รายงานวิจัยฉบับสมบูรณ์

การพัฒนาวิธี loop-mediated isothermal amplification (LAMP) สำหรับการตรวจเชื้อ monodon baculovirus (MBV) เชื้อ *Vibrio parahaemolyticus* และ *V. cholerae*

Development of loop-mediated isothermal amplification (LAMP) detection methods for monodon baculovirus (MBV) *Vibrio parahaemolyticus* and *V. cholerae*

โดย รศ. ดร. ปรินทร์ ชัยวิสุทธางกูร และคณะ

กุมภาพันธ์ 2554

สัญญาเลขที่ DBG5280001

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง “**การพัฒนาวิธี loop-mediated isothermal amplification (LAMP) สำหรับการตรวจเชื้อ monodon baculovirus (MBV) เชื้อ *Vibrio parahaemolyticus* และ *V. cholerae*”** ได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย ตามสัญญาเลขที่ DBG5280001 ระยะเวลาดำเนินการ 2 ปี ตั้งแต่วันที่ 2 มีนาคม 2552 ถึงวันที่ 1 มีนาคม 2554 ผู้รับทุนขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย ที่ให้การสนับสนุนอย่างเต็มที่ทำให้โครงการวิจัยสำเร็จลุล่วงไปได้ และขอขอบคุณบุคคลต่อไปนี้ที่มีส่วนสำคัญให้โครงการสำเร็จได้แก่ ผู้ร่วมวิจัยคือ ศ. ดร. ไพศาล สิทธิกรกุล (ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ) ผู้ช่วยวิจัยคือ น.ส. ชุติมา ศรีสุข (ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ) และนิสิตปริญญาเอก น.ส. ปิยะนุช พรหมภมร (ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ) รวมทั้งภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ ในฐานะสถาบันต้นสังกัดที่ให้ความสนับสนุนและอำนวยความสะดวกในโครงการวิจัย

บทคัดย่อ

รหัสโครงการ: DBG5280001

ชื่อโครงการ: การพัฒนาวิธี loop-mediated isothermal amplification (LAMP) สำหรับการตรวจเชื้อ monodon baculovirus (MBV) เชื้อ *Vibrio parahaemolyticus* และ *V. cholerae*

ชื่อนักวิจัย และสถาบัน: รองศาสตราจารย์ ดร. ปรินทร์ ชัยวิสุทธางกูร
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โครงการวิจัย:

การติดเชื้อ monodon baculovirus (MBV) ในกุ้งกุลาดำมีผลทำให้กุ้งที่ติดเชื้อเจริญเติบโตช้าและมีขนาดเล็กลงอย่างเห็นได้ชัด ส่วนในกรณีของการติดเชื้อแบคทีเรีย *Vibrio parahaemolyticus* จัดเป็นจุลชีพฉวยโอกาสที่ก่อให้เกิดโรคเมื่อกุ้งหรือสัตว์น้ำอยู่ในสภาวะเครียด นอกจากนี้ *V. cholerae* และ *V. parahaemolyticus* ยังเป็นสาเหตุสำคัญของการเกิดโรคอาหารเป็นพิษในมนุษย์ หากรับประทานอาหารทะเลที่ปนเปื้อนเชื้อดังกล่าว ดังนั้นการตรวจวินิจฉัยที่รวดเร็ว ถูกต้องและแม่นยำจึงเป็นสิ่งสำคัญ วัตถุประสงค์ของงานวิจัยนี้เป็นการพัฒนาวิธี loop-mediated isothermal amplification (LAMP) เพื่อใช้ในการตรวจวินิจฉัยการติดเชื้อ MBV เชื้อ *V. cholerae* และ *V. parahaemolyticus*

สามารถพัฒนาวิธี LAMP สำหรับตรวจการติดเชื้อ monodon baculovirus (MBV) หรือ *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) ในกุ้งกุลาดำได้สำเร็จ โดยมียีนเป้าหมายคือ polyhedrin gene พบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 63°C เป็นเวลา 60 นาที มีความไวในการตรวจ PemoNPV ประมาณ 50 อนุภาคต่อ 1 นาโนกรัมของ genomic DNA หรือ 150 อนุภาคต่อปฏิกิริยา จากการใช้ DNA ต้นแบบที่สกัดจากกุ้งที่ติดเชื้อ PemoNPV (โดยใช้ชุดสกัดกรดนิวคลีอิกของไวรัสที่มีขายเชิงพาณิชย์) พบว่าเทคนิค LAMP มีความไวประมาณ 0.7 เฟมโตกรัม ในขณะที่วิธี nested PCR มีความไวประมาณ 70 เฟมโตกรัม แสดงให้เห็นว่าเทคนิค LAMP มีความไวมากกว่า nested PCR 100 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ PemoNPV โดยไม่ทำปฏิกิริยาข้ามกับไวรัสชนิดอื่นๆ ของกุ้งได้แก่ ไวรัสหัวเหลือง (YHV) ไวรัสโรคทอร่า (TSV) ไวรัสจุดขาว (WSSV) และไวรัสที่ทำให้เกิดโรคแคระแกร็น 2 ชนิดได้แก่ PstDNA หรือชื่อเดิมว่า IHHNV และ PmDNV หรือชื่อเดิมว่า HPV

สามารถพัฒนาวิธี LAMP สำหรับตรวจการติดเชื้อ *V. cholerae* ทุกสายพันธุ์ได้สำเร็จ โดยมียีนเป้าหมายคือ *ompW* โดยพบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 65°C เป็นเวลา 75 นาที มีความไวในการตรวจ *V. cholerae* ใน culture ได้ 2.2 x 10^3 CFU/ml หรือ 8 CFU ต่อปฏิกิริยา มีและมีความไวในการตรวจ *V. cholerae* ที่ทำให้ปนเปื้อนในตัวอย่างกุ้ง (spiked samples) ได้ 2.2 x 10^4 CFU/g หรือ 20 CFU ต่อปฏิกิริยา ในขณะที่วิธี PCR มีความไวประมาณ 100 CFU ต่อปฏิกิริยา ดังนั้นวิธี LAMP จึงมีความไวสูงกว่า 5 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ *V.*

cholerae ทั้ง 16 isolates ที่ใช้ทดสอบ โดยไม่ทำปฏิกิริยาข้ามกับ non-*cholerae* *Vibrio* 28 isolates และ non- *Vibrio* 37 isolates

สามารถพัฒนาวิธี LAMP ควบคู่กับการตรวจ LAMP amplicons โดยการใช้ lateral flow dipstick (LAMP-LFD) สำหรับตรวจการติดเชื้อ *V. parahaemolyticus* ทุกสายพันธุ์ได้สำเร็จ โดยมียีนเป้าหมายคือ *tlh* โดยพบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 65°C เป็นเวลา 90 นาที มีความไวในการตรวจ *V. parahaemolyticus* ใน culture ได้ 120 CFU/ml และมีความไวในการตรวจ *V. parahaemolyticus* ที่ทำให้ปนเปื้อนในตัวอย่างกุ้ง (spiked samples) ได้ 1.8 x 10^3 CFU/g หรือ 3 CFU ต่อปฏิกิริยา ในขณะที่วิธี PCR มีความไวประมาณ 30 CFU ต่อปฏิกิริยา ดังนั้นวิธี LAMP-LFD จึงมีความไวสูงกว่า 10 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ *V. parahaemolyticus* ทั้ง 28 isolates ที่ใช้ทดสอบ โดยไม่ทำปฏิกิริยาข้ามกับ non- *parahaemolyticus* *Vibrio* 24 isolates และ non- *Vibrio* 35 isolates

คำหลัก: monodon baculovirus, *Vibrio parahaemolyticus*, *Vibrio cholerae*, loop-mediated isothermal amplification, LAMP, *Penaeus monodon* nucleopolyhedrovirus (PemoNPV)

Abstract

Project Code: DBG5280001

Project Title: Development of loop-mediated isothermal amplification (LAMP) detection methods for monodon baculovirus (MBV), *Vibrio parahaemolyticus* and *V. cholerae*

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Project Period: 2 March 2009 – 1 March 2011

Project Description:

Monodon baculovirus (MBV) is a viral pathogen causing stunt growth in black tiger shrimp *Penaeus monodon*. In the case of bacterial infection, *Vibrio parahaemolyticus* is an opportunistic pathogen causing vibriosis in shrimp or marine animals under stress condition. In addition, *V. cholerae* and *V. parahaemolyticus* can cause seafood-related illnesses in human after the consumption of *Vibrio*-contaminated food. Therefore, the rapid and accurate diagnosis of these pathogens is imperative. This research project aims to develop loop-mediated isothermal amplification (LAMP) detection methods for MBV, *V. cholerae* and *V. parahaemolyticus*.

In this report, a LAMP method was developed for detection of *Penaeus monodon* nucleopolyhedrovirus (PemoNPV), known previously as monodon baculovirus (MBV), using a set of six primers designed to specifically recognize the PemoNPV polyhedrin gene. The optimized time and temperature conditions for the LAMP assay were 60 min at 63 ˚C. The sensitivity of LAMP for PemoNPV detection was approximately 50 viral copies ng^{-1} genomic DNA (equivalent to 150 viral copies per reaction). Using a DNA template extracted from PemoNPV-infected shrimp by a viral nucleic acid kit, the detection limit of LAMP was 0.7 fg while that of nested PCR was 70 fg; therefore, the LAMP assay was 100 times more sensitive than nested PCR. The LAMP method did not amplify a product using nucleic acid extracted from shrimp infected with other viruses including yellow head virus (YHV), Taura syndrome virus (TSV), white spot syndrome virus (WSSV), *Penaeus stylirostris* densovirus (PstDNV) known previously as infectious hypodermal and hematopoietic necrosis virus (IHHNV), and *Penaeus monodon* densovirus (PmDNV) known previously as hepatopancreatic parvovirus (HPV).

A LAMP method for detection of *V. cholerae* was successfully developed. A set of five designed primers that recognized specifically the *V. cholerae ompW* gene was used. The optimized time and temperature conditions for the LAMP assay were 75 min at 65°C. The

LAMP method accurately identified 16 isolates of *V. cholerae* but did not detect 28 non-*cholerae Vibrio* isolates, and 37 non-*Vibrio* bacterial isolates. The sensitivity of LAMP for *V. cholerae* detection in pure cultures was 2.2 x 10^3 CFU ml^{-1} or equivalent to 8 CFU per reaction. In the case of spiked shrimp samples without enrichment, the detection limit for *V. cholerae* was 2.2 x 10^4 CFU g^{-1} or equivalent to 20 CFU per reaction while that of PCR was 100 CFU per reaction.

A loop-mediated isothermal amplification (LAMP) combined with amplicon detection by chromatographic lateral flow dipstick (LFD) assay for rapid and specific detection of *Vibrio parahaemolyticus* was successfully developed. Biotinylated LAMP amplicons were produced by a set of four designed primers that recognized specifically the *V. parahaemolyticus* thermolabile hemolysin (*tlh*) gene followed by hybridization with an FITC-labeled probe and LFD detection. The optimized time and temperature conditions for the LAMP assay were 90 min at 65°C. The LAMP-LFD method accurately identified 28 isolates of *V. parahaemolyticus* but did not detect 24 non-*parahaemolyticus Vibrio* isolates, and 35 non-*Vibrio* bacterial isolates. The sensitivity of LAMP-LFD for *V. parahaemolyticus* detection in pure cultures was 120 CFU ml^{-1}. In the case of spiked shrimp samples without enrichment, the detection limit for *V. parahaemolyticus* was 1.8 x 10^3 CFU g^{-1} or equivalent to 3 CFU per reaction while that of conventional PCR was 30 CFU per reaction.

Keywords: monodon baculovirus, *Vibrio parahaemolyticus*, *Vibrio cholerae*, loop-mediated isothermal amplification, LAMP, *Penaeus monodon* nucleopolyhedrovirus (PemoNPV)

หน้าสรุปโครงการ (Executive Summary)
ทุนวิจัยแบบมุ่งเป้า "การผลิตสัตว์น้ำเศรษฐกิจ"

ชื่อโครงการ การพัฒนาวิธี loop-mediated isothermal amplification (LAMP) สำหรับการตรวจเชื้อ monodon baculovirus (MBV) เชื้อ *Vibrio parahaemolyticus* และ *V. cholerae*

วัตถุประสงค์

เพื่อพัฒนาวิธี loop-mediated isothermal amplification (LAMP) สำหรับตรวจการติดเชื้อ monodon baculovirus (MBV) ในกุ้งกุลาดำ และเพื่อพัฒนาวิธี LAMP สำหรับตรวจการติดเชื้อ *Vibrio parahaemolyticus* และ *V. cholerae* ที่อาจพบปนเปื้อนในตัวอย่างกุ้งหรือตัวอย่างอาหาร

สิ่งที่ได้ดำเนินการ

สามารถพัฒนาวิธี LAMP สำหรับตรวจการติดเชื้อ monodon baculovirus (MBV) หรือ *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) ในกุ้งกุลาดำได้สำเร็จ โดยมียีนเป้าหมายคือ polyhedrin gene พบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 63°C เป็นเวลา 60 นาที มีความไวในการตรวจ PemoNPV ประมาณ 50 อนุภาคต่อ 1 นาโนกรัมของ genomic DNA หรือ 150 อนุภาคต่อปฏิกิริยา จากการใช้ DNA ต้นแบบที่สกัดจากกุ้งที่ติดเชื้อ PemoNPV (โดยใช้ชุดสกัดกรดนิวคลีอิกของไวรัสที่มีขายเชิงพาณิชย์) พบว่าเทคนิค LAMP มีความไวประมาณ 0.7 เฟมโตกรัม ในขณะที่วิธี nested PCR มีความไวประมาณ 70 เฟมโตกรัม แสดงให้เห็นว่าเทคนิค LAMP มีความไวมากกว่า nested PCR 100 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ PemoNPV โดยไม่ทำปฏิกิริยาข้ามกับไวรัสชนิดอื่นๆ ของกุ้งได้แก่ ไวรัสหัวเหลือง (YHV) ไวรัสโรคทอร่า (TSV) ไวรัสจุดขาว (WSSV) และไวรัสที่ทำให้เกิดโรคแคระแกร็น 2 ชนิดได้แก่ PstDNA หรือชื่อเดิมว่า IHHNV และ PmDNV หรือชื่อเดิมว่า HPV ผลงานนี้ได้รับการตีพิมพ์ดังนี้

Chaivisuthangkura P, Srisuk C, Rukpratanporn S, Longyant S, Sridulyakul P, Sithigorngul P. Rapid and sensitive detection of *Penaeus monodon* nucleopolyhedrovirus by loop-mediated isothermal amplification. J Virol Methods. 2009; 162: 188-193.

