Improving Isolation of White Spot Syndrome Virus on Mammalian Cell Line

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ABSTRACT

White spot syndrome virus (WSSV) is highly lethal and contagious viral infection of penaeid shrimp. Based on the morphological characteristics and genomic structures, the virus classified into Genus *Whispovirus* of the Family *Nimaviridea*. The virus is a large enveloped virus infecting shrimp and other crustaceans. A total of 360 samples distributed as (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 MDBK and Vero cell lines (as mammalian cell line) and with continuous supplementation of TN (Tris HCl and sod. Chloride) buffer.

A total of 16 and 18 WSSV isolates were obtained from 360 samples on Vero and MDBK respectively and a total of 22 isolates were obtained from when added TN to MDBK. Cells harvests was collected and the viral nucleic acid was examined by PCR. A total of positive nucleic acid obtained using PCR was 19, 19, 23. The virus isolates were confirmed using electron microscope that showed a rod-shaped, bacilliform viral particles. Addition of TN buffer improved isolation of WSSV on MDBK cells.

INTRODUCTION

White spot syndrome virus is a major problem in the shrimp aquaculture industry worldwide. Serious outbreaks often cause catastrophic losses in shrimp farming around the globe *(1)*. The vrions are rod-shaped to elliptical with a trilaminar envelope containing a circular double-stranded DNA genome of symmetrical particles of the Genus *Whispovirus* within the Family *Nimaviridea* (new family out from *Baculoviridea*) *(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 present in all shrimp growing regions *(1)*. WSSV has many infected sources as, it can survive in bird intestinal systems, and the birds may then defecate in aquaculture ponds, plants that repackage shrimp and the waste material may become available to seabirds or flying insects, carriers and also, infected microalgae used in feeding second stage of larval development have been shown to transmit the virus *(8)*. PCR method is the most popular diagnosis for detection of WSSV. This method provide an accurate diagnosis method for virus specific genomes *(9)*. Confirmation of WSSV infection may be made by electron microscope demonstration *(5)*.

MATERIAL AND METHODS

Samples Collection and Preparation

A total of 360 clinically affected animals distributed as (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 subcutecular 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 *(10)*.

Isolation of WSSV on Vero and MDBK with Supplementation of TN Buffer

Vero and MDBK cells were distributed into 24 well-flat bottom tissue culture plates by 500 µl (cells suspension and growth media). The plates were sealed and incubated

at 37°C for 24 hr. After examination the plates for the formation 60-80% cell confluence, the growth media was discarded and 100 µl from each sample was inoculated in each well. The inoculated plates were incubated at 37°C for 1 hr, for inoculums adsorption and then the inoculums was removed by washing the cells with culture medium prior the addition of maintenance medium (1000 µl / well). The tissue culture plates were incubated at 37°C with daily examination for recording the development of CPE. After 5 days, the inoculated cells were frozen and thawed for several times and harvested. The harvest prepared and passaged for 3 successive blind passages after addition of TN buffer to the inoculums by 70 µl harvest and 10, 20, 30, 40 µl 30 µl TN *(10).*

Detection and Identification of WSSV Nucleic Acid Using PCR

The technique was carried out as previously described by *(2).*

Detection and Confirmation of WSSV by Electron Microscopy

The technique was carried out as previously described by *(11).*

RESULTS

Isolation of WSSV on Vero and MDBK with Supplementation of TN Buffer

MDBK was more susceptible for replication of WSSV than Vero. The positive samples obtained from Vero cell without addition of TN was 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%). The positive samples obtained from MDBK cells without addition of TN was 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%).TN was added by 70 µl harvest and 10, 20, 30, 40 µl. Vero cell didn't accepted TN buffer while MDBK accepted it up to 40 µl /well and the best addition obtained at 30 µl to the inoculum. The inoculated cells showed CPE in the form of cell rounding, aggregation, and lysis (Fig. 1 & 2). CPE (cytopathic effect) was developed by 36 to 72 hr post inoculation and progressively increased on 2nd and 3rd passages even with all types of samples. The positive samples were, 9/120 (7.5 %), 6/120 (5%), 7/120 (5.83%) of *P. semesulacatus*, *P. japonicus* and *P. kerathurus*, respectively with a total of 22/360 (6.1%) (Table 1).

