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Establishment of pyrosequencing technology to detect White Spot Syndrome Virus (WSSV) in cultured aquatic animals

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ABSTRACT

This study aimed to establish pyrosequencing methods to detect white spot syndrome virus (WSSV). One pair of polymerase chain reaction (PCR) primers, and one pyrosequencing primer, were designed for WSSV. The pyrosequencing reaction system and conditions were optimized and a pyrosequencing method for detecting WSSV was successfully established. This method was able to specifically detect WSSV in eight viruses, with high sensitivity. The minimum detectable limit for nucleic acid was 23 copies/μL. The method was verified by detecting WSSV in 1881 batches of samples collected from domestic and imported shrimps. The detection results were more sensitive than conventional PCR. This research has therefore provided a new detection method for monitoring, and controlling aquatic animal virus diseases.

1. Introduction

White spot disease (WSD) is caused by infection with white spot syndrome virus (WSSV). WSSV has an extremely wide range of hosts, and can infect a wide range of aquatic crustaceans including marine, brackish and freshwater penaeids, crabs and crayfish (Maeda et al., 2000a). All decapod (order Decapoda) crustaceans from marine, brackish or freshwater sources are susceptible host species (Flegel, 1997; Lightner, 1996; Lo et al., 1997). WSD has been identified in crustaceans from China, Japan, Republic of Korea, South East Asia, South Asia, the Indian Continent, the Mediterranean, the Middle East and the Americas (Lo and Kou, 1998; Chotigeat et al., 2004).

The prevention and control of WSSV is a worldwide problem. WSSV can infect all types of farmed prawns and has a very high mortality rate, with up to 100% mortality at 3–10 days after the prawns first show clinical symptoms (Maeda et al., 2000b).WSSV is one of the main pathogens influencing aquaculture. With reference to the World Organisation for Animal Health (OIE) *Diagnostic Manual of Aquatic Animal Diseases* (Office International Des Epizooties, 2018), and entry-exit inspection and quarantine industry standards of the People's Republic of China (SN/T 1151.2-2011, 2011), polymerase chain reaction (PCR) method is the most widely applicable and effective method with which to detect WSSV. However, PCR cannot be used as the basis for disease diagnosis unless combined with DNA sequencing. As a conventional detection method of nucleic acid, PCR is time-consuming. If the concentration of the PCR product is too low to meet sequencing requirements, it will not be possible carry out sequencing, so the suspected positive samples cannot be fully diagnosed.

A sequencing method for detecting a short and conserved gene fragment of the pathogen must be established to be able to derive a full diagnosis; this method must be fast, simple, and meet the needs required for large-scale detection of WSSV. The pyrosequencing method can accurately measure sequences of short DNA fragments within a short space of time, and can simultaneously analyze 96 samples (Gruber et al., 2002; Nordstrom et al., 2000). This method is simple to operate and creates a standardized testing procedure. Using WSSV as a disease model, specific sequencing primers and fingerprint sequences were designed to optimize pyrosequencing conditions. A specific and accurate WSSV pyrosequencing technology system was successfully established, in order to provide a new detection method for monitoring, preventing and controlling aquatic animal virus diseases.

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2. Materials and methods

2.1. Virus strain and nucleic acid

WSSV Thailand strain, WSSV Guangxi strain, Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) Thailand strain, Infectious Myonecrosis Virus (IMV) VH0921 strain, *Macrobrachium rosenbergii* nodavirus (MrNV) Guangdong strain, Taura syndrome virus (TSV) ZHZC3 strain, Yellow Head Virus genotype 1 (YHV-1) Qingdao201603 strain and *Penaeus monodon*-type baculovirus (MBV) Xiamen201409 strain nucleic acid were provided by Liu Hong, a researcher at Shenzhen Entry-Exit Inspection and Quarantine Bureau. The nucleic acid of viruses were derived from infected shrimp tissues, The WSSV 060718 JN strain was preserved by the laboratory of Yantai Entry-Exit Inspection and Quarantine Bureau.