ผลงานนี้เป็นส่วนหนึ่งของผลงานวิจัยที่ได้รับรางวัล "ผลงานวิจัยดีเด่น (สาขาเกษตรศาสตร์และชีววิทยา) ประจำปี 2253" จากสภาวิจัยแห่งชาติ เรื่อง การแยกยีนโพลีฮีดรินของ *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) และการพัฒนาเทคนิค loop-mediated isothermal amplification เพื่อตรวจการติดเชื้อ PemoNPV ในกุ้งกุลาดำ

สามารถพัฒนาวิธี LAMP สำหรับตรวจการติดเชื้อ *V. cholerae* ทุกสายพันธุ์ได้สำเร็จ โดยมียีนเป้าหมายคือ *ompW* โดยพบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 65°C เป็นเวลา 75 นาที มีความไวในการตรวจ *V. cholerae* ใน culture ได้ 2.2 x 10^3 CFU/ml หรือ 8 CFU ต่อปฏิกิริยา มีและมีความไวในการตรวจ *V. cholerae* ที่ทำให้ปนเปื้อนในตัวอย่างกุ้ง (spiked samples) ได้ 2.2 x 10^4 CFU/g หรือ 20 CFU ต่อปฏิกิริยา ในขณะที่วิธี PCR มีความไวประมาณ 100 CFU ต่อปฏิกิริยา ดังนั้นวิธี LAMP จึงมีความไวสูงกว่า 5 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ *V. cholerae* ทั้ง 16 isolates ที่ใช้ทดสอบ โดยไม่ทำปฏิกิริยาข้ามกับ non-*cholerae* *Vibrio* 28 isolates และ non- *Vibrio* 37 isolates ผลงานนี้ได้รับการตีพิมพ์ดังนี้

Srisuk C, **Chaivisuthangkura P**, Rukpratanporn S, Longyant S, Sridulyakul P, Sithigorngul P. Rapid and sensitive detection of *Vibrio cholerae* by loop-mediated isothermal amplification targeted to the gene of outer membrane protein ompW. Lett Appl. Microbiol. 2010; 50: 36-42.

สามารถพัฒนาวิธี LAMP ควบคู่กับการตรวจ LAMP amplicons โดยการใช้ lateral flow dipstick (LAMP-LFD) สำหรับตรวจการติดเชื้อ *V. parahaemolyticus* ทุกสายพันธุ์ได้สำเร็จ โดยมียีนเป้าหมายคือ *tlh* โดยพบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 65°C เป็นเวลา 90 นาที มีความไวในการตรวจ *V. parahaemolyticus* ใน culture ได้ 120 CFU/ml และมีความไวในการตรวจ *V. parahaemolyticus* ที่ทำให้ปนเปื้อนในตัวอย่างกุ้ง (spiked samples) ได้ 1.8 x 10^3 CFU/g หรือ 3 CFU ต่อปฏิกิริยา ในขณะที่วิธี PCR มีความไวประมาณ 30 CFU ต่อปฏิกิริยา ดังนั้นวิธี LAMP-LFD จึงมีความไวสูงกว่า 10 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ *V. parahaemolyticus* ทั้ง 28 isolates ที่ใช้ทดสอบ โดยไม่ทำปฏิกิริยาข้ามกับ non- *parahaemolyticus* *Vibrio* 24 isolates และ non- *Vibrio* 35 isolates ผลงานนี้ได้รับการตีพิมพ์ดังนี้

Prompamorn P, Sithigorngul P, Rukpratanporn S, Longyant S, Sridulyakul P, **Chaivisuthangkura P.** The development of loop-mediated isothermal amplification combined with lateral flow dipstick for detection of *Vibrio parahaemolyticus*. Lett Appl. Microbiol. 2011 doi: 10.1111/j.1472-765X.2011.03007.x

รายงานโครงการวิจัยฉบับสมบูรณ์

ชื่อโครงการ การพัฒนาวิธี loop-mediated isothermal amplification (LAMP) สำหรับการตรวจเชื้อ monodon baculovirus (MBV) เชื้อ *Vibrio parahaemolyticus* และ *V. cholerae*

Development of loop-mediated isothermal amplification (LAMP) detection methods for monodon baculovirus (MBV), *Vibrio parahaemolyticus* and *V. cholerae*

1. Development of loop-mediated isothermal amplification (LAMP) detection methods for monodon baculovirus (MBV)

Introduction

Penaeus monodon nucleopolyhedrovirus (PemoNPV), known previously as monodon baculovirus (MBV) was first described in *Penaeus monodon* from Taiwan (Lightner and Redman 1981). PemoNPV is not a virulent pathogen; however, it can cause a significant reduction in the mean length of infected shrimp compared to uninfected shrimp (Flegel et al., 2004). PemoNPV infection can lead to considerable economic loss due to slow growth of the shrimp (Flegel et al., 1999) and may lead to secondary infections by *Vibrio* spp. and various protozoa under poor rearing conditions (Ramasamy et al., 2000; Vaseeharan and Ramasamy, 2003). PemoNPV has been found in assorted penaeid shrimp species (Manivannan et al., 2004) and in various parts of the world including Australia (Belcher and Young, 1998; Vickers et al., 2000), India (Madhavi et al., 2002; Vaseeharan and Ramasamy 2003) and Thailand (Fegan et al., 1991; Flegel 2006).

Traditionally, extensive PemoNPV infections were diagnosed by the presence of enlarged nuclei containing acidophilic occlusion bodies in tissue sections stained with hematoxylin and eosin (Flegel 2006). In addition, molecular techniques including *in situ* hybridization (Poulos et al., 1994) and polymerase chain reaction (PCR) have been developed for the detection of PemoNPV (Chang et al., 1993; Lu et al., 1993; Belcher and Young, 1998; Hsu et al., 2000). The PCR assays recommended by the OIE (Office International des Epizooties) reference manual consist of a nested PCR protocol developed by Belcher and Young (1998) and one-step PCR using primers that can detect PemoNPV from 10 geographical isolates (Surachetpong et al., 2005). Immunological based methods such as immunohistochemistry, dot blotting and western blotting using monoclonal antibodies (MAbs) specific to occlusion bodies of Thai isolates of PemoNPV were have also been developed for detection of the virus (Boonsanongchokying et al., 2006).

Loop-mediated isothermal amplification (LAMP) is a technique that can amplify up to 10^9 copies of target nucleic acid regions under isothermal conditions. LAMP assays employ the strand displacement activity of *Bst* DNA polymerase and utilized two sets of primers including two inner primers and two outer primers that are specific for six independent regions of the target sequence (Notomi et al., 2000). LAMP has been developed for the detection of assorted shrimp viruses including yellow head virus (YHV) (Mekata et al., 2006), Taura syndrome virus (TSV) (Kiatpathomchai et al., 2007; Kiatpathomchai et al., 2008; Teng et al., 2007), *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) (Pillai et al., 2006), white spot syndrome virus (WSSV) (Kono etal., 2004; Jaroenram et al., 2009; Mekata et al., 2009), infectious myonecrosis virus (IMNV) (Puthawibool et al., 2009), hepatopancreatic parvovirus (HPV) (Nimitphak et al., 2008) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Sun et al., 2006).

Recently, a novel polyhedrin gene of PemoNPV was isolated and characterized (Chaivisuthangkura et al., 2008). In this study, a LAMP assay for detection of PemoNPV infection in shrimp was developed based on the sequence of this PemoNPV polyhedrin gene. The sensitivity and specificity of LAMP was determined by comparison with that of the nested PCR method (Belcher and Young, 1998) recommended by the OIE.

Materials and methods

Samples infected with PemoNPV

Penaeus monodon post larvae at stage 10 (PL10) infected naturally with PemoNPV were kindly provided by Prof. Dr. T.W. Flegel, Centex shrimp, Mahidol University. The shrimps were stored at -70°C until DNA extraction. A portion of each sample was confirmed as positive for PemoNPV infection by a single-step PCR (Belcher and Young, 1998). The remaining portion was used for DNA extraction.

DNA preparation

Uninfected and PemoNPV-infected *P. monodon* post larvae were homogenized in lysis buffer (50 mM Tris-HCl, pH 9, 100 mM EDTA, 50 mM NaCl, 2% SDS; T. W. Flegel pers. comm.) and DNA was extracted from 200 µl of the homogenate using a high pure viral nucleic acid kit (Roche Molecular Biochemicals, Mannheim, Germany) as specified by the product manual. The concentration of extracted DNA in the samples was quantified by Quant-iT DNA assay kit (Molecular Probes, Eugene, OR, USA) using a fluorometer (Fluoroskan *Ascent* FL;

Labsystems, Helsinki, Finland) for detection of the fluorescence intensity. The extracted DNA was stored at -70°C until use.

Detection of PemoNPV by Nested PCR

The total DNA extracted from shrimp infected naturally with PemoNPV was used as a template for PCR amplification. The nested PCR condition were described in a previous report (Belcher and Young, 1998). Briefly, the primary PCR was carried out with primers MBV1.4F and MBV1.4R for 40 cycles using an annealing temperature of 65°C. The primary PCR amplicon size was 533 bp.

The nested PCR was further carried out with 0.5 μl of the primary PCR reaction as the template using nested primers MBV1.4NF and MBV1.4NR for 35 cycles and an annealing temperature of 60°C in a final volume of 50 μl. The nested PCR products (5 μl) were analyzed by electrophoresis on a 2% agarose gel. The nested PCR amplicon size was 361 bp.

LAMP primer design

A set of six primers for LAMP was designed according to the published sequence of PemoNPV polyhedrin coding region (GenBank accession no. **EU251062**) using Primer Explorer version 4 (http://primerexplorer.jp/elamp4.0.0/index.html). A forward inner primer (FIP), a backward inner primer (BIP), two outer primers (F3 and B3) and two loop primers (LF and LB) were used. The sequences of primers and their target locations are indicated in Table 1 and Figure 1.

Table 1
Primers for LAMP designed from polyhedrin gene of PemoNPV.

Primers	Gene position	Sequence (5′–3′)
F3	823–843	GTAACCAAGTCAACTGAAGAC
B3	1013–1032	GTCATTTGATGGCATCTCAT
FIP	849–871/TTTT/909–933	GAACCAAGTTCCGTTCATATCGTTG**TTTT**AAATCTAAATCCACACAACATGC
BIP	936–960/TTTT/992–1012	TATGCCAGAGTTAGAGAATGGAAAG**TTTT**AAGTGTCTGTTGTGTTGTTTC
LF	876–898	CAATTGGATTATCCCCTGGAGTC
LB	962–986	ATCATCTATTGCCTATGGAATCTGG

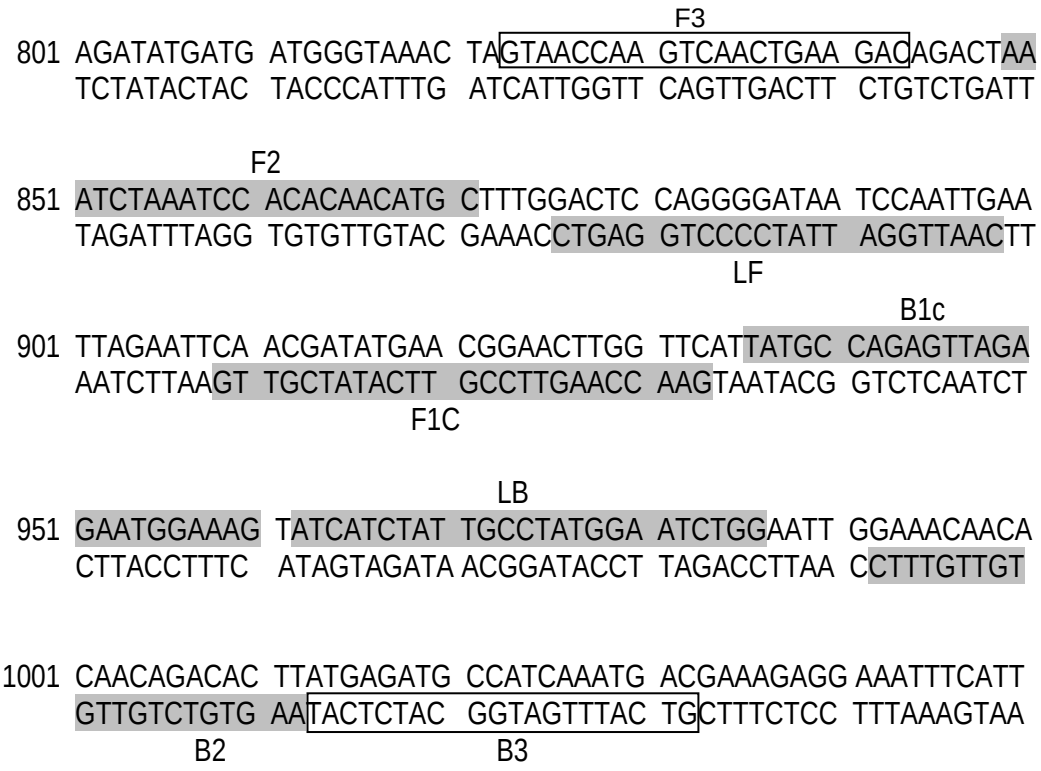

Figure 1. Partial nucleotide sequence of PemoNPV polyhedrin gene (GenBank accession no. EU251062) The sequences of primers FIP (F1c/TTTT/F2), BIP (B1c/TTTT/B2), LF and LB are shown with a grey background. The locations of F3 and B3 primers are shown in boxes.

