



Molecular Detection of White Spot Syndrome Virus from Farmed Shrimps in Saudi Arabia



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Abstract

Shrimp industry is one of the important sectors in the kingdom of Saudi Arabia and it has shown steep production and economic values. WSSV is a major pathogen which causing 100% mortality and economic losses in world wide. In KSA two most important species such as *Litopenaeus vannamei* and *Fenneropenaeus indicus* were cultured widely. Due to the sheer expansion of shrimp culture industry, WSSV occurrences was found in *F. indicus* and followed by WSSV outbreak, SPF *L. vannamei* was imported and cultured in KSA with stringent biosecurity measures implied by ministry of water, environment and agriculture. The present study was aims to identify the presence of WSSV in *L. vannamei* in selected area of KSA applying molecular method. Samples were collected from Jazan region it was fixed in 95% ethanol and stored in -80°C for the analysis. The collected samples were subjected to nucleic acid (DNA) extraction, followed by conventional and real time PCR. The obtained positive PCR products were purified, and it was sequenced. The sequences analyzed to see the similarities in NCBI blast. The present study found that the collected samples were shown a clear positive in *F. indicus* and *L. vannamei* in conventional and real time PCR analysis. The obtained sequences were shown 97% similarities with other WSSV isolates in Gen Bank. The obtained data from this study was found to be the presence of WSSV in KSA. PCR followed by sequencing was useful to identify the presence of WSSV in a shrimp project area. Furthermore, we suggest more molecular studies for prevalence of WSSV.

Introduction

The aquaculture in Saudi Arabia was feasible due to weather condition that allow culturing different subtropical and tropical species. In addition, it has unique location surrounding by red sea to the west with wide tracts of land and the persian gulf coasts to the east and comprise 80% of the Arabian Peninsula that provide an excellent locality for the sea food harvest. So, lack of fresh water makes mariculture is the best option to supply increasing demand for the sea food in Saudi Arabia [1]. Since, 1970 when the Ministry of Agriculture in cooperation with Food and Agriculture Organization (FAO) have established Jeddah Fisheries Research Centre were identify the potential species suitable in fresh and marine water in Saudi Arabia and the aquaculture industry was encouraged in the 1980s by providing governmental subsidies to the farmers [2]. Nile tilapia and sigan have been the first produced in aquaculture until 2000 when the shrimp produced in large quantities and planned to produce up to 100, 000 tons/year [3]. The Indian white shrimp *Fenneropenus indicus* was found to survive and grow well in the high saline water around the country. Saudi Arabia have an ambitious strategy (Vision 2030) by investing more in aquaculture as part of an economic plan aim to reduce the dependence on oil. The aquaculture in Saudi Arabia is an important industry where recently report from FAO stated the Ministry of Environment, Water and Agriculture in Saudi Arabia has invested 10.6 billion into aquaculture projects with the purpose to produce one million tonnes of fish in the next years (Chalil, 2012). In 2017 an updated

report for FAO account the capture of fishery production of the Saudi has been average of 68 000 tonnes during the last two years. Shrimps, mackerels, emperor fish and swimming crab are the main species caught [4]. In Saudi Arabia, there are 125 aquaculture farms and 56 of which are fully operational [5]. Worldwide, the major viral disease infecting shrimp are White Spot Syndrome Virus (WSSV). However, WSSV in Indian white shrimp (*F. Indicus*) were reported in Saudi Arabia [6]

and their origins, its virulent genotypes were determined [7,8]. Due to this WSSV outbreak the aquaculture production was decreased from 26 000 tonnes in 2009 and 2010 to 1000 tonnes in 2013 (Luis et al., 2016). As a result, all infected shrimp farming projects were closed [1]. In 2012, the ministry of environment, water and agriculture has perceived an idea to replace the currently cultured Indian white prawn *F. indicus* to the culture of Pacific white shrimp *L. vannamei* (specific pathogen free) they decided to import the *L. vannamei* (SPF) to Saudi Arabia [9]. The extensive studies were performed for environmental conditions, cohabitation, and feeding trials and samples were sent to the reference lab at Arizona University, and the results were negative for all the OIE/USMSFP listed pathogens of shrimp and in 2014 all the shrimp farms changed culture from *F. indicus* to *L. vannamei* (Luis et al., 2016). The main aims of the present study were to screening for the presence of WSSV in cultured shrimp species *L. vannamei* (SPF) by molecular based methods (conventional PCR and confirmed by

RT-PCR). The objective of this research was to identify the presence of WSSV in *L. vannamei* in selected area of Saudi Arabia applying molecular method.

