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DETECTION OF WSSV AMONG SOME EGYPTIAN CRUSTACEANS

By

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ABSTRACT

The current study was performed to fill up the gap in knowledge of WSSV in the Egyptian shellfish diseases literatures. Randomly 268 different shellfish samples including 28 *P. clarckii* was collected from Abbassa, Sharkeya city and 240 marine samples (100 *P. japonicus*, 100 *P. semisulcatus,* 20 *C. sapidus* and 20 *P. pelagicus*) were collected from Suez-Gulf, Suez city and Murrah Lake, Ismailia city. Although, most of the examined shrimp (*P. semisulcatus* or *P. Japonicus*) from both Suez Gulf and Murrah Lake were apparently healthy .yet, 4 *P. Japonicus* and 1 *P. semisulcatus* from Suez Gulf presented typical white spot lesions on their carapace. On the other side, all shrimp samples from Murrah Lake were apparently healthy. Also almost all examined Crabs and crayfish samples were apparently healthy. Molecular detection using regular PCR has revealed that 2/10 pools of *P. japonicus* exoskeleton from Suez Gulf were positive for WSSV and 1/10 pools of the same species from Murrah Lake was also positive for the virus. The condition was little optimistic in case of *P. semisulcatus* only 1/10 pools from Suez Gulf was positive and pools from Murrah Lake were negative. Controversially, the freshwater Crayfish from Abbassa, Sharkiya were all negative with the exception of only one sample out of 28. Moreover, Crabs from both Suez Gulf and Murrah Lakes were all negative for the virus supporting the well-known fact that Crabs are more immunologically competent than shrimp and crayfish.

Keywords:

WSSV-Marine shrimp - Crabs - Freshwater Crayfish - Suez Gulf - Murrah Lake).

INTRODUCTION

The annual total shrimp fisheries production from Egypt's Mediterranean and Red Sea coasts including the Suez Canal-Bitter Lakes, the coastal lagoons of Manzala, Burullus and Bardawil and inland lakes of Qarun through the entire period from 1988 to 1997 averaged 6,339 MT and represented less than 2% of the total fish and shellfish landings in Egypt **(Sadek *et al.*, 2002)**. The majority of shrimp fisheries production consists of small species (*Metapenaeus stebbingi,Trachypenaeus curvirostris, Parapenaeus longirostris and Solenocera crassicornis*),

while larger sized species (*P. japonicus, P. semisulcatus, P. kerathurus, P. latisulcatus and Metapenaeus Monoceros*) are caught only in small quantities **(Bishara, 1976; Sadek *et al.*, 2002)**. Shrimp culture in Egypt dates back to the early 1980s, where a first shrimp farm were established near Alexandria **(Sadek *et al.*, 2002).** Currently, all of shrimp hatcheries in Egypt rely on wild brood stock to produce the post larvae needed for stocking farms. This reliance on wild brood stock can result in lost production as a result of delays in capturing brood stock or variation in the natural abundance of shrimp. Also, reliance on wild brood stock has the risk of the spread of diseases. Disease has had a major impact on the shrimp farming industry. Since 1981, a succession of new viral pathogens has emerged in Asia and the Americas, causing mass mortalities and threatening the economic sustainability of the industry **(Walker and Mohan, 2009)**. Shrimp are arthropods and most shrimp viruses are either related to those previously known to infect insects (e.g., densoviruses, dicistroviruses, baculoviruses, nod viruses, and lute viruses) or are completely new to science and have been assigned to new taxa. Several important characteristics are common to shrimp viral diseases and distinguish them from most viruses of terrestrial or aquatic vertebrates. One important distinguishing feature is that most of the major pathogenic viruses cause very low level persistent infections that can occur at moderate to very high prevalence in apparently health shrimp populations **(Walker *et al.* 2001; Chakraborty *et al.*, 2002)**. Almost all shrimp pathogens are transmitted vertically (Egg shell contamination) and disease is the result of a massive viral amplification that follows exposure to various forms of environment or physiological stress **(Peng *et al.*, 1998; Cowley *et al.*, 2002; Sanchez-Martinez *et al.*, 2007)**. Stressors can include handling, spawning, poor water quality or abrupt changes in temperature or salinity. Shrimp viruses can also commonly be transmitted horizontally and, once viral loads are high and disease is manifest, horizontal transmission of infection is accompanied by transmission of disease. The third significant characteristic is a logical consequence of the former two in that shrimp commonly can be infected simultaneously or sequentially with multiple viruses **(Flegel *et al.* , 2004)** or even different strains of the same virus **(Hoa *et al.*, 2005)**. These characteristics present a very different landscape for the interaction of pathogen and host and significant challenges for diagnosis, detection, pathogen exclusion and the use of prophylactics in health management. White spot syndrome (WSSV) A DNA virus that represents the most critical worldwide threat to shrimp industry was first emerged in Fujian Province of China in 1992 **(Zhan *et al.*, 1998)**. It was soon after reported in Taiwan and