Determination of LAMP reaction condition

The LAMP assay was carried out in a total volume of 25 μl containing 40 pmol each of the inner primers (FIP and BIP), 5 pmol each of outer primers (F3 and B3), 20 pmol each of loop primers (LF and LB), 1.4 mM dNTP mix (Fermentas, Burlington, Canada), 8 mM $MgSO_4$, 0.8 M Betaine (Sigma-Aldrich, Saint Louis, USA), 8 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA), 1X of supplied buffer and 7 ng of total DNA template in a 1 μl sample volume. The reaction temperature was optimized at 60°, 63° and 65°C and LAMP was carried out for a pre-determined time (60 min). The LAMP reaction was terminated by heat inactivation at 80°C for 10 min. The LAMP products (2.5 μl) were analyzed by electrophoresis on a 2% agarose gel.

Construction of a recombinant plasmid harboring the PemoNPV polyhedrin gene

In order to determine the sensitivity of the LAMP method, the full-length polyhedrin gene of PemoNPV was amplified by primers MBVFBam (CG GGA TCC ATG TTC GAC GAT AGC ATG ATG) and MBVRXba (GC TCT AGA TTA TTC ATT TGT ATG ATG C) containing the

restriction sites *Bam*HI and *Xba*I (underlined) with *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA) using DNA isolated from PemoNPV-infected *P. monodon* PL as a template. The 1,383 bp PCR product was cloned into the cloning vector pCR-Blunt (Invitrogen) and transformed into *E. coli* Top10 cells. The recombinant plasmid was purified using the NucleoSpin Plasmid kit (Machery-Nagel, Düren, Germany) and the concentration of plasmid DNA in the sample was quantified using the Quant-iT DNA assay kit (Molecular Probes) and a fluorometer (Fluoroskan *Ascent* FL; Labsystems). The stock solution of plasmid DNA (2.9 ng μl^{-1}) harboring 5.2 x 10^7 viral copies ng^{-1} of the viral gene was stored at -70°C until further use.

Sensitivity of the LAMP method

The stock solution of recombinant plasmid harboring PemoNPV polyhedrin gene (5.2 x 10^7 viral copies ng^{-1} plasmid DNA) was diluted serially 10-fold with DNA extracted from normal *P. monodon* (3 ng μl^{-1}) to prepare stock solutions containing 5 x 10^4 – 0.5 viral copies ng^{-1} genomic DNA (1.5 x 10^5 – 1.5 viral copies μl^{-1}). The resultant DNA (1 μl) samples were used as templates for LAMP using the optimized conditions. The LAMP products were analyzed by electrophoresis on a 2% agarose gel.

Detection sensitivity of PemoNPV in shrimps by the LAMP assay and by nested PCR

To compare the detection limit of nested PCR to that of this LAMP method, reaction were performed using 10-fold serial dilutions (7 ng to 0.07 fg) of the total DNA extracted from a shrimp infected naturally with PemoNPV as the template. LAMP reactions were performed with 1 μl of the diluted DNA template for each dilution at the optimal temperature for 60 min and the resulting amplicon mixtures were analyzed by 2% agarose gel electrophoresis. The nested PCR was carried out as described above using the same amount of DNA template used in the LAMP assay. The nested PCR product was then analyzed by electrophoresis on a 2% agarose gel.

Specificity of the LAMP assay

The specificity of LAMP primers was determined using total nucleic acid extracted from shrimps infected with other viruses including infectious hypodermal and hematopoietic necrosis virus (IHHNV) now called Penaeus stylirostris densovirus (PstDNV), hepatopancreatic parvovirus (HPV) now called Penaeus monodon densovirus (PmDNV), Taura syndrome virus (TSV), white spot syndrome virus (WSSV), and yellow head virus (YHV) as verified by PCR or

RT-PCR. DNA extracted from uninfected *P. monodon* was used as a control template. The specificity of the LAMP method was compared to the standard nested PCR assay developed by Belcher and Young, 1998.

Results

Optimization of the temperature for PemoNPV detection by LAMP

To determine the optimum temperature in which to carry out the LAMP assay, 60-min reactions using three different temperatures, 60°, 63° and 65°C were performed. At all tested temperatures, the LAMP products displayed the ladder-like pattern characteristic of LAMP amplicons in agarose gels (Notomi et al., 2000). However, the LAMP products were clearest at 63°C (Fig. 2A lane 2). Therefore, the 63°C was selected as the temperature for the subsequent LAMP assays.

To determine the optimum duration for the LAMP assay, LAMP reactions were set up for four different incubation times including 15, 30, 45 and 60 min. As shown in Figure 2B, the LAMP amplicons were accumulated with all of these times; however, at 60 min the amplification products were the most clearly detected. Therefore, the reaction time of 60 min was chosen as an optimal duration for this LAMP assay.

Sensitivity of LAMP method

To determine the sensitivity of our LAMP method, DNA template containing the PemoNPV polyhedrin gene recombinant plasmid was diluted with normal shrimp DNA to give between 1.5×10^5 – 1.5 copies per reaction (5×10^4 – 0.5 viral copies ng^{-1} genomic DNA). As shown in Figure 3, the LAMP amplicons could still be clearly observed at 150 copies per reaction (i.e., equivalent to 150 viral particles assuming only one copy of the polyhedrin gene in the PemoNPV genome) or 50 viral copies ng^{-1} genomic DNA.

Comparison of sensitivity between LAMP and nested PCR

Using various amounts of DNA extracted from PemoNPV-infected shrimp as the template for LAMP and nested PCR (Fig. 4), the LAMP detection limit was found to be 0.7 fg of total DNA (Fig. 4A lane 8) whereas that of one-step primary PCR was 7 pg (Fig. 4B lane 4) and that of nested PCR was 70 fg (Fig. 4C lane 6).

Specificity of LAMP

LAMP assays were performed with DNA templates from *P. monodon* infected with YHV, WSSV, PstDNA and PmDNV, and DNA template from *P. vannamei* infected with TSV. As shown in Figure 5, no LAMP product was obtained with shrimp samples infected with any of these viruses. This result demonstrates that the LAMP method described here is specific to the detection of PemoNPV.

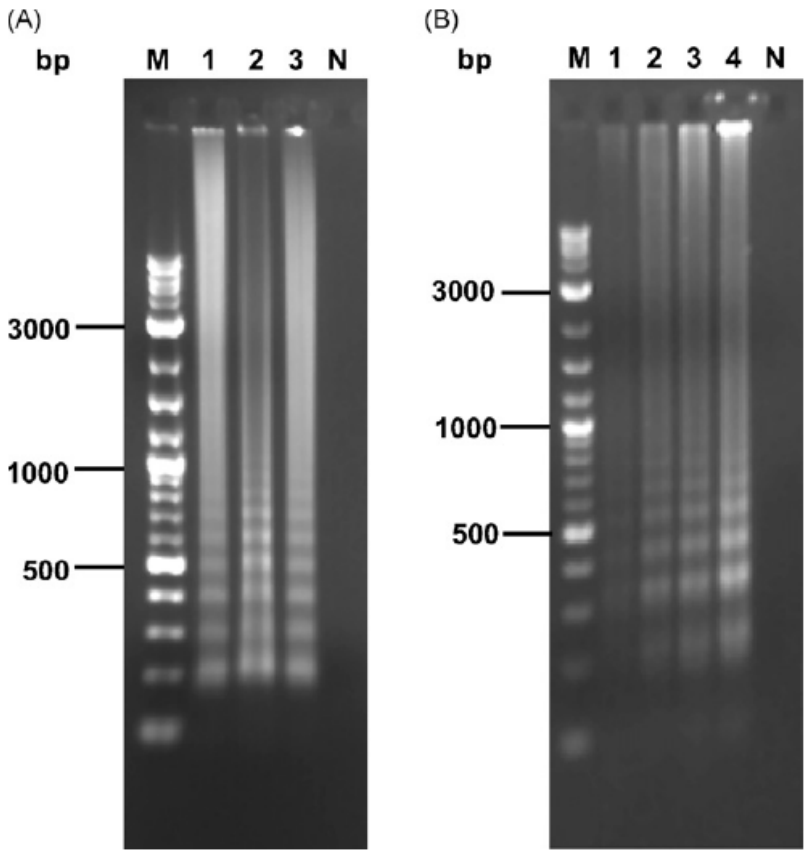

Fig. 2. Determination of LAMP conditions at different temperatures (A) and time (B) using 7 ng of DNA extracted from PemoNPV-infected P. monodon. (A) Temperature: lanes 1–3, the reaction was carried out at 60, 63, and 65˚C for 60 min, respectively. (B) Time: lanes 1–4, the reaction was carried out at 63˚C for 15, 30, 45, and 60 min, respectively; lane M: molecular marker; lane N: DNA extracted from normal *P. monodon* (negative control).

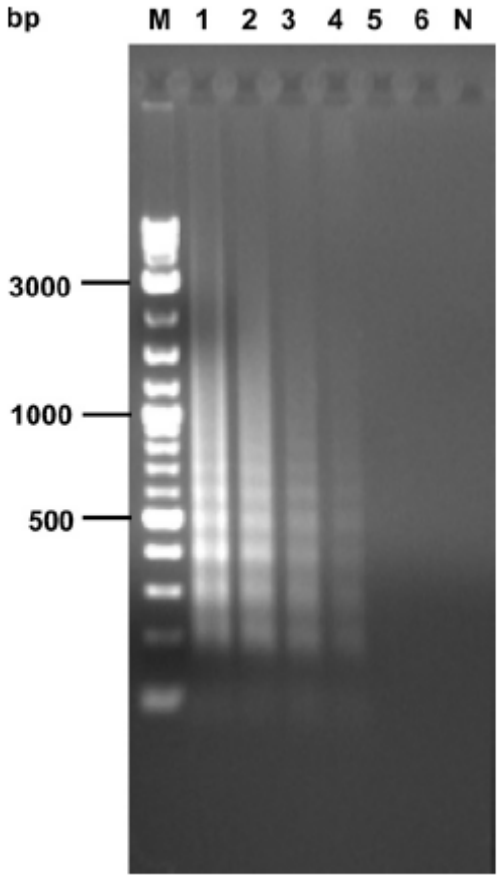

Fig. 3. Sensitivity of LAMP for the detection of PemoNPV. LaneM: molecular marker; lane N: DNA extracted from uninfected P. monodon (negative control); lanes 1–6: $1.5x10^{5}$ to 1.5 copies per reaction of full-length PemoNPV polyhedrin gene in recombinant plasmid.

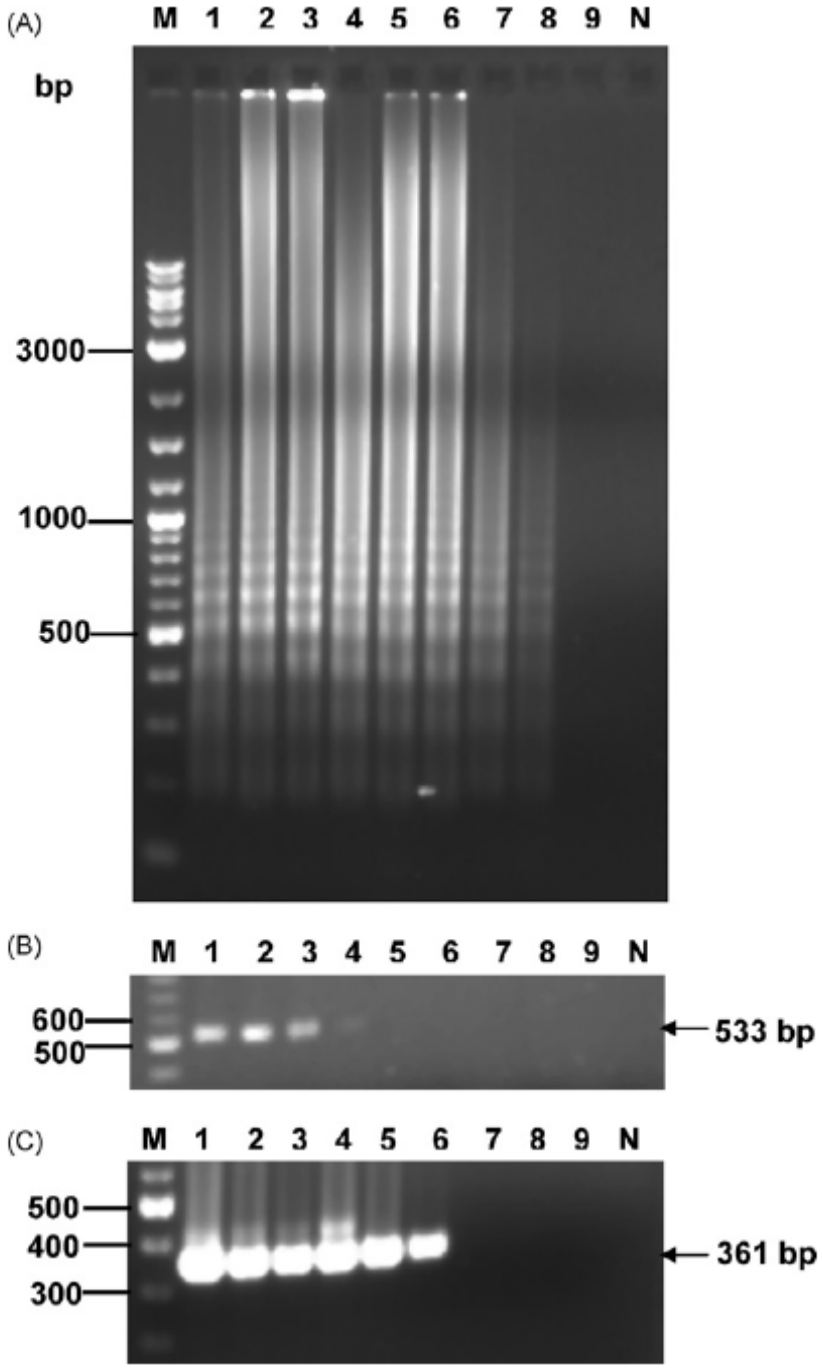

Fig. 4. Sensitivity comparison of LAMP (A) one-step primary PCR (B) and nested PCR (C) for the detection of PemoNPV infection in a DNA sample. Lane M: molecular marker; lanes 1–9: total DNA extracted from PemoNPV-infected *P. monodon* from 7 ng to 0.07 fg, respectively; lane N: no template control (negative control).