Materials and Methods

Samples were collected from Jazan region, fixed in 95% ethanol and stored in -80 °C for the analysis. The collected samples were subjected to nucleic acid (DNA) extraction, followed by conventional and real time PCR. The obtained positive PCR products were purified and it was sequenced. The sequences analysed to see the similarities in NCBI blast.

Sample collection

The samples used in the present study were obtained from moribund shrimps (*Fenneropenaeus indicus* and *Litopenaeus vannamei*) cultured in Jazan region, Saudi Arabia during epizootics in October, 2014 and August 2015 (Figure 1). The moribund shrimps were collected and dissected the pleopods at the farm site and fixed samples were transported to Fisheries Research Center, Jeddah, and it was stored in -80 °C for the downstream application [10,11].



Figure 1: Location of the sample collection site Jazan, Saudi Arabia. 1. 17°15'11.2"N 42°20'52.5"E

DNA extraction

The DNA extracted from fixed shrimp's tissue samples using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer instruction. Briefly, the ethanol fixed pleopod samples were dried and incised in to small pieces. Approximately 25mg of tissues was placed in 1.5ml microcentrifuge tube with 180µl Buffer ATL and 20µl proteinase K was added and it was mixed thoroughly. Samples were mixed till the complete mixing and 20µl of AL buufer was added, followed by 2nd round of mixing. Next, 200µl of 100% ethanol was added in the reaction and mixed again. Sample was loaded on DNeasy Mini spin column which was kept in 2ml collection

tube. The tubes were centrifuged in 8000rpm for 1 minute and flow through was discarded and placed the DNeasy Mini spin column in a new collection tube. In the column 500µl Buffer AW2 was added, and centrifuged for 3min at 14,000rpm subsequently discarded flow-through and collection tube. Finally placed the DNeasy Mini spin column in a clean 1.5ml microcentrifuge tube, and 200µl Buffer AE was added directly onto the DNeasy membrane and kept in a room temperature for 1 minute. The elution was carried out with centrifuging the DNeasy Mini spin column at 8000rpm in 1 minute. The eluted DNA was assessed by spectrophotometer (Nanodrop 2000, Thermo Scientific) and its quality and quantity were determined.

Nested PCR assay for the Confirmation of WSSV

Table 1: Primers used for PCR analysis of WSSV confirmation

PCR	Primer	Sequences 5' to 3'	Product Size
WSSV 1 st step	146F1	ACT-ACT-AAC-TTC-AGC-CTA-TCTA	1447bp
	146R1	TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A	
WSSV nested step	146F2	GTA-ACT-GCC-CCTTC-ATC-TCC-A	941bp
	146R2	TAC-GGC-AGC-TGC-TGC-ACC-TTG-T	
Decapod-specific primers	143F	TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG	848 bp
	145R	TTCAGN-TTT-GCA-ACC-ATA-CTT-CCC	

A nested PCR assay was developed for highly sensitive detection of WSSV. The extracted DNA was analyzed by nested PCR to confirm the WSSV infection with the modified PCR program based on OIE recommendation [12,13]. The 25µL of PCR reaction mixture was prepared according to the manufacturer instructions (ThermoFisher Scientific, USA). Each PCR reaction contains 1µL (100ng) of extracted DNA, 2.5µL of 10X dream taq green buffer (ThermoFisher Scientific, USA), 2.5µL of dNTPs mix, 1µL of each primer (5 micro moles), 0.4 µL of dream taq DNA polymerase enzyme and 16.6µL of nuclease free water. Thermal cycler conditions for the PCR reaction were as follows, initial denaturation at 94 °C for 3min, followed by 39 cycles of denaturation at 94 °C for 20 sec, annealing at 62 °C for 1 minute, extension at 72 °C for 1 minute and a final extension step at 72 °C for 3min. The product from the first step PCR was used as template for second step PCR. To verify the extracted

DNA Decapod-specific primers used as control reactions [14]. PCR products were analyzed by 1.2% agarose gel electrophoresis and the gel was visualized under UV trans-illuminator. The primer sequence details are listed in the Table 1.

