

ISOLATION OF WHITE SPOT SYNDROME VIRUS (WSSV) IN EGYPTIAN SHRIMP USING CONVENTIONAL PCR AND REAL TIME PCR (QPCR) TECHNIQUES

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Abstract: Shrimp aquaculture industry threatened by high mortality rates and severe economic losses as a result of white spot syndrome virus (WSSV) infection. Early-screening and diagnosis of WSSV are great strategies to decrease the economic losses of the disease on shrimp aquaculture. Therefore, this study was carried out to detect of white WSSV infected shrimp under using two molecular based methods, conventional PCR and qPCR. A total number of 90 samples of red (*Aristeus antennatus*) and gray (*Penaeus latisclcatus*) shrimp were collected from Kafr El-Sheikh and Alexandria governorates. External examination of shrimps collected from Kafr El-Sheikh Governorate revealed typical WSSV clinical signs (including loose and easily detached cuticle with appearance of small white spots (3 mm in diameter) and /or larger patches in the external surface of carapace and cephalothorax. The internal examination showed yellowish white, fragile and swollen hepatopancreas and swollen or shrunken lymphoid tissue. Red shrimp showed slightly obvious white spots without any internal lesions. PCR results confirmed the clinical investigation and postmortem (PM) examination and revealed presence of WSSV partial sequences with a size of 190 bp in shrimp samples from Kafr El-Sheikh Governorate. In contrast, samples collected from Alexandria (Borg El-Arab) gave negative results. The result of qPCR confirmed that obtained by conventional PCR and showed that all positive results of WSSV by conventional PCR gave cycle threshold (Ct) values ranged from 34.81 to 40.06. Our results concluded that, WSSV Diseases of shrimp attack shrimp markets of Kafr El-Sheikh Governorate. The conventional PCR and qPCR based methods for isolation and identification of shrimp WSSV, provided accurate results.

Key words: WSSV; PCR; qPCR; shrimp

Introduction

Shrimp are one of the greatest important food sources for human consumption because

shrimp have high levels of omega-3 fatty acids (1). The industry income related to shrimp species is about 50 billion dollars annually (2). Production declining of shrimp was observed

from 1994 to 1997 in India and from 1997 to 1998 in Asia (3-5). In Egypt, the white spot syndrome virus (WSSV) caused high losses in shrimp fields since 2009 (6). WSSV infects shrimp, lobsters, crayfish and crabs belonging to freshwater and marine crustaceans (2, 7).

WSSV is the only member of Whispovirus genus (belong to Nimaviridae family) (8). It is enveloped double stranded circular DNA virus with ovoid to bacilliform shape and a tail like end (9, 10). Shrimp aquaculture industry threatened by high mortality rates and severe economic losses as a result of WSSV infection. Early-screening and diagnosis of WSSV are great strategies to decrease the economic losses of the disease on shrimp aquaculture. Conventional polymerase chain reaction (PCR), *in situ* PCR, quantitative PCR, nested PCR as well as loop-mediated isothermal amplification (LAMP) (11-16) has been established for detection of WSSV. The WSSV causes serious economic losses because of high percent of mortality which leads to total crop losses through ten days or less under certain farming conditions (17). The host range of WSSV is at least 78 species, mostly to decapod crustaceans particularly shrimp (18). In coastal area of Egypt, industry depends on shrimp are gradually proceeded to cover the market needs, nevertheless the high risk shrimp farms infected with virus could be disturb the production (19).

To the best of our knowledge, only few researches conducted about WSSV in Egyptian shrimp. Consequently, the goal of this research article was early identification of WSSV in shrimp under Egyptian conditions using of molecular based methods (PCR and qPCR).

Materials and methods

Shrimp samples

A total of 90 shrimp samples were collected from two Egyptian Governorates located on Mediterranean Sea; Kafr El-Sheikh and Alexandria. The red shrimp (*Aristeus antennatus*) and gray shrimp (*Penaeus latisclcatus*) obtained from Kafr El-sheikh Governorate as well as gray shrimp (*Penaeus latisclcatus*) obtained from Borg El-Arab farm (Alexandria Governorate).