2.2. Main reagents and instruments

DNTPs, Ex*Taq* DNA polymerase, a Onestep PCR kit and a DL2000 Marker were purchased from TaKaRa (Shiga, Japan), DNA extraction kits and RNA extraction kits were purchased from Qiagen (Valencia, CA, USA). A pyro SQA Reagent DNA sequence analysis kit (96-well), and magnetic beads coated with streptavidin, were provided by Gene Biotechnology International Trade (Shanghai, China) Co., Ltd. Finally, the PYROMARKID pyrosequencing system was provided by Qiagen (Valencia, CA, USA).

2.3. Determination of DNA fingerprint sequence and design of sequencing primers

The relatively conserved the viral envelope protein VP28 gene in WSSV was used as a target for detection. The sequence of the VP28 gene of WSSV registered in NCBI was obtained and aligned with each other using MegAlign function in DNAStar 7.1 software. The most conserved segment of WSSV less than 20 bp was selected as the fingerprint sequence for pyrosequencing. Sequencing primers were designed in both forward and reverse directions of the fingerprint sequence. Primers and probes were synthesized by TaKaRa. Biotin-labeled universal primers were synthesized by Invitrogen. Primer F1: 5′-GATGAAAACCTCCGCA TTCCT-3′; primer R1: 5′-Biotin-TCGCTGTCAAAGGACACATCA-3′; sequencing primer S1: 5′-ACATCAGTCATCTTGAAGTA -3′.

2.4. PCR reaction for sequencing primers

DNA was extracted from tissue samples with a DNA extraction kit in accordance with the manufacturer's instructions. Approximately 2 μL of WSSV virus nucleic acid, 0.5 μL of forward sequencing primer, and 0.5 μL of biotin-labeled reverse primer were added into a reaction system prepared according to the PCR kit instructions, and diethyl pyrocarbonate (DEPC) water was added to 50 μL. PCR was then conducted as follows: 94℃ for 5 min; 94℃ for 30 s, annealing temperature of primer for 30 s, 72℃ for 30 s, 30 cycles; 72℃ for 10 min, and then storage at 4℃. PCR products were subsequently analyzed by agarose gel electrophoresis.

2.5. Pyrosequencing reaction

Approximately 50 μL of PCR products amplified by biotin-labeled sequencing primers were mixed with 200 μg of magnetic beads coated with streptavidin, and incubated at room temperature for 20 min to bind the PCR products with magnetic beads. The PCR products bound to magnetic beads were then absorbed by the vacuum pump of the sequencing instrument, and rinsed with 70% alcohol, denaturation buffer and washing buffer for 5, 5 and 10 s, respectively. The PCR magnetic bead products were then loaded into the reaction plate with the help of a vacuum pump, and mixed with 2 μL of sequencing primer by gently shaking, to release the magnetic beads. The samples were then dried in an oven at 80℃ for 2 min, then cooled at room temperature.

The samples were then loaded into the pyrosequencing meter using a reaction temperature of 28℃, an injection pressure of 600 mbar, an injection time of 8 ms, and a sequencing time of around 65 s. The sequencing primers were amplified by the addition of different dNTPs. Signals were released whenever there was a single dNTP bound, and collected by the pyrosequencing meter. Eventually, sequencing peaks were generated.

2.6. Specificity test

The DNA or cDNA templates of IHHNV, IMV, MrNV, TSV, YHV-1 and MBV were amplified by PCR using sequencing primers for WSSV, and the products were analyzed by pyrosequencing.

2.7. Analytical sensitivity

Using the WSSV DNA as a template, we amplified the full sequence of the VP28 gene, which was then transformed into a p GEM-T plasmid and used as a standard. After 10-fold dilution, the sensitivity of a series of standards was determined using the pyrosequencing method, and compared with that detected by conventional PCR.

2.8. Reproducibility test

The DNA of WSSV was conducted by the pyrosequencing reaction. In addition, the DNA or cDNA templates from IHHNV, IMV, MrNV, TSV, YHV-1 and MBV were amplified by PCR using sequencing primers for WSSV, and the products were analyzed by pyrosequencing.

