Incidence and genetic analysis of white spot syndrome virus (WSSV) in farmed shrimps (Penaeus indicus and Litopenaeus vannamei) in Iran

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Abstract
White spot syndrome virus (WSSV) is a double-stranded DNA virus that causes the most serious losses to shrimp farmers worldwide. Since the first WSSV incidence in Iran in 2001, it has caused several outbreaks of disease with high mortality and economic loss in the Iranian shrimp farming industry. During this study from May 2010 to November 2015, WSSV was also diagnosed as the causative agent of ten of fifteen unexplained mass mortality events (70-100%) in farmed shrimp (Penaeus indicus and Litopenaeus vannamei) in four provinces along the coastal areas of the Persian Gulf and the Gulf of Oman. Thus, this highlighted that WSSV outbreaks are continuing in Khuzestan, Bushehr, and Sistan and Baluchestan provinces, as well as the first outbreak of the virus in Hormozgan province in Iran. Sequence analysis of the amplified region using World Organization of Animal Health (OIE) recommended primer sets also confirmed the presence of WSSV genome. Sequence analysis of variable number of tandem repeats (VNTRs) within the coding regions of ORF75 of Iranian WSSV strains revealed two repeat patterns (11 and 5 RUs) that were identical to some Indian isolates, while they differed by deletion and insertion of one 45 bp RUs from the repeat patterns of reference genomes from Thailand (12 RUs) and South Korea (4 RUs), respectively. This suggests that India may be the main source of origin into Iran where movements of the virus are probably occurring via importation of live post-larvae from this country.

Introduction
White spot syndrome virus (WSSV) is an enveloped, large double-stranded DNA virus belonging to the family Nimaviridae (genus whispovirus; Vlak et al., 2005) that is the causative agent of a contagious disease (White Spot Disease; WSD) with high cumulative mortality (up to 100%) and rapid disease onset in more than 98 crustacean species including penaeid shrimps, crayfish, lobsters and crabs (Escobedo-Bonilla et al., 2008; Lo et al., 1996; Sánchez-Paz, 2010).

White spot syndrome (WSS) was first detected in China (Fujian province) in 1991 to 1992 and
then spread rapidly to other shrimp species in Asia and North America, and it has been listed as a notifiable disease by the World Organisation for Animal Health (Office International des Epizooties; OIE) since 1997 (Flegel, 1997; Lo et al., 2012; OIE, 2017).

WSSV infected decapods often show changes in behavior and pink to reddish-brown discoloration of the body with local lesions and white spots in the exoskeleton and epidermis (Park et al., 1998; Crockford, 2008; Lightner and Lo, 2008; Pazir et al., 2012). Nonetheless, subclinical forms of disease can occur in some decapods such as the crayfish, and the white spots can also be as a result of environmental stress factors or bacterial infection and the shrimps often die after the appearance of symptoms (Joseph et al., 2015).

Four WSSV complete reference genomes according to their geographical source including the WSSV-TW isolate (307.2 kb, Acc. No. AF440570) from Taiwan, the WSSV-CN isolate (305.1kb, Acc. No. AF332093) from China, the WSSV-TH isolate (292.9 kb, Acc. No. AF369029) from Thailand and the WSSV- KR (295.8kb, Acc. No. JX515788) from South Korea with 181-184 open reading frames (ORFs) coding for more than 50 proteins have been described (Van-Hulten et al., 2001; Yang et al., 2001; Chai et al., 2013).

Sequence comparison of genomes of geographical WSSV isolates often show very little variation, approximately 5% of the genome (Lo et al., 1999; Wang et al., 2000) including deletion associated with ORF14/15 and ORF23/24 and the variable number tandem repeats (VNTRs) associated with the 3 minisatellites, ORF 94, ORF 75 and ORF 125 consists of a 54 bp uniform repeat, a 69 bp uniform repeat and a compound repeat of 45 and 57 bp, respectively, that have been considered as valuable markers for epidemiological studies (Marks et al., 2004; Dieu et al., 2004).

