PRIMARY ISOLATION AND CHARACTERIZATION OF SPRING VIREMIA OF CARP VIRUS (SVCV) FROM CULTURED SILVER CARP)*HYPOPHTHALMIXIS MOLITRIX*) AND COMMON CARP (*CYPRINUS CARPIO* L) IN KAFR EL-SHIKH GOVERNORATE

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ABSTRACT

In the present study, Spring viremia of carp virus (SVCV) was identified from cultured common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmixis molitrix*) during disease out-break in a private fish farm in Egypt. The electron microscope was used to describe the viruse and determine their shape and form. Specific immunohistochemistery test was carried out using monoclonal antibody of SVCV and antimouse immunoglobulin to verify the presence of the SVCV. Histopathological investigation of the infected fishes was also carried out. The results revealed the presence of bullet-shape *Rhabdovirus*. The specificity of the isolated SVCV to antibody was proved. The clinical signs of infected fish included skin darkening, tail and fin rot, ulceration, hemorrhage on the abdomen, ascitis and redness of isthmus and head region. Postmortem lesions were congestion of all internal organs, distended gall bladder, hemorrhagic gas bladder, exophthalmia and swelling of the anal opening. Histopathological examination of the infected organs by the described virus revealed degenerative changes and focal necrosis in the involved cells of the internal organs. Polymerase chain reaction is good prognosis of the virus presence. The present results indicated that the virus infection was likely the cause the infection and was responsible for the mortalities and lesions during the outbreak. This result may be the first report on spring viremia among cultured fishes in Egypt but it needs further studies for specification and characterization of the virus.

Keywords: Silver carp, common carp, virus, *Spring viremia* of carp, disease, inclusion bodies, PCR.

INTRODUCTION

Aquaculture has become a major contributor to total fish production in Egypt. Therefore, aquaculture sector has witnessed a wide expansion during the past few years. As a result, aquaculture production increased from 139,400 tones in 1998 to reach 461,535 mt in 2005, representing 58% of total fish production (*GAFRD, 2007*).

Tilapias, carps and mullets represent over 95% of total aquaculture production. Typically, these fish species are cultured Simi intensively in polyculture systems. A number of disease outbreaks have been recorded recently in many fish farms throughout the country *(Saad, 2005*). The spring viremia of carp virus (SVCV) and *Rhabdovirus carpio* are the most important viral diseases affecting cultured fishes, causing high mortality (30-60%), especially among Common carp and Silver carp (*Kim et al., 2005*). It has been reported that SVCV and newly isolated strain of *Rhabodovirus carpio* cause high mortality in carps, both in acute or chronic form, during the spring of the year (Kim *et al., 2005; Saad , 2005 and Abou-Eissa 2007*).

However, studies on the effects of viral diseases on wild and farmed fishes are limited, particularly in tropical and subtropical regions of the world. This has been partially due to the increased water temperature and the very short life span of fish viruses in the aquatic environment. The high costs of examination concomitant with inaccessibility to tissue culture techniques and cell lines for examination of fish viruses make such studies more difficult (*Post, 1987*).

Thus, viral fish diseases in Egypt did not receive enough attention. The magnitude and impact of these viruses are still un known. Very few studies were conducted on isolation of SVCV from carp species in Egypt (Saad*, 2005; Abou-Easa, 2007*).

It is clear that more work is urgently needed on the identification, isolation, description of symptoms and prevention of the viral diseases infecting cultured and

wild fishes in Egypt. The present study describes the occurrence of Spring Viremia of Carp Virus (SVCV) from pond cultured Silver carp (Hypophthalmixis *molitrix*) and Common carp (*Cyprinus carpio* L) during disease outbreak in a private fish farm in Kafr-El-Shikh Governorate in Egypt, in order to determine the causes of mortalities among these fishes.

