Isolation and Identification of White Spot Syndrome Virus in Egyptian Marine Shrimp

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ABSTRACT

White spot syndrome virus (WSSV) is rod shaped, double-stranded DNA highly lethal and contagious viral infection of penaeid shrimp. A total of 360 shrimp samples (120 from each species; *Penaeid semesulacatus, P.japonicus* and *P.kerathurus*) were collected from Mediterranean Sea and Red Sea. The samples were prepared and the virus was isolated on Vero and Madian Darby Bovine Kidney (MDBK) cell lines. The MDBK cells were more susceptible for replication of WSSV than Vero cells. A total of 16 and 18 WSSV isolates were obtained from samples on Vero and MDBK cells respectively. A total of 18 and 19 WSSV positive nucleic acid were detected in Vero and MDBK cells respectively using PCR. The virus isolates were confirmed using electron microscope and histopathological examination of infected lesions was carried out. This is the first record of isolation of WSSV from Penaeid shrimp in Egypt.

INTRODUCTION

WSSV is highly lethal and contagious viral infection of penaeid shrimp. The virus is rod shaped, double-stranded DNA (dsDNA) classified within *Genus Whispovirus* within the *Family Nimaviridae (1)*. WSSV is reported to be 240-380 nm long and 70-159 nm diameter and nucleocapsid core is 1- 5 nm long and 95-165 nm diamter *(2)*. The virus is world wide, reported in Taiwan 1992, China, Japan and Korea 1993 *(3)*, Thailand, Indian and Malaysia in 1994 *(4)*. and in USA 1995 *(5)* and by 1996 it had severely affected East Asia and South Asia *(6)*, 1999 in Mexico and in 2000 in the Philippines *(7)*. Presently, it is known to be available in most known Penaeid shrimp growing regions *(1)*.

The virus has a wide host range among penaeid shrimp including *Penaeus monodon, Litopenaeus vannami, Fenneropenae indicus, Penaeid semesulacatus, P.japonicus* and *P.kerathurus* causing 100% mortality within days *(8)*. The virus is transmited horizontally through oral and water borne routes and vertically from infected mother prawns in shrimp hatcheries *(9)*.

Continuous cell lines would be used in diagnosis where it was used to measure WSSV titers and to observe virus replication *(10)*. Confirmation of WSSV requires more detailed analysis by PCR *(11)*. Further confirmation of WSSV infection may be made by electron microscope demonstration of WSSV in the appropriate target tissue types by the presence of the large rod shaped to somewhat elliptical virions *(5)*. Also, histopathology of WSSV is distinctive and can be used for diagnosis with moribund shrimp during outbreaks *(12)*.

In Egypt, a lot of studies on marine shrimp were carried out including bacterial, parasitic, management practices, environmental and biological problems but viral infection of shrimp still under investigation. Therefore, this work was directed to detect the white spot syndrome virus in Egyptian marine shrimp.

MATERIAL AND METHODS

Samples Collection and Preparation

A total of 360 clinically affected shrimp (120 from each species; *Penaeid semesulacatus, P.japonicus* and *P.kerathurus*) were collected from Mediterranean Sea and Red Sea. The samples included gills, pleopods, hepatopancreas, and subcuticular connective tissues mixed with cold TN buffer (20 mM Tris/HCl, 400 mM NaCl, pH 7·4). The tissues were homogenized, centrifuged, purified and the supernatants were collected and used in isolation *(13)*.

Isolation of WSSV on Vero and MDBK Cells

Vero and MDBK cells were cultured in 50 ml disposable plastic flasks in MEM using

10% newly born calf serum till a monolayer sheet was developed. The cells were treated by trypsin and distributed into 24 well-flat bottom cell culture plates as 500 ul of cells / well, incubated at 37°C for confluence formation, the growth media was discarded and 100 ul from each sample were added to each well, incubated at 37°C for 1 hr, then the inoculums were removed and maintenance medium (1000 ul/ well) was added, tissue culture plates were incubated at 37°C with daily examination for recording the development of CPE. The inoculated cells were frozen and thawed and passaged for 3 successive blind passages *(13)*.