Japan and has since become panzootic throughout shrimp farming regions of Asia and the Americas **(Walker and Mohan, 2009).** It is the most devastating disease of farmed shrimp with social and economic impacts over 15 years on a scale that is seldom seen, even for the most important diseases of terrestrial animals. Although first emerging in farmed kuruma shrimp (*Penaeus japonicus*), WSSV has a very broad host range amongst decapod crustaceans (e.g., marine and freshwater shrimp, crabs, lobsters, crayfish, etc.), all of which appear to be susceptible to infection **(Leu *et al.,* 2009)**. However, susceptibility to disease varies and some crustacean species such as Red swamp crayfish **(*Procambrus clarkii*)** have been reported to develop very high viral loads in the absence of clinical signs **(Yoganandhan and Hameed, 2007)**. All farmed marine (penaeid) shrimp species are highly susceptible to white spot disease, with mass mortalities commonly reaching 80-100 % in ponds within a period of 3 - 10 days **(Chou *et al.*,1995; Lightner *et al.,* 1998).** The early records of WSSV in Egypt, goes back to May 2006 when **Sharshar (2008)** had reported an emerging case of mass mortalities among collected freshwater crayfish (*Procambarus clarkii*) from Eshnawai and Kafr Salem El-Nakal drains in Tanta City, El-Gharbia Province. The author has concluded that, the white spots recorded on the cuticle of diseased crayfish was WSSV. This conclusion was based on achieved results after histopathological and PCR techniques were used for detection of WSSV in diseased crayfish. In 2010, the WSSV was also reported during the course of examinations of Red swamp crayfish samples collected from two diverse geographical locations at Sharkiya province (Personal communication with Dr. Alaa Eldin Eissa, the principle investigator of Cairo University funded research project researching the epidemiological roles of crayfish and other shellfishes in spread of viral diseases across the Egyptian aquatic habitat). Despite the alarming increase in intermittent eruptions of emerging viruses of shrimp and other crustaceans during the past two decades, yet, there are unexpected shortage literatures of investigating shrimp viruses through the Egyptian state. This fact necessitates swift and profound shift of interest of shellfish disease researcher to concentrate their diagnostic efforts on investigating these viral eruptions in both wild and cultured crustacean populations including shrimp. Thus the objective of the current study is the detection of WSSV among Egyptian crustacean populations.

MATERIAL AND METHODS

Sampling locations:

Marine shellfish samples (Shrimp and crabs) were collected from Attaka fishing port, Suez bay at Suez cityand Murrah Lake at Ismailia City, Egypt while freshwater crayfish samples were collected from an earthen pond based facilities at Abassa, Sharkiya.

Fig. (1): Attaka fishing port on Suez bay from where the Samples were randomly collected.

Fig. (2): MurrahLake at Ismailia city from where the Samples were randomly collected.

Sampling:

A. Shrimp.

A total number of 200 natural adult penaied marine shrimp (50 *Penaeus japonicas* and 50 *Penaeus semisulcatus*) were collected from Attaka fishing port,Suez bay at Suez cityand the same set of samples were equally collected from Murrah Lake at Ismailia City, Egypt.

B. Crab.

A total number of 20 adult Blue Crab (*Portugonus pelagicus*) were collected from Attaka fishing port, Suez bay at Suez cityand 20adult Chesapeake Blue Crab *(Canallectus sapidus)* were collected from Murrah Lake at Ismailia City, Egypt.