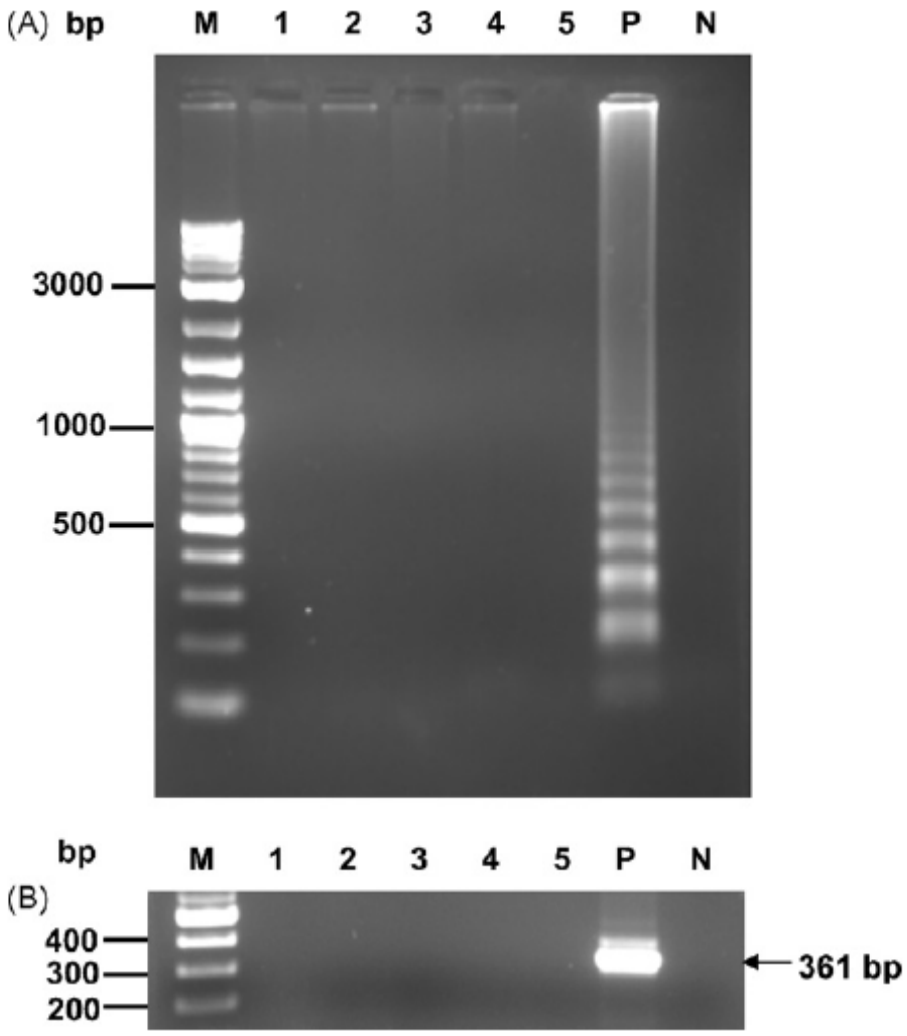

Fig. 5. Specificity tests of LAMP (A) and nested PCR (B) for PemoNPV detection. Total nucleic acid extracted from YHV-infected *P. monodon* (lane 1); TSV-infected *P. vannamei* (lane 2); WSSV-infected *P. monodon* (lane 3); PstDNV-infected *P. monodon* (lane 4) and PmDNV-infected *P. monodon* (lane 5). Lane M: molecular marker; lane P: PemoNPV-infected DNA sample (positive control); lane N: DNA extracted from normal *P. monodon* (negative control).

Discussion

In this study, a LAMP method targeting a recently reported polyhedrin gene was successfully developed for the detection of PemoNPV in shrimp. The LAMP, carried out at 63°C for 60 min, was faster than the typical method of nested PCR. Sensitivity of this approach was comparable to that of WSSV detection by LAMP combined with lateral flow dipstick (LFD) (100 copies; Jaroenram et al., 2009) and WSSV detection by real-time quantitative LAMP (100 copies; Mekata et al., 2009). In the case of IHHNV detection by LAMP agarose gel electrophoresis, a detection limit of 5 copies was reported; however, in this study the IHHNV

recombinant plasmid was diluted with sterile water instead of with uninfected shrimp DNA (Sun et al., 2006). When our tests were performed using sterile water dilution, a comparable detection limit of 8.5 copies was obtained (data not shown).

The LAMP detection limit for total DNA in this study (0.7 fg) was 10,000 times more sensitive than one-step PCR (7 pg) and 100 times more sensitive than nested PCR (70 fg). The sensitivity of LAMP in these experiments was comparable to that achieved in WSSV detection (1 fg; Kono et al., 2004). Other reports have determined that LAMP sensitivity is 10 times higher than nested PCR for both the detection of WSSV (Kono et al., 2004) and orf virus, a double-stranded DNA virus that causes an infectious skin disease in goats, sheep and other ruminants (Tsai et al., 2009).

A detection limit of 8 viral genome equivalents was previously reported in a study regarding PemoNPV detection using nested PCR (Belcher and Young, 1998). In that case, a hot phenol method was used to isolate the DNA from PemoNPV-infected shrimp to circumvent the co-purification of PCR inhibitors in total DNA extracted from whole postlarvae (Belcher and Young, 1998). However, in the present study a viral nucleic acid kit was used to isolate the total DNA from whole postlarvae; therefore, the inhibitors might still be present in the DNA and interfere with the LAMP reaction. This could cause a lower detection limit for the LAMP assay (150 viral copies).

As mentioned above, the total DNA of PemoNPV-infected shrimp was isolated by a viral nucleic acid kit in this study, and inhibitors may thus have been co-purified with the DNA sample. The higher sensitivity of LAMP (0.7 fg) compared to nested PCR (70 fg) could be due to the fact that the LAMP assay was less affected by extraneous shrimp biological substances in the DNA sample than nested PCR as previously reported (Kaneko et al., 2007; Yamazaki et al., 2008). These results demonstrate that LAMP could be used as an alternative method for PemoNPV detection when a typical DNA isolation technique is used.

In the present study, a pair of loop primers was included in the LAMP reaction in addition to the usual four LAMP primers to improve the sensitivity and accelerate the reaction (Nagamine et al., 2002). In the case of XSV detection in *Macrobrachium rosenbergii*, RT-LAMP with loop primers was 10,000 times more sensitive than conventional RT-PCR, whereas RT-LAMP without loop primers was equivalent to conventional RT-PCR (Pillai et al., 2006). The use of loop primers in LAMP reactions may also shorten the typical reaction time from 60 min to 30 min, as has been demonstrated for WSSV detection (Jaroenram et al., 2009). However, in the case of IHHNV detection, the LAMP method without loop primers still had 100 times more sensitivity than conventional PCR (Sun et al., 2006). Additionally, the use of lateral flow

dipstick detection instead of electrophoresis may also improve the sensitivity, as has been seen with the detection of Taura syndrome virus (Kiatpathomchai et al., 2007).

In conclusion, this study demonstrates an effective LAMP method for PemoNPV detection. This assay has a sensitivity threshold of approximately 150 viral particles per reaction and is 100 times more sensitivity than nested PCR when the total DNA of PemoNPV-infected shrimp was isolated using a viral nucleic acid kit. The developed LAMP method is also highly specific for PemoNPV detection making it well-suited for the rapid and accurate determination of PemoNPV infection from typically prepared DNA samples.

Acknowledgement

We would like to thank Prof. Dr. T.W. Flegel for kindly providing us with PemoNPV-infected *P. monodon* post larvae and Dr. W. Sukhumsirichart for kindly providing us with PmDNV-infected *P. monodon* DNA sample. This work was supported by The Thailand Research Fund (TRF), Thailand.

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2. Development of loop-mediated isothermal amplification (LAMP) detection methods for *Vibrio cholerae*

Introduction

Vibrio cholerae is generally recognized as a foodborne pathogen responsible for gastrointestinal disorders. *V. cholerae* belonging to O1 and O139 serogroups cause the serious form of the diarrheal disease known as cholera (Kaper et al. 1995). *V. cholerae* strains belonging to serogroups other than O1 and O139 referred to as the non-O1/non-O139 vibrios, which can be isolated from various aquatic or estuarine sources, have also been implicated as causative agents for human gastroenteritis (Morris 1990; Bagchi et al. 1993; Rudra et al. 1996). The prevalence of *V. cholerae* in seafood has been documented in several reports by various researchers (Karunasagar et al. 1992; Bagchi et al. 1993; Saravanan et al. 2007). The seafood contaminated with *V. cholerae* can act as reservoirs and may lead to the outbreak of diarrhea.

Therefore, an effective method for detection of all *V. cholerae* is imperative Traditional identification of *V. cholerae* is often achieved through biochemical tests which are time-consuming and laborious. Commercially biochemical identification systems are available; however, these systems may not always be accurate (O' Hara et al. 2003). Furthermore, several *Vibrio* species display similar biochemical characteristics which limit the identification of species by biochemical tests (Davis et al. 1981; Nishibuchi 2006). Several investigators have developed PCR-based techniques for detection of toxigenic strains of *V. cholerae* based on

various genes such as cholera toxin (*ctx*) gene (Shirai et al. 1991; Koch et al. 1993; Varela et al. 1994), and toxin-coregulated pilus (*tcpA*) gene (Keasler and Hall 1993; Rivera et al. 2001). Many researches have also developed conventional PCR techniques for detection of all *V. cholerae* based on various DNA regions such as regulatory protein (*toxR*) gene (Ghosh et al, 1997; Rivera et al. 2001), and outer membrane protein (*ompW*) gene (Nandi et al. 2000). A real time PCR method for detection of hemolysin (*hylA*) gene of all *V. cholerae* has also been developed (Lyon 2001). In the case of PCR identification of total *V. cholerae* targeted to *ompW* gene, the results demonstrated that all (100%) of the 254 *V. cholerae* strains tested were positive for *ompW* but all of the other *Vibrio* species or bacteria from other genera tested were found to be negative by the assay (Nandi et al. 2000).

Although PCR and real time PCR assays provide rapid and accurate identification of *V. cholerae*, they still require the thermal cycler. Recently, a novel technique called loop-mediated isothermal amplification (LAMP) has been developed and can amplify up to 10^9 copies of target nucleic acid regions under isothermal conditions (Notomi et al. 2000). LAMP has been developed for detection of various *Vibrio* species including cholera toxin-producing *V. cholerae* (Yamazaki et al. 2008a), *V. parahaemolyticus* (Yamazaki et al. 2008b; Nemoto et al. 2009), *V. vulnificus* (Ren et al. 2009; Han and Ge 2008), and *V. nigripulchritudo* (Fall et al. 2008).

In this study, the first LAMP assay for detection of *V. cholerae* was developed based on the sequence of *ompW* gene. The specificity and sensitivity in pure cultures and spiked shrimp samples were determined by comparison with that of PCR (Nandi et al. 2000).

Materials and Methods

Bacterial isolates, culture conditions, and DNA preparation

A total of 81 bacterial isolates including 16 *Vibrio cholerae* isolates, 28 non-*cholerae Vibrio* isolates, and 37 non-*Vibrio* bacteria were used in this study (Table 1). Various biochemical tests for identification of bacteria were performed in a conventional format. The 16s rRNA gene amplifications were also performed to confirm the bacteria identification as described in previous report (Weisburg et al. 1991). The origins and sources of all 44 *Vibrio* isolates tested were shown in Table 1. The other 37 non-*Vibrio* bacteria were obtained from clinical samples, food or environmental sources, as follows: four isolates of *Aeromonas hydrophila*, three isolates of *Aer. caviae*, three isolates of *Aer. sobria*, four isolates of *Pseudomonas aeruginosa*, two isolates of *Plesiomonas shigelloides*, one isolate each of *Aer. veronii*, *Aer. jandaei*, *Bacillus cereus*, *Escherichia coli*, *Edwardsiella tarda*, *Photobacterium damselae* subsp. *damsela*e, *P. damselae* subsp. *piscicida*, *Proteus vulgaris*, *Ps. stutzeri*, *Ps. chlororaphis*, *Ps. acidovorans*, *Ps.*

Table 1 Bacterial isolates used in this study, their sources, and specificity results of the LAMP and PCR assays