Detection and Identification of WSSV Nucleic Acid Using PCR

10 µl of PCR products were run on 1% agarose gel electrophoresis containing ethidium bromide at concentration of 0.5 µg/ml *(14)*.

Detection and Confirmation of WSSV Using Electron Microscopy

The technique was carried out as previously described by *(15)*.

Histopathological Examination of The Infected Lesions

Pathological examination of different organs of infected penaeid shrimp were examined *(5)*.

RESULTS

Isolation of WSSV on Vero and MDBK Cells

Isolation of WSSV on Vero Cells

The inoculated cells showed CPE in the form of cell rounding, aggregation, and lysis (Fig. 1). The CPE was developed by 48 to 96 hr post inoculation and progressively increased on 2nd and 3rd passages even with all types of samples.

The positive samples were 6/120 (5%), 5/120 (4.17%), 5/120 (4.17%) of *Penaeid semesulacatus*, *P. japonicus* and *P. kerathurus* respectively with a total of 16 /360 (4.4%) (Table 1).

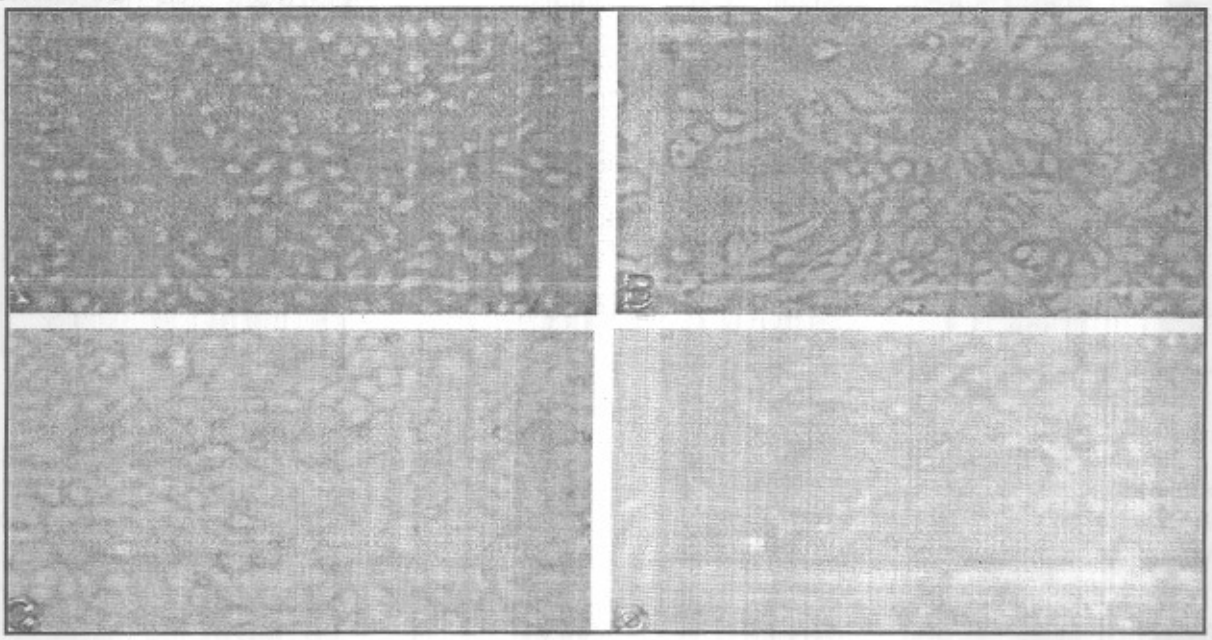

Figure 1: Isolation of WSSV on cell culture (Vero and MDBK cell lines)
(A) Normal Vero cells, showing confluent monolayer sheet. **(B)**Vero cells inoculated with collected sample, third passage showed stages of CPE. In form of a partial destruction of the cellular layer. The cells showed an apparent increase in size, diffuse cell rounding and different size parts of cell lysis. Examined by inverted microscope (X 40). **(C)** Normal MDBK cells, showing confluent monolayer sheet of cells. **(D)** MDBK cells inoculated with collected sample, third passage showed CPE. In form of destruction of the cellular layer, small cellular groups, some isolated round cells were observed and different size parts of cell lysis. Examined by inverted microscope (X 40).