Since the first outbreaks of WSSV in Indian white shrimp (Penaeus indicus) in 2001 (Abadan, Khuzestan province) farmers have changed to white leg shrimp (Litopenaeus vannamei) in some regions of Hormozegan, Khuzestan and Bushehr provinces, and the disease has caused a significant decline in shrimp farming in Iran (Rosenberry, 2002; Afsharnasab et al., 2014).

There are varying reports regarding the WSSV outbreaks in different provinces of Iran by 2012, with clinical signs, histopathological findings, electron microscopy and nested PCR using the IQ2000 kit (Afsharnasab et al., 2007; Pazir et al., 2012), and only in the study of Simrouni et al. (2014) the sequence of ORF94 of detected Iranian WSSV strains during 2010 to 2012 has been analysed. This study describes the incidence and genetic analysis of WSSV in P. indicus and L. vannamei in Iran during May 2010 to November 2015.

Materials and methods

Sample collection

The origin of the WSSV suspected shrimp samples analysed in this study is shown in Figure 1. Shrimp specimens, including farmed Indian white shrimp (P. indicus) and white leg shrimp (L. vannamei) were collected from 15 suspected WSSV outbreak farms (10 moribund shrimp from each outbreak) in four provinces along the Persian Gulf and Oman Sea in Khuzestan (Abadan city), Bushehr (Bushehr city), Hormozgan (Bandar -Abbas cities) and Sistan and Baluchestan provinces (Chabhar city).
**DNA Extraction**

DNA extraction was performed from approximately 20 mg of pooled homogenate samples of gills of the ten shrimp using Exgene™ Viral DNA/RNA kit (GeneAll, Korea) according to the manufacturer’s instructions. Concentration and purity were measured with a Nanodrop spectrophotometer (Thermo Scientific, US). Total DNA obtained was eluted in 50 μL of AE buffer and stored at -20°C until further analysis.

**PCR detection**

To screen for WSSV, the OIE recommended nested PCR method was performed using two primer pairs of 146F1-146R1 (first step) and 146F2-146R2 (second step), with the same PCR amplification protocol as follows: PCR reactions were carried out in 50 μL reaction mixture that consisted of 1 μL of template DNA, 1 μL of each primer, 5 μL of 10x reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 1 mM DTT, 50 pg/mL nuclease-free BSA), 200 pM of each dNTPs, and 2 units of Taq DNA polymerase made up to volume with sterile distilled water. The cycling conditions were one cycle at 94°C for 4 min, followed by 39 cycles of 1 min intervals at 94°C, 55°C, 72°C and finally extension was performed at 72°C for 5 min. The amplification products of the second step of nested PCR were resolved by electrophoresis using a 1% agarose gel and confirmed by sequencing. The used primers are listed in Table 2.

**Sequence analysis**

Sequence analysis of the amplified region by the OIE recommended primers (146F2-R2, second step) and variable regions of ORF75 WSSV.
genetic electrophoresis using a 1 % agarose gel, purified using Expin™ PCR SV kit (Gene all, Korea) and both DNA strands were sequenced and subjected to nucleotide sequence analysis by dideoxy chain termination method (Applied Biosystems, CA). The nucleotide sequences were aligned and analysed using the Geneious Pro 10.0.9 software.

**Analysis of VNTRs of ORF 75**

For all WSSV suspected samples, PCR assays were performed using primer pair (ORF75F-

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**Table 1.** Description of WSSV outbreaks in farmed shrimp in Iran (2010-2015).