Bacterial Species	Origin	*ompW* amplification		Source
		LAMP	PCR	
Vibrio cholerae O1, E1 Tor, Inaba (n=4)				
DMST22115	Stool	+	+	DMST
DMST22116	Stool	+	+	DMST
DMST22117	Stool	+	+	DMST
DMST22118	Stool	+	+	DMST
Vibrio cholerae O1, E1 Tor, Ogawa (n=4)				
DMST22125	Rectal swab	+	+	DMST
DMST22126	Rectal swab	+	+	DMST
DMST22127	Rectal swab	+	+	DMST
DMST22128	Rectal swab	+	+	DMST
Vibrio cholerae O139 (n=3)				
DMST22136	Stool	+	+	DMST
DMST22137	Stool	+	+	DMST
DMST22138	Stool	+	+	DMST
Vibrio cholerae non-O1, non-O139, non-O141 (n=5)				
DMST22140	Food	+	+	DMST
DMST22141	Food	+	+	DMST
DMST22142	Food	+	+	DMST
DMST22143	Stool	+	+	DMST
VCS4907014	*P. vannamei*	+	+	DBSWU
Vibrio alginolyticus (n=4)				
DMST22082	Stool	-	-	DMST
DMST22084	Food	-	-	DMST
DMSC14800	Seafood	-	-	DMSC
DBSWU Y16	Shrimp pond	-	-	DBSWU
Vibrio mimicus (n=3)				
DMST22088	Food	-	-	DMST
DMST22089	Food	-	-	DMST
DMST22090	Food	-	-	DMST
Vibrio shilonii VS4907012 (n=1)	*P. vannamei*	-	-	DBSWU
Vibrio campbellii (n=3)				
LMG21361	Unknown	-	-	GB (Belgium)
LMG21362	Unknown	-	-	GB (Belgium)
LMG21363	Unknown	-	-	GB (Belgium)
Vibrio harveyi (n=3)				
Centex639	Unknown	-	-	Centex
Centex1526	Unknown	-	-	Centex
LMG22888	Unknown	-	-	GB (Belgium)
Vibrio parahaemolyticus (n=5)				
VPdenmark	Unknown	-	-	
DMST23795 (O10:KUT)	Stool	-	-	DMST
DMST23797 (O10:KUT)	Stool	-	-	DMST
DMST5665 (O10:KUT)	Stool	-	-	DMSC
DMST15285	Unknown	-	-	DMST
Vibrio vulnificus (n=4)				
VVS4907001	*P. vannamei*	-	-	
VVS4907009	*P. vannamei*	-	-	
VVS4907011	*P. vannamei*	-	-	
MT1506	Unknown	-	-	DABU
Vibrio fluvialis (n=3)				
DMST22086	Stool	-	-	DMST
DMST22087	Stool	-	-	DMST
DMST24049	Stool	-	-	DMST
Vibrio anguillarum AVL01 (n=1)	Unknown	-	-	DABU
Vibrio ordalii Vib02 (n=1)	Unknown	-	-	DABU
Other non-*Vibrio* bacteria (n= 37)		-	-	

+, positive reaction; -, negative reaction

Centex: CENTEX Shrimp, Faculty of Science, Mahidol University, Thailand; DABU: Department of Aquatic science, Burapha University, Thailand; DMSC: Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand; DMST: Department of Medical Science, Ministry of Public Health, Thailand; DBSWU: Department of Biology, Faculty of Science, Srinakharinwirot University, Thailand; GB: Ghent University, Belgium

putida, *Ps. boreopolis*, *Ps. oleovorans*, *Ps. syringae*, *Ps. japonica*, *Ps. fluorescens*, *Salmonella* serotype Enteritidis, *Salmonella* serotype Typhimurium, *Staphylococcus aureus*, and *Yersinia ruckeri*.

An isolate of *Vibrio cholerae* non-O1/non-O139/non-O141 designated DMST 22142 (Table 1) was used for the assay of optimization and sensitivity testing with pure culture and spiked shrimp samples. All *Vibrio* isolates were cultured using thiosulfate citrate bile salt sucrose agar (TCBS agar; Difco) at 37°C for overnight. Non-*Vibrio* isolates were grown in tryptic soy agar (TSA; Difco) at 37°C overnight.

To extract bacterial DNA, a single loopful of culture on TCBS agar or TSA was used with QIAamp DNA mini kit (Qiagen) according to the manufacturer's specifications. The extracted DNA was stored at -70°C until use.

Polymerase chain reaction

The DNA extracted from bacterial samples was used as a template for PCR amplification. The PCR reaction conditions were described in a previous report (Nandi et al. 2000). Briefly, the PCR reaction was carried out with primers *ompW* (terminal sense; 5'-CAC CAA GAA GGT GAC TTT ATT GTG-3') and *ompW* (terminal antisense; 5'-GAA CTT ATA ACC ACC CGC G-3') for 35 cycles, each of which consisted of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s. The PCR amplicon size was 588 bp.

LAMP primer design

A set of five primers for LAMP was designed according to the published sequence of *ompW* coding region (GenBank accession no. **X51948.1**) using Primer Explorer version 4 (http://primerexplorer.jp/elamp4.0.0/index.html). A forward inner primer (FIP), a backward inner primer (BIP), two outer primers (F3 and B3) and one loop primers (LB) were used for LAMP method. The sequences of primers and the locations were indicated in Table 2.

Determination of LAMP reaction condition

The LAMP assay was carried out in a total of 25 μl volume containing 40 pmol each of the inner primers (FIP and BIP), 5 pmol each of outer primers (F3 and B3), 20 pmol of loop primer (LB), 1.4 mmol l^{-1} dNTP mix (Fermentas), 6 mmol l^{-1} $MgSO_4$, 0.8 mol l^{-1} Betaine (Sigma-Aldrich), 8 U of *Bst* DNA polymerase (New England Biolabs), 1X of supplied buffer and DNA

template. The reaction temperature was optimized at 60, 63 and 65°C and LAMP was carried out on pre-determined time (75 min) and terminated at 80°C for 10 min. The LAMP products (2.5 µl) were analyzed by 2% agarose gel electrophoresis.

Table 2

Primers for LAMP designed from *ompW* gene of *Vibrio cholerae*

Primers	**Gene position**	**Sequence (5'-3')**
F3	101-122	CGGTAGTACCTAATGACAGTAG
B3	292-313	GCAAATGTTTTGTTTCACCAAT
FIP	166-190/**TTTT**/126-150	CAAGCGTTAACCCTAAGTGGGTATT **TTTT**TAAAGTGTTAAACACTCAAAGTGAG
BIP	20-230/**TTTT**/273-291	ACATCAGTTTTGAAGTCCTCGCT **TTTT**ATCACCAAGGCTACCTAAC
LB	246-270	ACATAAGATTTCTACCTCTGGTGGT

LAMP and PCR specificity test

The 81 bacterial isolates as shown in Table 1 were used to investigate the LAMP specificity. DNA templates isolated from bacterial cultures described above were subjected to both LAMP and PCR amplification.

Determinations of sensitivities of LAMP and PCR with pure cultures

The sensitivity of the LAMP assay for *V. cholerae* in pure cultures was determined as previously described (Yamazaki et al. 2008a) with some modifications using known amounts of *Vibrio cholerae* non-O1/non-O139/non-O141 isolate DMST 22142. Briefly, a single colony on TSA supplemented with 1% NaCl was inoculated in 4 ml of tryptic soy broth (TSB; Difco) supplemented with 1% NaCl and incubated overnight at 37°C with agitation. Then 40 µl of TSB culture was transferred into a new 4 ml of TSB and incubated at 37°C with shaking at 225 rpm to obtain mid-log phase cells (OD 600 nm equal to 0.5). Serial ten-fold dilutions of the cultures were prepared with PBS (Phosphate-buffered saline).

For preparation of DNA from pure cultures, 100 µl of each dilution was centrifuged at 18,000 x *g* for 5 min, then the pellet was resuspended in 50 µl of 25 mmol l^{-1} NaOH and subsequently heated at 95°C for 5 min. After neutralization with 4 µl of 1 mol l^{-1} Tris-HCl buffer (pH 7.5), the suspension was centrifuged at 18,000 x *g* for 5 min. For LAMP assay, 2 µl of each supernatant was used as a template while for the PCR assay, 0.5 µl of each supernatant was used as a template. The sensitivity tests were conducted in triplicate, the last dilution with all three samples tested positive was considered as the detection limit.

In parallel, to enumerate the bacteria, 100 µl of each bacterial dilution was spread on TSA supplemented with 1 % NaCl in duplicate and incubated at 37°C for overnight. The colonies were counted at the dilution yielding 30 to 300 Colony Forming Units (CFUs), and the CFU ml^{-1} of bacterial suspension was calculated.

Determinations of sensitivities of LAMP and PCR with spiked shrimp samples

P. vannamei shrimp samples were purchased at a local market in Bangkok, Thailand. The shrimp samples were tested to be negative for *V. cholerae* according to the microbiological examination by enrichment in APW (Alkaline Peptone Water) for overnight and the shrimp homogenate was plating onto the TCBS agar. The DNA samples of the yellow colonies were extracted by a QIAamp DNA mini kit (Qiagen) and tested for the presence of *ompW* gene by PCR (Nandi et al. 2000). Only shrimp homogenates that were negative for *V. cholerae* were used in the following spiked shrimp experiments.

The sensitivity of the LAMP assay for *V. cholerae* in spiked shrimp samples was determined as previously described (Yamazaki et al. 2008a) with some modifications using known amounts of *Vibrio cholerae* non-O1/non-O139/non-O141 isolate DMST 22142.

Nine millilitres of APW were added to 1 g of the shrimp sample and homogenized thoroughly. Serial 10-fold dilutions of mid-log phase *V. cholerae* were prepared as described above. One hundred microlitres of each dilution of *V. cholerae* with known amounts was spiked into 900 µl of each of the shrimp homogenates and mixing well. The shrimp homogenate was centrifuged at 200 x *g* for 5 min to remove shrimp tissues. The supernatant was transferred to a new tube and centrifuged at 18,000 x *g* for 5 min. After removal of the supernatant, the pellet was resuspended in 100 µl of 25 mmol l^{-1} NaOH and the mixture was heated at 95°C for 5 min. After neutralization with 8 µl of 1 mol l^{-1} Tris-HCl buffer (pH 7.5), the suspension was centrifuged at 18,000 x *g* for 5 min.

For LAMP assay, 1 µl of each supernatant was used as a template while for the PCR assay, 0.5 µl of each supernatant was used as a template. The sensitivity tests were conducted

in triplicate, the last dilution with all three samples tested positive was considered as the detection limit.

Results

Optimization of the temperature for PemoNPV detection by LAMP

To determine the optimum temperature for LAMP assay, three different temperatures including 60, 63 and 65°C were used in the reaction for 60 min. At all tested temperatures, the LAMP products displayed the ladder-like pattern characteristic of LAMP amplicons in agarose gels (Notomi et al. 2000). However, at 65°C the LAMP products were clearest (data not shown). Therefore, the temperature at 65°C was selected for the subsequent LAMP assays.

To determine the optimum time for LAMP assay, five different reaction times including 30, 45, 60, 75 and 90 min were used in the LAMP reaction. The LAMP amplicons could be observed at 60 min. However, at 75 and 90 min the intensity of LAMP amplicons were stronger and clearly detected (data not shown). Therefore, the reaction time of 75 min was chosen as an optimal reaction time for LAMP assay.

Specificity of LAMP

To determine the specificity of LAMP assay for detection of total *V. cholerae*, various bacterial species were tested. As shown in Table 1, LAMP products were detected in all 16 *V. cholerae* isolates whereas no LAMP amplicons were revealed in 28 other *Vibrio* spp. isolates and 37 non-*Vibrio* isolates. All of the results of LAMP assay agreed with that of PCR (Table 1).

Sensitivity of LAMP and PCR methods

To determine the sensitivity of LAMP method, the DNA templates extracted from pure culture and spiked shrimp samples were used in the LAMP reaction. Based on initial inoculums of *V. cholerae* (2.2×10^{7} CFU ml^{-1}), the sensitivity of LAMP for detection of *V. cholerae* in pure culture was 2.2×10^{3} CFU ml^{-1} or 8 CFU per reaction while that of PCR was 20 CFU per reaction (Figure 1A).

For spiked shrimp samples, the sensitivity of LAMP was 2.2×10^{4} CFU g^{-1} or equivalent to 20 CFU per reaction while that of PCR was 100 CFU per reaction (Figure 1B).

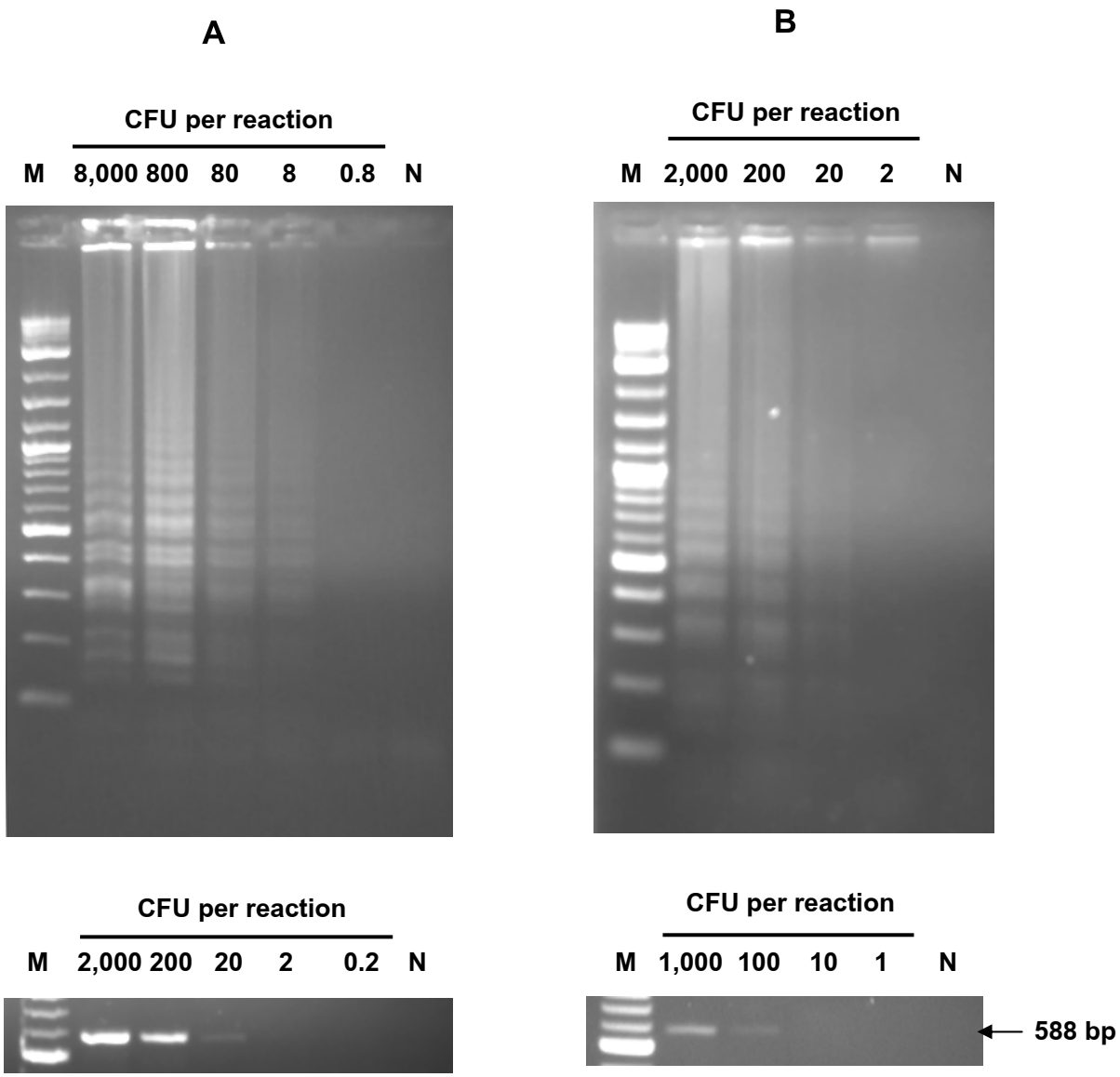

Figure 1. Sensitivity comparison of LAMP (top panel) and PCR (bottom panel) assays for *V. cholerae* detection in pure cultures (A) and spiked shrimp samples (B). The bacterial CFU per reaction was indicated on top of each lane. Lane M: molecular marker; lane N: no template control (negative control). LAMP amplicons of 2.5 μl out of 25 μl were loaded per lane, whereas PCR amplicons of 5 μl out of 25 μl were loaded per lane (A) and PCR amplicons of 10 μl out of 25 μl were loaded per lane (B).