Results

Confirmation of DNA by internal control primer (Decapod-specific primers)

Internal Control Decapod-specific (18s RNA) primers (143F 5'-TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG-3' and 145R 5'-TTCAGN-TTT-GCA-ACC-ATA-CTT-CCC-3' yielding an 848 bp amplicon (Figure 2). In this study decapod specific primers were employed to determine the integrity and specificity of PCR. The 18 S RNA sequence was specifically amplified in all PCR cases.

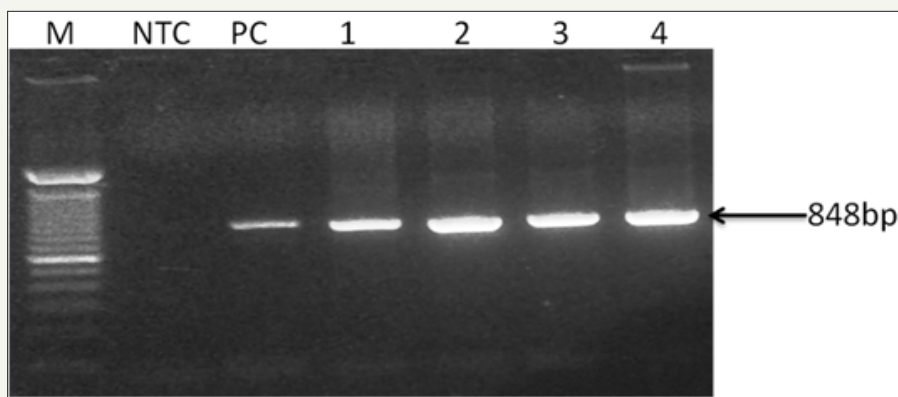


Figure 2: 0.8% Agarose gel photograph of PCR product, using Decapod-specific primers (OIE - Manual of Diagnostic Tests for Aquatic Animals, 2017) for the detection of virus in test and control Shrimp samples DNA. Captions: Lane_M, stander size molecular weight (100bp DNA ladder, Cat No; 15628019, Thermo Scientific, CA), L_{NTC}, Non- template Control, L_{PC}, Positive Control, L₁₋₄, *F. indicus* and *F. indicus* test samples.

Detection of WSSV by nested PCR (OIE)

The extracted DNA was eluted and stored at -20 °C. The DNA purity and concentration was determined by agarose gel

electrophoresis and spectrophotometer. The first-step and the second step (nested) PCR agarose gel electrophoresis showed different bands (Figure 3 & 4).