Detection and Identification of WSSV Nucleic Acid Using PCR

The positive harvested 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%) and the positive harvested 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%). The positive harvested MDBK-TN were 9/120 (7.5%), 6/120 (5%), 8/120 (6.39%) of *P. semesulacatus*, *P. japonicus* and *P. kerathurus,* respectively with a total of 23 /360 (6.39%) (Table 2). 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. 3).

Detection and Confirmation of WSSV by Electron Microscopy

The samples examined 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).

Table 1. Isolation of WSSV on Vero, MDBK and MDBK-TN

Cell lines / Species	Vero		MDBK		MDBK-TN	
	No.	+Ve (%)	No.	+Ve (%)	No.	+Ve (%)
P.semesulacatus	120	6 (5)	120	7 (5.83)	120	9 (7.5)
P.japonicus	120	5 (4.17)	120	5 (4.17)	120	6 (5)
P.kerathurus	120	5 (4.17)	120	6 (5)	120	7 (5.83)
Total	**360**	**16 (4.44)**	**360**	**18 (5)**	**360**	**22 (6.11)**

Table 2. Detection of WSSV Nucleic Acid Using PCR

Cell lines / Species	Vero		MDBK		MDBK-TN	
	No.	+Ve (%)	No.	+Ve (%)	No.	+Ve (%)
P.semesulacatus	120	7 (5.83)	120	7 (5.83)	120	9 (7.5)
P.japonicus	120	6 (5)	120	6 (5)	120	6 (58)
P.kerathurus	120	6 (5)	120	6 (5)	120	8 (6.67)
Total	**360**	**19 (5.28)**	**360**	**19 (5.28)**	**360**	**23 (6.39)**

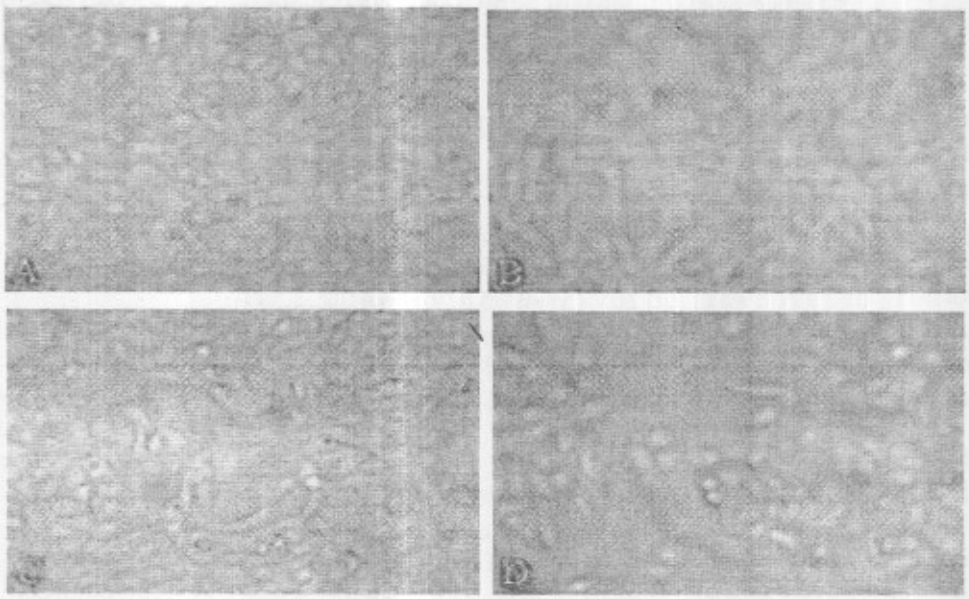

Figure 1. Monolayer MDBK Cells supplemented with TN

(A) Normal MDBK cells, showed confluent monolayer sheet of cells. **(B,C,D)** MDBK cells inoculated with Penaeid samples, showed CPE. In form of cell rounding, aggregation, destruction of the cellular layer and different size parts of cell lysis. It was examined by inverted microscope (X 40)