Discussion

In this study, a LAMP method targeting the *ompW* gene was successfully developed for the detection of *V. cholerae*. The LAMP, carried out at 65°C for 75 min, was faster than the typical method of PCR and more suitable for low-equipment setting laboratory.

The sensitivity of LAMP assay with pure culture in this study (8 CFU per reaction) was 2.5 times higher than that of PCR (20 CFU per reaction) and was comparable to that of LAMP assays for cholera toxin-producing *V. cholerae* detection (2.9 CFU per reaction; Yamazaki et al. 2008a), *V. parahaemolyticus* detection (2.0 CFU per reaction; Yamazaki et al. 2008b), *V. vulnificus* detection (20 CFU per reaction; Han and Ge 2008), and thermostable direct hemolysin (TDH)-producing *V. parahaemolyticus* detection (1.0 CFU per reaction; Nemoto et al. 2009).

In the case of spiked samples, the sensitivity of LAMP (20 CFU per reaction) was 5 times higher than that of PCR (100 CFU per reaction). However, the sensitivity of LAMP assay in this study was 10 times lower than that of LAMP assays for detection of cholera toxin-producing *V. cholerae* in spiked human feces (1.4 CFU per reaction; Yamazaki et al. 2008a), and *V. parahaemolyticus* in spiked shrimp sample (2.0 CFU per reaction; Yamazaki et al. 2008b). In the case of *Vibrio vulnificus* detection in spiked oyster sample, the detection limit without enrichment was 4 x 10^6 CFU per reaction (Han and Ge 2008), approximately 10^6 times lower than the detection limit of LAMP in this study. This discrepancy could be due to that oyster tissue matrix may contain certain inhibitors for LAMP assays. However, after enrichment in APW for at least 5 hours the LAMP assay was able to detect an initial *V. vulnificus* inoculum of 7 CFU g^{-1} of oyster tissue (Han and Ge 2008).

In the preliminary tests, various amounts of DNA templates including 1, 2, and 4 µl from spiked shrimp samples were used to assess the LAMP amplification without showing any inhibition in the reaction. The results showed that 1 or 2 µl of DNA template was enough to yield the LAMP amplification while that of 4 µl did not give the LAMP amplicons. In the case of PCR, even 1 µl of DNA template from spiked shrimp samples did not yield any PCR amplification whereas 0.5 µl of DNA template resulted in PCR amplicon. These results were in agreement with previous reports stating that LAMP assay was less susceptible to certain inhibitors present in the food components (Kaneko et al. 2007, Han and Ge 2008, Yamazaki et al. 2008a; Yamazaki et al. 2008b). Even though the sensitivity of the developed LAMP assay was comparable to that of PCR, the LAMP method was rapid and simple to perform.

In conclusion, the first LAMP method for detection of *V. cholerae* was successfully developed. The developed LAMP assay demonstrated high specificity for *V. cholerae* detection.

It had the sensitivity of approximately 8 CFU per reaction with pure culture and 20 CFU per reaction with spiked shrimp samples.

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3. The development of loop-mediated isothermal amplification combined with lateral flow dipstick for detection of *Vibrio parahaemolyticus*

Introduction

Vibrio parahaemolyticus is considered to be pathogenic for both humans and marine life. In humans, this organism has been reported in several countries and continents as a major cause of seafood caused illnesses after the consumption of raw or inadequately or improperly cooked seafood (Alam *et al*. 2000; Chiou *et al*. 2000; Daniels *et al*. 2000; Vuddhakul *et al*. 2000; McLaughlin *et al*. 2005). In aquatic animals*, V. parahaemolyticus* can cause serious diseases in fish, shellfish and penaeid shrimp leading to significant losses in aquaculture industries (Su and Liu 2007).

The identification and diagnosis of *V. parahaemolyticus* is usually performed by biochemical tests which are time consuming and several *Vibrio* species display similar biochemical characteristics that limit the identification of species by biochemical tests (Davis *et al*. 1981; Nishibuchi 2006). There are several reports on using PCR techniques targeting thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) which are known as major virulent factors of this bacteria (Lee and Pan 1993; Suthienkul *et al*. 1995). However, these virulent factors are found only in pathogenic strains. Most *V. parahaemolyticus* isolates

from the environment do not produce TDH or TRH. The *tlh* gene encoding the thermolabile hemolysin (TLH) was first cloned and characterized in 1986 (Taniguchi *et al*. 1986). This gene was detected in all tested strains of *V. parahaemolyticus* isolates from both clinical and environmental sources but was absent in other *Vibrio* species. Consequently, this gene is considered to be a species-specific gene in *V. parahaemolyticus*. Several studies have suggested using this gene as a target gene for identification of *V. parahaemolyticus* (Bej *et al*. 1999; Ward and Bej 2006; Nordstrom *et al*. 2007; Yamazaki *et al*. 2008b).

The PCR technique appears to be a rapid and accurate method for identification of *V. parahaemolyticus*. However, the thermal cycler is still required. Recently, a novel technique called loop-mediated isothermal amplification (LAMP) has been developed in which the synthesis of large amounts of DNA can be carried out in a short time with a high specificity under isothermal conditions (Notomi *et al*. 2000). The detection of LAMP products is usually performed by agarose gel electrophoresis, followed by ethidium bromide staining. To avoid this process and speed up the total time for the LAMP assay, LAMP combined with chromatographic lateral flow dipstick (LFD) has been conducted (Jaroenram *et al*. 2009; Nimiphak *et al*. 2010). This generic LFD dipsticks (Milenia Biotec) detect biotin labeled amplicons that has been hybridized with an FITC-labeled DNA probe complexed with gold-labeled anti-FITC antibody. This LFD technique does not require special instrumentation since the user simply dips the strip into the appropriate buffered LAMP.

In this study, LAMP-LFD targeting to *tlh* gene of *V. parahaemolyticus* was developed. The specificity and sensitivity in both pure cultures and spiked shrimp samples were determined by comparison with the PCR technique.

Materials and methods

Bacterial isolates and DNA extraction

A total of 87 bacterial isolates including 28 *V. parahaemolyticus* isolates, 24 non-*parahaemolyticus Vibrio* isolates and 35 non-*Vibrio* bacteria were used in this study (Table 1). Various biochemical tests for identification of bacteria were carried out in a traditional format. The 16s rRNA gene amplifications were also performed to verify the bacteria identification as described in previous report (Weisburg *et al*. 1991). All *Vibrio* isolates were cultured using TCBS agar while non-*Vibrio* isolate were cultured using tryptic soy agar (TSA; Difco) at 37°C overnight. The origins and sources of all 52 *Vibrio* isolates tested were shown in Table 1. The other 35 non-*Vibrio* bacteria were obtained from clinical samples, food or environmental sources, as follows: four isolates of *Aeromonas caviae,* four isolates of *Aer. hydrophila*, four

Table 1 Bacterial isolates used in this study

Bacterial isolates	Origin	*tlh* amplification LAMP	*tlh* amplification PCR	Source
Vibrio parahaemolyticus (n=28)				
DMSC5665 (O10:KUT)	Stool	+	+	DMSC
DMST15285	Food	+	+	DMST
DMST20245 (O1:KUT)	Unknown	+	+	DMST
DMST21243 (O1:K1)	Unknown	+	+	DMST
DMST21296 (O4:K12)	Chicken meat	+	+	DMST
DMST21308 (O1:K32)	Cattle fish	+	+	DMST
DMST21318 (O2:K28)	Pork	+	+	DMST
DMST21330 (O3:KUT)	Shrimp	+	+	DMST
DMST21332 (O11:KUT)	Stool	+	+	DMST
DMST22091 (O8:K21)	Stool	+	+	DMST
DMST22092 (O2:K3)	Food	+	+	DMST
DMST22093 (O2:K3)	Food	+	+	DMST
DMST22094 (O4:K34)	Stool	+	+	DMST
DMST23794 (O3:K54)	Stool	+	+	DMST
DMST23795 (O10:KUT)	Stool	+	+	DMST
DMST23796 (O2:K28)	Stool	+	+	DMST
DMST23797 (O10:KUT)	Stool	+	+	DMST
DMST23798 (O10:K52)	Stool	+	+	DMST
DMST23801 (O5:KUT)	Stool	+	+	DMST
DMST23802 (O4:KUT)	Stool	+	+	DMST
DMST23803 (O3:K54)	Stool	+	+	DMST
DMST23804 (O3:K7)	Stool	+	+	DMST
DMST23805 (O4:K68)	Stool	+	+	DMST
DMST23806 (O2:K28)	Stool	+	+	DMST
DMST23807 (O10:KUT)	Stool	+	+	DMST
CECT5034 (O10:KUT)	Spain	+	+	DABU
DABU (O10:KUT)	*Penaeus monodon*	+	+	DABU
VMARC (O5:K33)	Aquatic animal	+	+	VMARC
V. alginolyticus (n= 3)				
DMST22082	Stool	-	-	DMST
DMST22084	Food	-	-	DMST
DMSC14800	Seafood	-	-	DMSC
V. campbellii (n=3)				
LMG21361	Unknown	-	-	GB (Belgium)
LMG21362	Unknown	-	-	GB (Belgium)
LMG21363	Unknown	-	-	GB (Belgium)
V. cholerae (n=3)				
DMST22140	Food	-	-	DMST
DMST22142	Food	-	-	DMST
DMST22143	Stool	-	-	DMST
V. fluvialis (n=3)				
DMST22086	Stool	-	-	DMST
DMST22087	Stool	-	-	DMST
DMST24029	Stool	-	-	DMST
V. harveyi (n=3)				
Centex639	Unknown	-	-	Centex
Centex1526	Unknown	-	-	Centex
LMG22888	Unknown	-	-	GB (Belgium)

Table 1 (Continued)

Bacterial isolates	Origin	*tlh* amplification LAMP	PCR	Source
V. mimicus (n=3)				
DMST22088	Food	-	-	DMST
DMST22089	Food	-	-	DMST
DMST22090	Food	-	-	DMST
V. vulnificus (n=3)				
VVS4907001	*P. vannamei*	-	-	DBSWU
VVS4907009	*P. vannamei*	-	-	DBSWU
VVS4907011	*P. vannamei*	-	-	DBSWU
V. shilonii VS4907012 (n=1)	*P. vannamei*	-	-	DBSWU
V. anguillarum AVL01 (n=1)	Unknown	-	-	DABU
V. ordalii Vib02 (n=1)	Unknown	-	-	DABU
Other non-*Vibrio* bacteria (n=35)		-	-	

+, positive reaction - , negative reaction
LAMP, loop-isothermal amplification PCR, polymerase chain reaction
Centex: CENTEX Shrimp, Faculty of Science, Mahidol University, Thailand
DABU: Department of Aquatic Science, Burapha University, Thailand
DMSC: Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand
DMST: Department of Medical Science, Ministry of Public Health, Thailand
DBSWU: Department of Biology, Faculty of Science, Srinakharinwirot University, Thailand
VMARC: Veterinary Medical Aquatic Research Center, Chulalongkorn University, Thailand.
GB: Ghent University, Belgium

isolates of *Pseudomonas aeruginosa*, two isolates of *Plesiomonas shigelloides*, one isolate each of *Aer. jandaei*, *Aer. veronii*, *Bacillus cereus*, *Edwardsiella tarda*, *Escherichai coli*, *Photobacterium damselae* spp. *damselae*, *P. damselae* spp. *piscicida*, *Proteus vulgaris*, *Ps. acidovorans, Ps. boreopolis, Ps. chlororaphis, Ps. fluorescens, Ps. japonica, Ps. putida, Ps. oleovorans, Ps. stutzeri, Ps. syringae, Salmonella enterica* serotype Enteritidis, *Salmonella enterica* serotype Typhimurium, *Staphylococcus aureus* and *Yersinia ruckeri*.

An isolate of VP DMST22092 was used for the assay of optimization and sensitivity testing with pure culture. To extract bacterial DNA, a single loopful of culture on TCBS agar or TSA agar was used with QIAamp DNA mini kit (Qiagen) according to the manufacture's specifications. The extracted DNA was then stored at -70°C until use.