2.9. Detection of clinical samples

A total of 704 batches of domestic breeding shrimp samples, and 1811 batches of imported shrimp samples, were collected from 2014 to 2017, with at least 30 tails from each batch. Shrimps were mainly *Fenneropenaeus chinensis*, *Penaeus monodon Fabricius*, *Macrobrachium rosenbergii*, *Penaeus vannamei*, *Alpheus bellulus* and *Cherax quadricarinatus*. Fry, juvenile shrimps and adult shrimps were collected. The gills, hemolymph, stomach and abdominal muscle were taken from each shrimp. For fry, the whole shrimp was used as a sample; tissue was homogenized to extract DNA, and pyrosequencing used to detect WSSV. The results were compared with those of conventional PCR method as recommended by the OIE (2016) (Office International Des Epizooties, 2018). All samples were tested in duplicate.

3. Results

3.1. Determination of DNA fingerprint sequence and the design of sequencing primers

First, all of the WSSV VP28 gene(615bp) sequences in GenBank (AY324881.1, AY422228.1, AY682926.1, AY873785.1, DQ007315, DQ013883.1, DQ681069, EU414753.1, GQ328029.1, HM484380.1, HM484389.1, HM484390.1, KP219387.1) were aligned. This identified a nucleotide sequence at loci 176–188 (AGGTTGGATCAGG) which was highly conserved, and selected for use as a standard fingerprint sequence. Using this WSSV fingerprint, then specific sequencing primers were designed (Fig. 1).

3.2. PCR amplification of sequencing primers

First, the PCR conditions were optimized. In order to optimize the reaction temperature, five temperatures, 52, 54, 56, 58 and 60 °C, were selected as candidates. The results showed that pyrosequencing

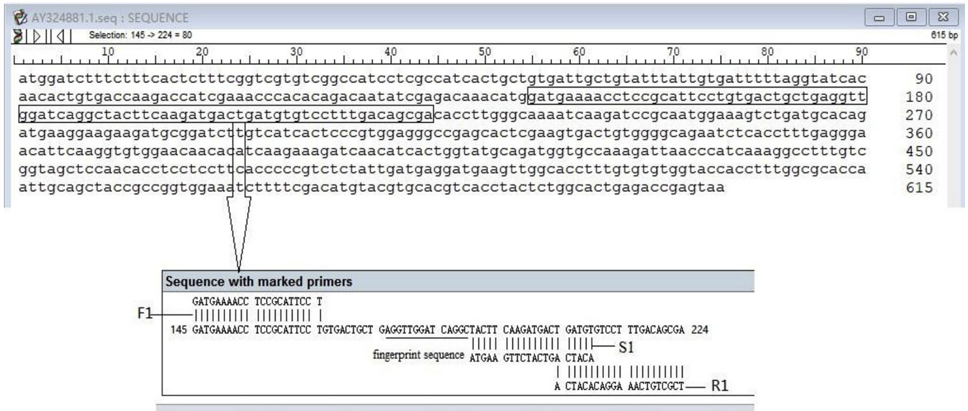

Fig. 1. Primers for the WSSV VP28 gene.
a:The results for PCR amplification of sequencing primers. Note: M. DL500 Marker; 1. WSSV Thailand strain; 2. WSSV Guangxi strain; 3. WSSV 060718 J N strain; 4. Negative control. b: Sequencing results for WSSV PCR products.

intensity remained stable between 54 and 56 °C; when the temperature was higher than 56 °C, the pyrosequencing intensity was weakened sharply. Therefore, the optimal temperature for PCR was determined to be 56 °C (data not shown).

94℃ for 5 min; 30 cycles of 94℃ for 30 s, 56℃ for 30 s, and 72℃ for 30 s; 72℃ for 10 min and finally, 4℃ termination.

Using this PCR method and the specific WSSV sequencing primers, it were able to successfully amplify the WSSV Thailand strain, Guangxi strain, 060718 J N strain and 150811 YT strain; the size of the target fragments (80 bp) matched that expected from the experimental design (Fig. 2a).

3.3. Pyrosequencing results

WSSV was amplified by PCR, and the products were analyzed by pyrosequencing; the sequence was confirmed as AGGTTGGATCAGG (Fig. 3b), consistent with the fingerprint sequence that was originally designed.

