中文摘要：

蝦類病毒性疾病一直是產業重要的議題，截至目前為止，尚無有效的疫苗及治療方法。目前在水產養殖上，僅能對於病毒性疾病進行預防的工作，以降低養殖上的風險。為了達到及早發現病源的目的，或更進一步隔絕病源進入養殖環境，一套有效率且準確的檢測平台就扮演了關鍵的角色。

以往的診斷技術不外乎是傳統病理的觀察，或是免疫相關等技術，然而這些技術常因其靈敏度不足，因而僅能在發病後用以確認病因，無法達到疾病預防的功效。面對日愈嚴重之病毒性疾病，唯有在病源進入養殖環境前，即有效加以攔阻，方可降低疾病造成之損失。為達成此目的，運用高靈敏度的分子檢測技術來發開相關疾病之檢測試劑是可行的。以核酸聚合？連鎖反應(polymerase chain reaction, PCR)**為例，其靈敏度可達到每個反應中有**10**個標的分子即可測出，可謂是相當強大的檢測工具。然而，**PCR**實驗室所需之儀器設備及專業的技術人員，並非一般養殖業者所能負擔與維持，而送檢雖可達到靈敏精確的要求，在時效上則無法達到產業需求，因此，也造成了大型養殖場可自行建構無特定病源**(specific-pathogen-free, SPF)**之養殖系統及相關檢測實驗室，而中小型養殖場則在缺乏有效之檢測工具下，隨時面臨著疾病的威脅。**

本研究即是著眼於解決中小型養殖產業之問題，運用恆溫核酸擴增技術(isothermal amplification)**來開發簡易、低成本與高精確性之分子檢測平台，來滿足中小型養殖業者之需求。研究中評估分枝型擴增法**(ramification amplification, RAM)**、依賴核酸序列擴增法**(nucleic acid sequence based amplification, NASBA)**及恆溫環形核酸擴增法**(loop-mediated isothermal amplification, LAMP)**等技術之可行性。同時，為了簡化核酸擴增後之繁瑣程序及避免汙染情形，即時定量的恆溫核酸擴增檢測方法也一併進行測試。結果顯示，利用**RAM**來偵測蝦類傳染性表皮與造血組織壞死症病毒**(Infectious Hypodermal and Hematopoietic Necrosis Virus, IHHNV)**，其靈敏度可達到每個反應中有**100**個目標分子即可檢出。利用**NASBA**來偵測蝦類陶拉病毒**(Taura Syndrome Virus, TSV)**，其靈敏度可達到每個反應中有**50**個目標分子即可檢出。利用反轉錄**LAMP**來偵測蝦類陶拉病毒，其靈敏度可達到每個反應中有**100**個目標分子即可檢出。而以上三種恆溫核酸擴增反應技術均可在三個小時以內達到**PCR**相當之結果，同時，在專一性與操作簡便上，也同樣達到預設的條件，因此，皆有機會開發成養殖業者使用之池邊檢測試劑，將來也許因應不同標的需求，搭配不同之技術平台。在於即時偵測與定量之考量，初期以**LAMP**搭配螢光共振能量轉移技術**(fluorescence resonance energy transfer, FRET)**開發即時定量檢測試劑，在評估其可行性後，開發搭配此試劑之儀器設備，藉以符合簡易、低成本與高精確性之檢測目的。

關鍵詞：核酸聚合？連鎖反應，無特定病源，恆溫核酸擴增技術，分枝型擴增法，依賴核酸序列擴增法，恆溫環形核酸擴增法，傳染性表皮與造血組織壞死症病毒，陶拉病毒，螢光共振能量轉移技術

Abstract:

Shrimp viral diseases are important issues in aquaculture industries. Until now, there have been not efficient vaccines or therapeutic strategies against these viral diseases. To face the threats, farmers tried to decrease cultivation risks with more efficient methods. However, they had no tools to early detect and prevent viruses into cultivation environment. For the purpose of pathogen detection before disease outbreak, a diagnostic platform with efficiency and accuracy plays an important role for health management of shrimp culture.

Traditional strategies, such as histochemistry or immune-related techniques, only could be used as disease determination because of their insufficient sensitivities. To pathogen prevention, however, applications of molecular diagnostic technologies are feasible strategies to conquer the defects of sensitivities and complexities of histochemistry and immuo-related techniques. Polymerase chain reaction (PCR), for example, is a powerful diagnostic tool that can detect even 10 molecules in the reaction. PCR related techniques had been used to establish the culture system of specific pathogen free (SPF) and daily diagnosis for large-scale or integrated farms. However, the needs of equipments and technicians for a PCR laboratory operating are too expensive for medium or small-sized farms. Although some diagnostic centers may provide services of PCR diagnosis, the time to get results is always too long. With so many difficults to get efficient diagnostic tools, the pathogenic threats are accomplished with these smaller farms.

In the study, some isothermal amplification techniques will be applied to develop a simple, economic and accurate molecular diagnostic platform to match farmers' needs. Isothermal amplification techniques, including ramification amplification (RAM), nucleic acid sequence based amplification (NASBA) and loop-mediated isothermal amplification (LAMP), will be evaluated the feasibilities. In addition, real-time detection of these products amplified by isothermal amplifications is also tested to avoid post-amplification procedures and simplify data interpretation. The results showed that the sensitivity of RAM in detecting IHHNV was 100 copies per reaction, and the sensitivity of NASBA and reverse transcription LAMP in detecting TSV was 50 and 100 copies per reaction, respectively. Three kinds of isothermal amplification methods could be achieved high sensitivities comparable to that of PCR at 3 hours. The specificities and simplicities of these methods are also verified under different conditions to match the purpose of on-site use. For the purposes of real-time detection and quantification, LAMP coupled with fluorescence resonance energy transfer (FRET) techniques is studied and the results are available for follow-up developments. For further utilities, a novel real-time isothermal amplification detection machine should be developed to fit the purpose of diagnosis with simple, economic and

accurate.

Keyword: PCR, SPF, isothermal amplification, RAM, NASBA, LAMP, IHHNV, TSV, FRET

Chapter 1. Introduction

Since 1970's, farmed shrimp production has been an almost exponential growth, therefore, management of farming environment has become more and more important. Shrimp production accounted for a trade value of USD 7.9 billion in 2001 (FAO, 2003), making it the most valuable marine product in the world trade. According to fishery statistics information from Food and Agriculture Organization (FAO), shrimp production globally was about 1.8 million tones and Pacific white shrimp *Litopenaeus vannamei* was the major species because of the better growth rate and disease resistance. Due to well control of entire life cycle, *L. vannamei* became the most popular farmed shrimp species around the world.

L. vannamei, also called *Penaeus vannamei*, is a decapods crustacean (Table 1), the order which included shrimps, lobsters and crabs. In 1997, Farfante and Kensley

Table 1. Taxonomy of *L. vannamei*

Phylum – *Arthropoda*
Class – *Crustacea*
Subclass – *Malacostraca*
Series – *Eumalacostraca*
Superorder – *Eucarida*
Order – *Decapoda*
Suborder – *Dendrobrachiata*
Infraorder – *Penaeidea*
Superfamily – *Penaeoidea*
Family – *Penaeidae*
Genus – *Penaeus*
Subgenus – *Litopenaeus*
Species - *vannamei*

renamed it as *L. vannamei* (Farfante and Kensley, 1997). *L. vannamei* is native to the Pacific coast of Mexico, Central and South America where ocean water temperature generally remained above 20℃ throughout the year. Because of its tolerance of wide range of environment, fast growth rate and disease resistance, more and more countries imported SPF *L. vannamei* and cultivated this potential species.

However, the international movement of live or frozen shrimp has led to the transfer and establishment of certain pathogens from one location to the others (Lightner, 1996a; Durand *et al*., 2000; AQUIS, 2000). Frozen commodity shrimps have been implicated as the route which White Spot Syndrome Virus (WSSV) was moved from Asia to the America, while Taura Syndrome Virus (TSV) was moved in the opposite direction with infected live broodstock from Central America (Nunan *et al*., 1998a; Tu *et al*., 1999; Yu and Song, 2000; Durand *et al*., 2000). There are now various pathogens threatening shrimp farming areas including viruses, bacteria, fungi and protozoa (Gabriel and Felipe, 2000). Among these pathogens, viruses are considered as the most significant factor that have caused massive mortalities around hatchery and grow-out ponds (Ownes, 2003). In 1974, the first shrimp virus, Baculovirus Penaei (BP) was isolated and identified from Northern Pink Shrimp, *Farfantepenaeus duorarum* (Couch, 1974). Until now, more than twenty viruses were isolated from cultured shrimp, including WSSV (Inouye *et al*., 1994; Takahashi *et al*., 1994; Chou *et al*., 1995; Huang *et al*., 1995a,b; Wangteerasupaya *et al*., 1995; Lo *et al*., 1996; Yan *et al*., 2004), TSV (Brock *et al*., 1995; Hasson *et al*., 1999), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) (Bell and Lightner, 1984), *P. monodon*-type Baculovirus (MBV) (Halder *et al*., 1989; Chang *et al*., 1993), Gill-associated Virus (GAV) (Cowley *et al*., 2002; Spann *et al*., 2003), Yellow Head Virus (YHV) (Nadala *et al*., 1997; Mohan *et al*., 1998; Tang and Lightner, 1999), Hepatopancreaus Virus (HPV) (Bonami *et al*., 1995; Sukhumsirichart *et al*., 1999) and Infectious Myonecrosis Virus (IMNV) (Tang *et al*., 2005; Poulos *et al*., 2006 a,b). Among these infectious viral diseases of cultured shrimp, WSSV, TSV, IHHNV and YHV are the major pathogens that caused massive economic losses (Vanpatten *et al*., 2004). These pathogens were spreaded from country to country by trade activities. With less information and diagnostics tools, the epidemics of pathogens were increasing and caused serious economic losses (Table 2) (Lightner, 2003).

The impacts caused by these viral diseases have inflicted those major shrimp farming countries, especially in Asia. Now China, Thailand, Vietnam, India and Indonesia are the major supplying and producing countries of cultured marine shrimp. To prevent viral pathogens spread by shrimp shipping and trading, sensitive and

accurate diagnostic methods are necessary for this purpose.

Table 2. Estimated economic losses since the emergence of certain diseases in penaeid shrimp aquaculture (modified from Lightner 2003).

Virus	Year of emergence to 2001	Product Loss (dollars)
WSSV - Asia	1992	4-6 billion
WSSV-Americas	1999	>1 billion
TSV	1991-92	1-2 billion
YHV	1991	0.1-0.5 billion
IHHNV*	1981	0.5-1.0 billion
* Includes Gulf of California fishery losses for 1989-1994.		

In general, there are many standard methods listed in OIE (Office International des Epizooties) manual of diagnostic tests for aquatic animals. These available diagnostic methods are based on gross and clinical signs, microscopy, immunohistochemistry and molecular diagnostics techniques. Using white spot disease (WSD) as an example, gross signs include the presence of white spots under the cuticle, reduction in feed intake, increasing lethargy, movement of moribund shrimp to the water surface and pond edges. The microscopic examination and molecular techniques are also available. Polymerase chain reaction (PCR) diagnostic method is according to Lo *et al.* (Lo *et al.*, 1996; Lo *et al.*, 1997), and is recommended for all situations where WSSV diagnosis is required. In this nested PCR based diagnostic method, a positive result showed in the first step implies a serious WSSV infection. When a positive result is obtained in the second amplification step only, a latent or carrier-state infection is indicated. Other molecular methods such as dot blot (Lightner, 1996a) and *in situ* hybridization (Flegel *et al.*, 1996; Lightner, 1996a) were also carried out.

As all diagnostic methods showed above, PCR technique has been applied on numerous pathogen detections and shrimp pathology researches. Many research groups reported diagnostic protocols on the detection of WSSV by PCR (Takahashi *et al.*, 1996; Maeda *et al.*, 1998; Tapay *et al.*, 1999; Otta and Karunasagar, 2003). Although PCR method has been widely applied for pathogen diagnosis, other nucleic acids based diagnostic methods are still studied to be more advanced, such as simplicity, sensitivity, accuracy or cost. Isothermal amplification methods are developed to satisfy some of the purposes.

Since 1990, several concepts of isothermal amplification strategies had been

developed and practiced to be a more potential tool for pathogen diagnosis, including self-sustained sequence replication (3SR) system (Guatelli *et al*., 1990), nucleic acid sequence based amplification (NASBA) (Compton, 1991), strand displacement amplification (SDA) (Walker *et al*., 1992), rolling circle amplification (RCA) (Fire and Xu, 1995), loop-mediated isothermal amplification (LAMP) (Notomi *et al*., 2000) and ramification amplification (RAM) (Zhang *et al*., 2001). The 3SR system is useful for the detection and nucleotide sequence analysis of rare RNAs and DNAs. NASBA is targeting on RNAs by using AMV reverse transcriptase, RNase H and T7 RNA polymerase. SDA requires restriction enzyme cleavage of the DNA sample prior to amplification. RCA and RAM use the same strategy to amplify the circular targets by specific primers and polymerases with strand displacement activity. LAMP employs a DNA polymerase and a set of specific primers that recognized a total of six distinct sequences on the target DNA. All of these isothermal amplification strategies contain single or multiple enzymes, special design of primer-set and simple equipments, and are easy to operate. Each strategy owns its innovation of DNA synthesis. For example, PCR used heat denaturation of double-stranded DNA products to promote the next round of DNA synthesis. 3SR and NASBA eliminate heat denaturation by using a set of transcription and reverse transcription reactions to amplify target sequences. SDA eliminates the heat denaturation step by employing a set of restriction enzyme digestions and strand displacement DNA synthesis with modified nucleotides as substrate. In this research, some isothermal amplification methods, such as RAM, NASBA and LAMP, will be practiced to detect different shrimp viruses. These methods employ different strategies in cycling DNA synthesis. By these researches, we try to develop a platform for pond site diagnostic system with powerful amplification methods.

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Chapter 2. Detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Litopenaeus vannamei* by ramification amplification (RAM) assay

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Background:

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) was first identified in 1981 to be the cause of mass mortality in blue shrimp *Litopenaeus stylirostris* (also known as *Penaeus stylirostris*) (Lightner *et al*., 1983a, b). In Pacific white shrimp *Litopenaeus vannamei* (also known as *Penaeus vannamei*), IHHNV causes growth reduction and runt deformity syndrome (RDS) rather than high mortality (Kalagayan *et al*., 1991). The economic loss caused by IHHNV infection in the USA from 1981 to 2001 was estimated to reach $US 0.5 to 1.0 billion (Lightner, 2003). According to morphological and genetic characteristics, IHHNV is classified as a member of the *Parvoviridae* family (Bonami *et al*., 1990; Mari *et al*., 1993) and is closely related to *densoviruses* of the genus *Brevidensovirus* (Shike *et al*., 2000). IHHNV has a non-enveloped icosahedral particle 22 nm in diameter, which contains linear 4.1 kb single-stranded DNA (Berns *et al*., 1995). Analysis of the genomic sequences of IHHNV collected in the USA between 1982 and 1997 showed that there was a low level of variation among these isolates (Tang and Lightner, 2002).

To avoid cross-species infection by IHHNV, routine surveillance of IHHNV is crucial for shrimp farmers and quarantine services. Recently, several PCR methods were developed for IHHNV detection in shrimp tissues (Lightner *et al*., 1994; Poulos *et al*., 1995) as well as in culture system detritus, mud and seawater (Glover *et al*., 1995). Quantitative real-time PCR IHHNV detection kits have also been developed (Dhar *et al*., 2001; Tang and Lightner, 2001). Although PCR-based methods have been commonly accepted in laboratories, they are generally not suitable for on-site pathogen detection due to their high cost and technical requirements.

Ramification amplification (RAM), a novel DNA amplification method, was first described in 1998 by Zhang *et al*. (1998). RAM is an isothermal amplification method that utilizes a circularizable oligodeoxyribonucleotide probe (C probe) as an amplifiable target. The C probe is a synthetic oligonucleotide with a sequence complementary to the specific target sequence. Upon hybridization to the target DNA, the 5' and 3' ends of the C probe are drawn together and can be ligated to form a closed circular molecule (Nilsson *et al*., 1994). Under isothermal condition, the circular C probe is subsequently amplified with a pair of C probe-specific primers, driven by *Bst* DNA polymerase with high strand-displacement activity. This

amplification is also named hyperbranched rolling circle amplification or cascade rolling circle amplification.

After RAM was first demonstrated by Zhang *et al*. (1998), it has been widely applied to DNA, RNA or protein based diagnosis and single nucleotide polymorphism analysis (Barany 1991; Tyagi *et al.*, 1996; Lizardi *et al.*, 1998; Thomas *et al.*, 1999; Schweitzer *et al.*, 2000; Wiltshire *et al.*, 2000). Other isothermal amplification methods, such as loop-mediated isothermal amplification (LAMP), were also reported to detect targets at the fentogram range under isothermal conditions (Kono *et al.*, 2004). However, RAM includes a ligation step that offers better specificity over other isothermal amplification strategies (Zhang *et al.*, 1998). If mismatch occurs at the ends of the C probes at the annealing stage, the ligation step in RAM would not proceed to allow the subsequent signal amplification step. Furthermore, RAM yields more than 10^6 copies of amplicons within 1 h from 20 copies of C probes (Zhang *et al.*, 1998), and shows a level of amplification efficiency competitive with that of other amplification methods such as PCR and LAMP.

In this approach, an IHHNV RAM method was established and evaluated for the detection of IHHNV in *L. vannamei*. By modifying the amplification conditions, an IHHNV RAM detection system was set up and further developed into a point-of-operation diagnostic kit.

Materials and methods:

a. **Sample preparation and DNA extraction.** Shrimp *L. vannamei* samples were collected from shrimp farms in Taiwan. For DNA extraction, 1 gill (about 20 mg) was ground thoroughly in 500 µl of Lysis Buffer (GeneReach Biotechnology Corp., Taichung, ROC). After incubation at 95°C for 10 min, samples were centrifuged at 12,000 × *g* for 10 min to remove the cell debris. Subsequently, 200 µl of supernatant was precipitated with 400 µl of 95% ethanol and centrifuged at 12,000× *g* for 5 min. The pellets were air-dried and stored at -20°C.

b. Circular C probe preparation. The sequences of the C probe and probe IHH-3227 are listed in Table 3. To prepare circularized C probe, 10^{11} copies of the 10^6 bp C probe were incubated with excess amounts of the 54 bp IHH-3227 oligonucleotide at 55°C for 1 h to allow the C probe to anneal to IHH-3227. Subsequently, T4 DNA ligase was added and the ligation reaction was performed at 37°C for 30 min. Circular C probes that were twisted around the IHH-3227 were purified by the Gene-Spin 1-4-3 DNA extraction kit (Promega, Wisconsin, USA) in order to remove the salts, enzymes and linear oligonucleotides, dissolved in 1x TE buffer, and stored at -20°C.

c. Coupling of biotin-labeled probes to streptavidin-coated beads. Streptavidin-coated magnetic beads (Promega, Wisconsin, USA) were used to immobilize the biotin-labeled capture probes NCA2-b and NCA3-b (Table 1). Briefly, 0.5 µM of probes were incubated with 2.5 mg ml^{-1} streptavidin-coated magnetic beads in 5× saline sodium citrate (SSC) buffer for 10 min at room temperature, followed by several washes in distilled water to remove unbound probes.

Table 1. Oligonucleotides used in IHHNV RAM assay

C probe	5'-GCTTCCTTAGTTGATAGTTGATTATTATCTGCTAACCAAGA GCAACTACACTCGGAATTCATTAGGTTACTGCGATTAGCACA CGCATTTTGCCAATGTTACGTCG-3'
IHH-3227	5'-GACTACATAATCAACTATCAACTAAGGAAGCC GACGTAACATTGGCAAAATGGA-3'
RAM-F	5'-GTGTAGTTGCTCTTGGTTAGA-3'
RAM-R	5'-ATTCATTAGGTTACTGCGATTAGCACA-3'
NCA2-b	5'-CCCAACTTGTGACCGTACAATCAA-3'
NCA3-b	5'-ACACTAGAAATTGGTATGGCGGAGAA-3'
106BD-b	5'-GCCAATGTTACGTCGGCTTCCTTAGTT-3'

C probe: circularizable oligonucleotide.
IHH-3227: synthesized IHHNV target oligonucleotide.
RAM-F, RAM-R: amplification primers of the circular C probe.
NCA2-b, NCA3-b: capture oligonucleotide coated on the magnetic beads.
106BD-b: biotin-labeled oligonucleotide used for the detection of RAM products in dot blot analysis.

RAM assay. A schematic of the IHHNV RAM assay is provided in Fig. 1. Initially, 5 µl of DNA extract, 10^9 copies of C probe, and 5 µg of magnetic beads coated with the IHHNV-specific probes NCA2-b/NCA3-b were allowed to hybridize in a total of 50 µl 5× SSC and 7.5% dextran sulfate at 55°C for 1 h. After washes twice with 50 µl 0.5× SSC, the mixture was added to a 10 µl ligation mixture containing 0.5 unit of T4 DNA ligase (Promega, Wisconsin, USA), incubated at 37°C for 10 min and washed twice with 50 µl 0.5× SSC. Subsequently, 20 µl of the RAM mixture (300 µM dNTP, 20 mM Tris-Cl [pH 8.8], 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 3 mM $MgSO_4$, 0.1% Triton X-100), 1.2 µM of each primer and 6.4 units of *Bst* DNA polymerase (New England Biolabs, Ontario, Canada) were added. The resulting mixture was incubated at 63°C for 60 min, followed by inactivation of the enzymes at 80°C for 10 min. The amplified products were analyzed by electrophoresis on a 2% agarose gel.