C. Crayfish.

A total number of 28 adult Red swamp crayfish *(Procambrusclarckii*) were collected from earthen pond based aquaculture facilities at Abassa, Sharkiya governorate, Egypt.

Clinical examination of collected shellfishes:

Clinical examination was adopted on shrimp, crab and crayfish samples that showed possible disease signs as well as those apparently healthy. Shellfish exoskeleton was cleaned with cotton soaked in 70 % ethyl alcohol. Clinical examination of the collected shellfish was done by naked eye for any possible body abnormalities using the method adopted from **Lightner and Redman (1998)**. To apply PM examination on visually inspected shellfish samples, cleaning the surface of the cuticle by cotton soaked in 70 % ethyl alcohol then separation of carapace from underlying connective tissue using sterile scissors and forceps were done in clean space or under safety cabinet. Cutting out the Carapace sagittal to expose hepatopancreas for color / consistency and gills , foregut , midgut , hind gut , musculature , periopodes , pleopods , uropod , telson , gonads and genital organs for any deviation in size , color and consistency **(Lightner and Redman, 1998).**

Sample Processing:

The collected live samples were stored in an insulated ice-box till transferred to the Fish Diseases and Management Laboratory (FDML), Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Cairo University for further processing. Samples were collected during winter and onset of spring and summer seasons of 2016/ 2017then processed for PCR according to protocols described by **Lightner and Redman, (1998)** for marine shrimp/crab samples and **Eissa *et al*., (2012)** for Red swamp crayfish. All collected shellfishes were cleaned, washed three times in sterile distilled water and dried thoroughly with sterile towels before dissection and collection of exoskeleton and internal organs. The carapace of the examined Shrimp /crab /crayfish were split sagittally using a surgical scalpel and scissors. The selected tissues were finely homogenized using sterile homogenizer till consistent homogenates were obtained. Homogenates were further diluted using Hanks balanced salt solution (HBSS: Sigma Chemical Co, St. Louis, MO, USA) (4 HBSS /1 homogenate). Haemolymph samples from adult Shrimp/crab /crayfish were collected using sterile syringes then aliquoted into 1 ml microfuge tubes. Diluted homogenates / hemolymph were stored at - 80 °C freezer till processed for DNA extraction and PCR detection of WSSV.

DNA extraction:

A total amount of 200 µl of Shrimp / crab / crayfish tissue homogenate and 200 µl haemolymphwere collected in a 1.5 ml microfuge tube with 600 µl lysis solution (100 mMNaCl, 10mMTris/HCl, pH 8, 25 mM EDTA (Ethylene diamine tetra-acetic acid), 0.5% SLS (sodium N-laurylsarcosinate), and 0.5 mg ml-1 proteinase K added then tubes were incubated at 55 ◦C for 8hrs. DNA extraction was further proceeded according to the method adopted from both Van **Hulten *et al.*, (2001)**. Total DNA of shrimp tissues was extracted by following the manufacturer instructions of the DNeasytissue extraction Kit (Qiagen, MD 20874USA - Catalogue # 69506).

Amplification:

The VP26F/VP26R primer pair, which targets to WSSV VP26 ORF **(Van Hulten *et al.*,2000),** was used to detect WSSV in samples.VP26 vp26F 5' ATGGAATTTGGCAACCTAACAAACCTG 3'; VP26 vp26R 5' GGGCTGTGACGGTAGAGATGAC 3'. That was targeting (304bp) the complete ORFs of structural viral proteins of WSSV VP26. Briefly, The PCR mixture (100 µl) contained 2 µg of DNA,100 pmol of each primer, 1 × PCR buffer, 2 mM MgCl2,200 µM dNTP, and 2.5 units of Taq polymerase (Invitrogen™, ThermoFisher Scientific, USA). The PCR was performed according to the published protocol using gradient thermal cycler (SimpliAmp, ThermoFisher Scientific, USA-Catalogue # A24811) with an initial denaturation at 95°C for 5 minutes and followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and elongation at 72°C for 30 seconds. The PCR amplification was completed using a final elongation step at 72°C for 5 minutes then incubation at 4°C.