Polymerase chain reaction

The DNA extracted from bacterial samples was used as a template for PCR amplification. The PCR reaction conditions were described in previous reports (Taniguchi *et al*. 1986; Bej *et al*. 1999) with some modifications. Briefly, the PCR reaction was carried out with primers L-tl (5'-AAA GCG GAT TAT GCA GAA GCA CTG-3') and R-tl (5'-GCT ACT TTC TAG CAT TTT CTC TGC-3') for 30 cycles, each of which consisted of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The PCR amplicon size was 450 bp.

Primer designed for LAMP

A set of four primers were designed for LAMP to target six distinct regions on *tlh* gene according to the sequence data submitted to GenBank (thermolabile hemolysin gene: M36437) using Primer Explorer ver. 4 (http://primerexplorer.jp/elamp_4.0.0/index.html). A forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3) were used for the LAMP method. To detect LAMP products by LFD, FIP was 5'-labeled with biotin (Bio Basic Inc., Canada) for the use in subsequent LFD steps. The sequences of primers and the locations are indicated in Table 2.

Table 2 Primers for loop-mediated isothermal amplification (LAMP) designed from *tlh* gene of *V. parahaemolyticus*

Primer	Gene position	Sequence (5'-3')
F3	1093-1112	GCGCAAGGTTACAACATCAC
B3	1281-1300	GCGTGACATTCCAGAACACA
Forward inner primer	1120-1139/TTTT/1163-1182	CGCGTTCACGAAACCGTGCTTTTTGATA CTCACGCCTTGTTCGA
Backward inner primer	1248-1265/TTTT/1195-1216	TTGGACATCAACCGCTCATCGTTTTTGA CGCTGCACACTCAGAG
Forward inner primer-biotin	1120-1139/TTTT/1163-1182	CGCGTTCACGAAACCGTGCTTTTTGATA CTCACGCCTTGTTCGA
FITC probe	1226-1247	FITC-ACATGTACACCCACGCATTGCG

Optimization of LAMP conditions

The LAMP assay was performed in a total of 25 µl of reaction mixture containing 40 pmol each of the inner primers (FIP and BIP), 5 pmol each of the outer primers (F3 and B3), 1.4 mmol l^{-1} dNTP mix (Fermentas), 6 mmol l^{-1} $MgSO_4$, 0.8 mmol l^{-1} Betaine (Sigma-Aldrich), 8U of *Bst* DNA polymerase (New England Biolabs) and 1x of supplied buffer and DNA template. The reaction temperature was tested for the optimal temperature and time combination at 60, 63 and 65°C for 90 min, followed by 80°C for 10 min to terminate the reaction. The products were analyzed by 2% agarose gel electrophoresis.

Biotin-labeled LAMP conditions

In order to confirm the results obtained from agarose gel electrophoresis, the biotin-labeled LAMP reactions were carried out along with normal LAMP. In this condition, the biotin-labeled was added at the 5' end of the FIP inner primer but other primers and components used were the same as those described earlier

Design and optimization of FITC-labeled probe for lateral flow dipstick (LFD) assay

A DNA probe was designed from the *tlh* gene sequence between the FIP and BIP regions (Table 2). The DNA probe was labeled with FITC at the 5' end and synthesized by Bio Basic Inc., Canada. According to the test protocol, after the biotin-labeled LAMP reaction was finished without heating inactivation, 5µl of DNA probe solution at 3 different concentrations (200, 20 and 2 pmol) were added to the biotin-labeled LAMP products before hybridization at 63°C for 5 min. Subsequently, 8 µl of the hybridized product was added to 120 µl of the assay buffer in a new tube (Milenia Genline HybriDetect 2T, Gießen, Germany). After that, the LFD strip was dipped into the mixture and the test result appeared after 5 to 10 min. The concentration of DNA probe that gave the strongest signal on the test line was determined to be the optimal concentration for LFD assays.

Specificity of LAMP and PCR detection

The 87 bacterial isolates as shown in Table 1 were used to investigate the LAMP specificity. DNA templates isolates from bacterial cultures describe earlier were subjected to both LAMP and PCR amplification.

Determination of sensitivities of LAMP and PCR in pure culture

The sensitivity of the LAMP assay for the detection of *V. parahaemolyticus* in pure culture was determined as previously described (Yamazaki *et al*. 2008b) with some modifications using known amounts of *V. parahaemolyticus* DMST22092. Briefly, a small number of cells from a single culture on TCBS agar was inoculated in 4 ml of tryptic soy broth (TSB; Difco) supplemented with 2% NaCl and incubated overnight at 37°C. Then, 40 µl of TSB culture was transferred into a new 4 ml of TSB and incubated at 37°C with shaking at 225 rev min^{-1} to obtain mid-log phase cells (OD 600 nm equal to 0.5). Serial 10-fold dilutions of the cultures were prepared in PBS (Phosphate-buffer saline solution).

For preparation of DNA from pure culture, 100 µl of each dilution was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 18000 g for 5 min. After removal of the supernatant, the pellet was resuspended in 50 µl of 25 mmol l^{-1} NaOH and the mixture was subsequently heated at 95°C for 5 min. After neutralization with 4 µl of 1 mmol l^{-1} Tris-HCl buffer (pH 7.5), the suspension was centrifuged at 18000 g for 5 min. Two µl of each supernatant were then used as a template DNA for LAMP and PCR assays. The sensitivity tests were conducted in triplicate, the last dilution with all three samples tested positive was considered as the detection limit.

In parallel with the latter, so as to count the bacteria, 100 µl aliquots of appropriate dilutions were spread on TSA supplemented with 2.5% NaCl in duplicate and incubated overnight at 37 °C. The bacterial colonies were counted at the dilution yielding 30-300 colony forming units (CFUs), and then the CFU ml^{-1} of bacterial suspension was calculated.

Determinations of sensitivities of LAMP and PCR with spiked shrimp samples

Penaeus vannamei shrimp samples were purchased at a local market in Bangkok, Thailand. To confirm that the shrimp samples were negative for *V. parahaemolyticus*, the samples were enriched in alkaline peptone water (APW) for overnight and the shrimp homogenate was then plated onto the TCBS agar and the DNA samples of the shrimp homogenate were extracted and tested for the presence of the *tlh* gene by PCR and LAMP assay.

The sensitivity of the LAMP assay for *V. parahaemolyticus* in spiked shrimp samples was determined as previously described by Yamazaki *et al*. (2008b) with some modifications using known amounts of *V. parahaemolyticus* DMST22092. Nine milliliters of APW were added to 1 gram of the shrimp sample and homogenized thoroughly. Serial ten-fold dilutions of mid-log phase *V. parahaemolyticus* were prepared as described earlier. One hundred microlitres of

each dilution of *V. parahaemolyticus* was spiked into 900 µl of each shrimp homogenate. After mixing well, each homogenate was centrifuged at 200 g for 5 min to remove shrimp tissue. The supernatant was transferred to new centrifuge tubes and centrifuged at 18 000 g for 5 min. After removal of the supernatant, the pellet was resuspended in 100 µl of 25 mmol l^{-1} NaOH, then the mixture was heated at 95 °C for 5 minutes. After neutralization with 8 µl of 1 mmol l^{-1} Tris-HCl buffer (pH 7.5), the suspension was centrifuged at 18000 g for 5 min. Two µl of each supernatant was then used as a template DNA for LAMP and PCR assays. The sensitivity tests were conducted in triplicate, the last dilution with all three samples tested positive was considered as the detection limit.

Results

Optimization of the temperature for *Vibrio parahaemolyticus* detection by LAMP

In order to determine the optimal conditions for LAMP assay, an isolate of *V. parahaemolyticus* DMST 22092 was used for an assay of both optimum temperature and time. To determine the optimum temperature for the LAMP assay, three different temperatures of 60, 63 and 65°C were used to carry out the reaction for 60 min. The LAMP products from all tested temperatures displayed the ladder-like pattern characteristic on agarose gel due to its characteristic structure. However, the LAMP product amplified at 65°C showed the clearest amplification of DNA when compared to the others. Therefore, the temperature of 65°C was chosen for the subsequent LAMP assays (data not shown).

To determine the optimum time for LAMP amplification, three different reaction times of 30, 60 and 90 min were used. The LAMP amplicons could be observed at 30 min; however, the LAMP product revealed the strongest signal at 90 min (data now shown). Therefore, the reaction time of 90 min was selected as an optimal reaction time for the LAMP assay.

The appropriate amount of FITC-labeled probe

To evaluate the appropriate amount of FITC-labeled probe in the hybridization reaction, 200, 20 and 2 pmol of DNA probe were tested. The results showed that the 20 pmol of DNA probe yielded the highest intensity of purple color at the test line (data not shown). Therefore, the concentration of 20 pmol was used for all subsequent assays.

Specificity of LAMP-LFD

To determine the specificity of LAMP-LFD for detection of *V. parahaemolyticus*, various bacterial isolates shown in Table 1 were tested in this study. The results indicated that the

LAMP assay can detect all 28 isolates of *V. parahaemolyticus* but did not show any positive results for 24 other *Vibrio* spp. isolates and 35 non-*Vibrio* isolates. In addition, all the results of LAMP assay agreed with that of PCR.

Sensitivity of LAMP-LFD and PCR methods

In order to determine the detection limit of the LAMP-LFD assay for *V. parahemolyticus*, bacterial genomic DNA of 10-fold serial dilution extracted from pure culture and spiked shrimp samples were used in the LAMP reaction. Based on the initial inoculums of *V. parahemolyticus* (1.2×10^7 CFU ml^-), each LAMP product was analyzed using both electrophoresis and dip stick. The results showed that the sensitivity of LAMP for the detection of *V. parahaemolyticus* in pure culture was 120 CFU ml^- or equivalent to 0.4 CFU per reaction while that of PCR was 4 CFU per reaction (Figures 1a and 2a). For spiked shrimp samples, the sensitivity of LAMP was 1.8×10^3 CFU ml^- or 3 CFU per reaction, while that of PCR was 30 CFU per reaction. (Figures 1b and 2b).

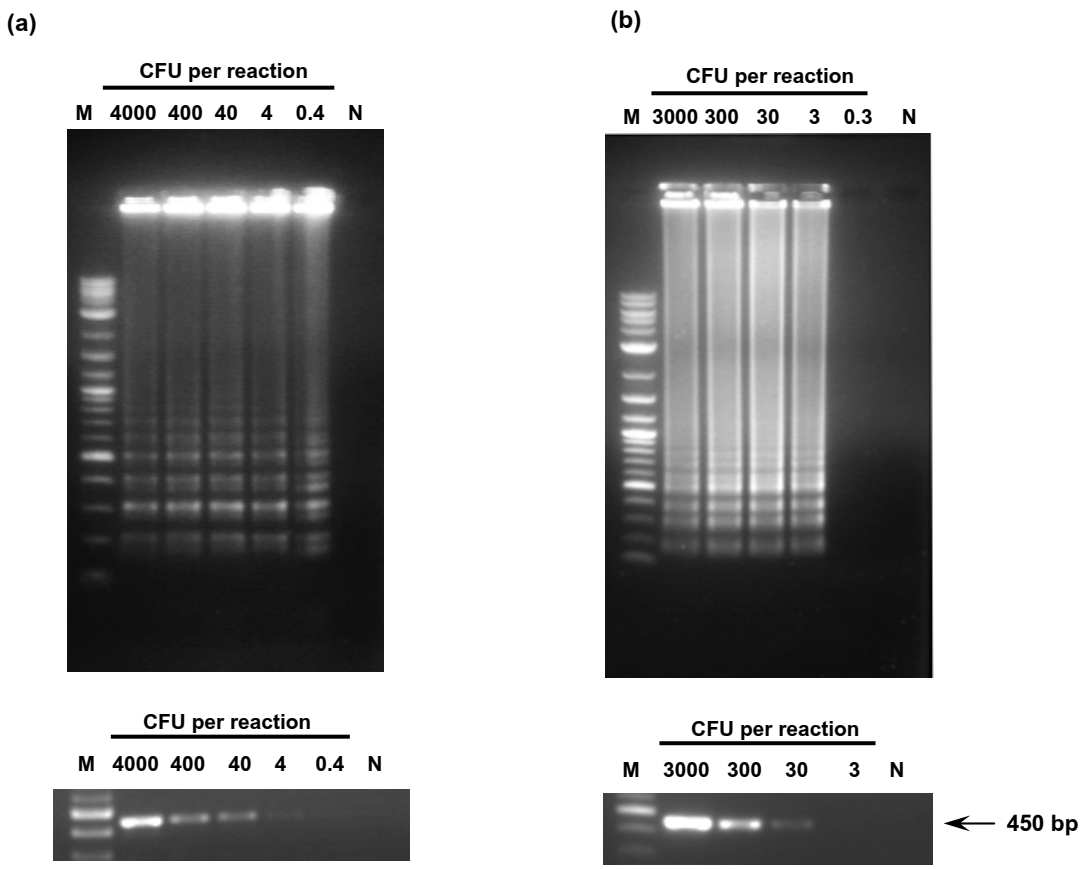

Figure 1 Sensitivity comparison of LAMP (top panel) and PCR (bottom panel) assay for the detection of *V. parahemolyticus* in pure cultures (a) and spiked shrimp samples (b). The bacterial CFU per reaction was indicated on the top of each lane. 2.5 µl of LAMP amplicons from 25 µl were loaded per lane, while 5 µl of PCR product from 50 µl were loaded per lane. Lane M: molecular marker and lane N: negative control.