Table 3. PCR detection in relation to the penaeid species

Spcies / Cell Lines	P.semesulacatus		P.japonicus		P.kerathurus		Total	
	No.	+Ve (%)	No.	+Ve (%)	No.	+Ve (%)	No.	+Ve (%)
Vero	120	7 (5.83%)	120	5 (4.17%)	120	6 (5%)	**360**	**18 (5%)**
MDBK	120	7 (5.83%)	120	6 (5%)	120	6 (5%)	**360**	**19 (5.28%)**

The WSSV-specific amplicon is 1447 bp and the sensitivity is approximately 20,000 copies of the template.The specific amplicon from this reaction illustrated in Fig. 2.

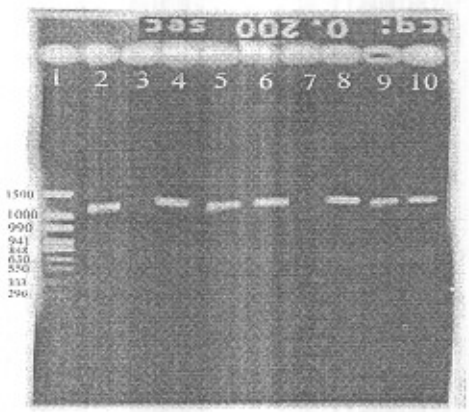

Fig. 2: Detection of WSSV nucleic acid using PCR products
All products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.
Products: Lane 1, molecular size marker (1-1.5bp).
Lane 2: Control +Ve. Lane3: Control-Ve. Lane 4:+Ve
Lane 5: +Ve. Lane 6: +Ve Lane 7: -Ve
Lane 8: +Ve Lane 9: +Ve Lane 10: +Ve

Detection and Confirmation of WSSV Using Electron Microscopy

The samples examined by electron microscope showed a rod-shaped, bacilliform with notable tail-like projection at one end of the particle, enveloped viral particles and measured about 120–150 by 30–50 nm in size (Fig. 3).

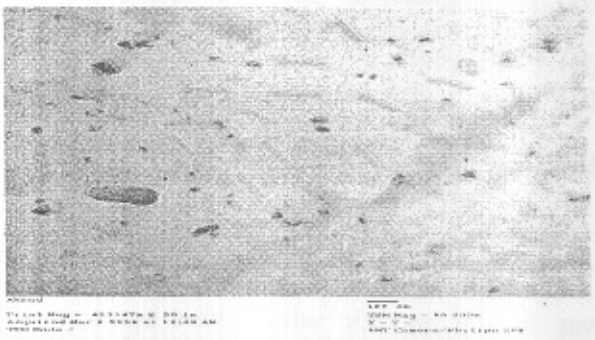

Fig. 3: Electron microscopic Detection
showed a rod-shaped, bacilliform enveloped viral particles and notable some have a tail-like projection at one end of the particle. It measure about 120–150 by 50–70 nm in size.

Histopathological Examination of Infected Lesions with WSSV

Histopathological findings of penaeid shrimp infected with WSSV were enlarged the infected-cell nuclei with characteristic bright red intranuclear inclusion bodies. Some ruptured nuclei showed several eosinophilic inclusions in their cytoplasm were seen in the hepatopancreas. The hepatopancreas showed acute liver necrosis with lymphocyte infiltrations and congestion of the hepatic blood vessels. Diffuse vacuolations of the hepatic cells was apparent. The pancreatic acinar cells were necrotic particularly adjacent the liver necrosis (fig. 4).