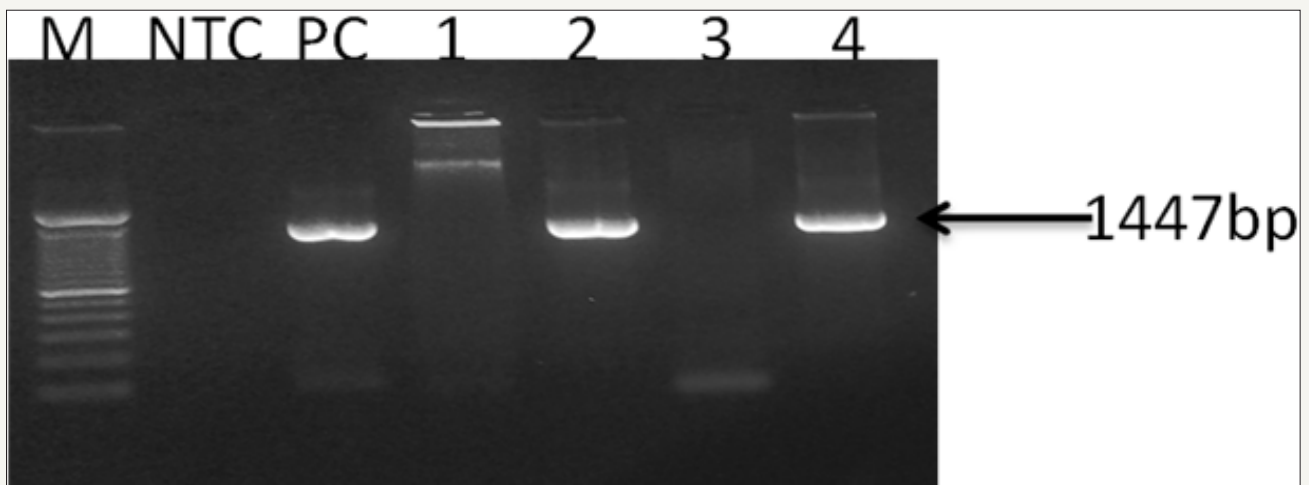


Figure 3: 0.8% Agarose gel photograph of first-step PCR for the confirmation of WSSV (OIE - Manual of Diagnostic Tests for Aquatic Animals, 2017). Captions: Lane_M, stander size molecular weight (100bp DNA ladder, Cat No; 15628019, Thermo Scientific, CA), L_{NTC}, Non template Control, L_{PC}, DNA Positive Control, L₁, *F. indicus* negative control, L₂, *F. indicus* positive, L₃, *L. vannamei* SPF, L₄, *L. vannamei* positive.

Table 2: Conditions (Target, Reporter and Quencher) used for the amplification and confirmation of WSSV from *F.indicus* and *L.vannamei* by IQ 2000 real time kit method.

Sample Name	Target Name	Reporter	Quencher	Ct	Quantity	Ct Threshold
NTC	WSSV	FAM	NFQ-MGB	Undetermined	-	0.092604
<i>Findicus</i> WSSV +ve	INTERNAL CONTROL	VIC	NFQ-MGB	19.95179176	-	0.159602
<i>Findicus</i> WSSV +ve	WSSV	FAM	NFQ-MGB	16.55850792	5462238720	0.092604
SPF	INTERNAL CONTROL	VIC	NFQ-MGB	17.4312458	-	0.159602
SPF	WSSV	FAM	NFQ-MGB	Undetermined	-	0.092604
<i>L.vannamei</i> WSSV+ve	INTERNAL CONTROL	VIC	NFQ-MGB	17.93489838	-	0.159602
<i>L.vannamei</i> WSSV+ve	WSSV	FAM	NFQ-MGB	21.09466743	24833562	0.092604

BLAST analysis (Table 3 & 4) (Figure 7 & 8)**Table 3:** The Nucleotide similarity and identity of WSSV isolate from *F.indicus* showed in BLAST analysis.

Description	Max Score	Total Score	Query Cover	E Value	Ident	Accession No
White spot syndrome virus consensus sequence isolate WSSV-chimera, complete genome	2078	2078	89%	0.0	97%	MG264599
White spot syndrome virus isolate FSL39, partial genome	2078	2078	89%	0.0	97%	MF784752
White spot syndrome virus isolate WSSV-AU, complete genome	2078	2078	89%	0.0	97%	MF768985
White spot syndrome virus strain CN04, complete genome	2078	2078	89%	0.0	97%	KY827813
White spot syndrome virus strain MEX2008, partial genome	2078	2078	89%	0.0	97%	KU216744
White spot syndrome virus strain CN01, complete genome	2078	2078	89%	0.0	97%	KT995472
White spot syndrome virus strain CN03, complete genome	2078	2078	89%	0.0	97%	KT995471
White spot syndrome virus strain CN02, complete genome	2078	2078	89%	0.0	97%	KT995470
White spot syndrome virus isolate EG3, complete genome	2078	2078	89%	0.0	97%	KR083866

Table 4: The Nucleotide similarity and identity of WSSV isolate from *L.vennamei* showed in BLAST analysis.

Description	Max Score	Total Score	Query Cover	E Value	Ident	Accession
White spot syndrome virus consensus sequence isolate WSSV-chimera, complete genome	2187	2187	93%	0.0	97%	MG264599.1
White spot syndrome virus isolate FSL39, partial genome	2187	2187	93%	0.0	97%	MF784752.1
White spot syndrome virus isolate WSSV-AU, complete genome	2187	2187	93%	0.0	97%	MF768985.1
White spot syndrome virus strain CN04, complete genome	2187	2187	93%	0.0	97%	KY827813.1
White spot syndrome virus strain MEX2008, partial genome	2187	2187	93%	0.0	97%	KU216744.1
White spot syndrome virus strain CN01, complete genome	2187	2187	93%	0.0	97%	KT995472.1
White spot syndrome virus strain CN03, complete genome	2187	2187	93%	0.0	97%	KT995471.1
White spot syndrome virus isolate BAN_SH_AL-1_2014 VP664 gene, partial cds	2187	2187	93%	0.0	97%	KJ773998.1
White spot syndrome virus, complete genome	2187	2187	93%	0.0	97%	AF369029.2