Conventional PCR assay

Shrimp samples were prepared for DNA extraction. Carapace and abdominal segments of declining shrimp as well as the control shrimp were split using a scalpel. A part of gills, internal organs, muscle and cuticle are preserved in -20°C for extraction of DNA. Total DNA was extracted according to the instructions of Gene-spin TM Viral Extraction Kit (Intron, South Korea, Cat. No. 1715) and as previously described (20). The PCR reaction mixture was 25 µl which consisted of 12.5µl of 2X master mix (0.1U/µl *Taq* polymerase, 500 µM dNTP, 20mM Tris-HCl (pH8.3), 100mM KCl, 3mM MgCl₂ and Stabilizer and enhancer), 1 µl of 10 pmol of each primer, and 2 µL of template DNA (50 µg/ml). The primers were designed as previously described (19). Amplification was performed in a thermocycler (Bio-Rad, C - 1000). The amplified products were examined on 1.5% agarose gel. Ten µL of amplified product, negative control were injected into the well then run with 50 bp DNA ladder in 1X TAE electrophoresis buffer (5 volts/cm² for 45min.). At the end of the run of electrophoresis, the gel was captured by a gel documentation system. The expected DNA fragments were 190 base pairs (bp) in length.

Real time PCR assay

A single tube qPCR reaction was adjusted according to the kit manual instruction. In brief, the reaction mixture was 25µl which consisted of 12.5µl of 2X SYBR Green qPCR Master Mix, 1 µl of 10 pmol of each primer (5-AATGGTCCCGTCCTCATCTCA-3) as well as (5-GCTGCCTTGCCGGAATT-3) specific for WSSV (15), and 2 µL of template DNA (50 µg/ml). PCR was conducted in an eppendorf thermal cyler amplification was performed in a thermocycler (Real time PCR-Agilent Technologies - Stratagene MX300P). Early denaturation at 94 °C for 5 min, then cyclic condition was 35 cycles at 94 °C for 30 sec, annealing at 54 °C for 1 min as well as extension at 72 °C for 1 min. The final extension at 72 °C for 10 min (15, 20). The melting temperature for all obtained products was 80°C indicating

the specificity of primers annealing to the template.

Results

Results of gross pathology

The collected shrimps collected from Kafr El-Sheikh Governorate were suspected to be infected with White Spot Syndrome virus (WSSV) based on the following main symptoms: loose and easily detached cuticle with appearance of white spots (3 mm in diameter) in external surface carapace and cephalothorax (Fig.1). These spots were not easy to be removed and in some region, they collected forming large patches of different sizes with whitish circular spots. The internal examination of these shrimps revealed yellowish white, fragile and swollen hepatopancreas and swollen and shrunken lymphoid tissue (Fig.1). In addition to accumulation of the fluids that caused swelling of bronchiostegites. On the other hand, red shrimp showed slightly obvious white spots without any internal lesions (Fig.1).

Results of conventional PCR

PCR results confirmed the clinical investigation and postmortem (PM) examination and revealed presence of WSSV

partial sequences with a size of 190 bp in shrimp samples which had WSSV gross lesions obtained from Kafr El-Sheikh Governorate (Fig. 2). In contrast, samples collected from Alexandria (Borg El-Arab) gave negative results. PCR products of infected shrimp samples of different species revealed that 54 out of 60 shrimp samples from Kafr El-sheikh Governorate were PCR positive. Meanwhile, 30 samples collected from Borg El-Arab Alexandria Governorate gave negative (-ve) results (Fig. 2).

Our results cleared that, there was a significant differences of the incidences of WSSV ($P < 0.01$) among the type of shrimp and among different regions. The results cleared that, the degree of WSSV infected shrimp from Kafr El-sheikh markets showed high incidences to WSSV infection however, no disease incidence were recorded in Alexandria gray shrimp (Table 1).

qPCR results of WSSV in shrimp samples

The 90 samples were examined with real time PCR using specific primers for WSSV. The result showed that all positive results of WSSV by conventional PCR gave cycle threshold (Ct) values ranged from 34.81 to 40.06 (Table 2, Fig. 3).