Gel Electrophoresis

In two separate 0.1 ml microfuge tubes, Five microliters of PCR product in 5µl of 1X TAE Buffer and 1 µl (6X bromophenol) loading dye and 0.5µl of DNA Ladder in 9.5µl of 1X TAE Buffer plus 1 µl loading dye were consistently mixed then 10µl of from each tube were loaded into a 1.5% agarose gel with ethidium bromide and electrophoresed at 100 V for 30 -32 minutes. The resultant bands were viewed using UV Trans-illuminator (Spectronics Corporation, NY, and USA - Catalogue #TVC-312R). Gel pictures were documented using a digital camera (RX100 V. The premium 1.0-type sensor compact camera with superior AF. Performance, Saint Diego,CA,USA).

Histopathology:

Tissuesamples from muscle and hepatopancreas were fixed in buffered formalin 10 % .Tissues was processed according to the protocol of **(Bell and Lightner, 1988).**

RESULTS

Clinical Examination:

Shrimp.

Clinically, most of the examined shrimp (*P. semisulcatus* or *P. Japonicus*) from both Suez Gulf and Murrah Lake were apparently healthy. However, 4 *P. Japonicus* and 1 *P. semisulcatus* from Suez Gulf presented typical white spot lesions on the carapace. On the other side, all shrimp samples from Murrah Lake were apparently healthy.

Fig. (3): A. Tiny whitish spots on the crusts of the middle abdominal segments of Suez Gulf P. japonicus. B. Mutiple white spots of different sizes scattered on the carapace of P. japonicus from Suez Gulf.

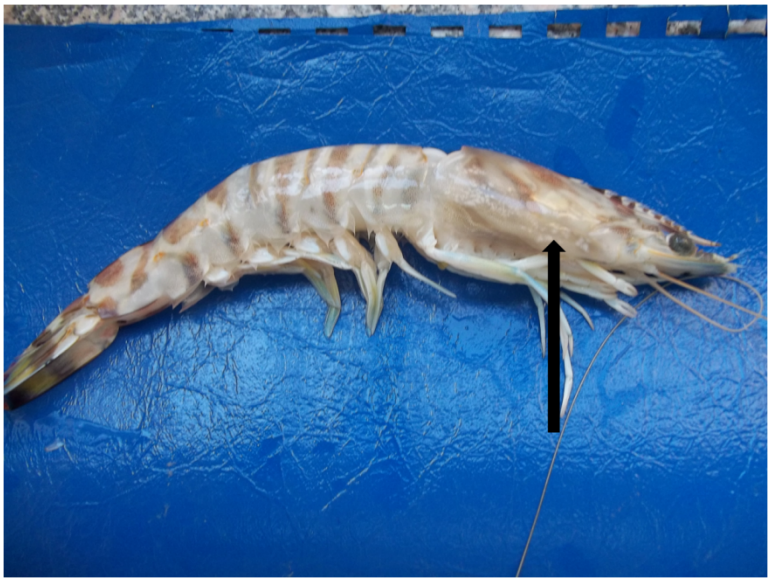

Fig. (4): Lateral view of P. japonicus showing typical multiple whitish spots of different sizes scattered all over the carapace.

Crabs:

All examined crabs collected from both Suez Gulf and Murrah Lake were apparently healthy with no record of lesions or parasites either externally or internally.

Crayfish:

Almost all examined crayfish samples from Abassa, Sharkiya were apparently healthy.

Histopathology:

Histopathologic ally, the clinically affected *P. japonicus* have shown few basophilic intra-nuclear inclusions within Inter-muscular connective tissue. Hepatopancreatic cells were severely degenerated, vacuolated and disorganized and necrotized in some cases with peri-capsular edema.