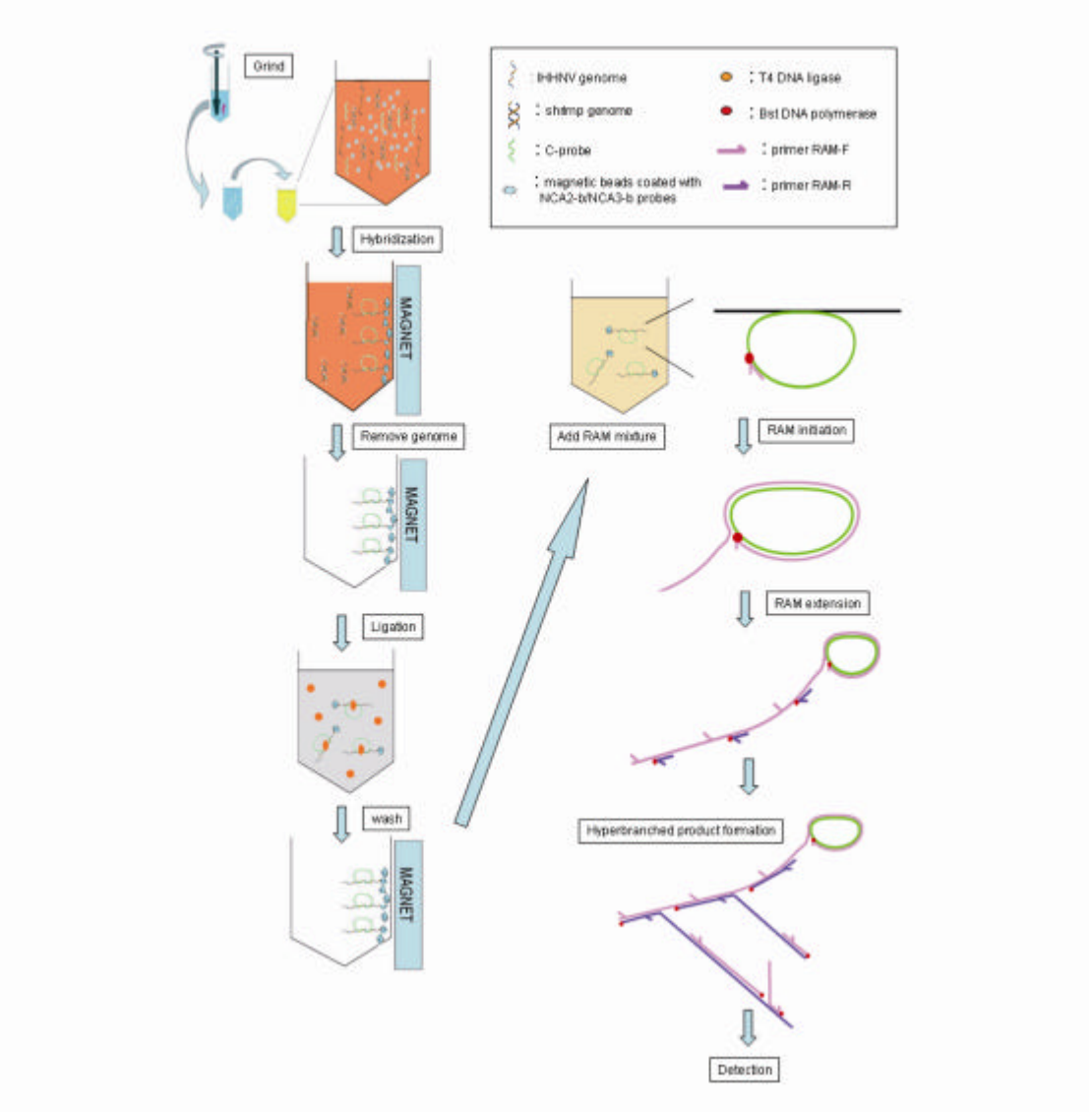

Fig. 1. Schematic IHHNV RAM assay. Samples were ground thoroughly and lysed by lysis buffer, then C probes and magnetic beads coated with capture probes were added to the lysate. The mixture containing IHHNV genome, C probe and magnetic beads coupled with capture probes was formed after hybridization. Shrimp genome and unbound C probes were removed by a washing step. Ligation of C probe ends was achieved by T4 DNA ligase. An additional washing step removed non-ligated C probes prior to addition of RAM reaction mixture and Bst DNA polymerase to the ligated complex. Numerous concatemerized hyperbranched repeats, derived from the C probe, were formed through DNA polymerization and strand displacement and detected by agarose gel electrophoresis or dot blot assay.

d. **PCR for IHHNV detection.** For comparative studies of the sensitivity of the RAM assay, the PCR-based IQ2000 IHHNV detection system (GeneReach

Biotechnology Corp., Taichung, ROC) was used. The reaction conditions for the IQ2000 IHHNV detection system included 10 cycles of 94°C for 20 s and 70°C for 20 s; 35 cycles of 94°C for 20 s, 56°C for 20 s and 72°C for 30 s, followed by an incubation at 72°C for 30 s and 20°C for 30 s. After the reaction was completed, samples were analyzed by electrophoresis on a 2% agarose gel. To clarify the specificity of the primer sets of IHHNV RAM, samples were prepared from white spot syndrome virus (WSSV)-infected shrimps, and the PCR-based IQ2000 WSSV detection system (GeneReach Biotechnology Corp., Taichung, ROC) was used. This system was a nested PCR that included 2 steps. The first run was 5 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s, followed by 15 cycles of 94°C for 15 sec, 62°C for 15 s and 72°C for 20 s. The nested run was 25 cycles of 94°C for 20 s, 62°C for 20 s and 72°C for 30 s followed by incubation at 72°C for 30 s and 20°C for 30 s. After the reaction was completed, the samples were analyzed by electrophoresis on a 2% agarose gel.

e. **Dot blot assay.** RAM products (1 µl) were spotted onto a nylon membrane (Millipore, Massachusetts, USA) and fixed by UV cross-linking at 0.12 J cm^{-2} in a UV Crosslinker Box (Spectroline, New York city, USA). The membrane was then hybridized with the biotin-labeled probe 106BD-b (Table 1) at 63°C in hybridization buffer (5× SSC) for 1 h, washed twice in wash buffer (1× SSC), incubated with 10 µg ml^{-1} streptavidin-alkaline phosphatase conjugate (Promega, Wisconsin, USA) in blocking buffer (100 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.3% Tween-20) at 25°C for 10 min and washed twice in 1× SSC. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Promega, Wisconsin, USA) diluted 100-fold in alkaline phosphatase buffer (100 mM Tris-Cl pH 9.0, 150 mM NaCl, 5 mM $MgCl_2$) was added to the membrane and incubated at 25°C for 10 min.

Results:

A. **Optimization of IHHNV RAM assay:** In the RAM assay, the *Bst* DNA polymerase plays an important role in the production of the ramification products. *Bst* DNA polymerase, a thermo-stable DNA polymerase with high strand displacement activities, was first isolated from *Bacillus stearothermophilus* and used for DNA sequence analysis (Mead *et al*., 1991). Magnesium ion (Mg^{2+}), a known cofactor of *Bst* DNA polymerase, is crucial to the activity of the enzyme. The effect of Mg^{2+} concentration on a *Bst* DNA polymerasemediated ramification reaction was investigated by using the circularized C probes as standards. The optimal concentration of Mg^{2+} required to detect 10^3 copies of template in the reaction was determined to be 3 mM. Other components such as dimethyl

sulfoxide (DMSO) and formamide had been reported to enhance the specificity of the ramification reaction (Cheng *et al*., 1994; Varadaraj and Skinner 1994). However, no significant difference was detected regardless of DMSO or formamide addition in the test reaction. The final RAM buffer contained 20 mM Tris-Cl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 3 mM $MgSO_4$ and 0.1% Triton X-100.

To estimate the sensitivity of the ramification step alone without involving the ligation step, serial 10-fold dilutions (10^1 to 10^7 copies) of circularized C probe were used as templates. The sequences of oligonucleotides used in this study are listed in Table 3. The DNA ladders of the hyperbranched RAM products were analyzed by electrophoresis on a 2% agarose gel. RAM products were obtained with 10^7 down to 10^1 copies of the circular targets (Fig. 2). This result revealed that the ramification step achieved high degrees of sensitivity.

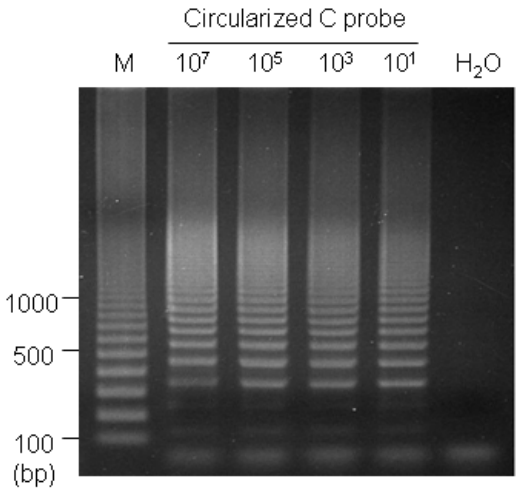

Fig. 2. Detection of circularized C probe amplified by *Bst* DNA polymerase. Variable numbers of copies (10^7, 10^5, 10^3, 10^1) of circularized C probe were used. The RAM reaction, using RAM-F and RAM-R primers, was performed at 63°C for 60 min. Amplified products were detected by electrophoresis on a 2% agarose gel. M: 100 bp DNA ladders; H2O: no circularized C probe added.

B. **IHHNV RAM assay targeting an artificial oligonucleotide:** Based on the published IHHNV genome sequence (GenBank accession number AY355308), the oligonucleotide sequence of IHH-3227 was designed to serve as an artificial template to set up the IHHNV RAM assay. IHH-3227 (10^1, 10^3 and 10^5) was used as the initial template and 10^9 copies of the linear C probe were added to the IHHNV RAM reaction. RAM products were analyzed by electrophoresis. The results showed that as few as 10^1 copies of the IHH-3227 target led to the

production of expected IHHNV RAM products (Fig. 3) and demonstrated the feasibility and sensitivity of the IHHNV RAM reaction.

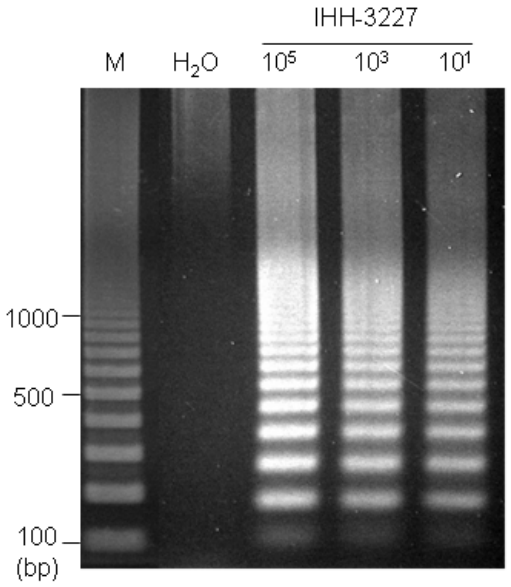

Fig. 3. Detection of a synthetic oligonucleotide target by IHHNV RAM assay. Variable numbers of copies (10^5, 10^3, 10^1, 0) of IHH-3227 and 10^9 copies of linear C probe were added in each test. After initial hybridization and ligation steps, ramification amplification using RAM-F and RAM-R primers was performed (see 'Materials and methods'). RAM products were detected by electrophoresis on a 2% agarose gel. M: 100 bp DNA ladders; H_2O: no IHH-3227 added.

C. **Sensitivity and specificity of IHHNV RAM assay compared with IHHNV PCR:** The sensitivity of the IHHNV RAM assay was evaluated by using the IQ2000 IHHNV diagnostic system. The IQ2000 diagnostic kit is a PCR-based IHHNV detection system with high sensitivity and specificity. Serial 10-fold dilutions (10^1, 10^2, 10^3 or 10^4) of DNA extracted from *L. vannamei* were examined. Products were observed with a DNA sample diluted as much as 10^4-fold in both the RAM and PCR reactions (Fig. 4A, 4B). RAM products were analyzed by dot blot assay to confirm that ladders were C probe-dependent amplicons (Fig. 4C).

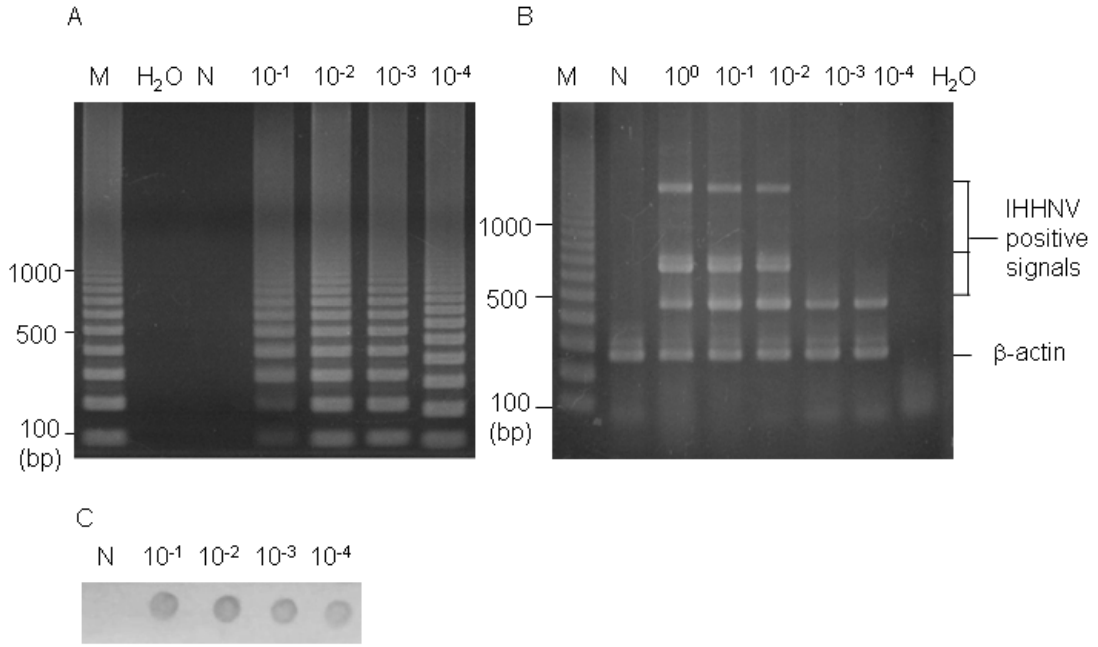

Fig. 4. Comparison of sensitivity between IHHNV RAM assay and IQ2000 IHHNV detection system. DNA extracts of various dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) were prepared from *Litopenaeus vannamei* and amplified by (A) IHHNV RAM assay or (B) IQ2000 IHHNV detection system. Products were analyzed by electrophoresis on a 2% agarose gel. (C) IHHNV RAM products were further confirmed by dot blotting analysis. M: 100 bp DNA ladders; H2O: no DNA extract added; N: negative control (yeast tRNA).

To clarify the specificity of this primer set, 2 WSSV-infected shrimps (Fig. 5A), 2 IHHNV-infected shrimps (Fig. 5B) and 2 negative samples were assayed by IHHNV RAM. Only IHHNV infected samples showed positive results after IHHNV RAM assay (Fig. 5C).

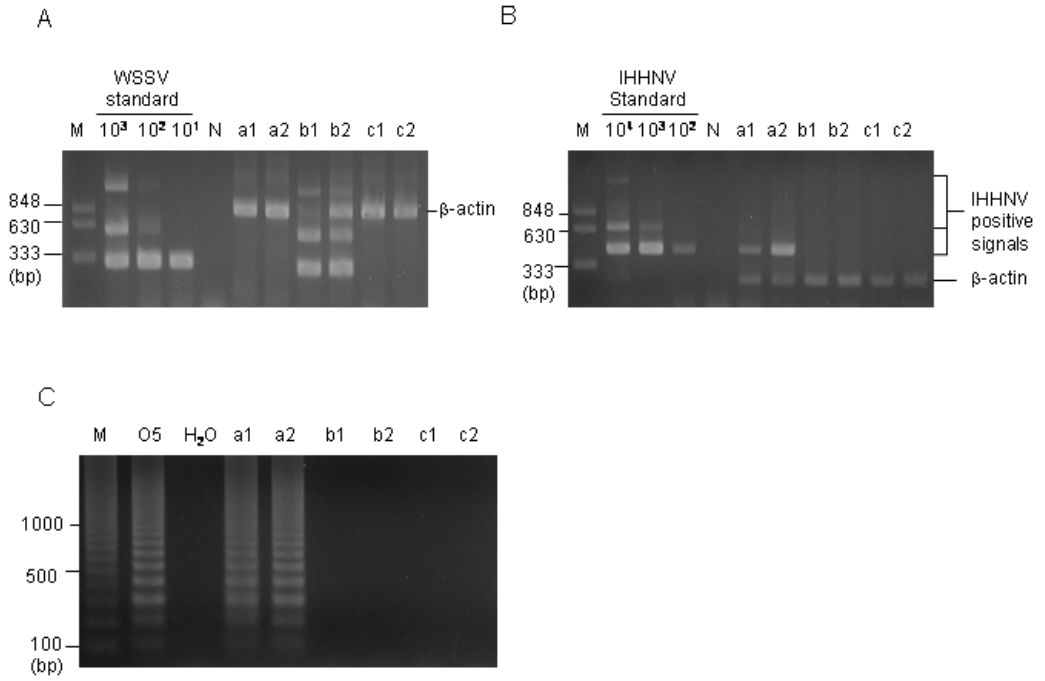

Fig. 5. Specificity of IHHNV RAM assay. Samples were prepared from Litopenaeus vannamei and assayed by (A) IQ2000 WSSV detection system, (B) IQ2000 IHHNV detection system and (C) IHHNV RAM assay. The standard group contained various plasmid copies containing either WSSV or IHHNV partial sequences; a1 and a2 samples were from IHHNV infected shrimps; b1 and b2 samples were from WSSV infected shrimps; c1 and c2 samples were from WSSV- and IHHNV-negative shrimps. Products were analyzed by electrophoresis on a 2% agarose gel. M: DNA ladders; H_2O: no DNA added; O5: 10^5 copies of circularized C probe alone; N: negative control (yeast tRNA).

D. **Sensitivity of IHHNV RAM assay using shrimp samples:** To further evaluate the sensitivity of the IHHNV RAM assay, shrimp samples were collected from shrimp culture ponds and diagnosed using the IQ2000 IHHNV detection system. Out of 4 test samples, 2 samples (#27 and #13) were 10-fold serially diluted and determined by the IQ2000 IHHNV PCR method to be lightly infected (Fig. 6A), whereas the other 2 samples (N1 and N2) were assessed by the same method to be IHHNV-negative (Fig. 6B). A similar degree of sensitivity was obtained with the IHHNV RAM assay (Fig. 6C). These results indicate that RAM offers a feasible method for sensitive detection of IHHNV in the field.

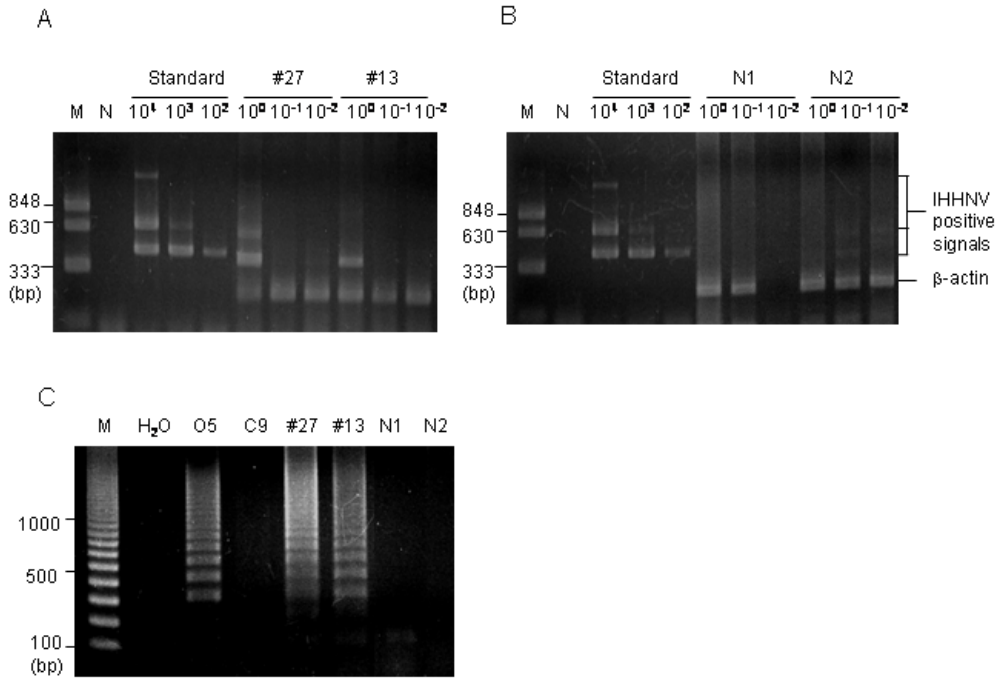

Fig. 6. Detection of IHHNV infection from farmed *Litopenaeus vannamei* samples by RAM assay. (A) IHHNV-infected *L. vannamei* DNA samples were diagnosed by IQ2000 IHHNV detection system. The standard group contained variable numbers of plasmid copies (10^4, 10^3, 10^2) containing IHHNV partial sequences; #27 and #13 groups were DNA samples prepared from L. vannamei at various dilutions (10^0, 10^{-1} or 10^{-2}). (B) IHHNV-negative L. vannamei DNA samples were analyzed by IQ2000 IHHNV detection system. M: DNA marker; N: negative control without DNA added. N1 and N2 groups were DNA samples prepared from *L. vannamei* at various dilutions (10^0, 10^{-1} or 10^{-2}). (C) The same samples used in (A) were subjected to IHHNV RAM assay. RAM products were analyzed by electrophoresis on a 2% agarose gel. M: 100 bp DNA ladders. H_2O: No DNA added; N: negative control (yeast tRNA); O5: 10^5 copies of circularized C probe alone; C9: 10^9 copies of linear C probe alone.