MATERIALS AND METHODS

Fish sampling and examination

Twenty naturally infected carps (10 Common carp and 10 Silver carp), with an average body weight of 30±5g, were collected from a private fish farm in Kafr El-Shikh Governorate, Egypt. The fish were transported alive to the Fish **Disease** Laboratory, Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University, Egypt, in plastic bags containing oxygenated water. The samples were processed and examined immediately after arrival. The fish were subjected to clinical, bacteriological, mycological and parasitological examinations, according to Bucke and Finaly *(1979) and Morrison et al. (1981).*

Viral identification

Samples from fish liver, kidney, spleen, muscles, intestine, heart, brain and gas bladder were collected and mixed with sterile sand and grinded in sterile mortar, freeze and thawed for about 3 times. Three drops of mycosatation (1000 IU/ml), Penicillin (1000 mg/ml), streptomycin and nystatin (500 IU/ml) were added to the supernatant. The supernatant was collected in small flasks and filtered through Millipore filter 0.22 and kept in the refrigerator at -20 C until used (*Bucke and Finaly, 1979; Saad, 2005*).

Electron microscope investigation

Fish samples for electron microscope examination were prepared from collected organs and fixed in 5% glutraldehyde, as described by *Jerome et al. (1995)*

Immunohistochemistry studies

Immunohistochemistry studies were carried out on samples preserved in formalin paraffin by using Spring viremia of carp virus (SVCV) and anti-mouse IgG, according to *Kirenan (2003).*

Histopathological examination

Specimens from brain, liver, kidney, muscles, intestine, and gas bladder were collected from infected fish, fixed in buffered formalin saline 10% and used for histopathological examination, according to *Culling (1983).*

Tissue culture

The primary cell line preparation was made from the ovary of mature Common carp, according to the steps described by *Habashi (1980).* The supernatant of tissue suspension was added to the tissue culture to study the cytopathic effect (CPE) of viruse as reported by Zhang and *Congleton (1994)* and Habashi and Saad *(2005).*

Polymerase Chain Reaction

The PCR was applied on the samples that proved to be +ve for S.V.C.V. with immunohistochemistry according to method implied by *Oreshkova et al. (1999).*

The steps of PCR were as following

Extraction of RNA from infected tissue extracts by using TRIZOL isolation kits. RNA was quantified and assessed for purity by measurement of OD260nm & OD280 nm using the U.V fiber-optic Spectrophotometer. RNA integrity was assessed by non-denaturing agarose gel-electrophoresis 2%. RNA was reverse-transcribed into cDNA samples & PCR was performed using ready to go RT-PCR beeds, Amersham-Bio-Sciences Kit, Primer 1 0.5 λ (25 picomol), Primer 2 0.5 λ (25 picomol), RNA 2 λ (2 δ), autoclaved H2O to 50 λ (47 λ) and R.T PCR program.

The five control beads included in the kit are packaged in red 0.5 ml microcentrifuge tubes. Each contains one room- temperature-stable bead containing 1 ng of rabbit globin mRNA and 8 pmol each of two globin- specific PCR primers. A control mix bead can be used to evaluate the performance of the RT-PCR Beads by adding the dehydrated control mix to a tube of RT-PCR Beads and performing RT-PCR.

The amplified product was visualized by electrophoresis using 10 – 20 % of total DNA in 2 % agarose gel and followed by staining with etidium bromide.

RESULTS

Clinical signs

The clinical signs and post-mortem lesions of naturally infected common carp and silver carp included general infection as nervous and respiratory manifestation, skin darkening, tail and fin rot, ulceration, hemorrhage on the abdomen, ascitis and redness of isthmus and head region. On the other hand, postmortem lesions included congestion of all internal organs, distended gall bladder, hemorrhagic gas bladder, exophthalmia and swelling of anal opening (Fig 1, 2, 3, 4, 5, 6 and 7). Moreover, the mortality rate was about 5% in the infected farm.

Bacteriological, mycological and parasitological investigation

The results of bacteriological, mycological and parasitological examination proved to be negative in all examined fish.