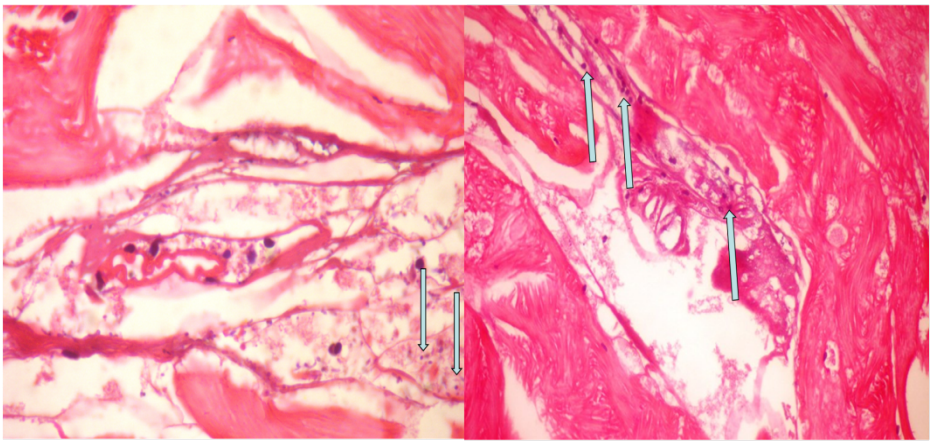

Fig. (5): Clinically affected *P. japonicus* with few basophilic intra-nuclear inclusions within inter-muscular connective tissue.

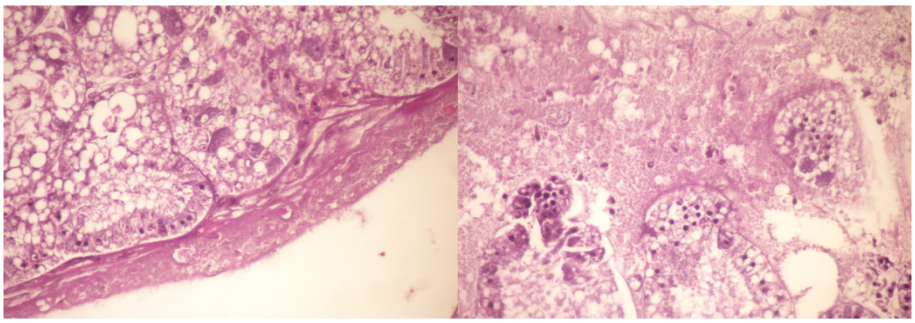

Fig. (6): *P. japonicus* with severely degenerated, vacuolated and disorganized and necrotized hepatopancrease with peri-capsular edema.

B. WSSV (VP26 ORF):

P. japonicas.

Upon using the PCR technique utilizing the specific 304 bp band of the VP26 ORF gene of WSSV was detected in 2 out of 10 pools (10/50) of Suez Gulf exoskeleton samples, while 1 out of 10 (5/50) Murrah Lake exoskeleton samples were positive for the virus.

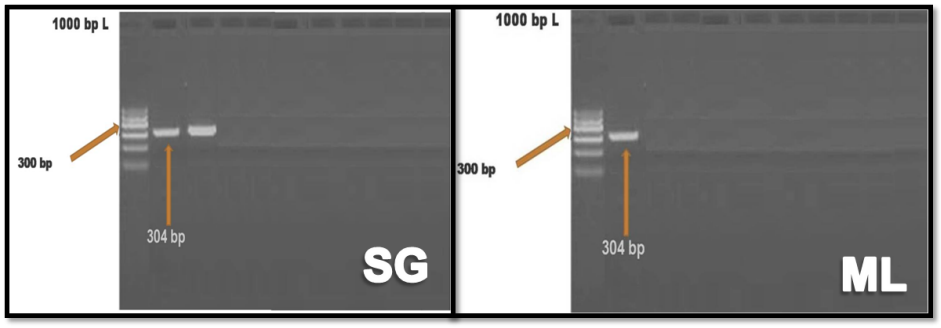

Fig. (7): PCR results for both Suez Gulf (SG) and Murrah Lake (ML) *P. japonicus* .samples documenting the specific 304 bp band of VP26 ORF gene for WSSV.

P. semisulcatus:

The specific 304 bp band of the VP26 ORF gene of WSSV was detected in only 1 out of 10 pools (5/50) of Suez Gulf exoskeleton samples while all Murrah Lake samples were negative for the virus.