Discussion:

In this study, a RAM assay was developed as a simple and sensitive IHHNV detection method. Optimal conditions for the ramification step were established in order to generate detectable products from 10^1 copies of an artificially generated circular C probe and its specific primers (Fig. 2). Subsequently, an IHHNV-specific RAM assay was developed after coupling with a target-specific ligation step using a

synthetic linear target. Detectable products were observed from as few as 10^1 copies of the artificial target (Fig. 3). The sensitivity of the IHHNV RAM assay was similar to that of the IQ2000 IHHNV detection system when DNA extracted from IHHNV-infected *L. vannamei* was used as targets (Figs. 4 & 6).

The selection of DNA polymerases is important for successful RAM reaction. The hyperbranched DNA products are formed in RAM reaction as a result of the polymerization, strand displacement and high processing activities of DNA polymerases (Fig. 1). However, when the C probes hybridize onto the targets, the topological constraint at the hybridized area seems to interfere with subsequent strand extension by some DNA polymerases (Baner *et al.*, 1998). The Klenow fragment of *E. coli* DNA polymerase I cannot pass through the hybridized area between the C probe and the target (Baner *et al.*, 1998). Although the phi 29 DNA polymerase, isolated from *Bacillus subtilis* bacteriophage phi 29, was reported to possess the ability to efficiently elongate long single-stranded DNA (Lizardi *et al.*, 1998; Zhang *et al.*, 2001a), the sensitivity of RAM achieved by the phi 29 DNA polymerase was 1000-fold lower than that of the *Bst* DNA polymerase, possibly owing to low intrinsic strand displacement activity (Zhang *et al.*, 2001a). Therefore, the *Bst* DNA polymerase appears to be the optimal choice for now.

The *Bst* DNA polymerase, which possesses high strand displacement activity and lacks 5'-3' exonuclease activity, was used to amplify the C probe signal in this study. To optimize reaction conditions for the *Bst* DNA polymerase-mediated ramification step, the circularized C probes were used as templates to bypass the ligation step. Without available information on the optimal concentrations of divalent cations for the *Bst* DNA polymerase, different concentrations of Mg^{2+} were added to the commercial standard buffer to attain the optimal RAM sensitivity. The concentration of Mg^{2+} was proved to be critical at this step. A 3 mM concentration of Mg^{2+} generated a 1000-fold increase in RAM sensitivity compared with a 2 or 4 mM concentration (data not shown). The suitable range of Mg^{2+} concentrations is thus narrow. Although magnesium ions were reported to be superior to manganese and calcium ions in mediating the activities of DNA polymerase (Chien *et al.*, 1976), replacement of magnesium ions by other divalent ions has not yet been excluded.

Since DMSO and L-proline were reported to facilitate DNA elongation step and stabilize the polymerase-template complex (Zhang *et al.*, 2001b) for DNA replication by *Bst* DNA polymerase, these components were tested separately in the RAM reaction mixture. However, no significant enhancement of RAM sensitivity was observed (data not shown). The effect of buffer components may be dependent on target DNA characteristics, such as DNA secondary structure or the inclusion of GC-rich regions.

The ligation reaction is another critical step of RAM assay. Without a successful ligation step, the C probes would not be circularized to allow RAM to proceed. In this study, T4 DNA ligase was used to link the junction formed after the C probes had perfectly annealed on the targets. T4 DNA ligase is an ATP-dependent DNA ligase that ligates DNA probes onto a DNA or RNA template (Nilsson *et al*., 2000; Nilsson *et al*., 2001). The quantity of circularized probes formed on targets is one of the key factors that determined the success of the RAM reaction. However, if the amounts of nonspecific circularized C probe products exceeded detection limits, false-positive results would be observed. Kuhn & Frank-Kamenetskii (2005) recently reported that non-template ligation of DNA by T4 DNA ligase generated very low yield that resulted in a background signal or false-positives. The fidelity of T4 DNA ligase is 1 to 2 orders of magnitude greater than that of thermostable ligases from thermophilic bacteria (Luo *et al*., 1996; Tong *et al*., 1999). Therefore, thermostable DNA ligases such as *Thermus thermophilus* (*Tth*) ligase and *Thermus aquaticus* (*Taq*) DNA ligase may be the suitable alternatives to achieve higher fidelity of the RAM reaction.

To avoid false diagnosis, the concentration of C probes needs to be optimized. In this study, the initial amounts of linear C probes (10^9 copies) could potentially result in the formation of template-independent ligation products by T4 DNA ligase. However, prior to the ligation step, magnetic bead-based sandwich hybridization was used to capture IHHNV genome and the C probe through capture probes. The hybridization and washing steps prior to the ligation step should greatly reduce the amount of free C probes and eliminate the possibility of creating detectable amounts of false-positive products.

Many features of the IHHNV RAM assay, such as its time efficiency, simplicity and sensitivity, suggest that it is a potential platform for the diagnosis of IHHNV and other pathogens. Moreover, RAM assay is a feasible method that may allow shrimp farmers to detect shrimp pathogens on-site without requirements for expensive thermocyclers and complex protocols.

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Chapter 3. Rapid and sensitive detection of Taura syndrome virus using nucleic acid-based amplification.

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Background:

Taura syndrome virus (TSV) was first reported from *Litopenaeus vannamei* in Ecuador in 1992 (Lightner, 1995). TSV disease has spread widely *via* infected broodstock throughout America and Southeast Asia, causing severe economic losses in the shrimp aquaculture industry in the last decade (Brock, 1997; Tu *et al*., 1999; Robles-Sikisaka *et al*., 2002; Chang *et al*., 2004). TSV is known to cause high mortality in *L. vannamei*, *Penaeus schmitti*, and *P. seiferus*, while sparing *P. stylirostris*, *P. monodon*, *P. japanicus*, *P. duorarum*, *P. chinensis*, and *P. aztecus* (Brock, 1997; Overstreet *et al*., 1997). TSV infection leads to 60–90% mortality in *L. vannamei*; the survivors become chronically infected but present no symptoms (Lightner, 1996b).

TSV, a member of the *Dicistroviridae*, contains a single-stranded, positive-sense RNA genome that is about 10,205 nt long and encodes 2 non-overlapping open reading frames, ORF1 and ORF2 aura syndrome. ORF1 and ORF2 are predicted to encode the nonstructural and structural polyproteins, respectively. Phylogenic analysis based on the nucleotide sequences of CP2 suggested that there are 3 lineages, namely the Americas, Belize, and SE Asia, among the various geographic TSV isolates (Robles-Sikisaka *et al*., 2002; Tang and Lightner, 2005). In the CP1 and CP2 regions, low genetic variation (0 to 0.24% for CP1 and 0 to 0.35 or 0 to 5.6% for CP2 nucleotide sequences) was observed among the isolates analyzed. Due to the low-fidelity nature of the RNA-dependent RNA polymerase, new isolates of TSV that are able to replicate freely in a new species, such as the one described by Chang *et al*. (2004) in *P. monodon*, are expected to keep emerging.

Sensitive and specific detection of the causative agents is a prerequisite for effective disease prevention and management. Methods developed for TSV detection and characterization include histological analyses, immunoassay, *in situ* hybridization, reverse transcription-polymerase chain reaction (RT-PCR), and real-time RT-PCR (Lightner, 1995; Mari *et al*., 1998; Nunan *et al*., 1998a; Poulos *et al*., 1999, Castagna *et al*., 2004; Tang *et al*., 2004). Among the methods described above, diagnosis of potential TSV carriers or TSV outbreaks at early stages relies mainly on sensitive and specific platforms, such as the nested and real-time RT-PCR (Romano *et al*., 1995; Nunan *et al*., 1998a; Tang *et al*., 2004). However, because of the extensive technical training and expensive equipment involved, these systems have not been widely

adopted by farm operators for sensitive and timely on-site diagnosis of TSV.

With the advantage of requiring only simple heating devices, various isothermal nucleic acid amplification methods providing detection sensitivity and specificity comparable to those of PCR have been developed lately (Demidov, 2002; Cook, 2003; Savan *et al*., 2005; Zhang *et al*., 2006). These methods include rolling-circle amplification, loop-mediated isothermal amplification, and nucleic acid-based amplification (NASBA), to name a few. NASBA is a single-step, enzyme-based RNA amplification reaction (Kievits *et al*., 1991; Deiman *et al*., 2002), employing 3 enzymes: avian myeloblastosis virus reverse transcriptase (AMV-RT), ribonuclease H, and T7 RNA polymerase. Two target-specific primers, including one with a 5'-extension containing the promoter sequence for T7 RNA polymerase, have been designed to amplify the RNA target in exponential kinetics through double-stranded cDNA intermediates. The amplicons are mainly single-stranded RNA, with a polarity opposite that of the target. Since the RNA synthesis step does not require a primer, the maximal yield of the NASBA amplification process could exceed the initial primer levels by at least 1 order of magnitude (Deiman *et al*., 2002). Being extremely rapid, highly sensitive, and specific (Lanciotti & Kerst 2001), NASBA is most suitable for amplification of single-stranded RNA targets and has been applied to the detection of RNA viruses, such as human immunodeficiency virus, avian influenza virus, enteroviruses, La Crosse virus, parainfluenza virus, and astrovirus, to name just a few (Hibbitts *et al*., 2003; Tai *et al*., 2003; Lambert *et al*., 2005; Yao *et al*., 2005; Brown, 2006).

For aquaculture animal pathogens, NASBA has been used for the detection of *nodaviruses*, in which a real-time NASBA was developed (Starkey *et al*., 2004). Since real-time detection requires expensive apparatus and reagents, such assays are unsuitable for the purpose of simple on-site detection. The ultimate aim of this study was to develop a quick and easy point-of operation diagnostic system for pathogens, by coupling isothermal NASBA with solid-phase platforms that are user friendly and affordable for amplicon detection. Therefore, we report here on the development of NASBA assays for the detection of TSV, as well as the development of post-amplification solid phase based detection platforms through the use of target specific capture and detection probes for NASBA products. The sensitivity and specificity of this system were compared to those of RT-nested PCR.

Materials and methods:

a. Sample collection. *L. vannamei* samples were collected from farms in Taiwan. TSV-positive samples were identified by using the IQ2000 TSV Detection and

Prevention System (IQ2000 TSV DPS; GeneReach Biotechnology Corp., Taichung, ROC). Gill-associated virus (GAV)- and infectious myonecrosis virus (IMNV)- positive shrimp samples were kindly provided by Dr. Peter Walker (CSIRO, Australia) and Dr. Donald V. Lightner (University of Arizona, USA), respectively. Yellow head virus (YHV)-positive samples were collected from the SE Asian countries.

b. Nucleic acids. Although TSV appears to be present in the lymphoid organ at the highest levels, it has been shown that pleopod sampling provides sufficient sensitivity for TSV detection, allowing noninvasive screening of costly broodstock (Tang *et al*., 2004). RNA was extracted from the pleopods or gills of shrimp samples using the RNA Extraction Kit (GeneReach Biotechnology Corp., Taichung, ROC), following the protocols provided with the kit. The plasmid constructs, pTSV containing a 350 bp fragment from the TSV VP2 gene region and pGFP containing the green fluorescent protein gene, were purified using the Midi-V100 Ultrapure Plasmid Extraction System (Viogene-Biotek, Taipei, ROC).

c. Primers and oligonucleotide probes. The sequences of the oligonucleotide primers for the NASBA and the probes for detection are listed in Table 1.

Table 1. Nucleotide sequences of primers and probes used in this study. The T7 promoter sequence is undlerlined (NASBA: nucleic acid sequences based amplification; OIE: Office International des Epizooties)

Primer or probe	Sequence (5'-3')	Function
TSVF2	CATTGTGGCTGTGGAATAA	NASBA primer
T7 TSVR2	AATTCTAATACGACTCACTATAGGGAG AAGGAAGCCGAATAGAATTTAGGT	NASBA primer with T7 promoter
TSVF3-biotin	CCTGGCATTGAGATGAACTTGCAGTC TGAAGCTCGAGCTA	Detection probe
TSVR5	CTCATATGCGTTGACTTTCGT	Capure probe
TSVR4	AGCTTCAGACTGCAAGTTCATCTCAAT	Hybridization control capture probe
9195	TCAATGAGAGCTTGGTCC	OIE RT-PCR primer
9992	AAGTAGACAGCCGCGCTT	OIE RT-PCR primer

The cDNA sequences encoding the TSV capsid proteins of different lineages available in GenBank (AF277378, AF277675, AF406789, AY355309, AY355310, AY355311, AY590471, AY826052, and AY826056) were aligned and compared. Design of the primers was based on conserved sequences within the VP2 gene. Standard BLASTN nucleotide-nucleotide search confirmed the specificity of the designed primers. Primers were purchased from BioBasic (Canada) or synthesized at GeneReach Biotechnology Corp., and purified through high-affinity purification or gel purification before use. The TSVF3-biotin primer was labeled with biotin at its 3'-end.

d. RT-nested PCR. For RT-nested PCR analysis, the IQ2000 TSV, YHV, GAV, and IMNV DPS were adopted for the detection of the corresponding viruses. RNA extracts and standards were diluted in yeast tRNA (40 ng/µl). Briefly, 2 µl of RNA samples was mixed with 7 µl of First PCR Premix, 0.5 µl of RT enzyme, and 0.5 µl of Taq DNA polymerase. The cDNA was synthesized at 42°C for 30 min, followed by 15 of the first PCR cycles. Subsequently, 14 µl of Nested PCR Premix and 1 µl of *Taq* DNA polymerase were added to the first PCR reaction products, followed by 30 cycles of PCR. The products were separated by electrophoresis in a 1.5% agarose gel, stained in ethidium bromide, visualized under UV light, and documented by using the AlphaImager Imaging System (Alpha Innotech, California, USA).

e. OIE TSV RT-PCR. The sampling and TSV RT-PCR detection procedures were carried out as described in an OIE (Office International des Epizooties) manual (OIE, 2003). Briefly, the GeneAmp EZ rTth RNA PCR Kit (Applied Biosystems, California, USA) was used for the RT-PCR reactions that include 10 µl of the total RNA extracted from tissue and 0.46 µM each of Primers 9,195 and 9,992 (Table 1), which amplify a 231 bp sequence of the TSV genome. The 50 µl reaction also contains 300 µM each of the dNTPs, 2.5 U of rTth DNA polymerase, 2.5 mM manganese acetate, 5 mM bicine, 11.5 mM potassium acetate, and 8% (w/v) glycerol, pH 8.2. After overlaying the reaction with 50 µl of light mineral oil, reverse transcription was carried out at 60°C for 30 min, followed by inactivation at 94°C for 2 min. The subsequent PCR reaction included 35 cycles of denaturation at 94°C for 45 s and annealing/ extension at 60°C for 45 s and a final extension step at 60°C for 7 min; 10 µl of the amplified products were analyzed in a 2.0% agarose gel in 0.5x TBE (Tris, boric acid, ethylene diamine tetra-acetic acid [EDTA]) and visualized as described above.

f. NASBA. The NASBA amplicons derived from Primers T7 TSVR2 and TSVF2 were expected to be 209 nt in length. Reaction conditions for NASBA were modified slightly from those described by Jean *et al*. (2002a). Briefly, the NASBA

reactions were performed in a final 20 µl reaction volume containing 2 µl of the extracted RNA, 40 mM Tris-HCl (pH 8.5), 15 mM $MgCl_2$, 50 mM KCl, 15% (v/v) dimethyl sulfoxide, 5 mM dithiothreitol, 0.5 mM of each deoxyribonucleoside triphosphate, 2 mM of each nucleoside triphosphate, 50 µg $µl^{-1}$ bovine serum albumin, and 0.2 µM of each primer. The mixture without enzymes was incubated at 65°C for 5 min and 42°C for 5 min before the addition of 2.5 U of RNase H (USB, Ohio, USA), 12 U of the RNase inhibitor (Takara, Shiga, Japan), 40 U of the T7 RNA polymerase (USB, Ohio, USA), and 8 U of AMV-RT (Promega, Wisconsin, USA), followed by a designated incubation period at 41°C. Yeast tRNA (40 ng/µl) was used to dilute RNA samples, to avoid losses of targets at extremely low concentrations.

g. **Denaturing agarose gel electrophoresis and Northern blotting.** NASBA amplicons were denatured in 1× RNA sample buffer (95% formamide, 0.5 mM EDTA) at 65°C for 2 min, and subsequently resolved in a 1.5% agarose gel containing 3.7% formaldehyde. After staining with ethidium bromide, the bands were visualized under UV light and subsequently transferred to Hybond-N membrane (Amersham Biosciences, Connecticut, USA) in 20x SSPE overnight. After 1 wash with 5x SSPE at room temperature, the membrane was UV cross-linked for 2 min in HL-2000 HybriLinker (UVP, California, USA) and dried at 63°C. Hybridization was carried out in Dr. Hyb buffer (DR. Chip Biotech. Hsinchu, ROC) containing 0.025 µM biotin-labeled TSP-3 at 63°C for 30 min. After 2 washes in 1x SSPE at 42°C, the membrane was rinsed with 0.5%(w/v) blocking reagent (Roche Diagnostics, USA) in Buffer I (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.3% [v/v] Tween 20) at room temperature, followed by the addition of strepavidin-alkaline phosphatase conjugate (Promega, Wisconsin, USA) diluted 1:2000 in Buffer I containing 0.5% (w/v) blocking reagent. The incubation was carried out at room temperature for 10 min. After 1 wash with 1× SSPE and 1 with Buffer II (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), color was developed by the addition of 0.5 mg ml^{-1} NBT and BCIP in Buffer II. Color was allowed to develop in the dark for no more than 10 min. And the reaction was stopped by rinsing the membrane with water.

h. **Dot blotting.** The NASBA products in 1x RNA sample buffer and the DNA controls in TE buffer were denatured at 95°C for 2 min, diluted in 6x SSPE buffer, and spotted on the Hybond-N filter. The subsequent UV cross-linking, hybridization, and detection procedures were carried out as described above for Northern blotting. Different concentrations of the pTSV plasmid DNA were included as positive controls for the detection steps.

i. **Microtiter plate detection of NASBA products.** NASBA amplicons were

captured by an amplicon specific primer coated on the wells of the FluoroNunc microtiter plate Module MaxiSorp Surface and detected by a TSV-specific, biotin-labeled primer. Briefly, 0.5 nmol of the capture probes, namely TSVR4 and TSVR5, were immobilized on the well in coating buffer (0.045 M Na_2CO_3/$NaHCO_3$), dried at 63°C for 5 min, and subsequently UV cross-linked (1,200 x 100 μJ cm^{-2}). The wells were sequentially washed in Buffer I and water. Then, 5 μl of the NASBA products mixed with 45 μl of 0.025 μM biotin-labeled TSP-3 in the hybridization buffer was added to the wells of the microtiter plate which were coated with capture probe in advance. After 30 min of hybridization at 63°C, the wells were washed 2 times with 1x SSPE at room temperature. Subsequently, the wells were blocked with Buffer I containing 0.5% (w/v) blocking reagent at room temperature, followed by the addition of strepavidin-alkaline phosphatase conjugate diluted 1:2000 in Buffer I containing 0.5% (w/v) blocking reagent at room temperature for 10 min. After 1 wash with 1x SSPE and 1 with Buffer II, 50 μl of 0.5 mg ml^{-1} NBT and BCIP in Buffer II was added. Color was developed in the dark for no more than 10 min before the reaction was stopped by rinsing the wells with water.

Results:

A. **Detection of TSV by NASBA.** The *L. vannamei* samples collected in Taiwan were first screened for TSV infection on the basis of the high sensitivity of the IQ2000 TSV DPS. RNA prepared from these samples was used as the template for the following NASBA tests. Using analysis by denaturing agarose gel electrophoresis, a single band corresponding to the expected size of 209 nucleotides (Fig. 1A) was detected only from the TSV(+) shrimp RNA extract, and not from a TSV(-) shrimp RNA extract, yeast tRNA, or ddH_2O. The band was confirmed by Northern blotting using the TSVF3-biotin probe (Fig. 1B) to represent the TSV NASBA amplicons. The specificity of the Northern-blotting analysis was validated by the presence of signals only with the pTSV DNA control, but not with pGFP, a plasmid with unrelated sequences (Fig. 1B).