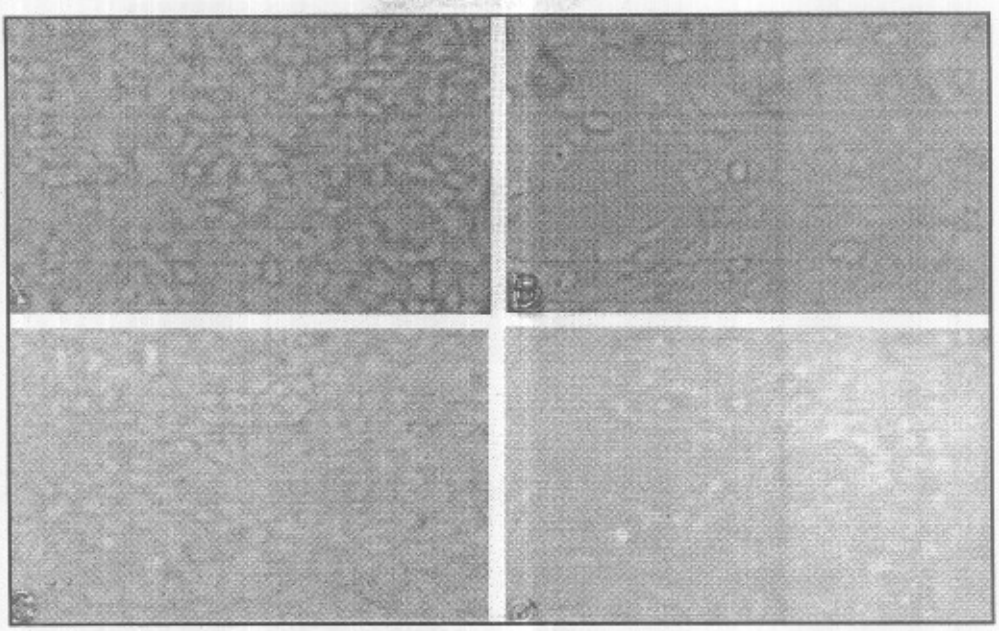

Figure 2. Isolation of WSSV on Vero and MDBK cell lines

(**A**) Normal Vero cells (**B**) Inoculated Vero cells showed CPE(partial destruction of the cellular layer, diffuse cell rounding and different size parts of cell lysis). (**C**) Normal MDBK cells (**D**) Inoculated MDBK cells CPE. It was examined by inverted microscope (X 40).

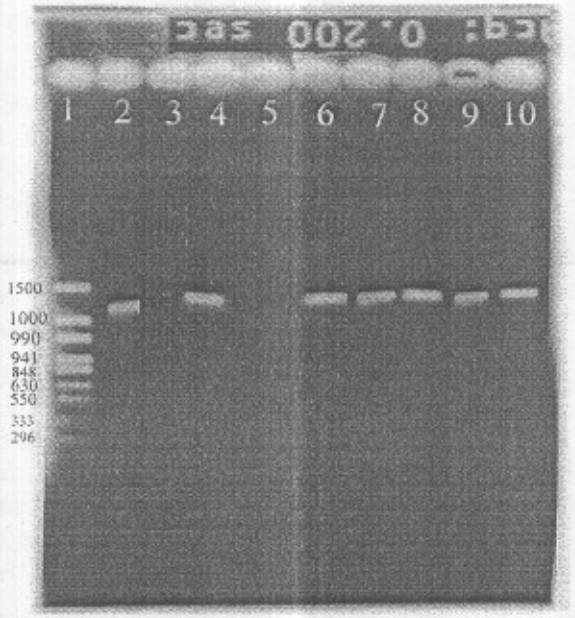

Figure 3. Detection of WSSV Nucleic Acid Using PCR products

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	Lane 3:	Control -Ve.	Lane 4:	+Ve
Lane 5:	–Ve	Lane 6:	+Ve	Lane 7:	+Ve
Lane 8:	+Ve	Lane 9:	+Ve	Lane 10:	+Ve