Electron microscope examination

From 20 fish samples examined (10 Common carp and 10 Silver carp), 18 samples (one Common carp and 17 Silver carp) contained virions viral particles; the helecks (bullet) shape which indicates the occurrence of SVCV. Moreover, the ultra-changes in the liver included vacuolated cytoplasm with lacking of organelles and undifferentiated nuclear inclusion resembling the virus-like particles. The viral particles replaced the cytoplasmic organelles. The Euchromatine-like intranuclear

inclusion has electron dense particles with clear halo (Core). Both virus particle aggregates in the nucleus and nuclear membrane can be identified by the presence of nucleocapside or envelope (Virions).

The kidneys showed vacuolated nucleus with dark dense body of virion attached to nuclear envelops and vesicles surrounding virion with virus like particle. Also brain showed intranuclear inclusion body with absence of myaline sheath; with high magnification there was capside particle with (visible core and homogenous electron dense and empty capside) especially in mixed infection. Large quantities of virus nucleocapsids with matrix viral precursors materials were found. In the brain, the tunica granulosa cell nuclei forming intra-nuclear inclusion bodies and vacuolated cytoplasm were recognized. The viral particles replaced the cytoplasm organelles, whereas the myline sheaths were absent (Figs. 8, 9 and 10). The air bladder of infected fishes contained intranuclear inclusion bodies with viral nucleocapsids.

Immunohistochemistry

Immunohistochemical examination of anti-SVCV (anti-Rh.C.) antibodies monoclonal was used on paraffin section of different organs of silver carp and common carp. The degree of infection intensity was recorded according to the positively degree, as follows: –ve, negative; ++ve, moderate; +++ve, marked; ++++ve, strong and +++++ve, intense.

In the liver, the hepatopancreatic cells exhibited the main common features which represent marked positive signs of anti-SVCV detection in all exocrine pancreatic cells. Meanwhile, the main characteristics of the virus detected by immunohistochemical of anti-virus SVC. anti-body was observed in the kidney of silver carp and common carp. The proximal tubular cells have a large amount of virus precipitate. On the other hand, there was a moderate to mark positively of anti-virus infection detected in the epithelial cells and sub-mucosa of the air bladders (marked in the epithelial cell and moderate in the sub-mucosa). The brain

showed strong positive reaction in granulosa cell membrane. In the mean time, the intense positively was found in the nerve fibers. In the muscles, the immunohistochemical detection of SVC antigen virus illustrated marked positive brown granulation in most of the myofibriler cells, and also in most of the cloudy swelling muscle fibers (Figs. 11 and 12).

Cell line formation and Cytopathic effect (CPE) due to viral infection

The ovarian-cultured cells formed a monolayer sheet, 48 hr post-incubation. The cells were spindle-shaped, with oval nucleus. The number of cells increased vigorously and a confluent monolayer sheet was formed within 48- 72 hr (Fig. 13)

The Cenotaphic effect (CPE) occurred within 3-4 days post infection by fish suspension, where cells were changed from spindle-shape to round shape. They were then detached to form plaque like structure, leading to death and detach of these cells. The degree and severity of CPE increased with increasing the concentration of the virus and decreasing time for the virus to cause CPE, and vice versa (Fig. 14).

Histopathological changes in infected fishes

The histopathological changes induced through natural infection of both common carp and silver carp by the isolated viruses (SVCV) were similar. These changes included vacuolar degeneration and necrosis of hepatic cells, congestion of the liver sinusoids and portal tract, vacuolated cytoplasm and appearance of nuclei of hepatopancreatic cells in the liver. In the kidney, degenerative haematopiotic tissue, necrosis and fibrosis were recognized in renal tubules and glomeruli. The brain showed dissolution of neural substance through the loss of stainable substances within the cytoplasm and chromatolysis. The nuclei were shifted to eccentric position against the margin of the cells. Severe congested blood vessels were found around distrusted gas bladder. Moreover, the intestine showed mild inflammatory reaction and hemorrhage with edema of submucosa. Muscular tissues showed hyaline degeneration and cloudy swelling of muscular

fibers, while the basophilic nuclei deposit between disappeared myofibrilar striation and the myofibrile exhibited pale eosinophilic color (Figs. 15, 16 and 17).