B. **Dot-blotting analysis of TSV NASBA products:** Based on the Northern-blotting analysis of the TSV NASBA amplicons, no cross-reactive signals could be picked up by the biotin-labeled, TSV-specific primer (Fig. 1B). In order to simplify the detection protocols for the TSV NASBA amplicons, a dot-blotting analysis was developed. The results (Fig. 1C) showed that using the TSV-specific TSVF3-biotin probe, positive signals were obtained only from the NASBA reaction of the TSV(+) RNA and pTSV, the positive control DNA for the detection steps, among

the samples tested. In the following experiments, different concentrations of the positive hybridization control, pTSV, were included to ensure that satisfactory degrees of detection sensitivity were reached.

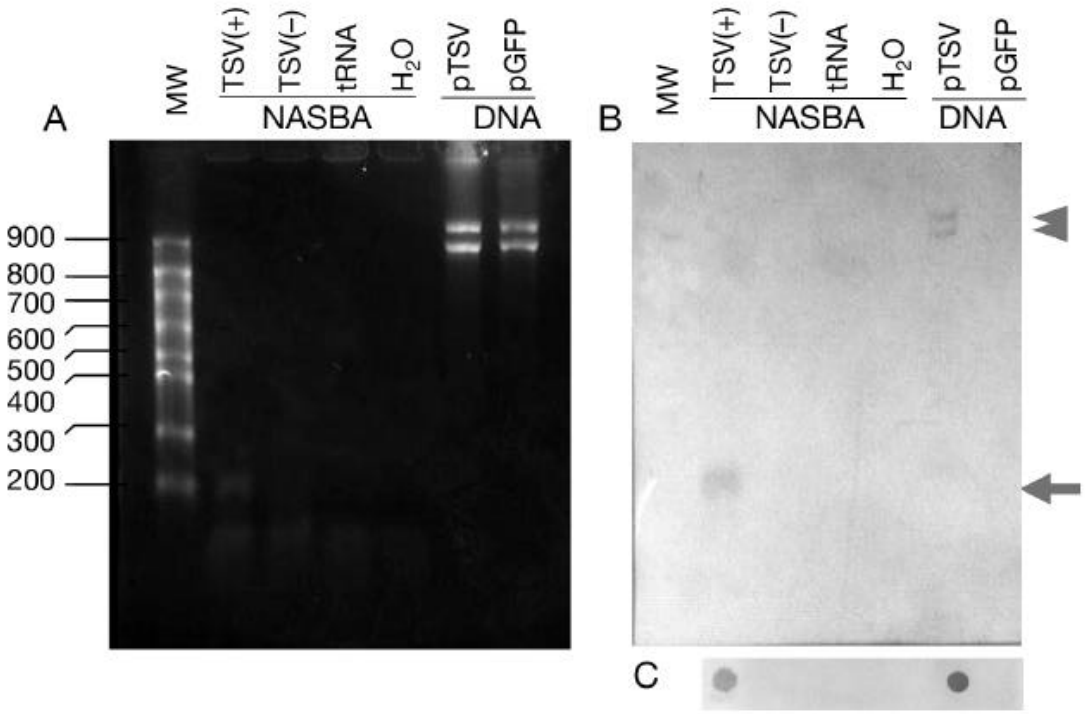

Fig. 1. Gel and Northern-blotting analysis of TSV (Taura syndrome virus) NASBA (isothermal nucleic acid-based amplification) products. Products of the TSV NASBA reactions using primers TSVF2 and T7TSVR2 were separated on (A) a 1.5% denaturing agarose gel and identified by (B) Northern blotting or (C) dot-blotting analyses using the biotin-labeled detection primer TSVF3-biotin. pTSV and pGFP plasmid DNA were included as positive and negative controls, respectively, for the detection steps (arrow: TSV NASBA product; arrowheads: pTSV; MW: RNA molecular weight markers [bp]; TSV[+]: TSV-positive shrimp RNA; TSV[–]: TSV-negative shrimp RNA; tRNA: yeast tRNA; H_2O: DEPC-ddH_2O).

C. **Optimal incubation time for TSV NASBA:** Several conditions for TSV NASBA were varied to optimize its performance. For example, the reaction was stopped after different periods of time and analyzed for the production of TSV NASBA amplicons (Fig. 2). Based on the results, no signals were derived from yeast tRNA and ddH_2O alone after 120 min. On the other hand, after 30 min at 41°C, TSV NASBA produced enough copies of amplicons from the TSV-positive RNA to be vaguely detected by dot blotting. However, the NASBA amplicons could be

detected without ambiguity after 50 min of incubation. The intensity of the signal increased with time, suggesting that the production of NASBA amplicons increases with incubation time between 50 and 120 min of incubation. Therefore, the NASBA reactions were carried out at 41°C for 90 to 120 min in the following tests.

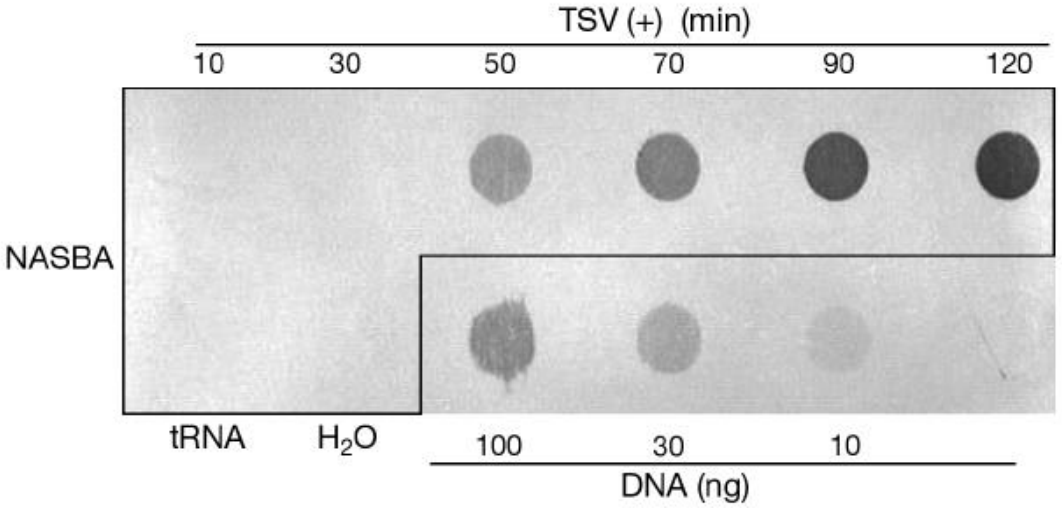

Fig. 2. Effects of amplification time variation on the TSV NASBA reaction. TSV NASBA reactions using TSV(+) shrimp RNA as template were carried out for 10, 30, 50, 70, 90, or 120 min at 41°C. The NASBA negative controls, namely yeast tRNA and DEPC-H2O, were incubated for 120 min. NASBA products were subsequently identified by dot blotting using the biotin-labeled detection probe as described in 'Materials and methods', and the pTSV plasmid DNA (100, 30, and 10 ng) was included as a positive control.

D. Comparison of sensitivity between NASBA/dot blotting and RT-nested, PCR-based TSV assays: To evaluate the feasibility of developing NASBA as an on-site TSV detection platform, the performance of the TSV NASBA/dot-blotting system was compared with a commercially available TSV RT-nested PCR system, the IQ2000 TSV DPS. This system has been applied to routine SPF (specific pathogen-free) broodstock screening at numerous large-scale shrimp farms. First, comparative analysis between the IQ2000 TSV DPS and the OIE TSV RT-PCR detection system, described as one of the standard screening methods for TSV in the 'Manual of diagnostic tests for aquatic animals (OIE 2003), was performed to verify the sensitivity of the IQ2000 TSV DPS. A TSV(+) shrimp RNA was 10-fold serially diluted and subjected to both assays carried out in parallel. The results showed that the end point of detection was at around a 10^4-fold dilution

with the OIE RT-PCR assay (Fig. 3A) and at about a 10^5-fold dilution with the IQ2000 TSV DPS (Fig. 3B), demonstrating that the latter is approximately 10 times more sensitive than the former.

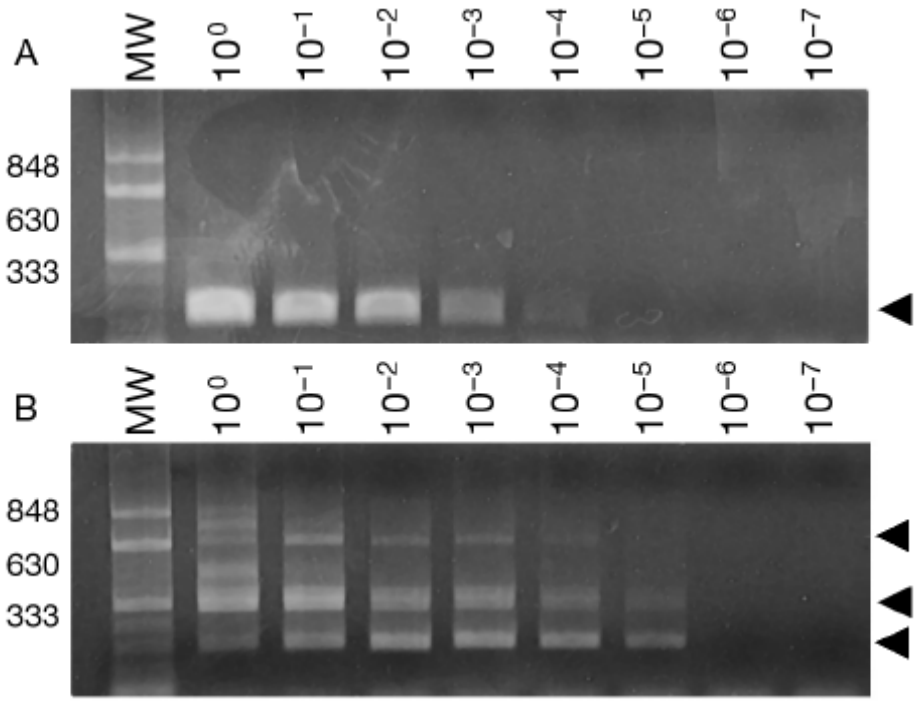

Fig. 3. Comparative sensitivity analysis between OIE (Office International des Epizooties) TSV RT-PCR and IQ2000 TSV DPS assay. A TSV-positive RNA sample prepared from shrimp tissues was 10-fold serially diluted (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) in yeast tRNA and amplified by either (A) the OIE TSV RT-PCR detection method or (B) the commercially available RT-nested, PCR-based IQ2000 TSV DPS. The PCR products were detected by electrophoresis in a 1.2% agarose gel (arrowheads: TSV-specific PCR products; MW: DNA molecular weight markers [bp]).

With the sensitivity of the IQ2000 system confirmed, we compared the sensitivity of the TSV NASBA established by us side by side with that of the RT-nested, PCR-based IQ2000 TSV DPS. A series of 5-fold dilutions of TSV(+) RNA extract was prepared in a yeast tRNA solution, and the same tRNA was included as the negative control. Replicate analyses were performed. The NASBA/dot-blotting analysis gave significant positive amplicon signals at 6,250-fold dilution (Fig. 4A), while the controls worked properly. With the IQ2000 TSV DPS carried out in parallel, amplification of the positive controls (103, 102, and 101 copies) led to the production of the typical ladders of 1 to 3

TSV-specific PCR products (arrowheads; Fig. 4B). Within a 3-log range of target copies, higher amounts of targets would result in the production of more bands. Meanwhile, no products were detected in the negative control reaction (tRNA; Fig. 4B), and the band of around 650 bp (arrow; Fig. 4B) is the RT-PCR internal control signal derived from shrimp RNA. The end point of sample dilution to obtain positive RT-nested PCR signals fell on the 31,250-fold dilution (Fig. 4B), demonstrating that the TSV NASBA assay was about 5-fold less sensitive than the TSV RT-nested PCR assays.

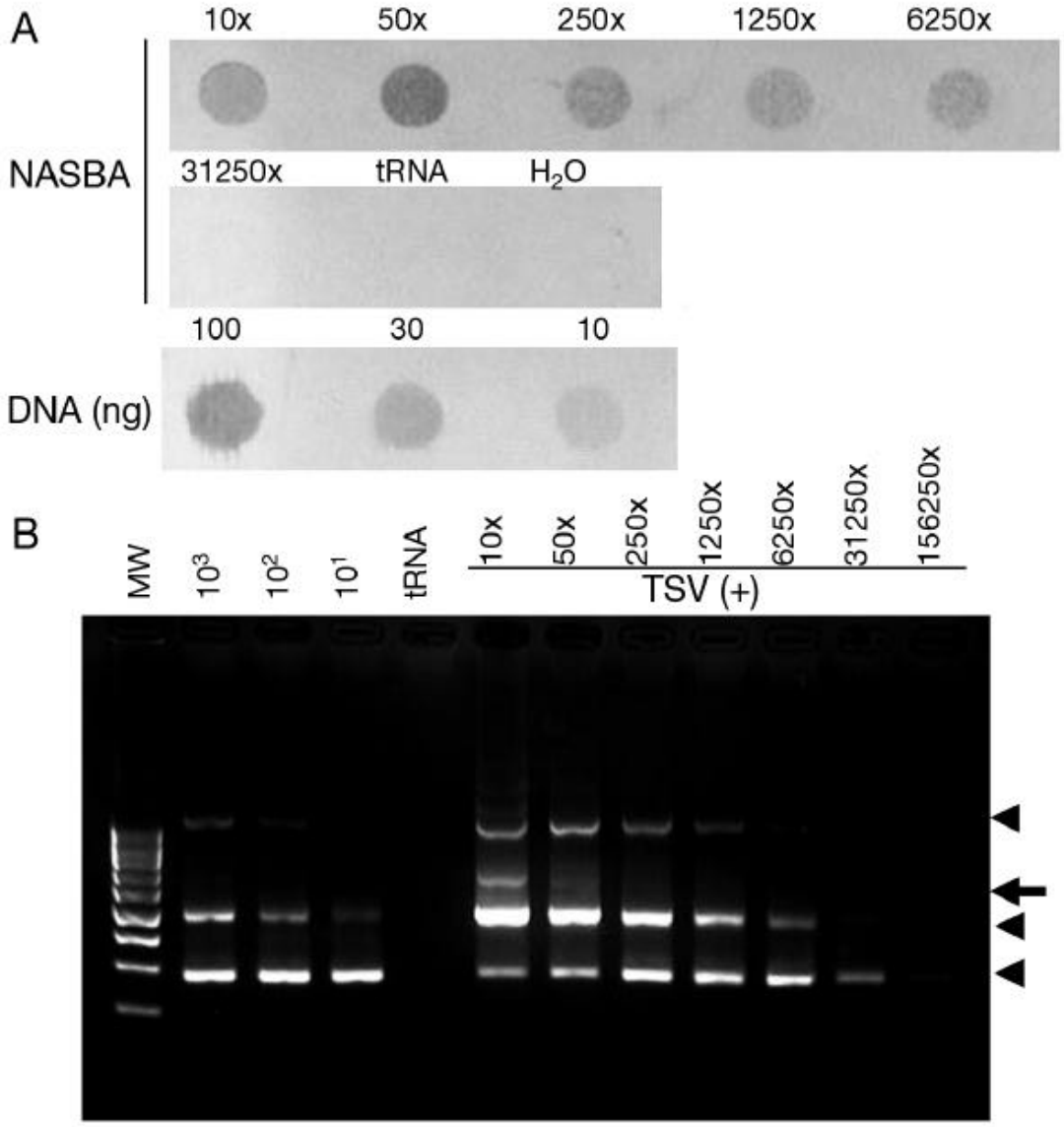

Fig. 4. Comparative sensitivity analysis between TSV NASBA and RT nested, PCR-based IQ2000 TSV DPS reactions. The same RNA extract isolated from a TSV(+) shrimp was 5-fold serially diluted (10x, 50x, 250x, 1250x, 31,250x,

156,250x) in yeast tRNA and amplified by the NASBA and the RTnested, PCR-based IQ2000 TSV DPS. Both yeast tRNA (tRNA) and DEPC H_2O (H_2O) were included as negative controls. (A) TSV NASBA. NASBA amplicons were analyzed by dot blotting using a biotin-labeled detection probe. pTSV plasmid DNA (100, 30, and 10 ng) was used as a positive control for the detection steps. (B) RT-nested PCR amplification (the IQ2000 TSV DPS). The products were detected by electrophoresis in a 1.2% agarose gel. The 10^3, 10^2, or 10^1 copies of (+) controls were provided by the kit (MW: 100 bp DNA molecular weight markers; arrowheads: TSV-specific PCR products; arrow: internal control signal).

For the subsequent TSV NASBA reactions in this study, serial dilutions of aliquots of the same TSV(+) RNA sample were included to monitor the sensitivity of the assays.

E. Specificity of TSV NASBA: To further investigate the specificity of the TSV NASBA system, samples positive for several non-target RNA viruses were confirmed to be TSV-negative in advance. Fig. 5B and 5C shows an example of the screening. In this case, an IMNV(+) sample undiluted or 10-fold diluted (1x or 1/10x, respectively) produced IMNV-specific PCR product ladders using the IQ2000 IMNV DPS. The positive controls (2×10^3, 2×10^2, and 2×10^1) diluted in yeast tRNA gave typical ladders of the IMNV-specific PCR products (Fig. 5B). When the same IMNV(+) sample was subjected to TSV detection by the IQ2000 TSV DPS, only the internal control signals were detected (arrow; Fig. 5C), indicating that the IMNV(+) RNA is TSV negative. Similarly, the YHV(+) and GAV(+) samples were confirmed to be TSV negative (Fig. 5C). When these YHV(+), GAV(+), and IMNV(+) RNA extracts were subjected to the TSV NASBA/dot-blotting analysis, no TSV NASBA products were detected (Fig. 5A), demonstrating that the TSV NASBA reaction established is indeed TSV specific.

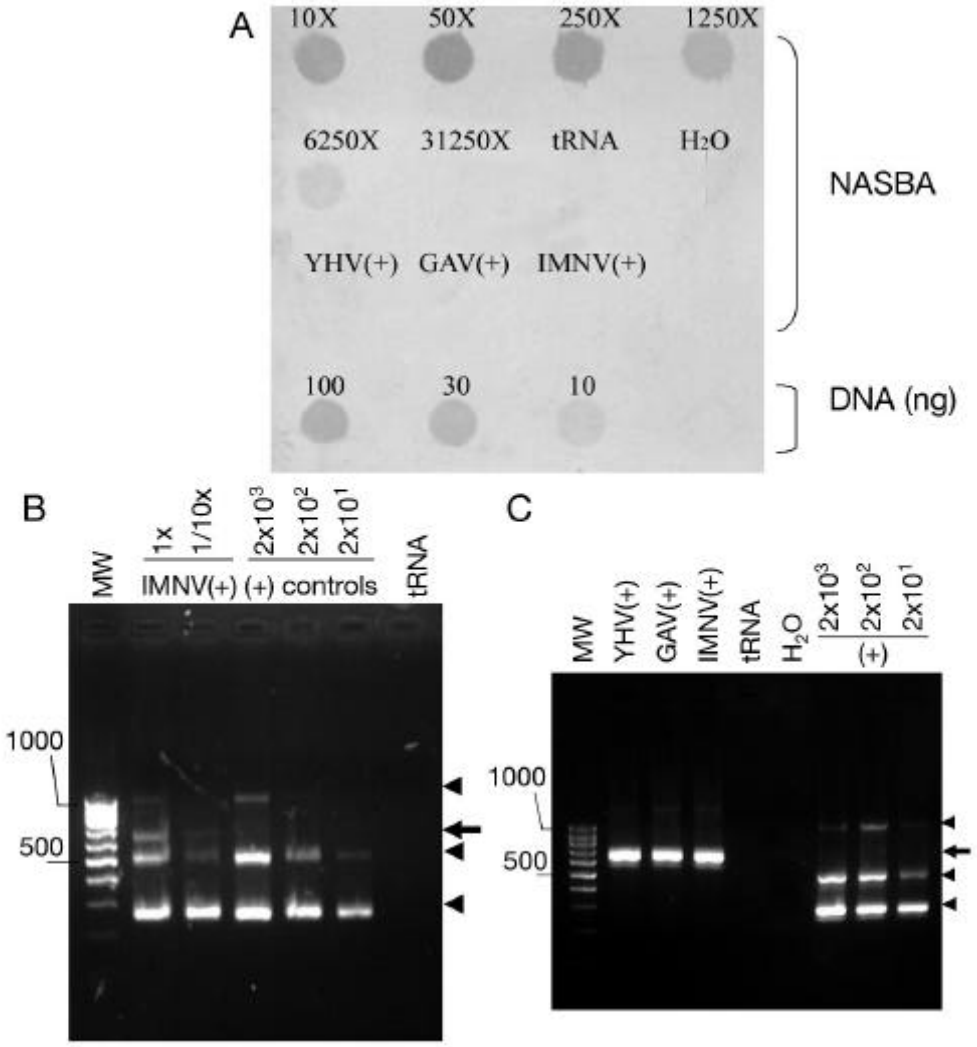

Fig. 5. Specificity of the TSV NASBA reaction. (A) TSV NASBA RNA of YHV(+), GAV(+), or IMNV(+) shrimp, together with 10x, 50x, 250x, 1,250x, 6,250x, or 31,250x dilutions of TSV(+) shrimp RNA, and the negative controls (yeast tRNA and DEPC-H2O) were amplified by TSV NASBA. The pTSV plasmid DNA was included as a positive control for the detection procedures. TSV-specific targets were detected by dot blotting as described in 'Materials and methods'. (B) IMNV RT-nested PCR amplification. The IMNV(+) RNA was identified by using the IQ2000 IMNV DPS. The IMNV(+) RNA, undiluted (1x) or diluted 10-fold (10x), as well as 2 x 10^3, 2 x 10^2 or 2 x 10^1 copies of the positive controls provided by the kit were analyzed. The products were analyzed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. (C) TSV RT-nested PCR. The YHV(+), GAV(+), IMNV(+) RNA samples, together with the negative (yeast tRNA and DEPC H_2O) and positive (TSV[+]) controls, were assessed by the IQ2000 TSV DPS (MW: 100 bp DNA molecular weight markers; tRNA: yeast tRNA; arrowheads:

virus-specific PCR products; arrows: internal control signal).

