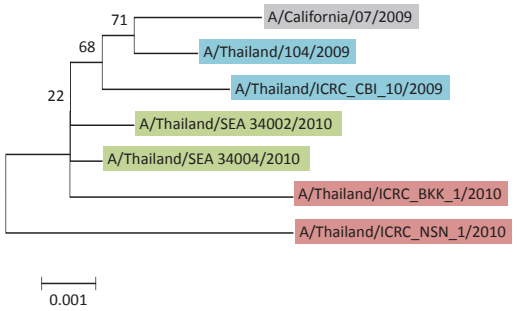

Figure 3. Phylogenetic tree based on *HA* gene nucleotide sequences

Table 1. History of virus isolates used in this study

Virus	Month at specimen collection	Epidemic wave No.	Place of origin	Passage history
A/Thailand/104/2009	Apr 2009	1	Imported case from Mexico	MDCK5
A/Thailand/ICRC_CBI_10/2009	June 2009	1	Chonburi, Thailand	MDCK5
A/Thailand/SEA 34002/2010	Feb 2010	2	Bangkok, Thailand	MDCK5
A/Thailand/SEA 34004/2010	Feb 2010	2	Bangkok, Thailand	MDCK4
A/Thailand/ICRC_BKK_1/2010	Aug 2010	3	Bangkok, Thailand	MDCK5
A/Thailand/ICRC_NSN_1/2010	Sep 2010	3	Nakornsawan, Thailand	MDCK6

Conclusion

This study demonstrated significant antigenic change of H1N1pdm to the point that the existing antibody arose from the first epidemic wave or vaccination might not be able to protect against the current circulating strains. The virus strain to be incorporated as a component of vaccine, therefore, should be reconsidered to match with the current strains which underwent antigenic change dramatically.

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