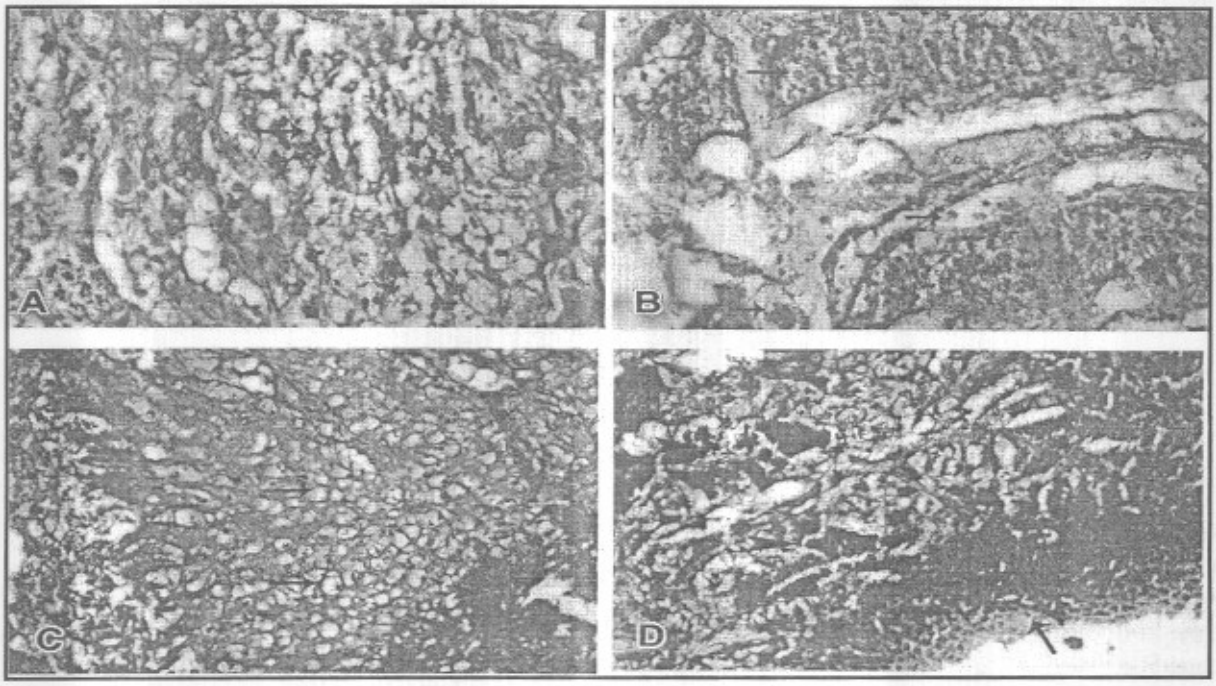

Figure 4:

(A) Hepatopancreas showing hemorrhages and eosinophilic intranuclear inclusion bodies (arrow). H&E x 200

(B) Eosinophilic intranuclear inclusion bodies in hepatopancreas x 300

(C) Subcuticular connective tissue showing spongiosis and ballooning degeneration (arrow) with increased mucous cells and necrosis (arrow head). H&E x400

(D) Subcuticular connective tissue showing hyperplasia of the epidermal cells (arrow) and focal calcification (arrow head). H&E x 300

The subcuticular connective tissues was focally necrotic with severe spongiosis, ballooning degeneration and increased mucous cells. Small areas of erosion or necrosis were seen. The necrotic areas were invaded by numerous lymphocytes. The dermis showed edema, leukocytes infiltrations and fibrous connective tissue proliferation. Focal epidermal hyperplasia, dermal calcification that represented by basophilic granular or homogenous material and necrosis in the underlying muscle fibers were noticed (Fig. 4).

The gills revealed mild hyperplasia of the covering epithelium and pillar cells particularly at the tips of the filaments, areas of necrosis with complete sloughing the epithelium were noticed besides several basophilic bacterial colonies (epicommensal organisms). Some gills showed fusion of its

filament-epithelium, telangiectasis and hemorrhages with few lymphocyte infiltrations were seen (Fig. 5).

The pleopods were focally necrotic and infiltrated by few lymphocytes. Fibrosis and organized thrombi were recorded besides intense infiltrations with lymphocytes. The supported muscle fibers showed necrosis and edema (Fig. 5).