Results of polymerase chain reaction

The results of RNA concentration were measured by gel electrophoresis and compared with Key of known DNA. The results of RT-PCR expressed in Fig. (18), in which we made transformation of RNA to DNA forming cDNA to make PCR to express the level of band and its diameter and size according to the amount of virus in sample suspension.

Moreover, M-Gene was detected by gell-electropherosis as A-470 base pair, also the results of RNA extraction was detected in all examined samples by viral M-Gene and no band detected –ve with CTR lane.

DISCUSSION

The present study revealed that, the clinical signs and post-mortem lesions in common carp and silver carp during disease outbreak appeared in the form of congestion, ulceration in gill cover and abdomen and redness of the tail, fins and mouth. Infected fishes exhibited hemorrhagic spots on the eye, with clear exophthalmia, inflammation of the anal opening, erosion, gill paleness and skin darkening. The darkening of the skin may have been due to that the viruses have stimulated the spleen melanocytes, leading to the increase in melanin pigment secretions, which are generally deposited under the skin, causing the darkening of the body surface (*Post, 1987*).

Postmortem lesions in the form of redness, hemorrhage of gas bladder and congestion of all internal viscera, the nervous manifestations which appeared in the infected fish may have been due to the destructive effect of the viruses on internal organs, especially brain, as has been reported by *Saad (2005)*.

Tissue culture inoculation confirmed the presence of the virus by appearance of cellular vacuolation appeared in the ovary cell culture within 3–4 days post-infection by samples of the infected common carp *(Bucke and Finlay, 1979)*.

The electron microscope (EM) is considered as an important tool for characterization and diagnosis of SVCV, as suggested by *Bekesi* and *Szabo* (*1979*). In support, *Bucke* and *Finlay* (*1979*) reported that, by using Electron microscope, the virus particles in common carp were identified as extra-cellular, bullet-shaped bodies with a diameter of approximately 70 nm. In the present study, EM examination revealed that the liver was characterized by vacuolated cytoplasm, undifferentiated nuclear inclusion-like viral particles transmitted from the nucleus to the cytoplasm. Therefore, EM examination confirmed the occurrence of SVCV infection in silver carp and common carp.

The presence of both electron dense inclusions and clear halo (core), which may indicate the presence of SVCV, as appeared in infected tissues, and bullet shape of rhabdovirus may suggest the outbreak of carps in the present study may have been caused by viral infection. However,

The severity of infection and concentration of SVCV in the different tissues of infected fishes in the present study, as indicated from the immunohistochemistry studies, agreed with the results of *Clerx et al. (1978) and Saad (2008).* Those authors found that the brown labeling granules were depended mainly on the level of antigen antibody reaction (SVCV antigen reacts with the SVCV antibodies). They attributed this to the destructive action of the virus,

The respiratory manifestations attributed to SVCV affecting gills and causing paleness of the gills and destruction of the gill lamellae *(Post, 1984).*

Meanwhile, the other PM lesions may be attributed to the effect of virus on inhibition of protein and DNA synthesis as well as destruction of the cells of the internal organs. *(Bjorklund et al., 1997).*

Also congestion of internal organs may be due to that, the SVCV are intra-cellular microorganism and mainly affecting the tunica intema of blood vessels leading to oozing of blood out side the blood vessels causing hemorrhagic appearance and petechial hemorrhage. Moreover, the distance between the cells

of blood vessels was increased leading to congestion, ascitis, exophthalmia and hemorrhage in all internal organs especially haemopiotic organs. *(Post, 1984).*

Moreover, *Ahne et al (2002) and Hoffmann et al. (2002)* reported that the clinical signs of the virus infection are external and internal hemorrhages, peritonitis and ascitis. They also stated that affected fish with SVCV showed destruction of tissues in the kidney, spleen and liver, leading to hemorrhage, loss of water-salt balance and impairment of immune response.