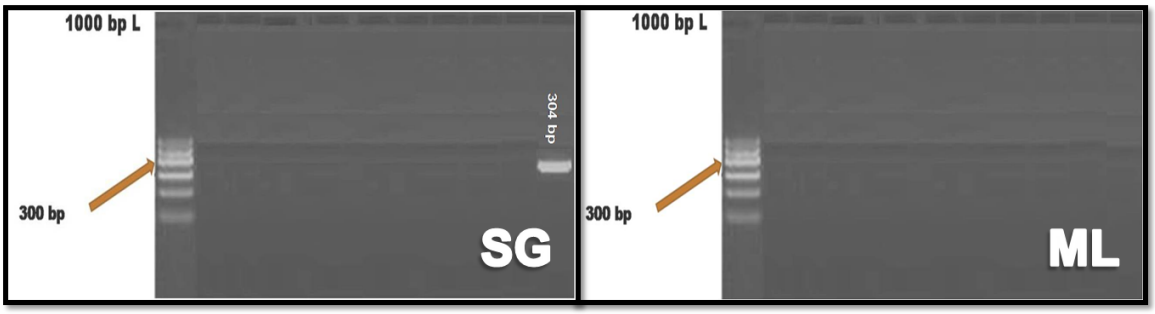

Fig. (8): PCR results for Suez Gulf (SG) (1 positive pool) and Murrah Lake (ML) (negative pools) of *P. semisulcatus* samples documenting the specific 304 bp band of VP26 ORF gene for WSSV.

Crabs:

All tested crab samples were negative for the specific 304 band of the VP26 ORF gene of WSSV.

Crayfish:

Upon using the PCR technique utilizing the specific 304 bp band of the VP26 ORF gene of WSSV was detected in only 1 out of 28 individual crayfish sample from Abassa, Sharkiya.

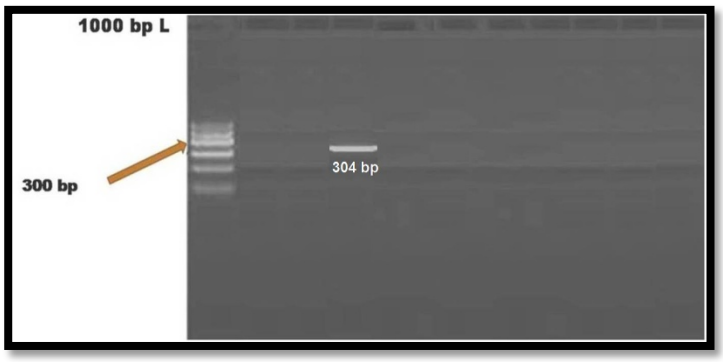

Fig. (9): PCR results for crayfish from Abassa, Sharkiya documenting the specific 304 bp band of VP26 ORF gene for WSSV.

Total and differential prevalence of WSSV infection among different examined species.

The molecular detection (PCR) of WSSV using specific oligonucleotide primers targeting WSSV VP26 ORF in a total 268 shellfish pooled samples from different marine and freshwater species revealed the following results.

The WSSV prevalence among shrimp and crab samples belonging to Suez gulf was 20% for *P .japonicus*, 10 % for *P. semisulcatus* and 0% for *Pr. Pelagicus*. The WSSV prevalence among shrimp and crab samples belonging to Murrah Lake was 10 % for *P. japonicus*and 0% for both *P. semisulcatus* and *C. sapidus*. The WSSV prevalence among freshwater crayfish (*P. clarkii*) samples belonging to Abassa, Sharkiya was 3.6% the recorded differential prevalence of WSSV infection among marine shrimp from different geographical sites was 75% for *P. japonicas* versus and 25 % for *P. semisulcatus*. In respected to tissue preference for WSSV infection, the PCR results indicated that exoskeleton pools were the most affected tissue compared to all other tested tissues samples. Geographically, the overall WSSV infection was clustered at Suez Gulf (63 %) followed by Murrah Lake (21 %) then Abassa, Shirkiya (16 %).