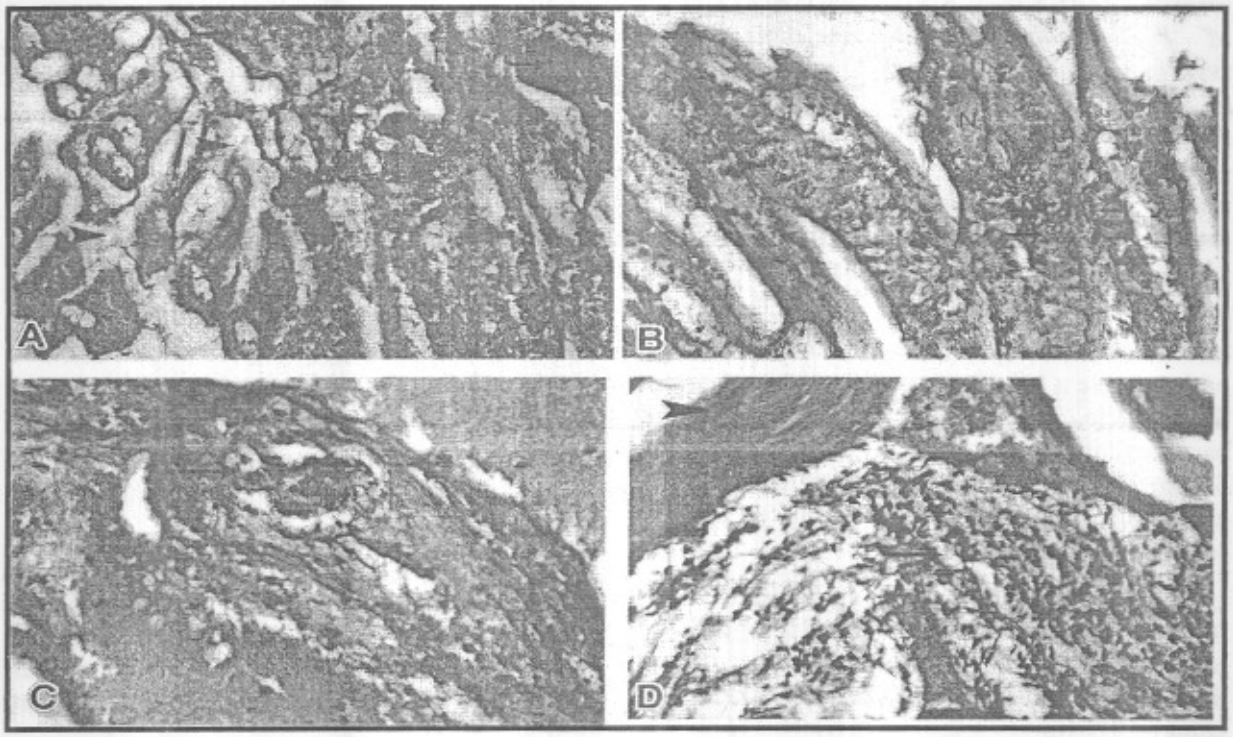

Figure 5:

(A) Gills showing necrosis (arrow) and edema among the gill filaments (arrow head), HE x 300.

(B) Gills showing fusion of the secondary lamellar epithelium (arrow) and necrosis (N), HE x 400.

(C) Pleopods showing fibrosis and organized thrombi (arrow), HE x 400

(D) Pleopods showing intense infiltrations with lymphocytes (arrow) adjacent necrotic muscles (arrow head), HE x 400.

DISCUSSION

Vero and MDBK cell lines as mammalian cells were used in the isolation of WSSV. The positive samples of Vero cells were 6/120 (5%), 5/120 (4.17%), 5/120 (4.17%) of *Penaeid semesulacatus*, *P. japonicus* and *P. kerathurus* respectively with a total of 16 /360 (4.4%) (Table 1). Similar results were recorded previously ***(13).*** While positive samples of MDBK cells were 7/120 (5.83%), 5/120 (4.17%), 6/120 (5%) of *P. semesulacatus*, *P. japonicus* and *P. kerathurus* respectively with a total of 18 /360 (5%) (Table 2). These results could be considered first recorded the isolation of WSSV on MDBK cells in Egypt.