F. NASBA detection of TSV in shrimp specimens: A total of 23 samples from 4 bathes (FITC, CTC, TP, and YS) of *L. vannamei* collected in Taiwan were tested by both TSV NASBA/dot-blotting analysis and IQ2000 TSV DPS (Table 2).

Table 2. Analysis of shrimp samples by TSV NASBA/dot-blotting analysis and the IQ2000 TSV Detection and Diagnosis Kit. Analysis of 4 batches (TC1: Tai-Chung 1; TC2: Tai-Chung 2; TP: Tai-Ping; YS: Yuan-Shin) of TSV samples collected in Taiwan. Different numbers of samples were analyzed concurrently by both the IQ2000 TSV Kit and TSV NASBA/dot-blotting assay.

Batch	No. of samples	IQ2000 kit positive	TSV NASBA postive
TC1	5	5/5	5/5
TC2	3	3/3	3/3
TP	10	2/10	1/10
YS	5	5/5	4/5
Total	23	15/23	13/23

In total, 8 of the 23 samples were determined to be TSV negative by both methods. Of the other 15 samples that were diagnosed as TSV positive by the IQ2000 TSV DPS, only 13 were designated as TSV positive by the TSV NASBA/dot-blotting analysis. The 2 samples designated TSV positive by the RT-nested PCR, but TSV negative by TSV NASBA/dot blotting were both categorized by the RT-nested PCR kit as 'weakly infected', i.e. these appeared to be samples with very low virus loads.

G. End-point detection of TSV by microtiter plate hybridization: As stated above, solid-phase detection is an attractive platform to be incorporated into a straightforward and cheap diagnostic system (Jean *et al.*, 2002a). Toward this end, the dot-blotting detection protocols were modified and applied to a microtiter plate format, in which NASBA amplicons were detected by a combination of a TSV NASBA amplicon specific capture probe and a biotin labeled detection probe. Similarly, positive signals can be visualized by the formation of the purple-colored precipitates. Moreover, a capture probe specific for the biotin-labeled detection probe was fixed on each well (arrows; Fig. 6B) to serve as an internal control for the detection steps. Specific detection of the TSV NASBA RNA amplicons was confirmed by the presence of signals with the TSV(+) samples and the absence of signals with the GFP RNA produced *in vitro* (arrowheads; Fig. 6B). Both dot-blotting and microtiter plate detection systems led to positive detection of the TSV NASBA amplicons of the TSV(+) RNA diluted up to 6,250-fold (Fig. 6A, B), indicating that these 2 simple systems offer similar degrees of detection sensitivity

for the TSV NASBA amplicons.

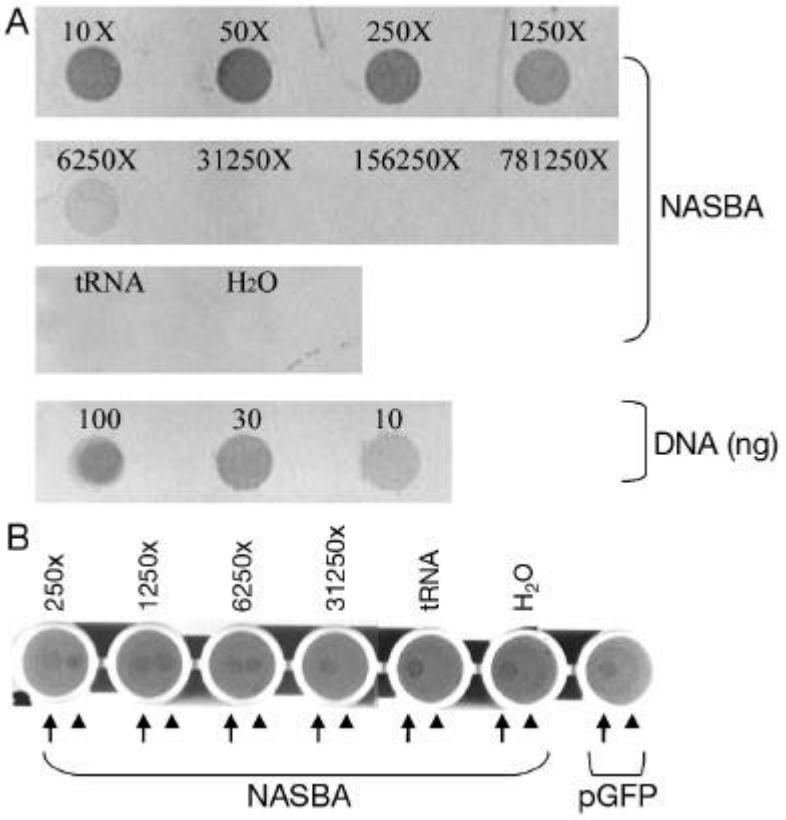

Fig. 6. End-point microtiter plate detection of NASBA amplicons, compared with dot-blotting detection. (A) Dot blotting of NASBA amplicons. NASBA reactions of serial dilutions of the TSV-positive RNA (10x, 50x, 250x, 1,250x, 6,250x, 31,250x, 156,250x, 781,250x), tRNA, and DEPC H_2O were spotted on filter paper and detected as described in 'Materials and methods'. The positive pTSV DNA controls (100, 30, and 10 ng) for the hybridization step were also included. (B) Microtiter plate detection of NASBA amplicons. The same NASBA amplicons as those analyzed in (A), together with a negative RNA control (*in vitro* RNA transcripts of the GFP gene; GFP RNA), were captured by the TSV-specific capture primer (TSVR5; arrowheads) immobilized in each well. The captured NASBA amplicons, in turn, hybridize with the biotin-labeled detection primer and allow subsequent visualization of the signal. The capture primer (TSVR4; arrows) was also immobilized on the well to interact with the biotin-labeled detection primer and thus to serve as a positive control for the detection steps.

Discussion:

NASBA is a robust amplification technology that is highly appropriate for RNA analyses and has been applied particularly to detecting various RNA viruses, as well as a number of other pathogens. This report describes the development of a NASBA/solid-phase detection assay for the rapid and sensitive detection of TSV in shrimp tissue specimens. The NASBA reactions were coupled with the simple colorimetric dot-blotting or microtiter plate method for the detection of TSV-specific amplicons (Figs. 1 & 6). No false-positive results were obtained with yeast tRNA or with non-target shrimp RNA viruses, namely GAV, YHV, or IMNV (Fig. 5), demonstrating a high degree of specificity of the TSV NASBA.

Various formats, including solid-phase hybridization, electrochemiluminescence, molecular beacon, enzymelinked gel assay, and fluorescence correlation spectroscopy have been developed for the detection of NASBA products (Deiman *et al*., 2002; Jean *et al*., 2002b). The high costs of equipment, chemicals, and reagents needed to perform real-time assays are prohibitive to the application of most of these formats at shrimp farms. On the other hand, solid-phase hybridization systems, in general, do not require any elaborate tools or expensive apparatus for signal generation, detection, and interpretation. Furthermore, the single-stranded RNA amplicons of NASBA are especially compatible with hybridizationbased detection systems using target-specific probes. To exclude the requirement of any instrument during the detection steps, the colorimetric alkaline phosphatase/ BCIP/ NBT system was adopted, to allow direct visualization of the signals. The dot-blotting system using a TSV-specific, biotin-labeled primer together with the strepavidin-alkaline phosphatase/BCIP/NBT signal amplification system was proven to be efficient for the detection of TSV NASBA products (Figs. 1, 2, 4, & 5). The sensitivity of the NASBA reaction reproducibly reached the same dilution end point (6250x; Figs. 5A & 6), providing evidence to support the reproducibility of the NASBA method. Moreover, colorimetric microtiter plate detection of NASVA products was later set up and shown to reach a sensitivity similar to that of the dot blotting assay (Fig. 6).

The TSV NASBA assay showed levels of sensitivity comparable to those of the PCR assays (Fig. 4, Table 2). The sensitivity of the TSV NASBA/colorimetric dotblotting method was determined using serial dilutions of TSV(+) RNA extracts prepared from shrimp tissues, and the results were compared against the commercially available RT-nested PCR assay, the IQ2000 TSV DPS, which was demonstrated to be about 10-fold more sensitive than the standard RT-PCR assay, the OIE TSV RT-PCR, as expected (Fig. 3). The detection limits of the TSV NASBA/solid-phase detection system were reproducibly 5-fold less sensitive than the RT-nested PCR assay (Fig. 4). Previously, a NASBA/slot blot detection system using a biotinylated oligonucleotide

probe reached detection sensitivity comparable to that of a RT-nested PCR/agarose gel electrophoresis detection system for enterovirus (Heim and Schumann, 2002; Guichon *et al*., 2004). Therefore, the sensitivity of the TSV NASBA can likely be improved through further adjustments of either the NASBA reaction or the detection protocols to reach higher levels of sensitivity. To enhance the sensitivity of the solid-phase detection of the NASBA amplicons, other signal amplification methods, such as branched DNA (Tsongalis, 2006) or dendrimers (Kim *et al*., 2003) will be tested.

Results of TSV screening among different batches of shrimp samples by the TSV NASBA and the IQ2000 TSV DPS were in close agreement with each other (Table 2). Thirteen out of 15 TSV RT-nested, PCR-positive samples also tested positive in the TSV NASBA analysis (Table 2). It is likely that levels of TSV RNA in the 2 samples tested negative were below the detection limits of the TSV NASBA, since they were also categorized as ‘weakly infected’ by the RT-nested PCR assay (data not shown).

Molecular diagnosis of shrimp virus pathogens is particularly important given the difficulty encountered in establishing shrimp cell lines and isolating shrimp viruses from the specimens in tissue culture. Rapid and sensitive detection of TSV can facilitate proper and timely disease management measures to be implemented following the confirmation of TSV cases in the field. Coupling with the end-point solid-phase detection formats described in this study, the TSV NASBA assays can be completed straightforwardly in about 3 to 4 h, meeting the demands for speedy diagnosis of aquaculture pathogens at custom and import-control checkpoints and at the farms. The NASBA assay has also been shown to tolerate field contaminants, such as cloacal, pharyngeal, and anal swabs, fecal samples, cage sweepings, and blood, that could interfere with assays, such as ELISA and RT-PCR (Shan *et al*., 2003), also making it feasible for environmental sampling.

Taken together, the isothermal TSV NASBA/solidphase detection systems have satisfactory sensitivity and specificity, providing an easy and simple approach for TSV detection.

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Chapter 4. Specific detection of reverse transcription-loop-mediated isothermal amplification amplicons for Taura syndrome virus by colorimetric dot–blot hybridization.

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Background:

Since Taura syndrome virus (TSV) infection in *Penaeus vannamei* in Ecuador was first reported in 1992 (Hasson *et al*., 1995), TSV has spread widely and caused severe economic losses in the shrimp aquaculture industry throughout the Americas and SE Asia countries (Hasson *et al*., 1995; Robles-Sikisaka *et al*., 2002; Tu *et al*., 1999; Tung *et al*., 2007). TSV is known to infect *P. vannamei*, *Penaeus schmitti*, *Penaeus seiferus*, *Penaeus stylirostris*, *Penaeus monodon*, *Penaeus japanicus*, *Penaeus duorarum*, *Penaeus chinensis*, and *Penaeus aztecus* (Hasson et al., 1995; Overstreet et al., 1997). In *P. vannamei*, approximately 73-83% TSV infection was reported to be fatal and the survivors became chronically infected and presented no symptoms (Hasson *et al*., 1995; Lightner, 1996b).

Efficient, rapid and timely disease diagnosis is critical for successful aquaculture management. For field diagnosis, the optimal detection system should be cheap, quick, and easy-to-operate besides meeting the requirements in specificity and sensitivity. Currently, PCR-based pathogen detection kits have been widely included in the biosafety protocols of large-scale shrimp farms. However, easy and rapid tests with satisfactory sensitivity and specificity are still not available for the small scale farmers.

Requiring only a simple heating apparatus, different isothermal nucleic acid amplification methods including ramification amplification, nucleic acid-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) have been developed to offer feasible platforms for rapid and sensitive detection of target nucleic acids in recently years (Compton, 1991; Notomi *et al*., 2000; Teng *et al*., 2006a,b; Zhang *et al*., 2001a). An isothermal TSV NASBA detection system has been developed for TSA by our group recently (Teng *et al*., 2006a). Although being sensitive and specific, application of NASBA to field diagnosis is impeded by that the reaction requires three enzymes. On the other hand, the strand displacement activities offered by a single enzyme, namely the *Bst* DNA polymerase large fragment, can drive autocyclic DNA synthesis through loop-forming primers in LAMP as described by Notomi *et al*. (2000). Furthermore, basic LAMP reaction involves four primers that target six separate DNA sequences on the target DNA, allowing the assay to reach higher levels of specificity than other nucleic-acid amplification platforms. The

amplicons are stem-loop DNA molecules containing different numbers of inverted repeats and a loop derived from the target sequences. LAMP-based detection assays have been reported for numerous aquaculture animal pathogens, including koi herpesvirus (Saito *et al*., 2005; Soliman and El-Matbouli, 2005), infectious hematopoietic necrosis virus (Gunimaladevi *et al*., 2005), viral hemorrhagic septicemia virus (Soliman and El-Matbouli, 2006), iridovirus (Caipang *et al*., 2004), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Sun *et al*., 2006), white spot syndrome virus (WSSV) (Kono *et al*., 2004), yellow head virus (YHV) (Mekata *et al*., 2006), *Macrobrachium rosenbergii* nodavirus and extra small virus (Pillai *et al*., 2006).

LAMP amplification products can be detected by gel electrophoresis, by naked-eye detection of a white precipitate of magnesium pyrophosphate, or by real-time assay with a fluorescent dye (Dukes *et al*., 2006). In addition, real-time turbidity detection of the magnesium pyrophosphate precipitate has been shown to be easy and rapid (Enomoto *et al*., 2005; Mori *et al*., 2004; Poon *et al*., 2006). However, these detection methods have a common drawback, i.e. the lack of specific identification of the amplicons. Recently, visual sequence-specific detection with the help of an UV illuminator was achieved after cationic polymer assisted precipitation of LAMP products that hybridize first with oligonucleotide probes labeled with different fluorescent dyes (Mori *et al*., 2006).

Different RNA extraction methods, such as the Trizol Reagent (Invitrogen, California, USA), QIAamp viral RNA mini kit, RNeasy Mini-Kit (Qiagen, Helden, Germany), and ISOGEN (Nippon Gene, Toyama, Japan), have been adopted for RT-LAMP assays described previously (Dukes et al., 2006; Gunimaladevi et al., 2005; Hong et al., 2004; Pham et al., 2005). Although most are straightforward protocols, they are less likely to be applied to field diagnosis because of the requirement of various apparatuses (such as a micro-centrifuge). The observation that LAMP reaction could proceed directly with samples collected in water or medium and ocular fluid for human herpesviruses suggested that LAMP assay is less sensitive to inhibitory substances in clinical samples (Enomoto *et al*., 2005; Kaneko *et al*., 2005). Therefore, a simple RNA-extraction procedure was developed for LAMP based on the guanidinium thiocyanate (GuSCN)-silica-based nucleic acid extraction method in which the RNA in biological samples was released by GuSCN and purified subsequently through silica hybridization (Boom *et al*., 1990).

In this report, aiming to establish a field diagnostic system involving no electronic devices other than a simple heating apparatus, an isothermal TSV RT-LAMP was established, followed by the development of a modified solid-phase hybridization assay that can specifically detect TSV RT-LAMP amplicons and a

simple RNA extraction protocol.

Materials and methods:

a. **Sample collection:** Samples of different shrimp *L. vannamei* and *P. monodon* that were infected with different viruses were collected from different countries (Table 2). TSV(+), WSSV(+), IHHNV(+), Monodon baculovirus (MBV)(+), and hepatopancreatic parvo-like virus (HPV)(+) samples were identified by using the corresponding IQ2000TM Diagnosis and Prevention System (IQ2000TM TSV DPS; GeneReach Biotechnology Corp., Taichung, ROC). The gill-associated virus (GAV)(+) and Mourilyan virus (MoV)(+) shrimp samples were kindly provided by Dr. Peter J. Walker (CSIRO, Australia), and the infectious myonecrosis virus (IMNV)(+) shrimp samples by Dr. Donald V. Lightner (University of Arizona, USA).

b. **RNA extraction:** For RNA extraction, gills (about 20 mg) homogenized in 500 µl of the Trizol Reagent (Life Tech, Gaithersburg, MD USA) with a disposable grinder were incubated at room temperature for 5 min. After $CHCl_3$ (100 µl) was added, the mixture was vortexed for 20 s and incubated at room temperature for 3 min. After centrifugation, RNA in the aqueous phase was precipitated using isopropanol (200 µl), rinsed with 75% ethanol, air dried, dissolved in DEPC-treated ddH_2O, and stored at -20℃. For simple RNA extraction in GuSCN, samples were homogenized in the designated concentrations of GuSCN with a disposable grinder. Supernatant was collected after large debris were allowed to sediment and used immediately for subsequent reactions.

c. **TSV reverse transcription-nested polymerase chain reaction-agarose gel electrophoresis (RT-nPCR-AGE) assay:** The TSV RT-nPCR reaction (i.e. the IQ2000TM TSV DPS) was assembled as described in the user's manual. The thermocycler program for the first RT-PCR was as follows: 1 cycle of reverse transcription at 42℃ for 30 min and denaturation at 94℃ for 2 min, 15 cycles of 94℃ for 20 s, 62℃ for 20 s and 72℃ for 30 s, and a cycle of 72℃ for 30 s and 20℃ for 20 s. And the program for the second PCR included 30 cycles of 94℃ for 20 s, 62℃ for 20 s and 72℃ for 30 s and one cycle of 72℃ for 30 s and at 20℃ for 20 s. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

d. **Plasmid construction:** Construction of pTSV, a plasmid containing the target cDNA of the TSV genome, was described previously (Teng *et al.*, 2006a). Plasmid DNA was purified using the Midi-V100 Ultrapure Plasmid Extraction System (Viogene-Biotek, Taipei, Taiwan) and quantified by UV spectrophotometry.

e. **TSV RT-LAMP:** The target sequences for the primers are located in the structural protein VP2-conding sequence based on the published sequence of TSV

(AF277675) (Table 1).

Table 1. Oligonucleotide primers used for RT-LAMP amplification of TSV and DBH detection

Primer name	Sequence (5'-3')
TSV-FIP	GTTGCAAGCTGTTCCTGCGTTTTCATAACGAC AGTTGGACATCTAGTG
TSV-BIP	GCGATGTGACTACGAGGTTACTGTTTTCCACA GCCACAATGCTCC
TSV-F3	GAATCTCTCTCTCGCGACGTT
TSV-R3	AATAATTGATGTCTGCTTAGCATTCA
TSD[a]	CTGTACGAGTACAAGCCACGCCCTT
rcTSD	AAGGGCGTGGCTTGTACTCGTACAG

[a]This probe is biotinylated at the 3'-end for DBH-based detection.

