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VIRAL STUDIES ON DISEASED *PENAEID* COLLECTED FROM SUEZ GOVERNORATE

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Abstract

The frequency of viral disease outbreaks in the shrimp aquaculture industry has markedly limited the development of shrimp aquaculture in the world. In the present study, primary tissue culture from *Penaeid semesulacatus* used for virological studies of diseased shrimp. A total of 70 adult symptomatically diseased shrimp of common Penaeid species were collected from El-Adabia,in Suez governorate. Gills, lymphoid organ, cuticular epithelium shrimp, and subcutecular connective tissue were prepared for isolation and histopathological examination which showed evidences of diseased agent invasive. On the tissue culture it was showed cytopathic effect developed by 48 hrs to 96 hrs post inoculation in 17 samples out of 70 samples as total collection. For more investigation harvested fluid from the tissue culture was negatively stained with phosphotungstic acid and examined by electron microscope that revealed presence of virus-like particles.

It can be concluded that, the wild shrimp isn't safe, can car rymany diseases and should be examined before used as breeder in the hatcheries.

Key words: Penaeid, shrimp and cell culture.

INTRODUCTION

The global population is predicted to expand from the current size to an estimated 7 billion to 9.1 billion by 2050. Aquatic food products, the fastest growing of the food-producing sectors, are predicted to supply more than 50% of this sector by 2015 (Stentiford and Kraaijeveld, 2012). The aquaculture industry continues to grow rapidly, particularly in the case of cultured marine shrimp. Indeed, cultured shrimp harvest was an estimated 3.5 million metric tons representing a sale value of $14.6 billion USD in 2009 (Moss *et al*., 2016).

Aquaculture practices have changed from a traditional extensive farming to semi-intensive or intensive systems that are more efficient production systems (Li *et al*., 2017). Shrimp adoption in culture systems worldwide, along with effective biosecurity measures and intensive farming techniques, have resulted in rapid growth of the global shrimp farming industry over the last four decades. This growth has been hampered only by pandemic viral diseases that have devastated harvests and yields; therefore, they have also greatly impacted the economics of the shrimp industry worldwide Flegel and Lo (2013).

The frequency of viral disease outbreaks in the shrimp aquaculture industry has markedly limited the development of shrimp aquaculture in the world. Thus developing a permanent shrimp cell line is absolutely imperative for the diagnosis and prevention of known and newly emerging prawn viruses, and for the analysis of interactions between viruses and their host cells as well as the mechanism of viral infection (Vinaya *et al*., 2018). However, work on the initiation of primary cell culture of shrimp tissues has accumulated much useful knowledge with respect to the optimal media, tissues, temperatures, osmolarities and pH values, etc., and the number of reports of successful primary cell cultures is on the increase. Types of tissues and organs from which the cells are derived were found to be influential for the survival time of primary shrimp cell cultures including lymphoid tissue (Toullec *et al*., 2017).

In the early era of shrimp farming expansion (1970s-1980s), *Penaeus monodon* was the major in culture species, and the industry relied on wild caught stocks to produce postlarvae (PL) and adult brood stock to supply seed for the industry (Moss *et al*., 2016). This is a high-risk practice because wild-caught animals can be carried diseases causative agents and introduced it into culture facilities. However, this method still used in Egyptian hatcheries and it may be the cause for appearing many diseased factors during rearing period.

The aim of present study is establishment of primary cell culture from marine shrimp using the lymphoid organ tissues of Suez shrimp (*Penaeid semesulacatus)* used for virological studies of diseased shrimp.

MATERIALS AND METHODS

Sample collection:

A total of 70 adult symptomatic diseased shrimp of common *Penaeid* species (*Penaeid semesulacatus, P.japonicus and P.kerathurus*) suffering from loose cuticle, soft shell or brown-reddish coloration were collected between March to June 2017 from El-Adabia in Suez governorate. The collected samples for histopathological examination were preserved in Davidson's buffer (330ml ethyl alcohol, 220ml formalin, 115ml glacial acetic acid and 335ml distilled water) , while the samples collected for isolation transported in ice box and preserved at -70 ºC. Additionally, healthy shrimp was collected as used for establish primary cell line.

External examination:

Clinical examination of affected shrimp was done out as described by **OIE, (2016**) to determine the clinical alteration on cuticle, eyes, pleopods, cephalothoraxes, abdomen and tail.