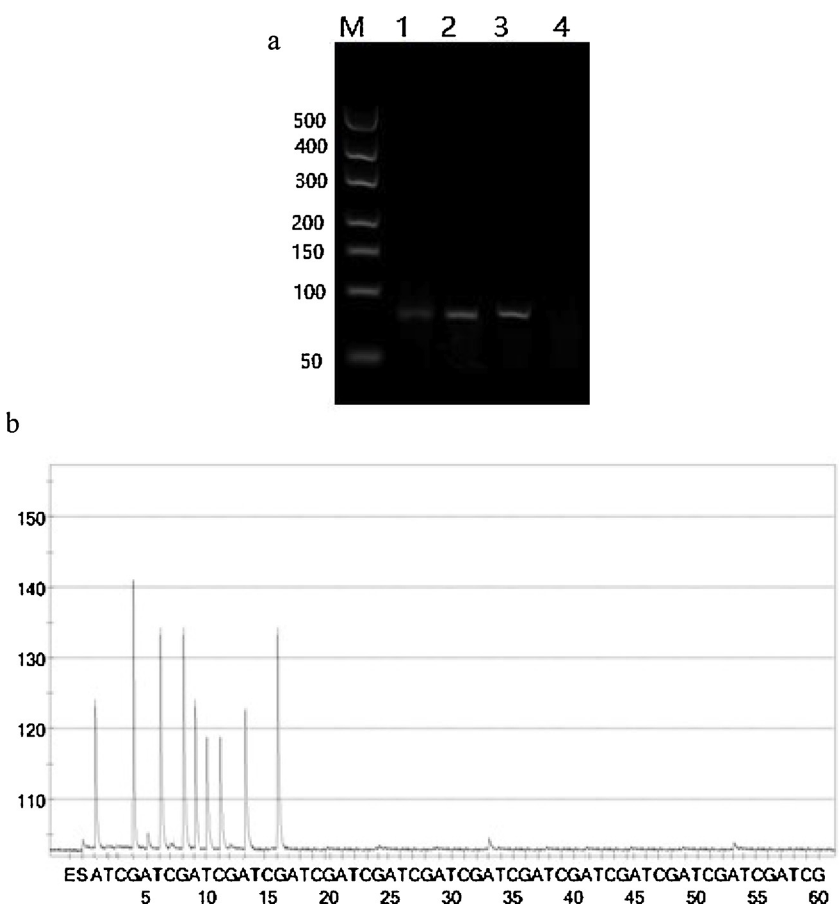

Fig. 2. The results of WSSV pyrosequencing.
Note: M. DL1000 Marker; 1-9. WSSV 2.3 × 10^6 copies/μL, 2.3 × 10^5 copies/μL, 2.3 × 10^4 copies/μL, 2.3 × 10^3 copies/μL, 2.3 × 10^2 copies/μL, 2.3 × 10^1 copies/μL, 2.3 × 10^0 copies/μL, 2.3 × 10^{-1} copies/μL.

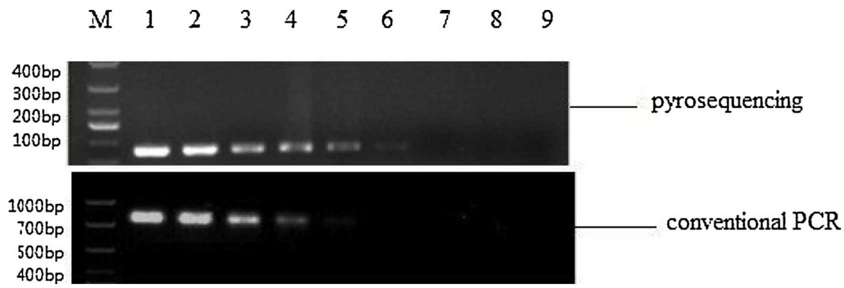

Fig. 3. Analytical sensitivity for the pyrosequencing of WSSV.

3.4. Specificity test

DNA or cDNA templates of IHHNV, IMV, MrNV, TSV, YHV-1 and MBV were amplified by PCR using specific sequencing primers for WSSV, and the products were analyzed by pyrosequencing. Amplified fragments of the expected size, and their corresponding peaks, were obtained from WSSV DNA templates using specific primers. No specific bands or peaks coincided with known sequences from other viruses, indicating that the primers had good specificity.

3.5. Sensitivity test

Following a series of 10-fold dilutions, the WSSV standard was detected using the pyrosequencing method. The minimum detectable amount of WSSV DNA was determined to be 23 copies/μL. The minimum detectable concentration of DNA for conventional PCR was 230 copies/μL; thus the pyrosequencing method was more sensitive than conventional PCR.