Primers were designed according to the criterions described by Notomi *et al*. (2000). The *Tm* values of F2 and B2 regions in the inner primers are between 60 and 65°C. The *Tm* values of F2 and B2 were lower than those of F1 and B1 in the inner primers and the *Tm* values of the outer primers (F3 and B3) lower than those of F2 and B2 in the inner primers. And the size of the final DNA amplicon (160 bp) is less than 300 bp. However, the sequence of the loop regions in the inner primers, i.e. 24 bp for FIP and 28 bp for BIP, are shorter than the recommended length of 40 bases or longer and there is a TT- instead of TTTT-spacer in the inner primers (Table 1). The 20-µl reaction mixture contained 2 µl of RNA extract, 360 µM dNTP, 400 mM betaine, 20mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 3 mM $MgSO_4$, 0.1% Triton X-100, 0.15 µM each outer primer, 1.2 µM each inner primer, 6.4 units of *Bst* DNA polymerase (BioMi, Taichung, ROC) and 30 units of Moloney murine leukemia virus reverse transcriptase (BioMi, Taichung, ROC). The reaction was carried out at the designated temperatures for 30-180 min, followed by heat inactivation at 80°C for 10 min. The products were analyzed by agarose gel electrophoresis or dot-blot hybridization (DBH) assay described below.

f. **DBH assay:** For DBH assay, 1 µl of test DNA was dotted on a Nylon membrane (Millipore, California, USA) and fixed by UV cross-linking at 0.12 J cm^{-2} in a UV Crosslinker (Spectroline, Westbury, NY, USA) or simply air dried. The membrane was then hybridized with the biotin-labeled detection primer (TSD) (Table 6) at 63 °C in Hybridization Buffer (5x SSC) for 60 min. Subsequently, the membrane was washed twice in Wash Buffer (1x SSC), followed by incubation with 10 µg/ml alkaline phosphatase-streptavidin conjugate (Promega, Wisconsin, USA) in Blocking Buffer (100mM Tris-HCl pH 8.0, 150 mM NaCl, 0.3% Tween-20) at 25

°C for 10 min and two washes in 1x SSC. Finally, the NBT/BCIP color development substrate (2.47 µg/ml nitro-blue tetrazolium chloride and 0.55 µg/ml 5-bromo-4-chloro-3-indolyl-phosphatase-*p*-toluidine (Promega, Wisconsin, USA) in Alkaline Phosphatase Buffer (100 mM Tris-HCl pH 9.0, 150 mM NaCl and 5 mM $MgCl_2$) was added to the membrane and incubated at 25°C until color precipitates appear.

g. Statistical analysis: Statistical analysis was performed using MedCalc 9.3.1 statistical software package (MedCalc Software, Mariakerke, Belgium). The inter-rater agreement statistic Kappa was calculated with 95% confidence interval to evaluate the agreement between RT-LAMP-DBH and RT-nPCR-AGE.

Results:

A. **Optimization of TSV RT-LAMP:** The optimal concentrations of the outer and inner primers were determined to be 0.15 µM and 1.2 µM, respectively. The reaction temperature of TSV RT-LAMP was determined by carrying out the reactions at 55-65°C for 90 min, and the products were analyzed by agarose gel electrophoresis. No visible product was detected with the tRNA negative control carried out at 60°C, and the typical ladder-like DNA pattern was detected in reactions containing RNA from a clinical specimen [TSV(+) RNA]. Significant levels of amplicons were produced at all temperatures tested (Fig. 1A). Considering the requirements of the primers and MMLV-RT, subsequent RT-LAMP reactions were all carried out at 60°C. Next, TSV RT-LAMP reactions containing either TSV(+) or TSV(-) shrimp RNA were performed at 60°C for varying periods of time to determine the optimal incubation time. Detectable amounts of RT-LAMP products were produced after 30 min and the reaction reached plateau after 1 h with this specimen [Fig. 1C, TSV(+)]. Since samples of lower TSV RNA loads may need longer incubation to accumulate detectable levels of RT-LAMP amplicons (Varga and James, 2006), the following TSV RT-LAMP assays were carried out at 60°C for 1.5 h to ensure optimal performance. The smeared products detected by agarose gel electrophoresis with the TSV(-) sample after 3 h of incubation (Fig. 1C) will be addressed below.

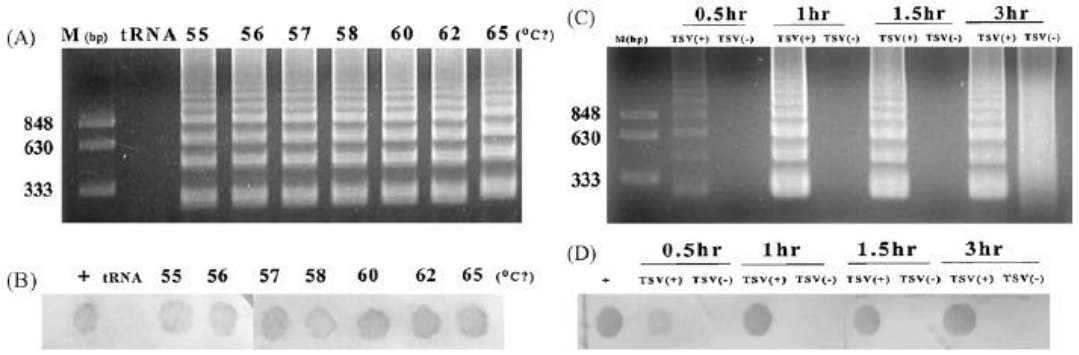

Fig. 1. Determination of TSV RT-LAMP conditions. (A-B) Effects of temperature on TSV RT-LAMP. RNA extracts prepared from TSV(+) shrimp were used to carry out RT-LAMP at different temperatures (55-65 ℃) and analyzed by agarose gel electrophoresis (A) or DBH assay (B). (C-D) Effects of incubation time on TSV RT-LAMP. RNA extracts prepared from TSV(+) and TSV(-) shrimp were used to carry out RT-LAMP for various lengths of incubation time at 60℃ and analyzed by agarose gel electrophoresis (C) or DBH assay (D). M (bp), molecular weight markers (base pair); tRNA, yeast tRNA; +, positive control for hybridization (TSV RT-LAMP amplicons prepared and confirmed previously).

B. **Specific detection of TSV RT-LAMP amplicons by colorimetric streptavidin-biotin DBH assay:** Notably, aberrant LAMP amplification products visualized as DNA ladders of different size by agarose gel electrophoresis have been detected occasionally with different sets of LAMP primers tested. The aberrant amplification products were mostly observed with samples containing none or very low levels of the target molecules. An example is shown in Fig. 1C [3 h-TSV(-)]. Therefore, in addition to testing different sets of RT-LAMP products and controlling primer quality to avoid the generation of any aberrant amplicons, specific detection of RT-LAMP amplicons was attempted. Toward this end, a chromogenic streptavidin biotin DBH assay for the detection of TSV RT-LAMP amplicons was developed. We tried to remove as many steps of the detection assay as possible to make it a user-friendly protocol without diminishing the detection sensitivity. The DNA denaturation step generally serves to unwind the double-stranded target DNA before probe hybridization in a DNA hybridization protocol. Removal of the DNA denaturation step was attempted by targeting the biotin-labeled detection primer (TSD) to the loop regions. Serial dilutions of different DNA samples were dotted on a membrane, UV cross-linked, and incubated sequentially with the biotin-labeled detection primer, the AP-streptavidin conjugate, and the chromogenic substrates for AP.

Oligonucleotide rcTSD (Table 1) is the exact complement fragment of the biotin-labeled TSD primer. The TSV RT-nPCR amplicons also contain the complementary sequence of TSD. No signal was developed with the TSV RT-nPCR amplicons without the denaturation step as expected (Fig. 2A), while visible purple spots could be discriminated directly by naked eyes with both rcTSD and TSV RT-LAMP products.

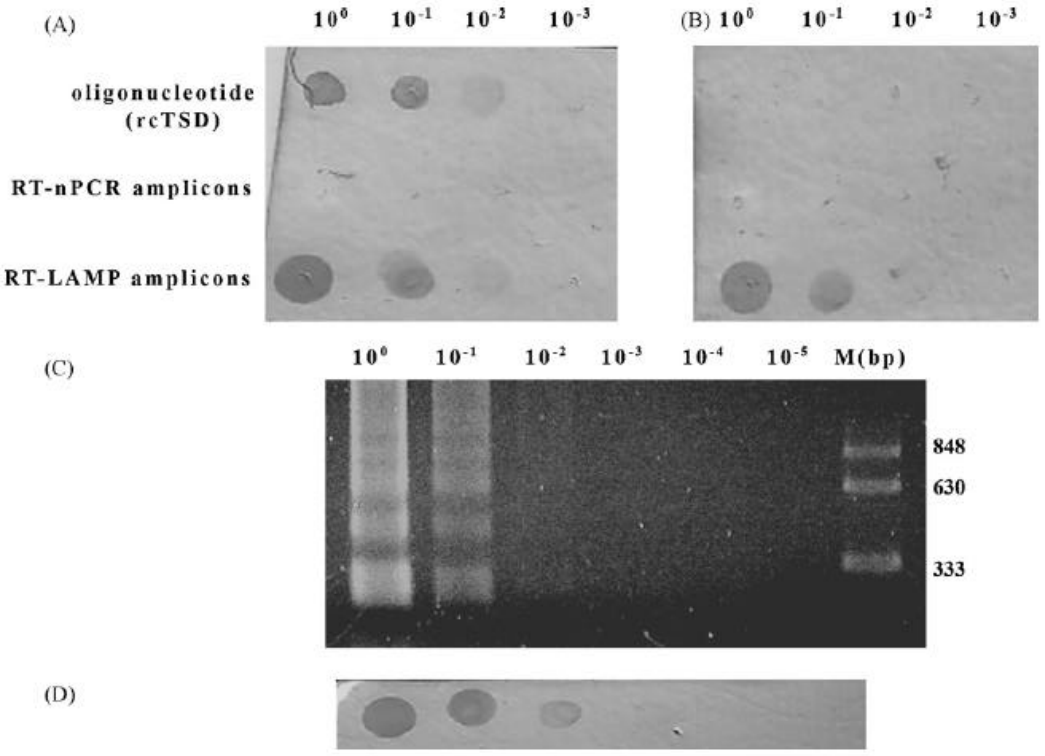

Fig. 2. Detection of TSV RT-LAMP amplicons by DBH. (A-B) Effects of DNA denaturation and UV cross-linking on DBH. Ten-fold serial dilutions (10^0 to 10^{-3}) of an oligonucleotide (rcTSD), TSV RT-nPCR amplicons, or TSV RT-LAMP amplicons were dotted on a membrane, followed by UV cross-linking (A) or no UV cross-linking (B). The membranes were hybridized sequentially with the biotin-labeled detection probe TSD and chromogenic substrates. (C-D) Agarose gel electrophoresis and DBH detection of TSV RT-LAMP amplicons. Serial dilutions (10^0 to 10^{-5}) of one TSV RT-LAMP reaction product were subjected concurrently to agarose gel electrophoresis (C) or DBH analysis (D). M (bp), molecular weight markers (base pair).

Our next target was to test whether the UV cross-linking step could be omitted. Hybridization of a duplicate membrane of the one shown in Fig. 2A was carried

out in parallel except that no UV cross-linking was performed and the membrane was air-dried instead. Positive signals were detected only with the TSV RT-LAMP amplicons (Fig. 2B). Notably, the detection sensitivity by DBH assay appeared to be quite similar with or without the UV cross-linking treatment (Fig. 2A and B; RT-LAMP amplicons). Detection sensitivity of the established DBH assay without UV cross-linking for LAMP amplicons was compared to that of agarose gel electrophoresis assay. Serial 10-fold dilutions of a TSV RT-LAMP reaction were subjected to both assays concomitantly. The DBH assay apparently does offer satisfactory sensitivity since the same dilution end point (10^{-3}) was reached by both methods (Fig. 2C and D). The specificity of the DBH assay for TSV RT-LAMP products is demonstrated by the absence of signals when samples with aberrant RT-LAMP products were applied. The smeared TSV RT-LAMP products detected with the TSV(-) RNA after three hours of incubation [Fig. 1C; 3 h-TSV(-)] did not react with the biotin-labeled probe in DBH analysis [Fig. 13D; 3 h-TSV(-)]. Similarly, DNA ladders of different patterns in Fig. 15B were not detected by DBH in Fig. 15C with the pTSV (10^{1}), tRNA and TSV(+) RNA (10^{-4} to 10^{-6}) samples. Therefore, additional levels of specificity were offered by the modified DBH assay at the post-amplicon detection step.

C. **Sensitivity of TSV RT-LAMP-DBH assay:** Currently, no standard cell culture method has been described for TSV detection. And the RT-nPCR-based IQ2000 TSV DPS was demonstrated previously to be about 10-fold more sensitive than the conventional RT-PCR assay recommended by OIE (Office International des Epizooties) for TSV detection (Teng *et al.*, 2006a). Serial 10-fold dilutions of a TSV(+) RNA were subjected to RNA RT-LAMP-DBH andRT-nPCR-AGEin parallel for comparison of detection sensitivity. For RT-nPCR-AGE, three expected products (arrow heads) are expected with the positive control (pTSV) and the largest product shows up only with higher concentrations of pTSV (Fig. 3A). Hence, with the positive controls showing detection limit at 10 copies and the TSV(-) shrimp RNA yielding only the internal control PCR product (arrow) (Fig. 3A), an optimal RT-nPCR-AGE reaction was achieved. In RT-LAMP, the positive control (pTSV) for the LAMP step and the negative controls [tRNA and TSV(-) RNA] also showed that TSV RT-LAMP worked properly (Fig. 3C). Under these circumstances, the same dilution end point, ie. 10^{-3} dilution of TSV(+) RNA, was reached by both RT-nPCR-AGE (Fig. 3A) and TSV RT-LAMP-DBH (Fig. 3C), demonstrating that the two assays provided comparable sensitivity.

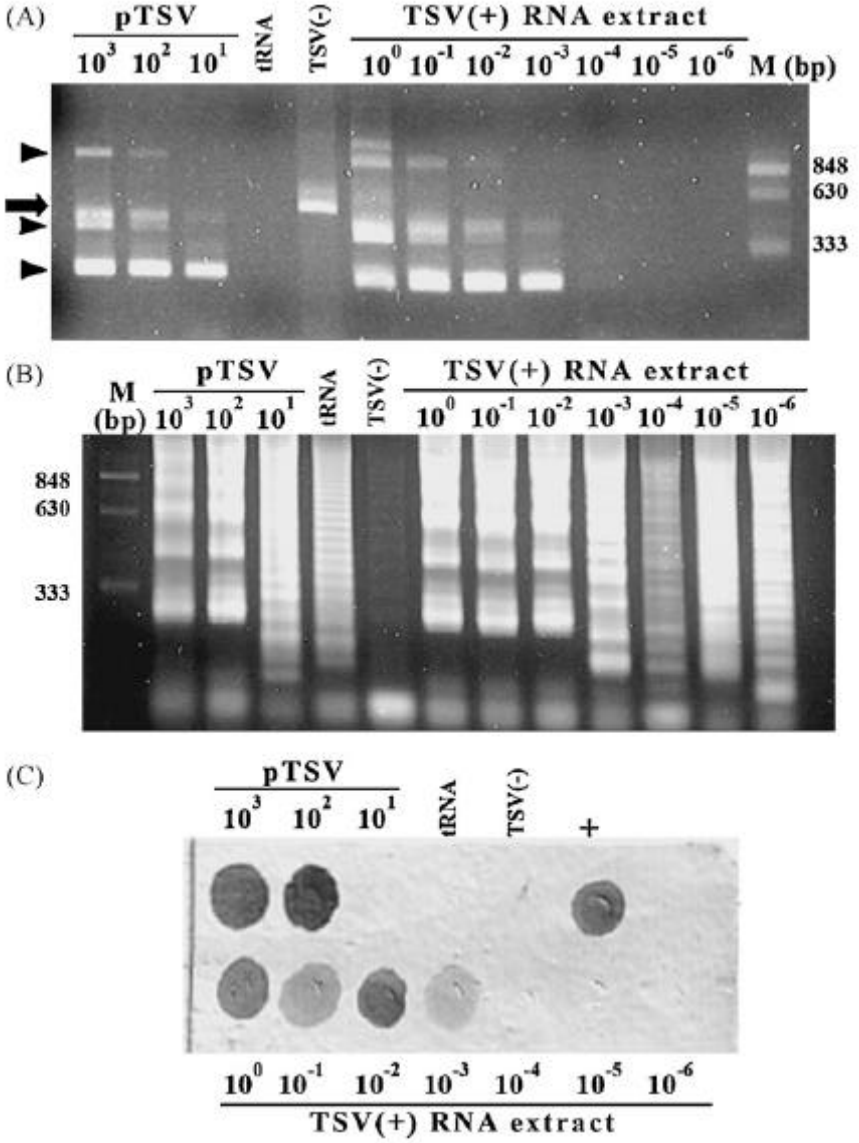

Fig. 3. Comparison of detection sensitivity between TSV RT-LAMP and TSV RT-nPCR-AGE. (A) TSV RT-nPCR-AGE analysis. TSV(+) RNA prepared from a TSV-positive shrimp was serially diluted in 10-fold increments (10^{0} to 10^{-6}) and subjected to TSV nested RT-PCR-AGE. (B-C) TSV RT-LAMP analysis. The same samples as in (A) were applied to TSV RT-LAMP analysis and analyzed by agarose gel electrophoresis (B) or DBH (C) in parallel. Controls included pTSV plasmid DNA, tRNA, and TSV(-) RNA. Arrow, internal control PCR product; arrowhead, TSV specific PCR products; M(bp), molecular weight markers (base pair); pTSV, a plasmid containing the cDNA of the target sequence (10^{1} to 10^{3} copies); tRNA, yeast tRNA; TSV(-) RNA, RNA extracted from a TSV negative shrimp; +, positive control for hybridization (TSV RT-LAMP amplicons prepared and confirmed previously).

D. **Specific detection of TSV by TSV RT-LAMP-DBH assay:** Cross-reactivity studies using RNA extracts from shrimp specimens infected with various viruses, including WSSV, IHHNV, MoV, IMNV, GAV, MBV, and HPV (Table 2), were carried out.

Table 2. Summary of different shrimp virus-positive specimens used in this study

Virus	Collection location	Host species
WSSV	Taiwan	*P. vannamei*
TSV	Taiwan	*P. vannamei*
GAV	Australia	*P. monodon*
YHV	Thailand	*P. monodon*
MBV	India	*P. monodon*
HPV	Thailand	*P. monodon*
NHPV	Mexico	*P. vannamei*
IMNV	Brazil	*P. vannamei*
MoV	Australia	*P. vannamei*

In RT-nPCR-AGE, production of the shrimp internal-control PCR product (Fig. 4A, arrow) alone indicated that these samples did not have detectable levels of TSV RNA. Similarly, in TSV RT-LAMP-DBH assay, positive signals were produced only with the positive control (pTSV) and the TSV(+) shrimp RNA, but not with the WSSV, IHHNV, MoV, IMNV, GAV, MBV, and HPV(+) RNA samples (Fig. 4B and C).

E. **Detection efficiency of TSV RT-LAMP:** Comparative evaluation between TSV RT-LAMP-DBH and RT-nPCR-AGE for TSV diagnosis was performed with a total of 125 *P. vannamei* samples collected in Taiwan. TSV was detected in 50 (40%) and 61 (48.8%) of the specimens by RT LAMP-DBH and TSV RT-nPCR-AGE (Table 3), respectively.

Table 3. Summary of TSV RT-LAMP-DBH and TSV RT-nPCR-AGE detection results for a total of 125 *P. vannamei* samples collected from farms in Taiwan for TSV

		TSV RT-LAMP-DBH		
		+	-	No.
TSV	+	50	11	61
RT-nPCR-AGE	-	0	64	64
	No.	50	75	125

+, TSV-positive; -, TSV-negative; No., number of specimens.

Among them, 50 and 64 were diagnosed TSV-positive and TSV negative,

respective, by both methods (Table 3), while 11 were positive by RT-nPCR-AGE but negative by RT-LAMP-DBH. The kappa test result for agreement between the diagnosis by RT-nPCR-AGE and RT-LAMP-DBH was 0.823, indicating a very good concordance between the two assays.

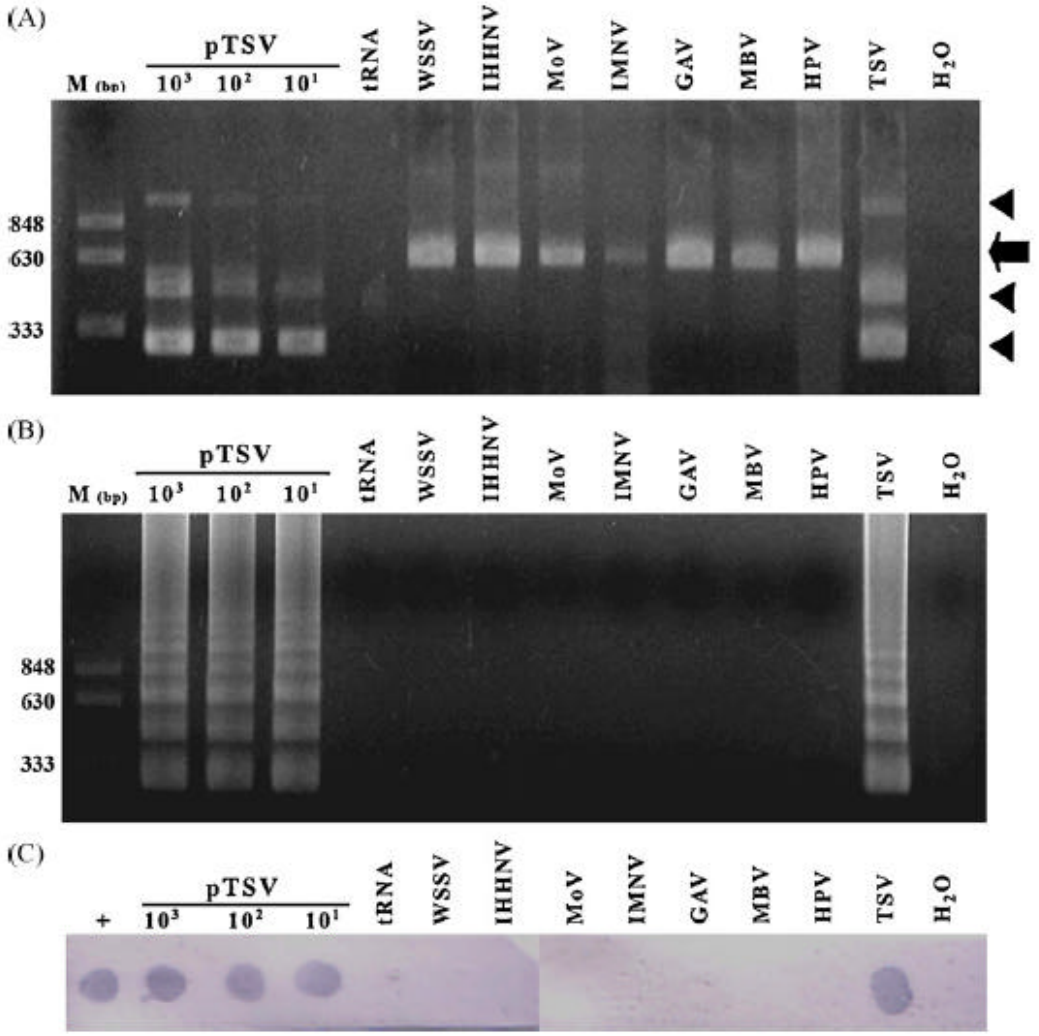

Fig. 4. Reactivity of TSV RT-LAMP-DBH with different shrimp viruses. (A) TSV RT-nPCR-AGE analysis. RNA prepared from WSSV, IHHNV, MoV, IMNV, GAV, MBV, HPV, or TSV(+) shrimp samples, pTSV plasmid, tRNA, or H_2O was amplified by TSV nested RT-PCR and assessed by agarose gel electrophoresis. (B-C) TSV RT-LAMP analysis. The same samples as in (A) were subjected to TSV RT-LAMP and detected by agarose gel electrophoresis (B) or DBH (C). M (bp), molecular weight markers (base pair); arrow, internal control PCR product; arrow head, TSV specific PCR products; pTSV, a plasmid containing a cDNA fragment of the target sequence; tRNA, yeast tRNA; +, positive control for hybridization (TSV RT-LAMP

amplicons prepared and confirmed previously).