<table>
<thead>
<tr>
<th>Province/ City</th>
<th>No. of farms outbreak / WSSV positives*</th>
<th>Shrimp species</th>
<th>Outbreak date</th>
<th>Acc. No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bushehr/ Bushehr</td>
<td>1/1</td>
<td><em>L.</em> vannamei</td>
<td>May 2010</td>
<td>KT894028</td>
</tr>
<tr>
<td>Sistan and Baluch-istan/ Chabahar</td>
<td>5/3</td>
<td><em>P.</em> indicus</td>
<td>May to June 2013</td>
<td>KT894030, KT894031 and KT894032</td>
</tr>
<tr>
<td>Sistan and Baluch-istan/ Chabahar</td>
<td>2/1</td>
<td><em>P.</em> indicus</td>
<td>May 2014</td>
<td>KT894040</td>
</tr>
<tr>
<td>Khuzestan/ Abadan</td>
<td>3/3</td>
<td><em>L.</em> vannamei</td>
<td>October to November 2015</td>
<td>KX492915, KX84738 and KX84739</td>
</tr>
<tr>
<td>Hormozgan/ Bandar-Abbas</td>
<td>4/2</td>
<td><em>L.</em> vannamei</td>
<td>October to November 2015</td>
<td>KX714112 and KX714113</td>
</tr>
</tbody>
</table>

* From each of the 10 farms experiencing outbreaks, 10 moribund shrimp were collected and each gill/pleopod homogenate sample tested was pooled from 5 shrimp.

**Table 2.** The primer sets used in this study.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>F-Primer (5’-3’)</th>
<th>R-Primer (5’-3’)</th>
<th>Annealing T (°C)</th>
<th>Amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>146F1-R1</td>
<td>ACTACTAACTTCAG-CCTATC</td>
<td>TAATGCGGTTGTAAAT-GTTCT</td>
<td>55</td>
<td>1447</td>
<td>(OIE, 2017)</td>
</tr>
<tr>
<td>146F2-R2</td>
<td>GTAACCTGCCCTTC-CATCGC</td>
<td>TACGGCAGCTGCTG-CACCTT</td>
<td>55</td>
<td>941</td>
<td>(OIE, 2017)</td>
</tr>
<tr>
<td>ORF75F-R</td>
<td>GAAGCAGTATCTCTA-ACAC</td>
<td>CAACAGGTCCGTA-AAAGAAG</td>
<td>49</td>
<td>Variable</td>
<td>(Dieu et al., 2004)</td>
</tr>
</tbody>
</table>
R) designed by Dieu et al. (2004, Table 2), for ORF 75 minisatellite consisting of a compound repeat of 45 and 57 bp. The PCR conditions and temperature profile for the ORF75 were the same as described previously for the OIE recommended primers except that the annealing temperature was lowered to 49°C (Table 2). PCR products were purified from 1% agarose gels using an ExpiP™ PCR SV kit (Gene all, Korea) and both DNA strands were sequenced using the primers used in the initial amplification. The sequences were analysed for the presence of tandem repeats using Tandem Repeats Finder (TRF) program (Benson, 1999). The nucleotide sequences and repeated units (RUs) were analysed and compared with the four complete reference genomes of WSSV at GenBank by the BLAST tool provided by the National Centre for Biotechnology Information (NCBI), (http://www.ncbi.nlm.nih.gov), BioEdit software, and by Geneious Pro 10.0.9.

Results

PCR Detection

PCR screening of the pooled samples collected from the 15 suspected WSD outbreaks on shrimp farms in Iran during 2010 to 2015 revealed 10 WSSV positive farms, including one outbreak in Bushehr province (Bushehr city, _L. vannamei_), four outbreaks in Sistan and Baluchistan province (Chabahar city, _P. indicus_), two outbreaks in farms of Hormozgan province (Bandar-Abbas city, _L. vannamei_), and three farms were WSSV positive in Khuzestan province (Abadan city, _L. vannamei_). The positive samples from infected shrimp farms showed the suspected clinical signs of WSD with high mortality (Figure 2), gave PCR products (second step) of approximately 941 bp (Figure 3) and were confirmed by sequence analysis. The presence of WSSV was not detected in samples from negative controls. The description of WSSV positive samples are shown in Table 1.