3.6. Reproducibility test

The re-extracted WSSV DNA, and the DNA or cDNA templates of IHHNV, IMV, MrNV, TSV, YHV-1 and MBV, were amplified by PCR using specific sequencing primers for WSSV, and the products were analyzed by pyrosequencing. Amplification fragments of expected size (80 bp), and their corresponding peaks, were obtained from the WSSV DNA template, while no specific bands, or peaks, coincided with known sequences from other viruses, indicating that our primers had good reproducibility

3.7. Detection of clinical samples

Detection results for clinical samples demonstrated that the number of WSSV positive samples was identical when compared with the pyrosequencing method and conventional PCR; in total, 276 batches were positive. Positive samples detected by PCR were confirmed to be WSSV-positive by sequencing (Table 1).

4. Discussion

The sensitivity of PCR is limited, and the mismatch of primers during amplification may lead to non-specific combination, resulting in false positives, false negatives and non-specific amplification. To guarantee the accuracy of such methods, the OIE and China entry-exit inspection and quarantine industry standards clearly state that PCR products must be sequenced; however, the length of time taken for sequencing is relatively long, generally 15–20 d. The pyrosequencing method established here takes a much shorter time, and the results can be obtained in 3–4 h; furthermore, this method provides a specific sequence and thus eliminates the risk of false positives. Pyrosequencing has been widely used in rapid detection and typing of many pathogens (De Battisti et al., 2013; Gharizadeh et al., 2005), It has also been used in molecular surveillance of influenza viruses by applying it to SNP detection, mutation screening, and reassortant virus identification (Deng et al., 2011).

In this study, the pyrosequencing method established herein is more analytically sensitive than conventional PCR, the detection time is significantly reduced because the results are not sequenced. The cost of pyrosequencing method is 1.5 times that of the PCR method. A total of 1881 samples were detected, and the detection results were compared with conventional PCR. The data showed that the pyrosequencing method had high accuracy and strong specificity, and is therefore an effective test method.

The core of the pyrosequencing method is to design specific sequencing primers and fingerprint sequences. According to existing literature, there is no strict requirement for the specificity of the 5′end of sequencing primers for pyrosequencing, but the 3′ end must be entirely complementary to the target nucleic acid sequence Ronaghi (2001). DNA melting temperatures (Tm) of sequencing primers at the 5′ end and 3′ end are consistent, so both ends of the primers can combine with the target template, thus improving the accuracy of detection. The fingerprint sequence is designed by comparing the sequence of various genes from viruses, and the most conserved sequence is selected. Because pyrosequencing technology in unable to detect long segments of DNA, the fingerprint sequence should be less than 20 bp in length.

By gathering WSSV gene sequences, the most conserved fingerprint sequence of WSSV could be identified and the most effective sequencing primers could be designed. Combined with the sensitivity of PCR, and

Table 1
Detection results for WSSV by pyrosequencing and conventional PCR.

Sampling position	Name of shrimp	Number	Positive number	Negative number	Positive rate
Yantai	*Fenneropenaeus chinensis*	118	25	93	21.1%
Qingdao	*Fenneropenaeus chinensis*	148	32	116	21.6%
Weihai	*Penaeus vannamei*	139	28	111	20.1%
Shenzhen	*Macrobrachium rosenbergii*	177	21	156	11.9%
Shanghai	*Penaeus monodon Fabricius*	162	42	120	25.9%
Thailand	*Penaeus vannamei*	496	61	435	12.3%
Indonesia	*Alpheus bellulus*	283	44	239	15.5%
Australia	*Cherax quadricarinatus*	358	23	335	6.4%

the accuracy of DNA sequencing technology, we successfully established a WSSV pyrosequencing method for the very first time. The sequencing primers and fingerprint sequences applied in this method are highly conserved, and can only amplify the target virus gene; they do not bind to other viruses and therefore show good specificity. The minimum detectable amount of nucleic acid was 23 copies/μL, more sensitive than conventional PCR, although the detection cycle was much shorter. Further tests showed that this new technology can detect WSSV quickly, sensitively, simply and specifically, and can be used for the early diagnosis and prevention of such diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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