A rapid method for simultaneously diagnosing four shrimp diseases using PCR-DNA chromatography method

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Keywords: Microsporidia, PCR, Vibriosis, White spot syndrome virus (WSSV)

Shrimp farming accounted for more than half of world production of shrimp in 2014 (Food and Agricultural Organization, 2017a). In shrimp farming, viral, bacterial, microsporidium and fungal diseases cause severe losses, so rapid methods for detecting them are needed.

Polymerase chain reaction (PCR)-based detection method is widely used as screening for many pathogenic bacteria due to its convenience and sensitivity. Common viral diseases include white spot disease (WSD) and infectious hypodermal and haematopoietic necrosis (IHHN). A common bacterial disease is acute hepatopancreas necrosis disease (AHPND). Conventional PCR detection methods targeting the agents that cause these diseases are recommended (World Organisation for Animal Health, 2016). Recently, Enterocytozoon hepatopenaei (EHP) was identified as the microsporidium that causes growth retardation in farmed shrimp (Tangprasittipap et al., 2013). Conventional PCR has also been used to detect EHP infection (Tang et al., 2015; Tangprasittipap et al., 2013). Conventional PCR requires agarose/acrylamide gel electrophoresis and a device for visualizing the gel.

Another method for detecting the PCR product is single-strand tag hybridization (STH) chromatographic printed array strip (PAS) method (Monden et al., 2014; Ohshiro, Miyagi, Tamaki, Mizuno, & Ezaki, 2016; Tian et al., 2014). In this method, multiplex PCR products can be visualized with high sensitivity within 15 min. Moreover, these chromatography strips are portable and ideal for field testing, as it eliminates the need for an electrophoresis equipment and preparation of gels that needs also require gel documentation equipment for viewing the PCR results. The method consists of a multiplex PCR in which multiple primer sets are tagged with a specific linker and biotin. After obtaining the PCR products, the PCR products then rise by capillary action in a tiny DNA chromatography strip to a point determined and hybridized by tag linker, stained with streptavidin-coated blue latex, which is visible to the naked eye (Figure 1).

In this study, we developed a detection system for four shrimp diseases (WSD, IHHN, AHPND and EHP infections) using multiplex PCR and STH chromatographic PAS, named PCR-DNA chromatography.

A total of 89 shrimp DNA samples of Penaeus monodon and Litopenaeus vannamei were collected in Thailand for this study. Total genomic DNA of these samples was extracted using tacob™ Nucleic Acid Automatic Extraction System (GeneReach Biotechnology Corp, Taichung City, Taiwan). For each of the DNA samples, each disease was detected by conventional PCR using KAPA2G Fast Multiplex Mix (Kapa Biosystems, Wilmington, MA, USA) or KAPA 2G Fast Hot Start Ready Mix with dye (Kapa Biosystems) referring to previous reports: WSD (Lo et al., 1996), IHHN (Tang, Navarro, & Lightner, 2007), AHPND (Tinwongger et al., 2014) and EHP infections (Tangprasittipap et al., 2013). We obtained 30 samples that were positive for white spot virus (WSV) which is the causative virus of WSD, seven samples that were positive for infectious hypodermal and haematopoietic necrosis virus (IHHNV) which is the causative virus of IHHN, 14 samples that were positive for toxin gene of a virulent strain of Vibrio parahaemolyticus of AHPND, 19 samples that were positive for EHP, 14 samples that were double-positive for IHHNV and EHP and samples that were negative for each of these diseases.

To develop PCR-DNA chromatography, we targeted WSD, IHHN, AHPND and EHP infections and 16S rRNA/tRNAVal/12S rRNA mitochondrial region (an internal control) using primers previously reported for WSD (Kiatpathomchai et al., 2005), IHHN (Tang, Navarro, & Lightner, 2007), AHPND (Tinwongger et al., 2014) and EHP infections (Tangprasittipap et al., 2013) and 16S rRNA/tRNAVal/12S rRNA (Pascoal, Barros-Velázquez, Cepeda, Gallardo, & Calo-Mata, 2008).
Table 1 shows the primer sets used for PCR-DNA chromatography. The primers targeting 16S rRNA/tRNAVal/12S rRNA were constructed on well-conserved regions of the mitochondrial DNA of the penaeid shrimp species, which make it possible to confirm the success of DNA extraction and to adapt our PCR-DNA chromatography to a variety of penaeid shrimp species. Each of the primers was labelled with a distinct tag-linker sequence and biotin (TBA Co., Ltd., Sendai, Japan) for STH chromatographic PAS.

Multiplex PCR was performed with 1 μl of shrimp DNA sample, 5 μl of SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio, Shiga, Japan), 300 nM each of WSD_136198 forward and WSD_136429 reverse primers, 200 nM each of IHHN_309 forward and reverse primers, 300 nM each of TUMSAT_Vp3 forward and reverse primers, 300 nM each of EN176 forward and reverse primers and 100 nM each of 16ScruC4 forward and 16ScruC3 reverse primers in a final volume of 10 μl by distilled water in a PCR tube. The condition for multiplex PCR was determined as: initial denaturation at
95°C for 2 min; 36 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 15 s.