Analysis of VNTRs of ORF 75

All WSSV positive samples tested by the OIE recommended assay gave amplicons for the ORF 75 region (Figure 4). In general, with the exception of the strain of Bushehr (KX650068) the number and repeat patterns for the ORF 75 were identical for samples from different locations. The sequence of _L. vannamei_ in Busheh province detected in 2010 (IR-2, KX650068) with the smaller amplicon (401bp) revealed a repeat pattern of 45×3, 57×1, 45×2 that contains 5 RUs: one 102 bp RU and four 45 bp RUs.

Sequencing of the ORF75 fragment from the other provinces with amplicons ranging from 734 to 794bp revealed a repeat pattern of 45×2, 57×1, 45×5, 57×1, 45×2, 57×1, 45×2, containing 11 RUs (Three 102 bp RUs and eight 45 bp RUs). The number and repeat patterns of the ORF 75 of Iranian strains (11 and 5 RUs) were identical to some Indian isolates previously described by Pradeep et al. (2008; Figure 5), while they differed by deletion and insertion of one 45 bp RUs from the repeat patterns of reference genomes from Thailand (12 RUs) and South Korea (4 RUs), (Figures 5 and 6).

Discussion

In Iran, WSD was first reported from _P. indicus_ in Khuzestan Province (Abadan city) in 2001 (Rosenberry, 2002). It quickly spread in the south of the country and caused several outbreaks with severe mortality in farmed _P. indicus_ and _L. vannamei_ in the Bushehr province (Bushehr city) in 2003, 2005, 2009 and 2010, and Sistan and Baluchestan province (Chabhar

Figure 2. WSSV outbreak in Iranian shrimp farms. (A) Mass mortality caused by WSSV in a shrimp farm in Iran (Khuzestan, Abadan). (B) White spots on the carapace of WSSV-infected shrimp (L. vannamei).

Figure 3. Nested PCR assay of WSSV by the OIE recommended primers in farmed shrimp in Iran. Lanes are M: marker; P: positive control; N: negative control; Lanes 1-5 are WSSV-infected samples from 4 provinces including: 1: Bushehr province (Bushehr city, L. vannamei); 2 and 3: Sistan and Baluchistan province (Chabahar city, P.indicus), 4: Hormozgan province (Bandar-Abbas city, L. vannamei); 5: Khuzestan province (Abadan city, L. vannamei). PCR products were analysed on a 1% agarose gel and visualised by ethidium bromide.

Figure 4. PCR amplification of WSSV ORF 75 according to Dieu et al. (2004) from farmed shrimps in Iran during 2010 to 2015. M: marker; Lanes are 1, 2, 4, and 6-12 are WSSV-infected samples: 1 and 2: Hormozgan province (Bandar-Abbas city, L. vannamei); 4, 6, 7 and 8: Sistan and Baluchistan province (Chabahar city, P. indicus); 9: Bushehr province (Bushehr city, L. vannamei); 11, 12 and 13: Khuzestan province (Abadan city, L. vannamei). Lanes 3 and 5 are negative controls. PCR products were analysed on a 1% agarose gel and visualised by ethidium bromide.
Figure 5. Schematic alignment of different genotypes of WSSV from Taiwan (TW, 1842 bp, used as reference genome), China (CH, 1515 bp), Thailand (TH, 1323 bp), South Korea (KR, 849 bp), India (IN-1 and IN-2, variable) and Iran (IR-1 and IR-2, Variable), showing number and order of tandem repeat units (RUs) within the ORF75 (45bp and (45+57) 102 bp). Dashed lines indicate the deleted sequences. IR-2 (KX650068) is the WSSV-ORF75 sequence detected from L. vannamei in Busher province in 2010 (with the smaller amplicon (401bp). IR-1 represents the other sequences (9 strains) with larger amplicon ranged 734 to 794bp from (L. vannamei and P.indicus) in Sistan and Baluchistan, Hormozgan, and Khuzestan provinces.

Figure 6. Multiple sequence alignment of Iranian strains of WSSV with five strains from the USA, Taiwan, China, Thailand, and South Korea, based on the ORF75. The RUs (45 bp) are underlined and dashed lines indicate the deleted sequences.