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1  CGAGCAITTTGTATCTCTTTGGCATTGGCCGCGCCAGAGTCT
  M A F C I L F G I R P P Q K S
46  TCCATGGAGAAATAGAGCCGCCCTTATCAGGCCACAGSCTT
  S H E E I R A P P Y Q A N K L
91  ATTAGTGACAACATCAGTGATGAACATGTCAGATGATCTCT
  I S D K H Y V H N M S K I D S
136  AGAGTACAGGATCTTCCCTCTTAGAAGGTTAGGATGGACT
  R V T G S S L L K K V S E W T
181  GAATGAGATGAATCTCACTTAAATGGAACATTTGACCATCA
  E M R M N S N F N G T F E P S
226  AGACTGCCCTCTCCAACTCTGGCATGACAGGCGAGAGTCAAC
  R L A L S N S G M T T A G V N
271  CTGAGCTTATTGTCAACCAATAATGCAAGAGTGTACTAGGA
  L D V I V K P N N A R S V L G
316  ATATTGGAATGTATCGCCAGCTGTGCAAGCCGCGAGCCGAG
  I L E C H R Q H V C T A D A K
361  GGAATCTGCTCTCAGCCATCCAGCCGCTTCCAGCCACCGAT
  G T V A S A M P A V F Q A T D
406  GGAACGGTAAAGATCTGAATGATCCAGATGCTCTGCCAAGG
  G N G N E S E L I Q N A L P R
451  AACAGATACATCAAAAGAGCACAATGACCTCAAATCTGTGTG
  N R Y I Q K S T M N N A Q T V V
496  TTGTCTAATGTTTGGAGCACTATGCGGATCTTGGAAAGGTT
  F A N V L E Q L I A D L G K V
541  ATCGTGAAGCACTGGCCGACCATGCTGATCTGTACAGAA
  I V N E L A G T I A E S V P E
586  AGCGTATGAAACCAAGAAATGATGATAGACTAGGCTCT
  S V Y E N T K E M I D R L G S
631  GAGGACCTCTCAATCTAATAATGAGGAGTAGAATCAATG
  D D L F K S N N N G G V E S M
676  GATTATGAGATAGGAAACCACTCCAGCAATGTGCGGCTCTC
  D Y E D S E T T S N N G P V L
721  ATCTCAGAGCCATGAAGATGCGCTATCACACACTAATTC
  I S E A M K N A V Y H T L I S
766  GGCAGGCGACTGGCCGAAATGATCACTGCTCATGCGCC
  G K A A R P E N V P F A S C A
811  AGCGCCCTCTCGCTTTGATTTCTCTGTCAAGGAGATACA
  S G P L A F D F L L S K G D T
856  TTCGAGAAAGAGCGGACAGAGTGCAGCAGCTGCCGATCC
  F E E K N A E Q G A A A V S
901  TCTACTATTCTCTCTCTCAACTACTCTCTGTAAGCAITTG
  S T Y S S S N T T L R K H L
946  GCTCGAGTTTTCGAGCCATCTCTTAA
  A R V F E A I S *
    
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Figure 7: Translated AA sequences from amplified WSSV gene from *F.indicus*.