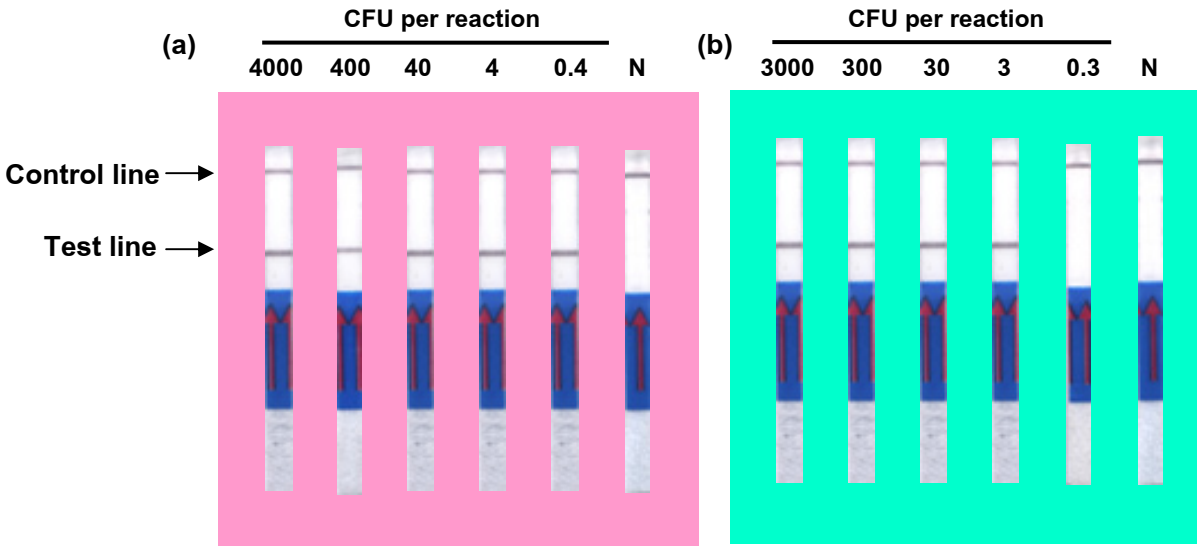

Figure 2 Sensitivity of LAMP combined with LFD for the detection of *V. parahaemolyticus* in pure culture (a) and spiked shrimp samples (b). The bacterial CFU per reaction is indicated on the top of each lane. N: negative control.

Discussion

V. parahaemolyticus is considered a major worldwide cause of seafood-associated bacterial gastroenteritis (Nishibuchi and DePaola 2005; Faruque and Nair 2006). In 2008 the Foodborne Diseases Active Surveillance Network (FoodNet) of the CDC's Emerging Infections Program on diseases caused by enteric pathogens commonly transmitted through food reported that the incidence of *Vibrio* infection has continued to increase. Of *Vibrio* infections, 52% were caused by *Vibrio parahaemolyticus* (CDC 2009). Therefore, the problem of *V. parahaemolyticus* contaminated food is a concern and an effective method to detect this bacterium is required.

In this study, the first LAMP-LFD method targeting the *tlh* gene of *V. parahaemolyticus* has been successfully developed. The optimal LAMP conditions are 65°C for 90 min. This is faster than the typical methods of PCR and biochemical tests.

The results obtained from this study revealed that the LAMP-LFD demonstrated a high specificity to *V. parahaemolyticus* by giving positive results to all tested isolates of this bacteria while yielding a negative result to non-*parahaemolyticus Vibrio* and non-*Vibrio* bacterial isolates. The sensitivity of LAMP-LFD with pure culture in this study was 0.4 CFU per reaction (120 CFU ml^{-1}) which seems a little high. However, the samples may, to some extent, contain DNA

derived from dead or viable but non culturable (VNC) cells (Hayashi *et al*. 2006) which may affect the sensitivity testing.

In the case of spiked shrimp samples, the sensitivity of LAMP-LFD (3 CFU per reaction) was 10 times higher than that of a conventional PCR assay (30 CFU per reaction). The sensitivity result of the developed LAMP-LFD assay was comparable to that of LAMP assays for cholera toxin-producing *V. cholerae* in spiked human feces (1.4 CFU per reaction; Yamazaki *et al*. 2008a), *V. parahaemolyticus* in spiked shrimp samples (2.0 CFU per reaction; Yamazaki *et al*. 2008b) and *trh*2-carrying *V. parahaemolyticus* in spiked shrimp samples (5.0 CFU per reaction; Yamazaki *et al*. 2010) and approximately five to seven times higher than that of LAMP assay for *V. harveyi* in added shellfish cultures (17.2 CFU per reaction; Cao *et al*. 2010) and *V. cholerae* in spiked shrimp sample (20 CFU per reaction; Srisuk *et al*. 2010).

The sensitivity of LAMP-LFD in both pure culture and spiked shrimp samples was 10 times higher than that of a conventional PCR assay. These results agreed with previous studies that demonstrated higher sensitivity of LAMP compared to that of PCR (Yamazaki *et al.* 2008b; Han and Ge 2008; Nemoto *et al*. 2009; Chen and Ge 2010; Liu *et al*. 2010).

Since the LAMP-LFD does not need gel electrophoresis, it is time saving and carcinogenic ethidium bromide does not have to be used. The LAMP-LFD products can be easily detected by dipping the membrane into some of the assay buffer which can reduce the total time for LAMP assays by 60 minutes. Furthermore, the specificity of the LAMP assay can be increased since the hybridization with a specific probe to LAMP amplicons is employed.

In conclusion, the first LAMP-LFD method for *V. parahaemolyticus* detection was successfully established. It had the sensitivity of approximately 3 CFU per reaction with spiked shrimp samples. The developed LAMP-LFD assay is a sensitive, rapid, simple and valuable tool for detection of *V. parahemolyticus* for both research and diagnostic purposes.

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Outputs ที่ได้จากงานวิจัย

มีผลงานวิจัยที่ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 3 เรื่องคือ

1. **Chaivisuthangkura P**, Srisuk C, Rukpratanporn S, Longyant S, Sridulyakul P, Sithigorngul P. Rapid and sensitive detection of *Penaeus monodon* nucleopolyhedrovirus by loop-mediated isothermal amplification. J Virol Methods. 2009; 162: 188-193. (impact factor = 2.077)

2. Srisuk C, **Chaivisuthangkura P**, Rukpratanporn S, Longyant S, Sridulyakul P, Sithigorngul P. Rapid and sensitive detection of *Vibrio cholerae* by loop-mediated isothermal amplification targeted to the gene of outer membrane protein ompW. Lett Appl. Microbiol. 2010; 50: 36-42. (impact factor = 1.64)

3. Prompamorn P, Sithigorngul P, Rukpratanporn S, Longyant S, Sridulyakul P, **Chaivisuthangkura P.** The development of loop-mediated isothermal amplification combined with lateral flow dipstick for detection of *Vibrio parahaemolyticus*. Lett Appl. Microbiol. 2011 doi: 10.1111/j.1472-765X.2011.03007.x (impact factor = 1.64)

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ภาคผนวก

บทความสำหรับการเผยแพร่

โครงการเรื่อง การพัฒนาวิธี loop-mediated isothermal amplification (LAMP) สำหรับการตรวจเชื้อ monodon baculovirus (MBV) เชื้อ *Vibrio parahaemolyticus* และ *V. cholerae*

การติดเชื้อ monodon baculovirus (MBV) ในกุ้งกุลาดำมีผลทำให้กุ้งที่ติดเชื้อเจริญเติบโตช้าและมีขนาดเล็กลงอย่างเห็นได้ชัด ส่วนในกรณีของการติดเชื้อแบคทีเรีย *Vibrio parahaemolyticus* จัดเป็นจุลชีพฉวยโอกาสที่ก่อให้เกิดโรคเมื่อกุ้งหรือสัตว์น้ำอยู่ในสภาวะเครียด นอกจากนี้ *V. cholerae* และ *V. parahaemolyticus* ยังเป็นสาเหตุสำคัญของการเกิดโรคอาหารเป็นพิษในมนุษย์ หากรับประทานอาหารทะเลที่ปนเปื้อนเชื้อดังกล่าว ดังนั้นการตรวจวินิจฉัยที่รวดเร็ว ถูกต้องและแม่นยำจึงเป็นสิ่งสำคัญ วัตถุประสงค์ของงานวิจัยนี้เป็นการพัฒนาวิธี loop-mediated isothermal amplification (LAMP) เพื่อใช้ในการตรวจวินิจฉัยการติดเชื้อ MBV เชื้อ *V. cholerae* และ *V. parahaemolyticus*

สามารถพัฒนาวิธี LAMP สำหรับตรวจการติดเชื้อ monodon baculovirus (MBV) หรือ *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) ในกุ้งกุลาดำได้สำเร็จ โดยมียีนเป้าหมายคือ polyhedrin gene พบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 63°C เป็นเวลา 60 นาที มีความไวในการตรวจ PemoNPV ประมาณ 50 อนุภาคต่อ 1 นาโนกรัมของ genomic DNA หรือ 150 อนุภาคต่อปฏิกิริยา จากการใช้ DNA ต้นแบบที่สกัดจากกุ้งที่ติดเชื้อ PemoNPV (โดยใช้ชุดสกัดกรดนิวคลีอิกของไวรัสที่มีขายเชิงพาณิชย์) พบว่าเทคนิค LAMP มีความไวประมาณ 0.7 เฟมโตกรัม ในขณะที่วิธี nested PCR มีความไวประมาณ 70 เฟมโตกรัม แสดงให้เห็นว่าเทคนิค LAMP มีความไวมากกว่า nested PCR 100 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ PemoNPV โดยไม่ทำปฏิกิริยาข้ามกับไวรัสชนิดอื่นๆ ของกุ้งได้แก่ ไวรัสหัวเหลือง (YHV) ไวรัสโรคทอร่า (TSV) ไวรัสจุดขาว (WSSV) และไวรัสที่ทำให้เกิดโรคแคระแกร็น 2 ชนิดได้แก่ PstDNA หรือชื่อเดิมว่า IHHNV และ PmDNV หรือชื่อเดิมว่า HPV

สามารถพัฒนาวิธี LAMP สำหรับตรวจการติดเชื้อ *V. cholerae* ทุกสายพันธุ์ได้สำเร็จ โดยมียีนเป้าหมายคือ *ompW* โดยพบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 65°C เป็นเวลา 75 นาที มีความไวในการตรวจ *V. cholerae* ใน culture ได้ 2.2×10^3 CFU/ml หรือ 8 CFU ต่อปฏิกิริยา มีและมีความไวในการตรวจ *V. cholerae* ที่ทำให้ปนเปื้อนในตัวอย่างกุ้ง (spiked samples) ได้ 2.2×10^4 CFU/g หรือ 20 CFU ต่อปฏิกิริยา ในขณะที่วิธี PCR มีความไวประมาณ 100 CFU ต่อปฏิกิริยา ดังนั้นวิธี LAMP จึงมีความไวสูงกว่า 5 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ *V. cholerae* ทั้ง 16 isolates ที่ใช้ทดสอบ โดยไม่ทำปฏิกิริยาข้ามกับ non-*cholerae* *Vibrio* 28 isolates และ non- *Vibrio* 37 isolates

สามารถพัฒนาวิธี LAMP ควบคู่กับการตรวจ LAMP amplicons โดยการใช้ lateral flow dipstick (LAMP-LFD) สำหรับตรวจการติดเชื้อ *V. parahaemolyticus* ทุกสายพันธุ์ได้สำเร็จ โดยมียีนเป้าหมายคือ *tlh* โดยพบว่าสภาวะที่เหมาะสมของปฏิกิริยาคือการใช้ อุณหภูมิที่ 65°C เป็นเวลา 90

นาที มีความไวในการตรวจ *V. parahaemolyticus* ใน culture ได้ 120 CFU/ml และมีความไวในการตรวจ *V. parahaemolyticus* ที่ทำให้ปนเปื้อนในตัวอย่างกุ้ง (spiked samples) ได้ 1.8 x 10^3 CFU/g หรือ 3 CFU ต่อปฏิกิริยา ในขณะที่วิธี PCR มีความไวประมาณ 30 CFU ต่อปฏิกิริยา ดังนั้นวิธี LAMP-LFD จึงมีความไวสูงกว่า 10 เท่า นอกจากนี้เทคนิค LAMP ที่พัฒนาขึ้นมีความจำเพาะต่อ *V. parahaemolyticus* ทั้ง 28 isolates ที่ใช้ทดสอบ โดยไม่ทำปฏิกิริยาข้ามกับ non- *parahaemolyticus Vibrio* 24 isolates และ non- *Vibrio* 35 isolates

ผลงานวิจัยที่ได้รับทุนสนับสนุนจาก สกว. ในโครงการวิจัยชุดนี้ ได้รับการตีพิมพ์จำนวน 3 บทความในวารสารนานาชาติ ดังนี้:

1. **Chaivisuthangkura P**, Srisuk C, Rukpratanporn S, Longyant S, Sridulyakul P, Sithigorngul P. Rapid and sensitive detection of *Penaeus monodon* nucleopolyhedrovirus by loop-mediated isothermal amplification. J Virol Methods. 2009; 162: 188-193. (impact factor = 2.077)

ผลงานนี้เป็นส่วนหนึ่งของผลงานวิจัยที่ได้รับรางวัล “ผลงานวิจัยดีเด่น (สาขาเกษตรศาสตร์และชีววิทยา) ประจำปี 2253” จากสภาวิจัยแห่งชาติ เรื่อง การแยกยีนโพลีฮีดรินของ *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) และการพัฒนาเทคนิค loop-mediated isothermal amplification เพื่อตรวจการติดเชื้อ PemoNPV ในกุ้งกุลาดำ

2. Srisuk C, **Chaivisuthangkura P**, Rukpratanporn S, Longyant S, Sridulyakul P, Sithigorngul P. Rapid and sensitive detection of *Vibrio cholerae* by loop-mediated isothermal amplification targeted to the gene of outer membrane protein ompW. Lett Appl. Microbiol. 2010; 50: 36-42. (impact factor = 1.64)
3. Prompamorn P, Sithigorngul P, Rukpratanporn S, Longyant S, Sridulyakul P, **Chaivisuthangkura P.** The development of loop-mediated isothermal amplification combined with lateral flow dipstick for detection of *Vibrio parahaemolyticus*. Lett Appl. Microbiol. 2011 doi: 10.1111/j.1472-765X.2011.03007.x (impact factor = 1.64)