Postmortem examination (PM):

The post mortem investigation was done on live and freshly dead shrimp to examine all internal organs including gills, lymphoid organ, hepatopancreas, midgut, and hindgut for detection the abnormalities. The examination was done according to OIE, (2016).

Sample preparation:

Gills, lymphoid organ, cuticular epithelium, and subcutecular connective tissue were taken from the shrimp, mixed with cold (TN) buffer (20 mM Tris/HCl, 400 mM NaCl, pH 7·4) by 1:8 and homogenized. The homogenates were centrifuged at 1700 rpm for 10 min at 4 °C to pellet cellular debris. The supernatant solution was further clarified by two rounds of centrifugation at 5000 rpm for 10 min at 4 °C followed by two rounds of centrifugation at 13000 rpm for 10 min at 4 °C Xu *et al*. (2016).

Disinfection of used shrimp for established primary cell line:

The animals were sacrificed by immersion in crushed ice and disinfected in 800 mg L)1 sodium hypochlorite solution prepared in ice cold sea water (salinity 15 g L)1) for 10 min. Subsequently, they were washed five times in sterile ice cold sea water, dipped in 70% alcohol prepared in distilled water, rinsed again in ice cold sea water, and used for the removal of tissue aseptically **OIE (2016**).

Primary cell culture preparation:

Primary culture of shrimp lymphoid organ is prepared according to the method of Xu *et al*. (2016). Lymphoid organs were obtained from health adult shrimp (40-50 g) (Photo. 1). It was washed three times in cold antibiotic-buffered mixture consisting of 1000 IU/ml penicillin, 1000 µg/ml streptomycin, 250 µg/ ml gentamicin, 250 µg/ml fungizone, and 1M phosphate buffered saline (PBS). After a final wash and further incubation for 10 min in cold antibiotic-buffered solution, the lymphoid organs were then minced to small fragments 1 mm3. Four tissue fragments were transferred to each well in 24-well plates and 1 ml of the culture medium containing 2x L-15 supplemented with 15% FCS, 1% glucose, 5 g/L NaCl, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml fungizone, was added to each well. Plate was then sealed and incubated at 28ºC until 70- 80% confluent monolayer was formed.

Virus isolation:

After examination the plates under the inverted microscope for the formation of about 70-80% cell confluence, the growth media was discarded and 30 µl from each sample was inoculated in each well. The inoculated plates were incubated at 28°C for 1 hr, and then washed with culture medium prior the addition of maintenance medium. The tissue culture plates were incubated with daily examination for recording the development of cytopathic effect (CPE). After 5 days, the inoculated cells were frozen and thawed for several times then harvested the product Xu *et al*. (2016).

Electron microscopic examination:

Harvested fluid from the cell lines was centrifuged at 3000 rpm/ 5min, one drop from the supernatant put on the collodion-coated grids for one minute and then negatively stained with 2% phosphotungstic acid (pH 7.0). The grids put on clean filter paper for removing the excess stain for 1 hr till complete dryness. Then fixed on the electron microscope holder and examined by transmission electron microscope (TEM) OIE (2016).

Histopathological examination of infected lesions:

Fixation procedures was done with Davidson's (5-10% volume: weight) via needle and syringe and into the live shrimp. After fixation, the samples passed in ascending concentration of ethyl alcohol (80%, 90%, 95% and 100%). Samples was cleared by using of xylene in two separated baths and embedded in paraffin then placed in embedding molds to be sectioned by microtome. Five-micron thick paraffin sections were prepared. Staining of the sections was occurred by deparaffinizing, stained with hematoxylin and phloxine/eosin then examined by the light microscope (**Bancroft *et al*., 1996**).

RESULTS

External examination:

The collected shrimp showed loose cuticle, soft shell, and expansion of the red chromatophores, overall pale reddish coloration making tail fan with pleopods distinctly red and black gills (Photo. 2). There is loss many of pleopods (Photo. 3).

Postmortem examination:

The examined shrimp showed soft shells, black gills, focal destruction of subcuticle (Photo. 4), gut filled with reddish brown content and soft musculature (Photo. 5).

Primary cell culture:

Primary culture prepared from lymphoid organ was showed difference in viability after established that may be attributed to the weak binding of cells to the substrate after the first day (Photo. 6).

Virus isolation:

The observed changes began with a partial destruction of the cellular monolayer. Next, small cellular groups and some isolated round cells were observed. The cells showed an apparent increased in size, diffuse cell rounding aggregation and different parts of cell lysis (Photo. 7). The cytopathic effect (CPE) was developed by 48 hr post inoculation and progressively increased on 2^{nd} and 3^{rd} passages. The CPE was showed in 17 samples out of total collection.