Furthermore, the sequence variation of ORF 194 has been reported in some strains of WSSV in *L. vannamei* from Khuzestan and Bushehr Provinces in 2010 to 2012 (Simrouni et al., 2014). During this study from 2010 to 2015, WSSV was also diagnosed as the causative agent in ten of fifteen outbreaks, including one outbreak in the Bushehr province (*L. vannamei*, 2010), four farms in Sistan and Baluchistan province (*P. indicus*, 2013 and 2014), three farms in Khuzestan province (*L. vannamei*, 2015), and two outbreaks from Hormozgan province (*L. vannamei*, 2015) that the last one is the first report of WSSV outbreaks in this province.

In the present study, all WSSV positive samples were detected by World Organization of Animal Health recommended nested PCR and ORF 75 was also sequenced to reveal the phylogenetic relationship of detected strains.

The primer sets 146F1-146R1 and 146F2-146R2 developed by Lo et al. (1996) and recommended by the OIE were able to amplify the WSSV isolates from Taiwan (Lo et al., 1996), Japan and Bangladesh (Ayub et al., 2008) but not from *Penaeus monodon* in the Philippines (Natividad et al., 2006), and *Cherax quadricarinatus* in Australia (Claydon et al., 2004). Therefore, our results revealed that both the primer sets recommended by the OIE can be considered for PCR screening of WSSV in different species of farmed shrimps in Iran.

Moreover, sequence analyses of the amplified region of Iranian strains revealed the closest identity with the sequence of four previously reported genomic references of WSSV from Taiwan (AF440570), Thailand (AF369029), South Korea (JX515788) and China (AF332093).

WSSV positive shrimp samples were also analysed for the variable number of tandem repeats (VNTRs) within the coding regions of ORF75 and compared to other WSSV strains from various geographic areas.

The number and repeat patterns of the ORF 75, except for the strain of Bushehr (IR-2, KX650068) with 5 repeat units (RUs), were identical to shrimp samples from various different locations within Iran, including three provinces along the coastal areas of the Persian Gulf and the Gulf of Oman, and different host species, with 11 RUs (IR-1). The observed repeat patterns of IR-1 and IR-2 were different by deletion and insertion of one 45 bp RUs from the repeat pattern of reference genomes from the Thailand (12 RUs) and South Korean (4 RUs) strains, respectively, that may be explained by the genomic mutations for adaptation to different environmental conditions (Waikhom et al., 2006). The origin of the Bushehr strain (IR-2 KX650068), the cause of high morbidity and mortality in some farmed shrimps in this region is unknown. However, it may also have been imported from East Asian countries as brood shrimp and live feed are frequently imported inside this province from some East Asian countries. More work is required to clarify the source and species variation of this strain of WSSV.

However, the number and repeat patterns of ORF 75 of Iranian strains of WSSV were iden-
tical to those previously described from India with the product size ranging between 320 and 778 bp (Pradeep et al., 2008). Similarly, sequence analysis of ORF 194 of Iranian strains detected from Khuzestan and Bushehr provinces in 2010 to 2012 (Simrouni et al., 2014), showed 3 and 6 RUs with G and T SNPs in position 48 which were also identical to Indian isolates suggesting that the Iranian WSSV strains may have originated from India or at least they have the same origin (Thailand and/or South Korea) via ballast water or illegal importation of live post-larvae (PL).

In conclusion, PCR screening of shrimp samples using OIE recommended primer sets revealed ten of fifteen outbreaks caused by WSSV, including the first outbreak of the virus in Hormozgan province (L. vannamei, 2015). In addition, the number and repeat patterns of Iranian strains showed close identity to each other and some Indian isolates. Considering the WSD outbreaks detailed in this study and previous reports, it can be suggested that the virus remained as the main pathogen problem in Iranian shrimp farms. In addition, it can be speculated that the current plans and strategies of the Iran Veterinary Organization may not be adequate to prevent further disease outbreaks and that a new revised plan may be necessary.

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**References**