Table 1: Incidences of isolated WSSV among examined shrimp after conventional PCR

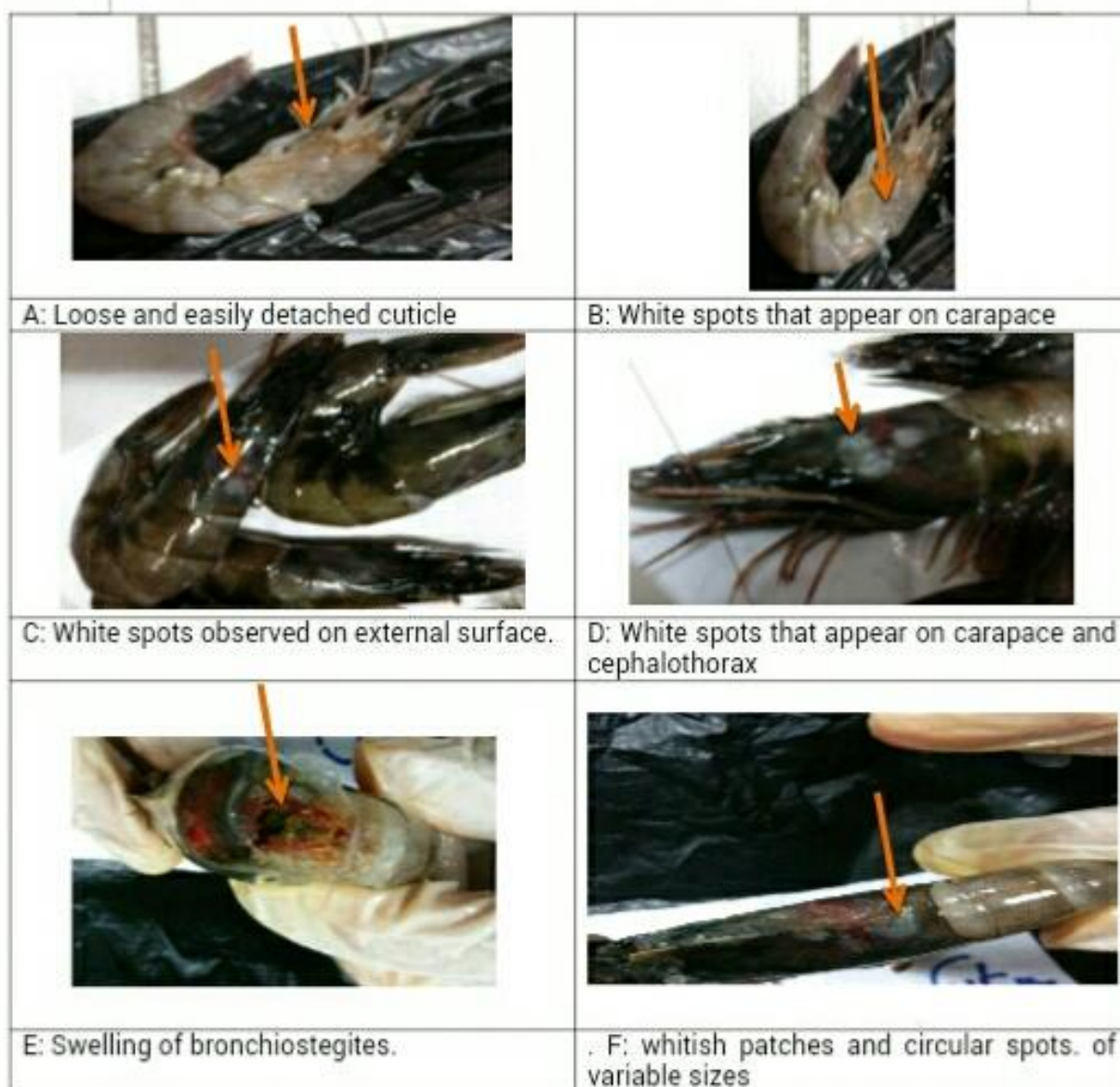
Number of sample	Number of positive sample	Locality	Site of DNA extraction	Appearance of symptoms	Results	% of infection
15	14	Kafr-Elsheikh	Cuticle of red shrimp	Have symptoms	+ve (WSSV)	93.3 %
15	13	Kafr-Elsheikh	Cuticle of gray shrimp	Have symptoms	+ve (WSSV)	92.9 %
15	13	Kafr-Elsheikh	Internal organs of gray shrimp	Have symptoms	+ve (WSSV)	92.9 %
15	14	Kafr-Elsheikh	Internal organs of red shrimp	Have symptoms	+ve (WSSV)	93.3 %
15	0	Alexandria	Cuticle of gray shrimp	No symptoms	-ve (WSSV)	0
15	0	Alexandria	Internal organs of gray shrimp	No symptoms	-ve (WSSV)	0