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1  0GAGCAITTTGTATCTCTTTGGCATTGGCCGCGCCAGAGTCT
  M A F G I L F R I R P Q K F
46  TCCATGGAGAAATAGAGCCGCCCTTATCAGGCCACAGSCTT
  S M E I R A T P Y Q A N K L
91  ATTAGTGACAACATCAGTGATGAACATGTCAGATGATCTCT
  I S D K H Y V H N M S K I D S
136  AGAGTACAGGATCTTCCCTCTTAGAAGGTTAGGATGGACT
  R V T G S S L L K K V S E W T
181  GAATGAGATGAATCTCACTTAAATGGAACATTTGACCATCA
  E M R M N S N F N G T F E P S
226  AGACTGCCCTCTCCAACTCTGGCATGACAGGCGAGAGTCAAC
  R L A L S N S G M T T A G V N
271  CTGAGCTTATTGTCAACCAATAATGCAAGAGTGTACTAGGA
  L D V I V K P N N A R S V L G
316  ATATTGGAATGTATCGCCAGCTGTGCAAGCCGCGAGCCGAG
  I L E C H R Q H V C T A D A K
361  GGAATCTGCTCTCAGCCATCCAGCCGCTTCCAGCCACCGAT
  G T V A S A M P A V F Q A T D
406  GGAACGGTAAAGATCTGAATGATCCAGATGCTCTGCCAAGG
  G N G N E S E L I Q N A L P R
451  AACAGATACATCAAAAGAGCACAATGACCTCAAATCTGTGTG
  N R Y I Q K S T M N N A Q T V V
496  TTGTCTAATGTTTGGAGCACTATGCGGATCTTGGAAAGGTT
  F A N V L E Q L I A D L G K V
541  ATCGTGAAGCACTGGCCGACCATGCTGATCTGTACAGAA
  I V N E L A G T I A E S V P E
586  AGCGTATGAAACCAAGAAATGATGATAGACTAGGCTCT
  S V Y E N T K E M I D R L G S
631  GAGGACCTCTCAATCTAATAATGAGGAGTAGAATCAATG
  D D L F K S N N N G G V E S M
676  GATTATGAGATAGGAAACCACTCCAGCAATGTGCGGCTCTC
  D Y E D S E T T S N N G P V L
721  ATCTCAGAGCCATGAAGATGCGCTATCACACACTAATTC
  I S E A M K N A V Y H T L I S
766  GGCAGGCGACTGGCCGAAATGATCACTGCTCATGCGCC
  G K A A R P E N V P F A S C A
811  AGCGCCCTCTCGCTTTGATTTCTCTGTCAAGGAGATACA
  S G P L A F D F L L S K G D T
856  TTCGAGAAAGAGCGGACAGAGTGCAGCAGCTGCCGATCC
  F E E K N A E Q G A A A V S
901  TCTACTATTCTCTCTCTCAACTACTCTCTGTAAGCAITTG
  S T Y S S S N T T L R K H L
946  GCTCGAGTTTTCGAGCCATCTCTTAA
  A R V F E A I S *
    
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Figure 8: Translated AA sequences from amplified WSSV gene from *L.vennamei*.

Discussion

Worldwide, white spot syndrome virus (WSSV) is considered one of the most pathogenic and devastating threat to the commercial shrimp farming. In addition, the virus affect other fresh and marine crustaceans such as Crayfish, Crabs and lobsters. In fact, WSSV is highly virulent in shrimp farms, particularly in shrimp culture type within few days it can spread leading to 100% mortality. In Saudi Arabia, the WSSV was first reported in 2010 affected cultured *F. indicus* species and resulting in massive economic losses (Kathy et al., 2012). To date, several reports have been published describing the economic losses of WSSV in Saudi Arabia.

Ministry of Environmental, Water and Aquaculture as long-term solution instead *F. indicus* infected species to SPF *L. vannamei* resistant to WSSV with offers many benefits to shrimp industry. Epidemiological studies and precise information on the economic impact of WSSV throughout the country is not available and is required to overcome the expected disaster and disease outbreaks. The results of the present study showed a clear WSSV DNA positive band from *F. indicus* and *L. vannamei* samples by conventional PCR. The positive samples were further detected and confirmed by RT-PCR analysis. The detected positive WSSV in the samples were sequenced and were showed 97% similarities with other WSSV sequences published in GenBank. Contrary to the previous believes, the presence of WSSV was confirmed in KSA. However, the result could postulate that the WSSV is more common in KSA than previously thought and could have economic impacts on the local investment. The virus could introduce to the country through the imported shrimps (Kathy et al., 2012). The result of the examined samples by RT-PCR, could indicate that the current technique is robust, sensitive and highly specific molecular technique for the detection of WSSV therefore, it is recommended to detect the virus in infected shrimp at field level.

Conclusion and Recommendations

The analysed data from the current study indicate the infectivity of WSSV to the shrimps in KSA. Conventional, real time PCR followed by sequencing was potent to clarify the presence of WSSV in shrimp cultivated area given an alarming for industrial disaster. Based on our findings of this study, we conclude that PCR based technique is a good measure for accurate measurement of DNA molecule in sequence specific manner. Furthermore, we suggest more molecular-based studies for prevalence WSSV among shrimp farms in KSA to identify the risk factors of the coming disease outbreaks.

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