Electron microscopic examination:

Visualization under TEM of centrifuged tissue culture harvest of the samples revealed presence of virus-like particles, enveloped icosahedrons measuring 60-70nm in diameter, with capsomers layer 10-15 nm in thickness and nucleoid 20-30nm, (photo. 8).

Histopathological examination:

Pathological examination of tissues collected from diseased penaeid shrimp showed moderate hyperplasia, sloughed areas and edema in gills (Photo 9 and 10). Hepatopancreas showed sever degenerative changes and sever vacuolar degenerative (Photo 10 and 12). Pleopod was showed area of necrosis in subcuticler epithelium (Photo 13). Thickness and deformity in cuticle of penaeid shrimp (Photo 14).

DISCUSSION

The appeared of loose cuticle, soft shell, expansion of the red chromatophores, black gills, signs of pleopods losses refereed to invasion of pathogenic agent to shrimp tissues as that recorded by Nakano *et al*. (1994) who cited sick shrimp often show red discoloration, usually involving the legs and tail fan. In severe cases, entire body surface, including appendages, is red

associated with a number of diseases and therefore can be used in isolation to identify a disease.

The pale hepatopancreas, soft musculature and focal necrosis of subcuticular tissues which showed in postmortem examination are general symptoms used as guide in follow up the disease and help in tissue samples collection so that the samples were collected from gills, pleopods, cuticular epithelium, and subcutecular connective tissue at adult shrimp stage which were suitable for studying virus infection and principal target tissues for most shrimp viruses that was supported by Tu *et al.* (1999) who cited shrimp viruses may infect all life stages but infection severity may be below detection limits in spawned eggs and in the larval stages, so these life stages may not be suitable samples for many viruses as Taura syndrome virus (TSV) detection or certification of TS disease freedom.

Development of stable shrimp cell lines promoted studies at the cellular and molecular levels of viral problems such as the underlying mechanisms of viral replication and cytopathology as a consequence of infection, an understanding of viral genomic function and genetics. Furthermore, established cell lines from various shrimp tissues and organs facilitate early viral detection. First attempt was made in shrimp cell culture by Erickson *et al.* (2002) who used L-15 medium for the cultivation of shrimp primary lymphoid and ovary cells cultures from *Penaeus monodon*.

Primary culture prepared from lymphoid organ of shrimp was established for virological studies of diseased shrimp. The cells was showed difference in viability after established that may be attributed to the weak binding of cells to the substrate after the first day and so, it was needed a longer incubation period to allow cells to attach firmly to the coated substrate. Cell viability decreased after seven days in culture (Photo. 6). It was established one week in culture then going to subculturing process.

After inoculation of collected samples on the established cell culture, the cells showed an apparent increased in size, diffuse cell rounding aggregation

and different parts of cell lysis. The developed CPE was developed by 48 hr to 96 hr post inoculation and progressively increased on 2nd and 3rd passages (Photo. 7) and that was similar recorded by Xu *et al.* (2016) when isolated a new pathogenic iridovirus from red-claw crayfish.

For more investigation of the formed CPE, harvested fluid from the cell lines was negatively stained with phosphotungstic acid and examined by TEM revealed presence of virus-like particles, enveloped icosahedrons measuring 60-70nm in diameter with capsomers layer 10-15 nm in thickness and nucleoid 20-30nm, (photo. 8). This particles similar picture of iridovirus Xu *et al.* (2016) and Taura syndrome virus (TSV) Tu *et al.* (1999). So that it needed more studies for confirmatory of the isolate.

The histopathology examination of infected lesions is distinctive and can be used for diagnosis with moribund shrimp during outbreaks, also used as diagnostic support for the virus infection. Pathological examination of different parts of penaeid shrimp showed moderate hyperplasia, sloughed areas and edema in gills. Sever degenerative changes and sever vacuolar degenerative in hepatopancreas. Areas of necrosis in subcuticler epithelium of pleopod. Thickness and deformity in cuticle. The histopathological findings for different examined anatomical sites for penaeid shrimp were showed in figures (9 to 14). This appearance was similar cited by Morales and Chavez (1999) in histopathological studies on wild broodstock of white shrimp *Penaeus vannamei* in the Platanitos area.