After completion of the multiplex PCR, 10 μl of developing buffer (TBA Co., Ltd.) and 1 μl of streptavidin-coated blue latex (TBA Co., Ltd.) were added to the 10 μl of PCR product. A DNA chromatography strip (TBA Co., Ltd.) was then directly placed in the PCR tube. After 10 min, the strip was removed and read. The appearance of blue lines on the strip indicates the binding of streptavidin-coated blue latex to the PCR amplicons, which are labelled with biotin. All the pathogen-positive samples showed a positive band at the position corresponding to each pathogen on the strip (Figure 2). All the non-infected samples showed a positive band only for 16S rRNA.
(Figure 2) which is thought to be because 16S rRNA was not amplified when there are many amplicons from pathogens.

To measure the sensitivity of PCR-DNA chromatography, we used different concentrations of positive plasmid templates. The target region of each pathogen was ligated into pGEM T-easy vector (Promega, Madison, WI, USA). The extracted plasmid sequences were analysed by ABI Genetic Analyzer 3130 (Applied Biosystems, Inc., Foster City, CA, USA). The plasmids were diluted from $10^3$ copies/μl to 1 copy/μl, and then, 1 μl of each sample was analysed by PCR-DNA chromatography. To compare PCR-DNA chromatography with conventional electrophoresis, 10 μl of multiplex PCR products was electrophoresed on a 2.5% agarose gel containing GelStar™ Nucleic Acid Gel Stain (Lonza, Basel, Switzerland). PCR-DNA chromatography could detect as few as 10 copies of WSD, AHPND and EHP infections and as few as 100 copies of IHHN (Figure 3a). These were the same sensitivities shown by conventional electrophoresis (Figure 3b). Therefore, this PCR-DNA chromatography could be an alternative to conventional electrophoresis.

Occurrence of dual infections has been reported (Food and Agricultural Organization, 2017b; Otta et al., 2014; Pazir et al., 2011); hence, it is important to have a comprehensive detection to apply the needed countermeasures. To determine whether PCR-DNA chromatography could detect all four diseases simultaneously, PCR-DNA chromatography was performed with four kinds of plasmids ($10^2$ copies) with 1 μl of non-infected shrimp DNA sample, or with pooled DNA samples from each infected shrimp. As a result, all target regions of the four pathogens were detected in one test (Figure 4a). We also found that we could replace SYBR® Premix Ex Taq™ (Tli RNaseH Plus) with KAPA2G Fast Multiplex Mix (Kapa Biosystems, Wilmington, MA, USA) or Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany) (Figure 4b). This shows that the sensitivity was not affected by multiplex PCR enzymes.

In addition to having the same sensitivity as conventional PCR for each of the four diseases, PCR-DNA chromatography produced clearer visual results and was able to detect four diseases at once. PCR-DNA chromatography detection takes less than 60 min (30–45 min for multiplex PCR and 10 min for DNA chromatography strip), while conventionally PCR method normally takes about 90–120 min just for PCR without visualization through electrophoresis and need to run PCR targeting different diseases individually. Therefore, PCR-DNA chromatography makes it possible to diagnose up to four shrimp diseases rapidly and inexpensively.

We were also able to detect IHHN in DNA samples from boiled shrimp, WSD in DNA samples from a crab: susceptibility species (Maeda et al., 1998; 2010) and AHPND in DNA samples from soil, respectively (data not shown). Thus, PCR-DNA chromatography can be used to screen pathogens in samples other than shrimp.

Because some IHHNV-related sequences are integrated into the shrimp genome (Saksmerprome et al., 2011; Tang & Lightner, 2006), current PCR methods for detecting IHHN may yield false-positive results. Therefore, screening for IHHN requires a larger target DNA region to exclude the integrated sequences. In this study, the DNA chromatography strip was able to detect five PCR amplicons. However, the strip could detect up to eight PCR amplicons. This flexibility should allow rapid and accurate IHHN screening by increase target DNA regions in the multiplex PCR system.

In conclusion, we expect PCR-DNA chromatography will be useful for diagnosing not only shrimp diseases, but also other fishery pathogens in laboratory or diagnostic centre due to its ease of use, speed and flexibility.

**FIGURE 4** Versatility of PCR-DNA chromatography. (a) Multiplex PCR with (1) four kinds of plasmids ($10^2$ copies) with 1 μl of non-infected shrimp DNA sample and (2) pooled DNA samples from each infected shrimp. (b) Same as A 1, except with KAPA2G Fast Multiplex Mix (1) or Qiagen Multiplex PCR Master Mix (2)

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How to cite this article: Koiwai K, Kodera T, Thawonsuwan J, Kawase M, Kondo H, Hiroi I. A rapid method for simultaneously diagnosing four shrimp diseases using PCR-DNA chromatography method. *J Fish Dis*. 2017;00:1–5. https://doi.org/10.1111/jfd.12732