F. **Simplification of RNA extraction method:** Boom *et al.* described a simple method for RNA extraction involving lysis of tissue in 4 M GuSCN and a subsequent silica particle-based RNA capture step which generally requires centrifugation (Boom *et al.*, 1990). In order to simplify the RNA extraction step for RT-LAMP, RT-LAMP was carried out using the GuSCN extract without the silica-assisted purification steps. First, detectable levels of the RT-LAMP amplicons were generated when one-tenth volume of 2 µl of TSV(+) shrimp gill crude extract prepared in 62.5, 125, or 250 mM GuSCN were added to a 20 µl RT-LAMP reaction (Fig. 5A and B), indicating that up to 25 mM GuSCN could be tolerated by RT-LAMP.

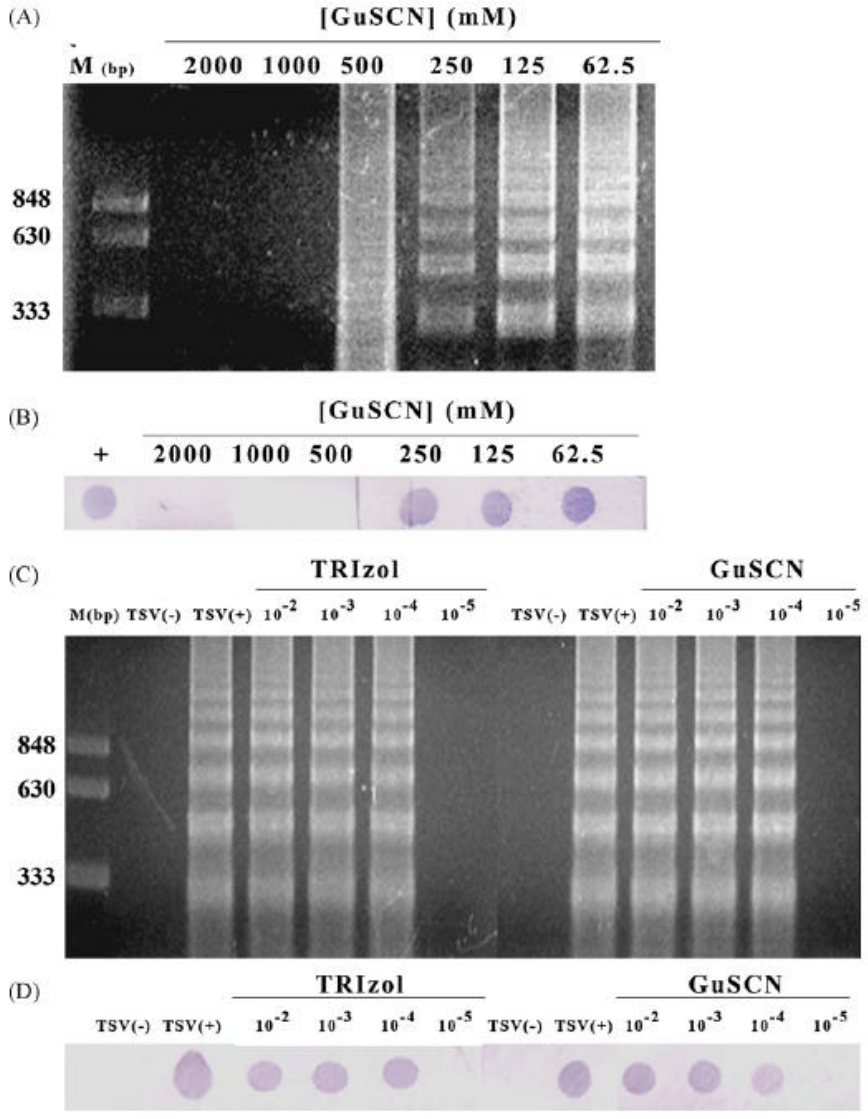

Fig. 5. GuSCN-based RNA extraction for TSV RT-LAMP. (A-B) Effects of concentrations of GuSCN on TSV RT-LAMP. After gill tissues were homogenized in different concentrations (6.25–2,000 mM) of GuSCN, 2 µl of the supernatant were applied to 20-µl TSV RT-LAMP reactions. The products were analyzed by agarose gel electrophoresis (A) or DBH (B). (C-D) Analysis of RT-LAMP reactions using RNA prepared with TRIzol Reagent or GuSCN. RNA from gill tissues was extracted by either the TRIzol Reagent protocol or by homogenization in 2 M GuSCN. After the RNA extracts were 10-fold serially diluted (10^{-2} to 10^{-5}), 2 µl each were applied to 20 µl TSV RT-LAMP reactions. The amplicons were analyzed by agarose gel electrophoresis (C) or DBH (D). M (bp), molecular weight markers (base pair); TSV(+), RNA prepared previously from another TSV-positive shrimp sample using TRIzol Reagent; +, positive control for hybridization (TSV RT-LAMP amplicons prepared and confirmed previously). TSV(-), TSV-negative shrimp.

In the optimized process, RNA was prepared in 2 M GuSCN to maximize RNA release and diluted at least 10^2 folds to make its final concentration 20 mM or less. RNA prepared this way was compared to RNA prepared by the TRIzol Reagent method. Detection limits of TSV RT-LAMP were reached at the same dilution (10^{-4}) of both RNA extracts (Fig. 5C and 5D), demonstrating that similar RNA extraction efficiency was achieved by both methods.

Discussion:

Timely detection of pathogen is important for aquaculture operators to take appropriate measures to prevent or manage disease outbreaks. This report describes the development of a diagnosis system in which a mixture of the reaction ingredients and an RNA lysate are incubated for 90 min to carry out RT-LAMP. Subsequently the amplicons are subjected to a modified DBH protocol that does not require the denaturation and UV cross-linking steps. At the end, positive purple spots can be detected easily by naked eyes with sensitivity comparable to RT-nPCR-AGE. Requiring only a simple heating machine, the turnaround time for the TSV RT-LAMP-DBH assay is estimated to be around 3.5 h.

Although the addition of loop primers had been demonstrated to accelerate the LAMP reaction (Nagamine *et al.*, 2002), our observation that the presence of loop primers resulted in unstable reactions and required systemic assessment and optimization of the reaction (data not shown) is in agreement with what has been described by Pillai *et al.* (2006). Therefore, a TSV RT-LAMP assay without loop primers was established, placing emphasis on reproducibility and reliability of the results. Design of the primers was based on the criterion described by Notomi *et al.*

(2000) excepted that shorter loops and a spacer of two instead of four T nucleotides are included in the inner primers (Table 1). The spacer in the inner primer apparently is not a critical element since inner primers without any spacer have also been shown to work in LAMP reaction (Hirayama *et al*., 2004; Hong *et al*., 2004; Mori *et al*., 2006).

The single-tube TSV RT-LAMP was carried out at one temperature. Although, the avian myeloblastosis virus RT is able to withstand high temperatures and has been preferentially adopted for single-tube RT-LAMP reactions reported previously (Fukuta *et al*., 2004; Hong *et al*., 2004), MMLV RT was used instead in this study to lower the final costs of the diagnosis system even though its optimal temperature for cDNA synthesis was described to be between 37 and 42℃ by the manufacturers. And similar amounts of amplicons were produced at temperatures ranging from 55 to 65℃ (Fig. 1A). Higher temperatures provide more stringent conditions for primer-target interaction than lower temperature in LAMP. Taking both RT and LAMP reactions into consideration, subsequent TSV RT-LAMP was set up at 60℃. Working at a suboptimal temperature for the MMLV RT enzyme, sensitivity of the assay was likely compensated by the 90-min incubation time that is longer than similar RT-LAMP reactions without loop primers (60 min) described previously (Ito *et al*., 2006; Mekata *et al*., 2006).

Interestingly, the UV cross-linking treatment apparently is critical for oligonucleotide rcTSD but not RT-LAMP amplicons to attach efficiently to the membrane (Fig. 2A and B). It is likely that the LAMP amplicons are quite long and bind to the membrane with much greater affinity than short oligonucleotides. Furthermore, the RT-LAMP amplicons, but not the RT-nPCR products, appeared to interact with sufficient amounts of the biotin-labeled detection primer to produce detectable signal (Fig. 2 and 3) without a preceding denaturation step. This could be attributed to that the detection probe was targeted to the loop region of the "dumbbell-like" amplicons. This principle was also proposed to play an important role for a recently described sequence-specific detection method for LAMP amplicons in which fluorescently labeled detection primers were derived from the loop region as well (Mori *et al*., 2006). However, an UV illuminator is required for optimal visual detection of the fluorescence emitted by the detection probe in this case. Instead, without any analysis apparatus involved, the modified DBH protocol described herein is ready to be combined with LAMP for field applications.

Aberrant DNA ladders in the absence or in the presence of very lowconcentrations of the target template were occasionally observed with different sets of primers tested for this study. One of the worst scenarios was intentionally presented in Fig. 3. And the appearance of these aberrant products of different sizes

was not reproducible. For example, the tRNA sample led to production of the aberrant products in Fig. 3B, but not in Figs. 1A and 4B. Therefore, false-positive interpretation for samples that lead to non-specific amplicons is of great concern. Results in Fig. 2A shows that without the denaturation and UV cross-linking steps, the colorimetric enzyme-linked DBH method reached similar detection sensitivity to gel electrophoresis analysis. The color spots are long-lasting and could be evaluated visually without the assistance of any image analysis equipments. Most importantly, in the case that uncharacteristic DNA ladders did occur, the detection probes allowed specific identification of the TSV RT-LAMP products [Fig. 1D, 3 h-TSV(-); Fig. 3C, pTSV(101), tRNA and TSV(+) RNA extract (10^{-4} to 10^{-6})] with satisfactory sensitivity by DBH. In addition, no TSV RT-LAMP-DBH signals were detected with the TSV(-) RNA samples prepared from *P. vannamei*, and *P. monodon* (Figs. 1, 3 and 4). The specificity of TSV RT-LAMP-DBH was also validated by that no detectable amplicons were generated with some important shrimp viral pathogens, including WSSV, IHHNV, MoV, IMNV, GAV, MBV, and HPV (Fig. 4).Taken together, the modified DBH assay is simple to perform and can specifically and sensitively detect the legitimate TSV RT-LAMP amplicons.

TSV RT-LAMP-DBH reached the same detection limit as TSV RT-nPCR-AGE (Fig. 3). Furthermore, a very good concordance (kappa value, 0.823) was found between TSV RTLAMP-DBH and TSV RT-nPCR-AGE for the detection of TSV in *P. vannamei* (Table 3). Overall, although being slightly less consistent in identifying shrimp samples containing low concentrations of TSV RNA targets than RT-nPCR-AGE, TSV RT-LAMP-DBH appeared to offer satisfactory detection sensitivity.

The GuSCN homogenization method offered RNA extraction efficiency comparable to that of the TRIzol Reagent extraction method (Fig. 5C and D). Requirement of RNA purity appears to be less stringent for RT-LAMP than for RT-nPCR. Up to 25mM RNA extract required further dilutions to work for RT-nPCR (data not shown). The observation agrees with a recent report stating that LAMP is less sensitive to inhibition by components in clinical samples than PCR and the DNA extraction step could be omitted (Kaneko *et al*., 2007). In addition, RT-LAMP reaction was reported not to be inhibited with increasing amounts of templates, whereas inhibition of RT-PCR was usually observed when larger amounts of template were used (Pillai *et al*., 2006).

Therefore, the TSV RT-LAMP-DBH assay described herein is relatively easy and simple to perform and does not require any sophisticated instrumentation. Since these studies were all carried out in a laboratory setting, further adjustments are underway to facilitate successful application of this system in field diagnosis.

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Chapter 5. Real-time detection of white spot syndrome virus (WSSV) by isothermal amplification

Background:

Real-time quantitative polymerase chain reaction (qPCR) technology for virus detection and quantification provides the advantages of high sensitivity and reproducibility. Numerous virus detection strategies were developed based on qPCR for virus monitoring and detection, such as human immunodeficiency virus type 1 (HIV-1) (Luo *et al*., 2005; Katsoulidou *et al*., 2006), hepatitis virus B and C (HBV, HCV) (Garson *et al*., 2005;Zhao *et al*., 2005; Candotti *et al*., 2004; Cook *et al*., 2004; Lindh and Hannoun, 2005), cytomegalovirus (CMV) (Guiver *et al*., 2001; Watzinger *et al*., 2004) and SARS-associated coronavirus (SARS-CoV)(Gut *et al*., 1999; Chui *et al*., 2005; Huang *et al*., 2005) . qPCR permits the detection of the amplicons generated during each amplification cycle and eliminates post-amplification handling to avoid contamination. In addition, qPCR offers a broad dynamic quantitative capacity across a more than seven-log range of targets (Chen *et al*., 2001; Jebbink *et al*., 2003; Lindh and Hannoun, 2005).

To real-time monitoring the increasing of amplicons, many detection formats and chemistries have been developed, including DNA intercalating dyes and target specific probes. DNA intercalating dyes such as ethidium bromide (EtBr) (Higuchi *et al*., 1992), YO-PRO 1 (Ishiguro *et al*., 1995), SYBR® Green I (FMC Bioproducts, Rockland, ME, USA) (Wittwer *et al*., 1997; Zipper *et al*., 2004), or BEBO (Bengtsson *et al*., 2003) intercalates into DNA double strand and emits fluorescence when excited by an appropriate light source. During the annealing and extension steps, the dye binds to the newly synthesized DNA strands leading to maximum fluorescence emission at the end of the elongation phase. As soon as the DNA is denatured during PCR cycling, intercalated dye molecules are released. The fluorescence signals recorded in each cycle reflect the amounts of PCR products generated during the amplification process (Morrison *et al*., 1998).

Compared to other real-time detection formats, systems based on the use of intercalating dyes are easier to establish and less expensive. However, the lack of probes in the assay results in lower specificity. Amplification and detection of specific PCR products are determined by the amplification primers and intercalating dyes. Dye molecules binding to non-specific PCR products or primer dimmers also contribute to overall fluorescence signal intensity, and may therefore lead to inaccurate quantification of target DNA. These artifacts may also result in the generation of fluorescence in the "No Amplification (NAC) and No Template (NTC) Controls", thus affecting the interpretation of results. It is essential to control the specificity of

amplified fragments at the end of PCR by melting curve analysis.

The other formats of real time detection systems are based on specific hybridization of one or two fluorescence-labeled oligonucleotide probes to the target sequences during amplification. Hydrolysis probes, such as TaqMan® probes, are now commonly applied to the detection of target DNA or RNA (Castelain *et al*., 2004; Hu *et al*., 2005). These probes are dual-labeled with a reporter dye (e. g. FAM or VIC) covalently bound to the 5'-end and a quencher dye (e. g. TAMRA or BHQ) covalently bound to the 3'-end. When probes hybridized on the target sequences, the light of reporters are suppressed by quenchers dyes by fluorescence resonance energy transfer (FRET) (Selvin, 1995). However, when the reporter dye is hydrolyzed by DNA polymerases that possess the function of 5'-exonuclease activity, the fluorescence emitted and can be detected specifically through a filter by a CCD camera. Beside hydrolysis of probes, another detection format frequently used for the detection of target sequences is based on hybridization of probes (Caplin *et al*., 1999). This method relies on the use of one or two oligonucleotide probes that hybridize to a sequence located between the amplification primers. Molecular beacons, one kind of hybridization probes, are oligonucleotide probes containing two flanking sequences of 5-7 nucleotides designed to be complementary to each other, and an intervening sequence complementary to the target of interest. The reporter and quencher dyes are labeled at the 5'- and 3'-end, respectively. Hairpin-loop structure is formed before molecular beacons anneal to target sequences during amplification. Lightcycler™ probes (LC probes), another kind of the hybridization probes, consist of two oligonucleotide probes. One is labeled with a donor dye at the 3'-end (e.g. Fluorescein, emitting green light), and the other is labeled with an acceptor dye at the 5'-end (e.g. LC Red 640 or LC Red 705, emitting red light). The probes are hybridized during the annealing step of PCR to the same strand at a distance of 1-5 nucleotides to bring the two dyes in close proximity. The donor dye is excited by an appropriate light source to emit fluorescence, and the emitting fluorescence is used as the excitation source of the receptor dye. If both probes are bound to the specific target sequences, FRET will be observed.

The detection formats mentioned above have advantages and specific limitations. Intercalating dyes are significantly cheaper than target-specific probes, but also bind to primer dimers and other nonspecific PCR products. Subsequent melting curve analysis increases the discriminative power of dye-based assays, but adds to the complexity of data analysis. Lacking of internal control is another limitation for intercalating dyes which are therefore less commonly used in clinical diagnostics. Target-specific probes offer more specificity and multiplicity that an internal control can be obtained in the same reaction. However, the cross-talk phenomenon of

fluorescence should be avoided in the development of multiplex detection.

In this study, fluorescence-labeled probes, such as molecular beacons and Lightcycler™ probes, will be applied to the detection of WSSV-specific sequences after loop-mediated isothermal amplification (LAMP).

Materials and methods:

a. **Sample preparation and DNA extraction.** Shrimp *L. vannamei* and *P. monodon* were collected from shrimp farms in Taiwan and China. For DNA extraction, 1 gill (about 20 mg) was ground thoroughly in 500 µl of Lysis Buffer (Genereach Biotechnology Corp., Taichung, ROC). After incubation at 95°C for 10 min, samples were centrifuged at 12,000 x *g* for 10 min to remove the cell debris. Subsequently, 200 µl of supernatant was precipitated with 400 µl of 95% ethanol and centrifuged at 12,000 x *g* for 5 min. The pellets were air-dried and recovered in 200 µl of ddH_2O or TE buffer and stored at -20°C.

b. **Real-time quantitative Polymerase chain reaction (qPCR) and real-time LAMP (qLAMP).** For qPCR analysis, the IQ REAL™ WSSV Quantitative System was used (Genereach Biotechnology Corp., Taichung, ROC). Briefly, 2 µl of DNA sample was mixed with 21 µl of real-time Pre-mix and 2 µl of Taq DNA polymerase. The condition was 40 cycles of 93°C for 15 sec, and 60°C for 1 min. The reaction was carried out in the ABI™ 7300 real-time PCR system. For qLAMP analysis, 2 µl of DNA sample was added to the qLAMP mixture containing 0.375 µM outer primers, 2 µM inner primers, 0.7 µM loop primers, 20 mM HEPES buffer (pH 7.9), 20 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 0.4 µl of *Bst* DNA polymerase, 0.4 M betaine and 0.375 mM dNTP. For different detection targets, primers were designed on the basis of target sequences available in GenBank. The reaction was carried out at 60°C for 120 min in the Roche LightCycler™ 1.5 real-time PCR system.

c. **Dot blot assay.** LAMP products (1 µl) were spotted onto a nylon membrane (Millipore) and fixed by UV cross-linking at 0.12 J cm^{-2} in a UV Crosslinker Box (Spectroline, New York city, USA). The membrane was then hybridized with the biotin-labeled probe at 63°C in hybridization buffer (5× SSC) for 1 h, washed twice in wash buffer (1× SSC), incubated with 10 µg ml^{-1} streptavidin-alkaline phosphatase conjugate (Promega, Wisconsin, USA) in blocking buffer (100 mM Tris-Cl pH 8.0, 150 mM NaCl and 0.3% Tween-20) at 25°C for 10 min and washed twice in wash buffer. Nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate substrate (NBT/ BCIP substrate) (Promega, Wisconsin, USA) diluted 100-fold in alkaline phosphatase buffer (100 mM Tris-Cl, pH 9.0, 150 mM NaCl and 5 mM $MgCl_2$) was added to the membrane and incubated at 25°C for 10 min.

d. **LC probes and molecular beacons**. The reference sequence of WSSV was GenBank accession number AF440570. The sequences of these probes are shown in Table 1.