Chi² = 11.14 **

** = Significant at ($P < 0.01$)

Table 2: Mean of Cycle Threshold of samples examined for WSSV using qPCR

Samples NO.	Site of DNA extraction	Mean of Cycle Threshold
1	Cuticle of red shrimp from Kafr-Elsheikh	40.06
2	Cuticle of gray shrimp from Kafr-Elsheikh	36.01
3	Internal organs of gray shrimp from Kafr-Elsheikh	34.81
4	Internal organs of red shrimp from Kafr-Elsheikh	37.60
5	Cuticle of gray shrimp from Alexandria	0.0
6	Internal organs of gray shrimp from Alexandria	0.0

**Figure 1:** White spot syndrome virus (WSSV) infection gross lesion on shrimp. A, B: from red shrimp. C- F: from gray shrimp

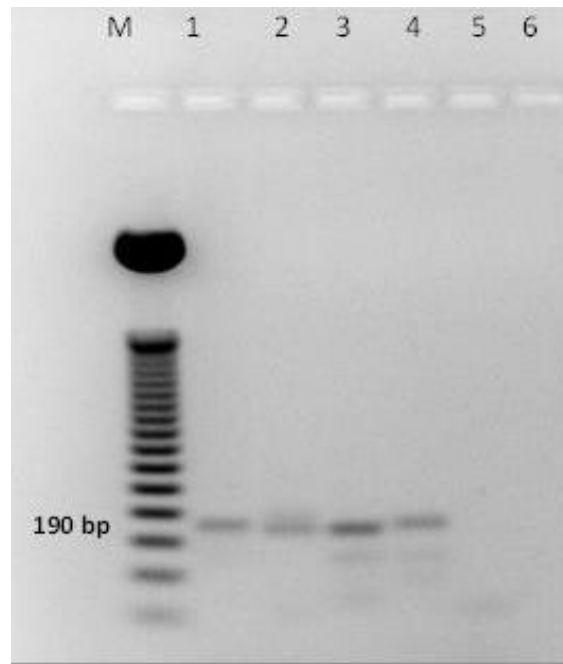


Figure 2: PCR results of WSSV on agarose gel. M= 50 bp ladder, 1= (sample from cuticle of red shrimp), 2= (sample from cuticle of gray shrimp), 3= (sample from internal organs of gray shrimp), 4= (sample from internal organs of red shrimp), 5= (sample from cuticle of gray shrimp), 6= (sample from internal organs of gray shrimp). 1-4 samples collected from Kafr EL-sheikh, 5-6 samples collected from Alexandria