It can be concluded that, the wild shrimp isn't safe and can carry many diseases. Thus it should be paid closer attention to take more effective measures for preventing the disease outbreaks and economic losses specially when used the wild shrimp as breeder in the hatcheries. Also, the need for development of strategies to prevent and control viral diseases is of tremendous interest and importance for the sustainability of this industry.

Photo. 1. Health adult shrimp used for establishment primary tissue culture.

Photo. 2. Distinctly red tail fan and pleopods with black gills.

Photo. 3. Signs of focal epithelial necrosis in pleopods leading to lose many of it.

Photo. 4. Black gills.

Photo. 5. Gut filled with reddish brown content and soft musculature.

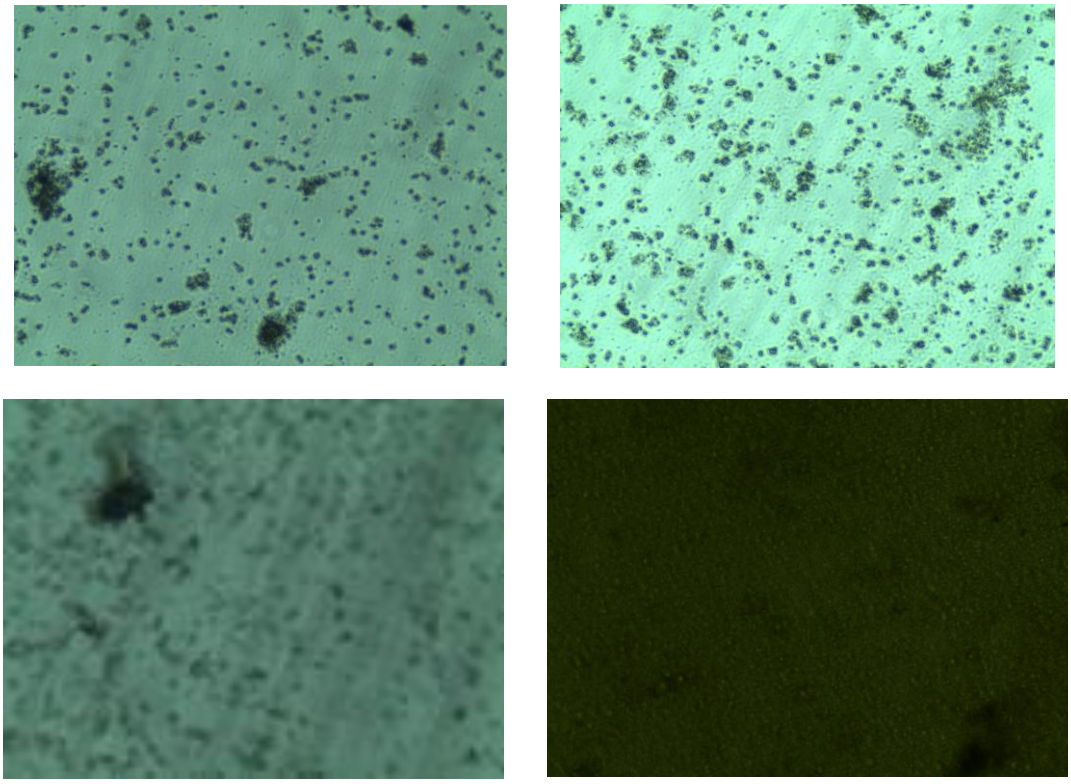

Photo. 6. Stages of formed primary tissue culture till formed confluent monolayer sheet. (X 100).

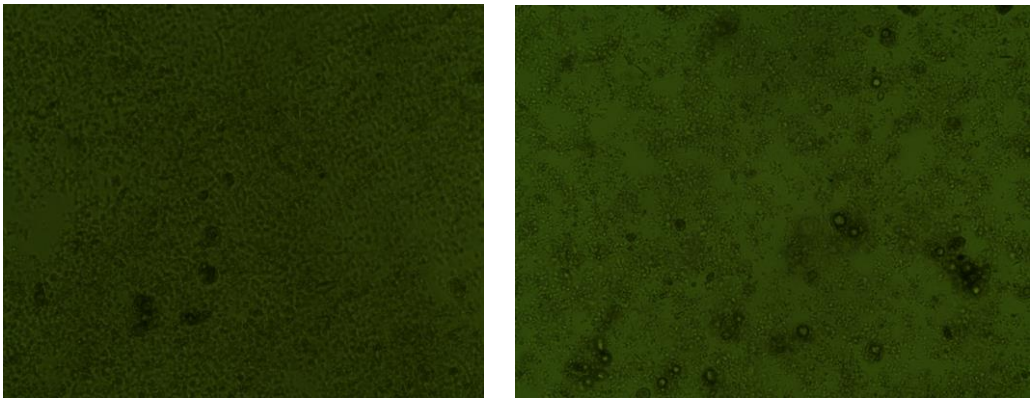

Photo. 7. The cells showed an apparent increased in size, diffuse cell rounding aggregation and different parts of cell lysis (X 100).