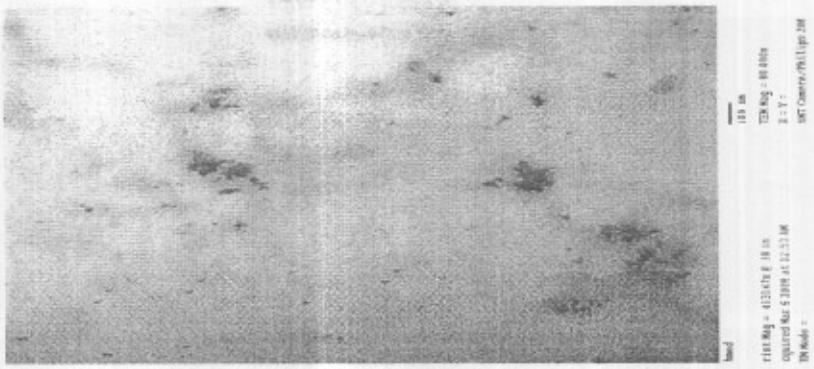

Figure 4. Electron microscopic Detection
showed a rod-shaped, bacilliform enveloped viral particles and notable some have a tail-like projection at one end of the particle. It measure about 120–150 by 50–70 nm in size.

DISCUSSION

The most usable media for the marine viruses isolation have been modified by adding supplements to commercially available media. So, TN buffer was used as supplement to the cell lines during isolation of WSSV *(12)*. MDBK was more better than Vero cells in the isolation of the virus and was more accepted.

That is the cause of using MDBK with the supplementation of TN buffer. Also, MDBK was used with the same buffer in studying of MDBK characterization *(13)*. The inoculated cells showed similar CPE but more rabid, the positive isolated samples were, 9/120 (7.5 %), 6/120 (5%), 7/120 (5.83%) of *P. semesulacatus*, *P. japonicus* and *P. kerathurus*, respectively with a total of 22/360 (6.1%) (Table 1).

These results indicated that, isolation of WSSV was improved on MDBK by adding TN buffer.

Application of PCR in identification of the virus WSSV isolate was by using primer 146F and 146R, WSSV-specific amplicon from this reaction is 1447 bp and 1.5 kbp DNA marker (Fig. 3). The positive harvest were 9/120 (7.5%), 6/120 (5%), 8/120 (6.39%) of *P. semesulacatus*, *P. japonicus* and *P. kerathurus*, respectively with a total of 23 /360 (6.39%).

The higher results obtained by PCR than isolation technique may 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 salinity in fresh water less than 0.21‰, in brackish water over 0.21 ‰ up to 30‰ and in marine water over 30‰ *(14)*. From this sides using of TN as salts supplementation of the MDBK cell lines were improved of the virus isolation and PCR detection results that due to the WSSV is a marine virus and need specific environmental osmotic pressure for improving its activity.

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 (Figure 3). This morphological characters of the WSSV was recorded by *(15)*.

TN improved WSSV isolation process and it is important to establish salts balance suitable for isolation of the marine viruses on the way Eagle's minimum essential medium that used for isolation of other viruses.

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الملخص العربى

تحسين عزل فيروس ظاهرة البقع البيضاء على خلايا محضرة من حيوانات ثديية

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**المعمل المركزى لبحوث الأسماك بالعباسة

يسبب فيروس ظاهرة البقع البيضاء نفوق عالي وسريع الانتشار فى جمبري المياه المالحة. بلأستناد إلى تركيب الفيروس تم تصنيفه تابعا لعائلة *Nimaviridea* .هذا الفيروس من الفيروسات ذات الغلاف ويستطيع أن يصيب جميع القشريات. جميع ٣٦٠ عينة جمبرى من الجمبري السويسى واليابانى و القزازى بواقع ١٢٠ عينة من كل نوع ثم تم تجهيز العينات وحقنها على الزرع الخلوى باستخدام خلايا , Vero. MDBKكنوع من الخلايا الثديية و تم تزويد تلك الخلايا بمحلول ملحى TN أثناء تمريرات العزل وكانت النتائج بالنسبة Vero ١٦ و بالنسبة MDBK ١٨و بالنسبة MDBK-TN ٢٢ عينة. كذلك تم الكشف عن الحامض النووي الفيروسي باستخدام تفاعل البلمرة المتسلسل وكانت النتائج هى ١٩و١٩و ٢٣ على التوالى من إجمالي ٣٦٠. كذلك تم التأكد من عدوى وعزل الفيروس من خلال تعيين الفحص بالميكروسكوب الإلكتروني وأظهرت وجود الفيروس بشكله العصوى. بهذا يعتبر TN محلول يحسن عزل فيروس ظاهرة البقع البيضاء .