Table 1. The sequences of probes used.

Name	Sequence
MB3	5'-FAM-CGGCAATCTCACAGCGGCTCTTGCACCGTTAGATTGCCG-BHQ1-3'
MB5	5'-FAM-GGCAATCTCACAGCGGCTCTTGCACCGTTAGATTGCC-BHQ1-3'
MB6	5'-FAM-CGGCAATCTACAGCGGCTCTTGCACCGTAGATTGCCG-BHQ1-3'
MB7	5'-FAM-CGGCAATCACAGCGGCTCTTGCACCGTTATTGCCG-BHQ1-3'
LC5	5'-LC640-CGTTCGCCATTGAAGCAGTGTTGGG-phosphate-3'
LC13	5'-CACCCACAGCGGCTCTTGC-Fluorescein-3'

e. Samples for specificity and sensitivity assay. The WSSV positive controls were DNA extracted from WSSV-infected shrimp and a TA-cloned plasmid containing target WSSV sequences. The negative control was DNA extracted from specific-pathogen-free (SPF) shrimp. Taura syndrome virus (TSV)-, infectious hypodermal haematopoietic necrosis virus (IHHNV)- and koi herpesvirus (KHV)-positive DNAs were prepared from diseased animals.

Result:

A. Optimization of LAMP reaction. The condition of WSSV LAMP was based on the reference published by Kono *et al.* Briefly, 10^6 copies/ µl of standard plasmid was used initially for LAMP condition optimization. The product was formed between at 55 and 65℃ after 120 min and the sensitivity was 10^2 copies/µl (data not shown). We then chose 60℃ and 10^3 copies/µl to test reaction time (50-120 min). LAMP products were detected after 60 min by dot blot assay (Fig. 1).

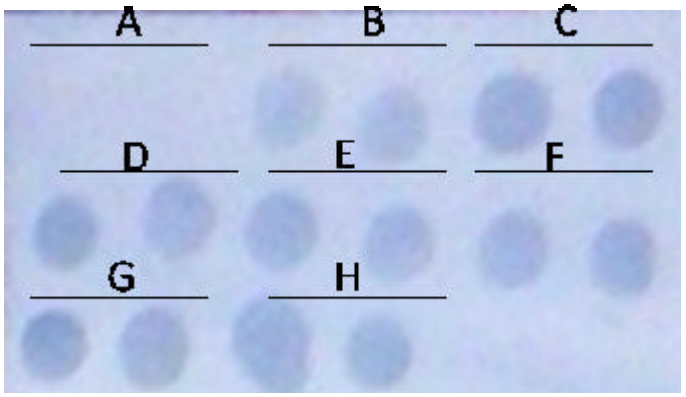

Fig. 1. Optimization of LAMP reaction time. Time course of LAMP reaction was tested by using 10^3 copies of WSSV standard plasmids. The range of reaction was from 50 min to 120 min at 60℃. Each time point was duplicated. A: 50 min. B: 60 min. C: 70 min. D. 80 min. E: 90 min. F: 100 min. G: 110 min. H: 120 min.

B. Optimization of qLAMP detection by molecular beacons. After optimization of WSSV LAMP condition, molecular beacons and LC probes (Table 1) were added at different concentrations to test for sensitivity. Nonspecific signals were detected by molecular beacons in TSV RT-LAMP or KHV LAMP which were included as the negative controls (Fig. 2).

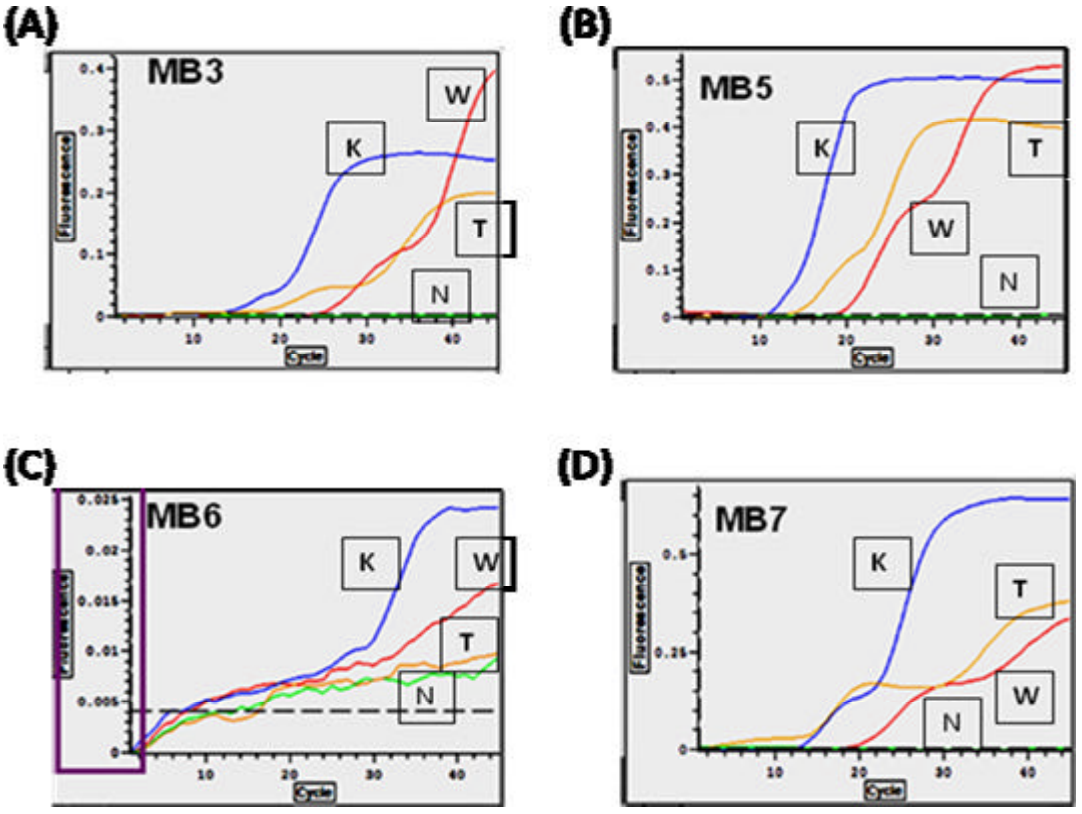

Fig. 2. Specificity analysis of different molecular beacons. (A) MB3, (B) MB5, (C) MB6 and (D) MB7 were designed according to WSSV specific sequences. The concentration of molecular beacons used in each reaction was 0.5 μM. Koi herpesvirus (KHV) LAMP (blue line, K), Taura syndrome virus (TSV) LAMP (brown line, T) and negative control (H_2O) (green line, N) were also tested by these molecular beacons. WSSV LAMP (red line, W) shown no significant differences with other LAMP reactions.

Different concentrations of molecular beacons were tested to eliminate the phenomenon, however, the strategy did not work. When we added the molecular beacons after LAMP reaction was finished, KHV LAMP product could not be recognized by MB5 and TSV LAMP product showd less signal than WSSV (Fig. 3). Because of undesirable sensitivities of the molecular beacons, LC probes were then tested.

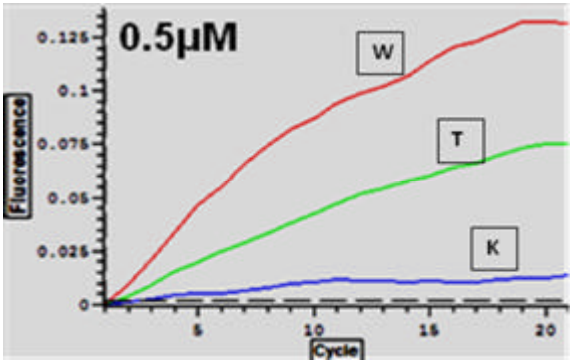

Fig. 3. Binding analysis of different LAMP products with molecular beacon MB5. 0.5 μM of MB5 was added directly in to WSSV (red line, W), TSV (green line, T) and KHV (blue line, K) LAMP products respectively.

C. Optimization of qLAMP detection by LC probes. WSSV-infected DNA sample L561 was serially diluted and quantified by the IQ REAL^{TM} WSSV Quantitative System. The viral load of L561 was compared and calculated by WSSV standard curve. After 10^5 dilution of L561 DNA extract, the signal was under the detection threshold (Fig. 4).

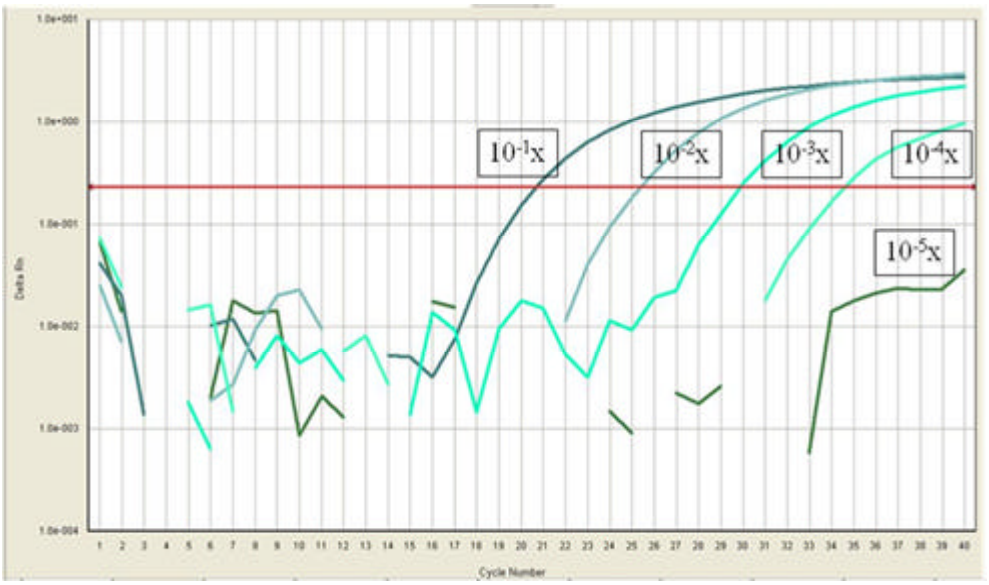

Fig. 4. Detection of WSSV by IQ real™ WSSV quantitative system. Shrimp L561 was WSSV infected tissue from *P. monodon*. After DNA extraction, 10-fold serial dilution was tested to confirm the sensitivity of real-time PCR system. The result showed that the signal of 10^5 diluted sample was below the detection threshold.

The same sample was also assayed by WSSV LAMP with LC probes added. The sensitivity of qLAMP was 25 copies/ µl by adding 0.2 µM LC probes in each reaction (Fig. 5). This condition was used to assay the specificity of qLAMP.

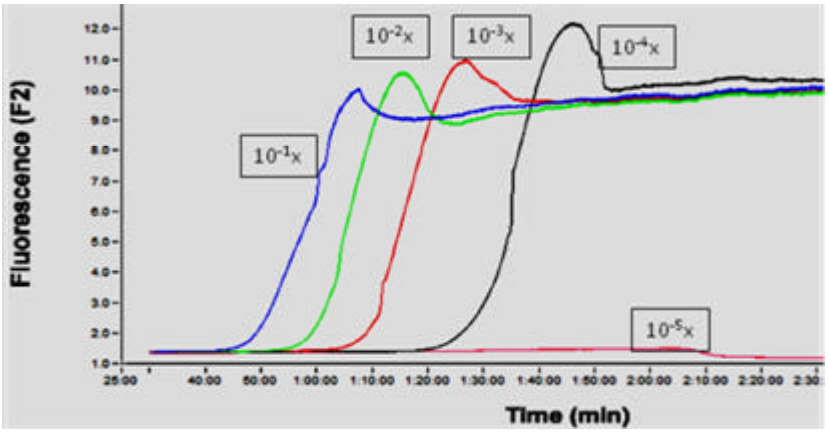

Fig. 5. Sensitivity analysis of WSSV with specific LC probes. WSSV infected sample was serial diluted and tested by WSSV LC probes. Real time PCR was also tested by using the same samples. WSSV LAMP by using LC probes could reach the sensitivity of 25 copies (10^{-4}x) in 90 min reaction time.

D. Specificity assay of qLAMP by LC probes. First, WSSV and TSV RT-LAMP using 10^5 and 10^4 copies of plasmids, respectively, and 0.2 µM LC probes were tested. Fluorescence signals raised in samples WSSV 10^5 and 10^4 and kept on basal level in samples TSV 10^5 and TSV 10^4 (Fig. 6).

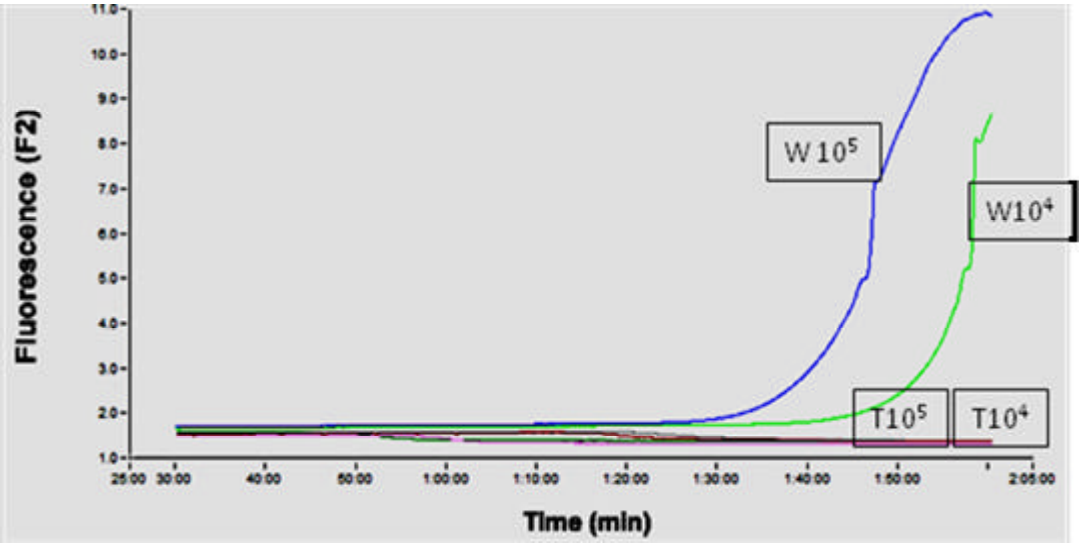

Fig. 6. Specificity assay of LC probes. WSSV and TSV LAMP using 10^5 and 10^4 copies of plasmids and 0.2 µM of LC probes were tested. Fluorescence signals raised in samples WSSV 10^5 (blue line, $W10^5$) and 10^4 (green line, $W10^4$) and kept on basal level in samples TSV 10^5 (red line, $T10^5$) and TSV 10^4 (black line, $T10^4$).

Then WSSV and IHHNV infected samples were used as templates. WSSV samples were analyzed by WSSV LAMP and IHHNV samples were analyzed by IHHNV LAMP. The same pair of LC probes was added in WSSV and IHHNV LAMP. Fluorescence signals raised by using WSSV infected samples and kept on basal level by using IHHNV infected samples (Fig. 7). By these assays, we concluded that the pair of LC probes was specific annealed with WSSV LAMP products and no cross-annealing with TSV or IHHNV LAMP products.

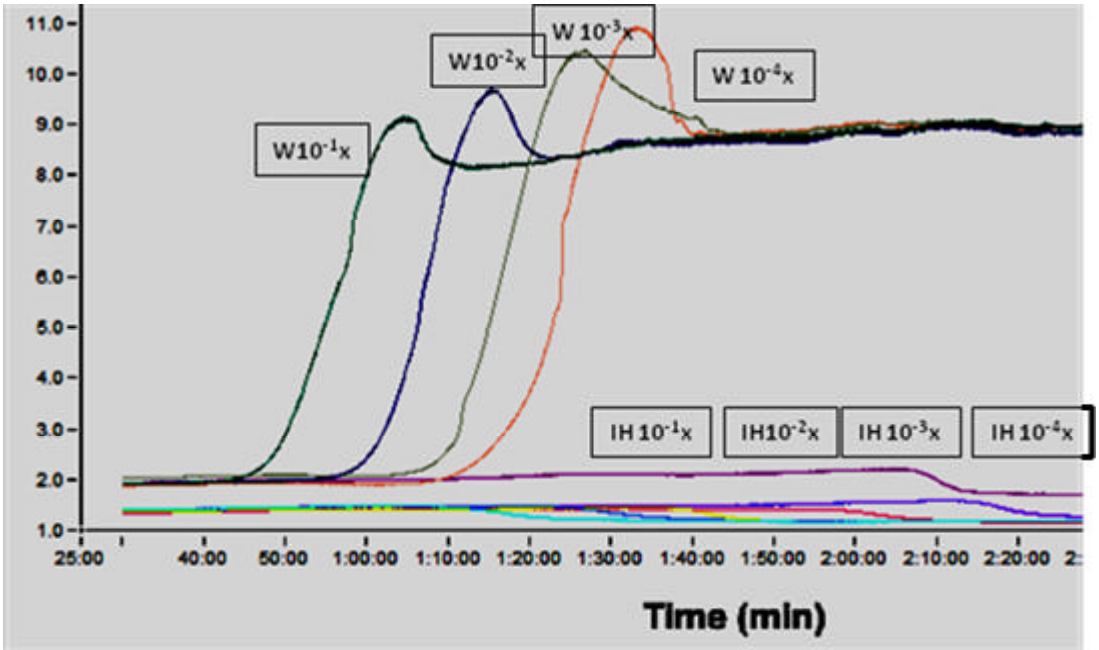

Fig. 7. Specificity analysis of LC probe for detecting WSSV and IHHNV infected samples. Serial dilutions of WSSV and IHHNV infected samples were used to test the specificity by using WSSV specific LC probe. Fluorescence signals raised by using WSSV infected samples and kept on basal level by using IHHNV infected samples.

Discussion：

Although a qLAMP strategy was published by increasing turbidity (Mekata *et al.*, 2008), a big challenge to avoid the disturbance of non-specific LAMP products was still existed. Here a WSSV qLAMP strategy with molecular beacons or LC probes was studied.

In the study, 10^3 of WSSV standard plasmids could be detected at 60 min by LAMP reaction (Fig. 1), and 10^1 of WSSV standard plasmids could be detected within 100 min reaction time (data not shown). These results showed high sensitivity as described before. The condition was then used and coupled with fluorescence probes for following assays. For specificity assay, the tests with LC probes showed more specific to detect LAMP products than that of molecular beacons (Fig. 2 and 3). There was not enough information to understand why the noise signals occurred. The hairpin structure of molecular beacons may be unwound when the non-specific LAMP products were existed in solution. Different modifications of loop sequences of molecular beacons will be tested for further studies.

For the consideration of better specificity with LAMP products, LC probes were combined in LAMP reaction to test the performances of quantification (Fig. 6 and 7).

By using WSSV infected *P. monodon* that infection load was quantified by real-time PCR as a template, the graphs of serial diluted samples showed the same patents and sensitivities comparable to real-time PCR assay (Fig. 5). If a standard curve of WSSV qLAMP could be established, absolute quantification will be available. However, analytic software should be also established to analyze the results between reaction time and quantity. If the software were available in the future, real time qLAMP with absolute quantification was a feasible way to substitute qPCR for pathogen diagnosis. Furthermore, the concept of multiplex detections could be developed with different fluorescent dyes labeling to distinguish viral and internal control signals. If the signal crosstalk between different fluorescent dyes could be eliminated, multiplex detection by isothermal amplification technologies will be a feasible strategy.

With these concepts and experimental data, qLAMP or other quantitative real time isothermal amplification methods possessed potential to become powerful on-site diagnostic tools. However, no commercial diagnostic products based on fluorescence probes were published until now. Several problems should be conquered to commercialize these kinds of products. (1) Target specific fluorescence probes were hard to design and optimization. Using the format of molecular beacon as an example, the loop region of molecular beacons should exactly anneal to the target sequence under reaction temperature, and in this temperature, the structure of stem region could be unwound to release the fluorescence signals. If the noise signals increased during reactions, false positive results will be observed. To avoid background signals, probe design is the key point. Some circumstances shall be avoided, such as the structure of hairpin loop unwound before annealing to target. Although some websites provided softwares to design molecular beacons, actual conditions should be tested by experiments, including probe concentration, signal intensity and probe specificity. (2) Target specific fluorescence probes were more expensive than intercalating dyes. More budgets should be considered when these probes were used in product developing. (3) Equipments for isothermal amplification by fluorescence probes that suitable for on-site use were unavailable. All these equipments for real time quantitative assay were expsnsive that could be only operated in laboratories. If these problems can be resolved in the future, real time isothermal technologies can be remarkable on the field of molecular diagnosis. Taken together, real-time isothermal amplification strategies were potential and feasible for developing on-site diagnosis kits.

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