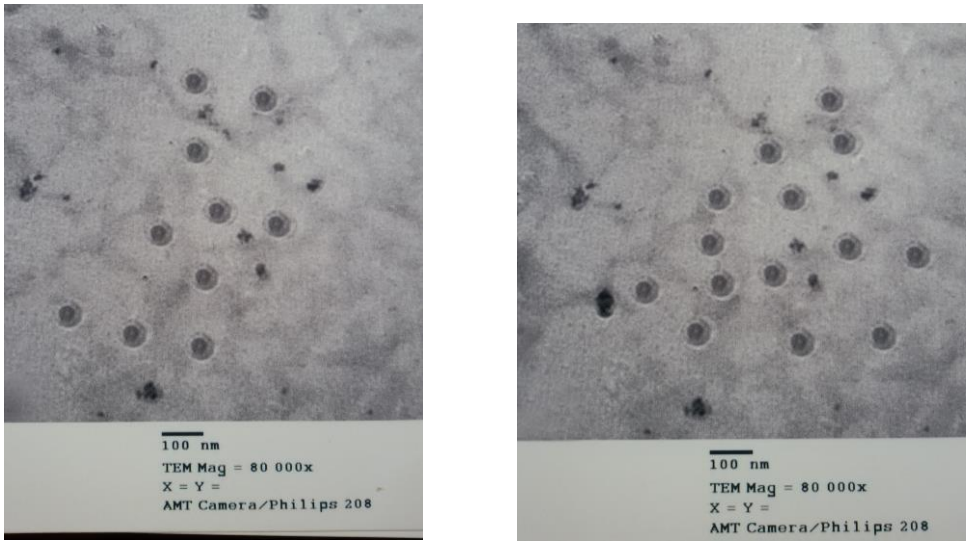

Photo. 8. Transmission electron microscopy of tissue culture harvest showing a large numbers of virion particles.

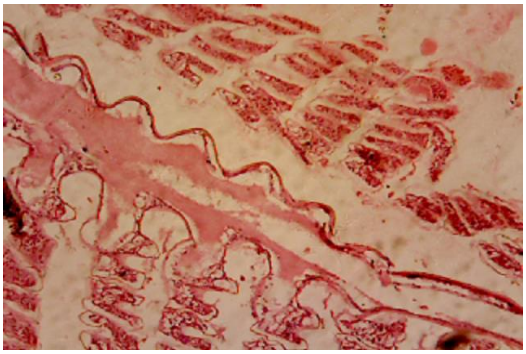

Photo. 9. Gills of penaeid shrimp showed edema. HE x 100.

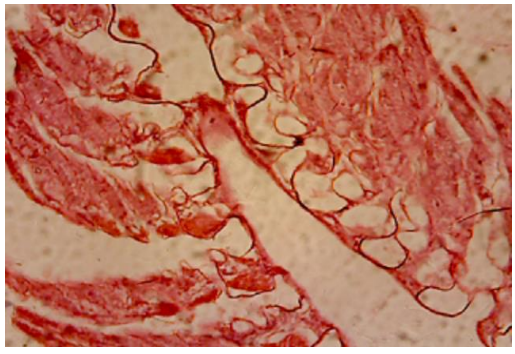

Photo. 10. Gills of penaeid shrimp showed moderate hyperplasia and sloughed areas. HE x 100.

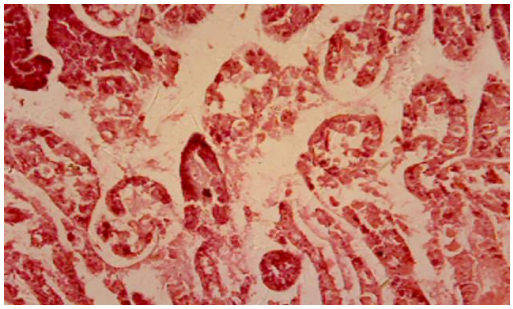

Photo. 11. Hepatopancreas of penaeid shrimp showed sever degenerative changes. HE x 100.