Application of PCR in identification of the virus isolate using primer 146F and 146R, WSSV-specific amplicon from this reaction is 1447 bp and 1.5 kbp DNA Ladder. The positive harvest of Vero cells were 7/120 (5.83%), 5/120 (4.17%), 6/120 (5%) of *P.*

semesulacatus, *P. japonicus* and *P. kerathurus* respectively with a total of 18 /360 (5%). The positive harvested of MDBK cells were 7/120 (5.83%), 6/120 (5%), 6/120 (5%) of *P. semesulacatus*, *P. japonicus* and *P. kerathurus* respectively with a total of 19 /360 (5.28%). PCR detecting result recorded 5.17% ***(17)***. The higher results obtained by PCR than isolation could be due to PCR detected the nucleic acid either with complete virus or even the virus product while isolation need complete active virus to be detected.

The examined harvest by electron microscope showed a rod-shaped, bacilliform with notable a tail-like projection at one end of the particle, double strained enveloped viral particles and measured about 120–150 by 30–50 nm in size (Fig. 4). These morphological characters of the WSSV was previously recorded by ***(8)***.

The histopathology examination of infected lesions of WSSV is distinctive and can be used for diagnosis of moribund shrimp during outbreaks and used as diagnostic support for the virus infection. Histopathological findings for different examined anatomical sites for penaeid shrimp were the infected-cell nuclei enlarged (karyomegaly) with characteristic bright red intranuclear inclusion bodies. This appearance was recorded by **Lightner** ***(5)***. The examined hepatopancreas showed acute liver necrosis with lymphocyte infiltrations and congestion of the hepatic blood vessels, diffuse vacuolations of the hepatic cells as a result of moderate glycogen and fat infiltrations. Interstitial aggregations of lymphocytes and erythrocytes were also noticed. The gills revealed mild hyperplasia of the covering epithelium and pillar cells particularly at the tips of the filaments, areas of necrosis with complete sloughing the epithelium. The subcuticular connective tissues was focally necrotic with severe spongiosis, ballooning degeneration and increased mucous cells with small areas of erosion or necrosis were seen. The pleopods were focally necrotic and infiltrated by few lymphocytes. Fibrosis and organized thrombi were recorded besides intense infiltrations with lymphocytes. Similar histopathological lesions were cited in Thailand ***(18)***.

The WSSV are present in diseased Egyptian marine shrimp. Control and prevention strategy should be designed to avoid prevalence of such disease in Egypt as well as it is useful to use the biosecurity to prevent and control the spreading of the virus.

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الملخص العربي

عزل وتصنيف فيروس ظاهرة البقع البيضاء فى جمبري المياه المالحة فى مصر

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**المعمل المركزى لبحوث الأسماك بالعباسة

فيروس ظاهرة البقع البيضاء هو فيروس عصوى الشكل يحتوى على خيطين مزدوجين من الحامض النووى الدوكسى ريبوز. يسبب الفيروس نفوق عالى وسريع الأنتشار فى جمبرى المياه المالحة. تم تجميع ٣٦٠ عينة من الجمبري السويسى والياباني و القزازى بواقع ١٢٠ عينة من كل نوع ثم تم تجهيز العينات وحقنها على الزرع الخلوى باستخدام خلايا Vero و MDBK. وكانت النتائج ١٦ و١٨ معزولة على التوالى من إجمالي ٣٦٠ عينة مما يوضح افضلية MDBK فى عزل الفيروس. كذلك تم الكشف عن الحامض النووي الفيزوسي باستخدام تفاعل البلمرة المتسلسل وكانت النتائج بالنسبة Vero ١٨ عينة إيجابية و بالنسبة MDBK 19 عينة إيجابية وتم التأكد من الكشف على الفيروس من خلال تعيين الفحص بالميكروسكوب الإلكتروني له و فحص أنسجة الجمبري المصابة به. بهذا يعتبر هذا البحث أول بحث يقوم بعزل وتصنيف فيروس ظاهرة البقع البيضاء فى مصر.