DISCUSSION

Disease has had a major impact on the shrimp farming industry. Since 1981, a succession of new viral pathogens has emerged in Asia and the Americas, causing mass mortalities and threatening the economic sustainability of the industry **(Walker and Mohan, 2009).** Shrimp are arthropods and most shrimp viruses are either related to those previously known to infect insects (e.g., densoviruses, dicistroviruses, baculoviruses, nodaviruses, and luteoviruses) or are completely new to science and have been assigned to new taxa .The continuous shortage of Egyptian shellfish harvest from fisheries has been steadily increasing throughout the past few decades. Different species of Suez Gulf marine shrimp were among the highly affected species. This fact has derived some native shrimp species to be listed among those vulnerable to jeopardy of threatened, endangered or even extinct species. These drastic impacts on Egyptian native shrimps were of multifactorial causes including but not limited to environmental pollution (chemical / biological), overfishing, biological invasions and climatic changes**. (Eissa and Zaki, 2011).** The most critical biological invasion was adopted by three main emerging viruses, white spot syndrome virus (WSSV), Taura syndrome virus (TSV) and Yellow head virus (YHV). The WSSV has emerged in the Egyptian marine shrimp populations at the late 1990s then swiftly reported in wild marine shrimp populations by late 2009 and 2012 **(Eissa *et al* 2012)** and surprisingly spread to freshwater shellfish populations such as Red swamp crayfish by late May 2006 **(Sharshar , 2008).** Hypothetically, the WSSV virus could have reached the Egyptian waters through the international maritime traffic across

the Suez Canal where the highly dynamic shipping process could have brought the WSSV infected shrimp species incidentally linked to ship bottom, or included within ballistic water or immigrating from the southeast Asian coasts through Indian ocean to Red sea then to Suez Canal and neighboring lakes. Also, migratory seabirds such as Sea gulls who predates on marine crustaceans might spread the virus trans-continently from geographically endemic places (e.g. Southeastern Asia) to new places **(Vanpatten *et al.,* 2004, Eissa *et al.,* 2012).** In the current study, *P. japonicus* collected from Suez Gulf have presented a 20 % prevalence of WSSV infection compared to 10 % prevalence of infection of those belonging to Murrah Lake. This difference could be attributed to higher levels of chemical pollution (crude oil, PCBs, PAHs and heavy metals) which suppress the immune system of shellfishes triggering them to be much liable for contracting infection than others from lesser polluted marine water bodies like Murrah Lake. This assumption could be supported by another striking result which emphasizes that another examined shrimp species (*P. semisulcatus*) was negative for the WSSV in Murrah Lake while presented 10 % prevalence of infection in Suez Gulf. Waterman and **Chace, (2012)** explained that the harder the crustacean shell the more resistant against pathogen invasion. They further, emphasized that, the crabs are much higher in their cuticle and cell mediated defense than other small crustaceans such as Shrimps and crayfishes. This interesting theory fully coincides with our findings pertaining to negative results of WSSV among crab's populations from both Suez Gulf and Murrah Lake. Consistent with the aforementioned Waterman and **Chase, (2012)** hypothesis, Red swamp freshwater crayfish presented very low prevalence of WSSV infection (3.6%). Clinically, the recorded white spot lesions on the carapace and crusts of cephalothorax /abdominal segments were consistent with the recorded WSSV standard clinical picture in paneid shrimps **(Eissa and Hosni, 2015).** WSSV infects mainly cells in tissues of ectodermal and mesodermal origins **(Wongteerasupaya *et al.,* 1995)**, while tissues of endodermal origin are refractory to WSSV infection. This theory explains why positive WSSV PCR results were limited to shrimp exoskeleton (Ectodermal origin) in our study. Similarly, the presence of intra-nuclear inclusions in the inter-muscular connective tissue of P. *japonicus* supported the theory that tissues originating from mesoderm (muscles) are also liable to WSSV infection. Since shrimp, as all Arthropods, possess an open circulatory system it is not surprising that, the hemocytes are also found in other tissues, which may explain why WSSV has been detected in several tissues **(Di Leonardo *et al*., 2005)**. This assumption could support the presence of some sort of

WSSV infection within hepatopancreatic tissues. In conclusion, the current study sheds the light on the magnitude of WSSV among wild shrimp, crabs and crayfish populations within the Egyptian open waters. Specifically, we declared that Suez Gulf shrimp populations were overly affected with WSSV than those of Murrah Lake. Selectively, *P. Japonicus* was more susceptible to WSSV than *P. semisulcatus*. We surprisingly found an evolutionary gifted resistance of two types of crabs (*P. pellagicus and C. Sapidus*) against WSSV natural infection. Presence of positive freshwater crayfish samples for WSSV confirms the critical epidemiological importance of such invasive aquatic species in spread and establishment of emerging viruses across the Egyptian aquatic habitats.

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