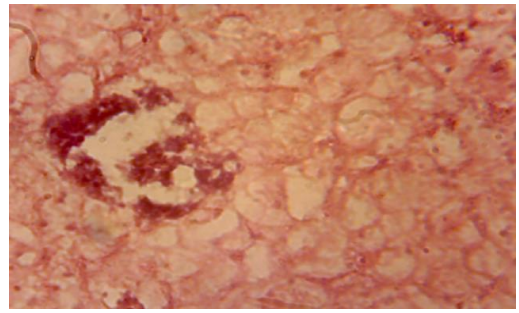

Photo. 12. Hepatopancreas of penaeid shrimp showed sever vacuolar degenerative. HE x 400.

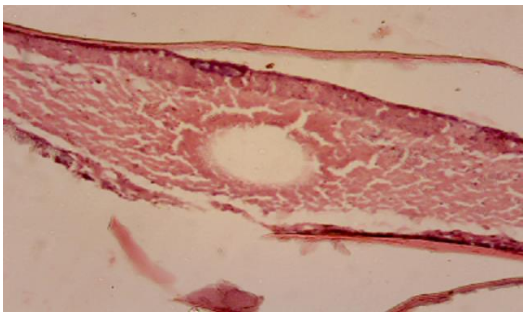

Photo. 13. Pleopod of penaeid shrimp showed area of necrosis in subcuticler epithelium. HE x 100.

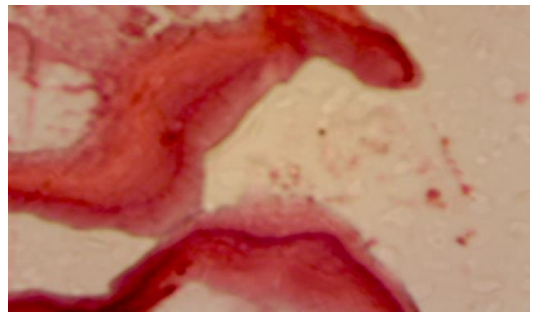

Photo. 14. Thickness and deformity in cuticle of penaeid shrimp. HE x 400.

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دراسات فيروسية على جمبرى مياه مالحة مصاب مجمع من محافظة السويس

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المعمل المركزى لبحوث الثروة السمكية بالعباسة – مركز البحوث الزراعية.

الملخـص العربـى

تعتبر الفيروسات التي تصيب جمبري المياه المالحة من المسببات المرضية القاتلة وسريعة الانتشار خلال أيام قليلة بين الأنواع المختلفة للجمبري. فى الوقت الذى تقوم المفرخات بجمع الأمات من البحر قبل موسم التفريخ ثم يتم إستخدامها لإنتاج اليرقات ولهذا كان من الضرورى دراسة مخاطر أن تكون تلك الأمات حاملة لأى فيروس. ولهذا تم تجميع عينات مختلفة من جمبري المياه المالحة من محافظة السويس تظهر عليه أعراض غير طبيعية من ضعف الهيكل الخارجى، إحمرار اقدام العوم، سواد الخياشيم وتم تحضير أجزاء من هذا الجمبرى شملت الخياشيم والطبقة تحت القشرية والجسم الكبدى البنكرياسى وأقدام العوم لحقنها على خلايا نسيجية أولية محضرة من الجسم الليمفاوى لجمبرى ظاهريا تبدو عليه علامات الصحة كما تم أخذ عينات أخرى وتحضيرها وذلك لفحص الأنسجة المصابة بالمجهر الضوئى. وبعد الحقن والتمرير على الخلايا الأولية ظهر بعض النشاط الفيروسى عليها. كما اظهر الفحص المجهرى للأنسجة وجود آثار للمسببات المرضية. ولتأكيد الدلائل المرضية تم أخذ عينات من محتوى النسيج الخلوى وفحصها بالميكروسكوب الإلكتروني النافذ لدراسة شكل الفيروس وقد بين الميكروسكوب الإلكتروني النافذ وجود جُسيمات تشبه الفيروسات فى تلك العينات تتميز بشكلها الكروي، تحاط بغلاف خارجي يتراوح حجم تلك الجُسيمات ما بين 60 إلى 70 نانوميتر.

فى الخاتمة، تخلص الدراسة إلى أنه من الضروري معرفة أن الجمبرى المصاد من المصادر الطبيعية من الممكن أن يكون حاملا لأمراض فيروسية وبالتالى من الخطأ أن يتم إستعماله كأمات فى المفرخات دون الفحص والتأكد من سلامته.