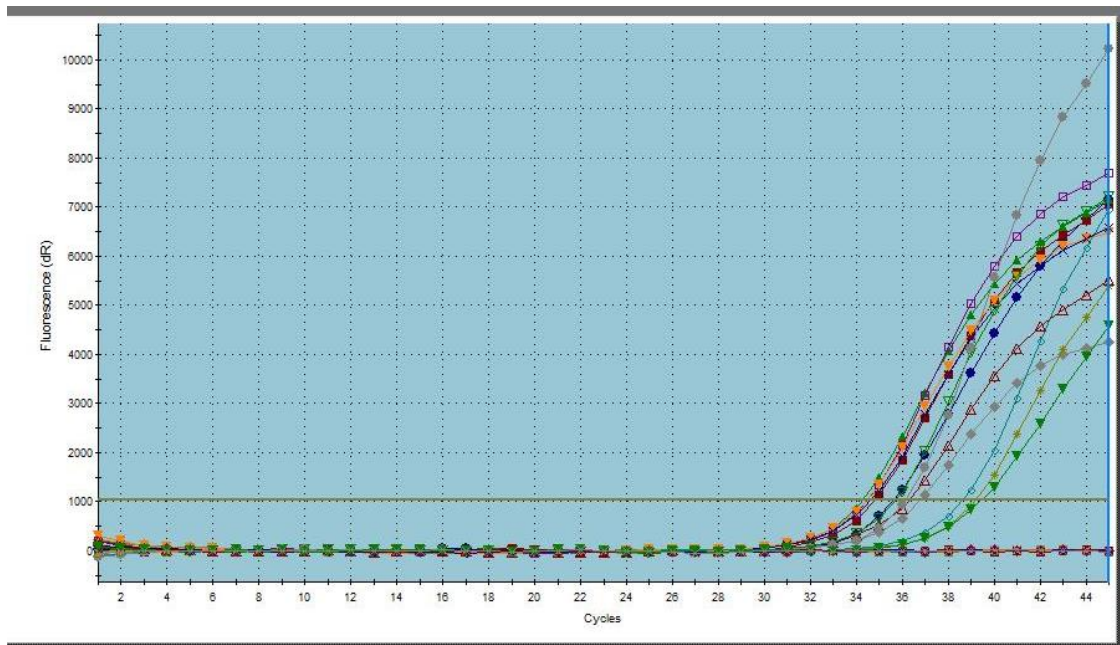


Figure 3: Amplification curves of real time PCR results of WSSV

Discussion

WSSV is one of the most serious shrimp disease which does not only attack shrimp farms in Egypt and causes economic losses but also infects other freshwater and marine crustaceans, mostly crayfish and crabs (1, 9).

The virus is so dangerous so that can lead to 100% mortalities during only 2-10 days from appearance of symptoms (2, 21). Little information and knowledges about the incidences and prevalence of this virus in shrimp aquaculture have been reported in Egypt. Although the shrimp aquaculture

became more advanced in Egypt, the infected shrimp threaten shrimp farming. Thus, control WSSV is required to avoid shrimp losses. The aim of this study was to throw the light on the occurrence and incidences of WSSV in some two species of the shrimp present in Egypt.

Our results revealed that 54 out of 60 shrimp samples collected from Kafr El-sheikh Governorate showed positive results for WSSV however, no positive results were obtained from the 30 total shrimp samples collected from Alexandria Governorate. These results supported by the results obtained by Megahed et al 2019, Eissa et al 2009 and Salama et al 2008 (6, 19, 22) and confirming WSSV identification among Egyptian shrimps.

Herein, gross pathological examination revealed presence of white spots on the shrimp body after removal of cephalothorax cuticle. Our results agreed with those obtained by (2, 23-26) who also found cuticle chromophores and calcium deposition. Although we did not find softening of exoskeleton, in many epizootics of this disease, this softening could be observed. Changes in the structural integrity of the exo and pro cuticle could be the most vital reason for WSSV syndrome (28-30). The obtained internal examination results agreed with (2, 27, 31) and proved that white spot virus infected hepatopancreatic sheath.

PCR (both conventional and real time) confirmed presence of WSSV in shrimps collected from Kafr El-sheikh Governorate. In the present study, 90 shrimp samples were examined with conventional PCR using specific primers of WSSV. The result showed that 54 out of 60 gray and red shrimp samples of Kafr El-sheikh Governorate were PCR positive. Meanwhile 30 gray shrimp samples collected from Borg El-Arab, Alexandria Governorate gave negative (-ve) results. Results from conventional PCR were further confirmed by qPCR which showed amplification curves with Ct values ranged from 34.81 to 40.06 in all conventional PCR positive samples. PCR has been applied for the diagnosis of WSSV infections in clinical samples and shown to be rapid, sensitive and specific diagnostic method (32-35).

Conclusion

To the best of our knowledge, this may be the first study to detect shrimp WSSV in Egypt using qPCR. The conventional PCR and qPCR based methods are successful methods for early identification of WSSV in clinical samples of infected shrimp that delivers accurate tool for identification of this virus.

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