Histopathological changes in different organs in the form of pyknosis with atrophy of hepatic cells and decreasing of eosinophilic granules of cytoplasm. Moreover, there was vacuolar degeneration with red color cellular content which indicate virus infection in the liver. The degeneration of haemopiotic tissues and necrosis of renal tubules in kidneys as well as congestion of blood vessels in air sacs may be attributed to the destructive effect of the virus *Way et al (2003)*

From the results it can be concluded that the clinical signs and mortality may be due to viral infection which recorded through electron microscope and immuno-histochemistry.

Polymerase Chain Reaction (PCR).

In order to isolat Spring viraemia of carp virus RNA. DNA probes have been constructed using reverse transcription-PCR amplification technique and cDNA cloning in plasmid and phage vectors used to isolate the viral RNA. The results of RT-PCR of 6 viruses through 2 % agarose gel then staining with ethidium bromide were correlated positively with the RNA level.

The sensitivity and specificity of viral RNA detection was assessed non-radioactive probes in infected cell culture and in tissues from dead fish. Viral RNA was more frequently detected in the brain and gills than in the abdominal organs *(Oreshkova et al.1996).*

According to *Oreshkova et al. (1996)* method we performed PCR technique as the, RNA concentration measured by spectrophetometer at wave length 260 & 280 the results of PCR. The present result showed that: Silver carp have highest RNA concentration than Common carp and the severity of the disease related to the amount of virus RNA in samples was gradually increased.

The M-Gene were designed according to *(Kiuchi & Roy, 1984)*, have bands of M. gene:

(M1 5'- CATGTCTACTCTAAGAAAGC) (M2 5'-ACCGAATCGATGAGGCACAT)

The present work detected by gell electrophoresis at 2: 4 % revealed the expression of the viral M. gene was positive detection in all examined samples, and no band was detected negative with CTR lane. Also its concentration was 47٦ bp. So that the results were confirmed that SVCV was given the same bands of M. gene.

Moreover, to the best knowledge of the authors it is the first record of spring viremia among cultured carp in Egypt.

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Legend of the Figures

Fig (1). Naturally infected common carp showing congestion of head region and eyes.

Fig (2). Naturally infected common carp showing hemorrhagic gas bladder surface.

Fig (3). Naturally infected common carp showing sever brain congestion.

Fig (4). Naturally infected silver carp showing congestion and uni-lateral exophthalmia.

Fig (5). Silver carp naturally infected showing slight congestion of all internal organs especially gas bladder.

Fig (6). Normal and naturally infected brains of silver carp. Infected brains show congestion due to viral infection.

Fig.(7). Silver carp gas-bladder showing congestion particularly in the posterior chamber.

Fig.(8). Electron micrograph of silver carp kidneys showing rounded form (x) and helices form of SVCV, indicating viral infection. (Formalin+ PTAX40,000).

Fig. (9). Electron micrograph of common carp liver, with high magnification showing the intranuclear inclusion bodies. The virus like particles replaced the chromatin and nuclear matrix, most virion are encapsulated electron dense particle with clear core(x). (Formalin+ PTAX40, 000).

Fig. (10). Electron micrograph of common carp brain, with para-crystalline of virus like particles in the cytoplasm).(PTAX 20,000).

Fig. (11). Paraffin section of air-sacs of common carp, naturally infected with virus, showing marked positive anti-SVCV in both layer of gas bladder. (DAB X 400).

Fig. (12). Paraffin section of silver carp brain, naturally infected with virus, showing strong positive of anti-SVCV in granulosa membrane and

nuclei (arrows) as well of blood capillary and nerve connective tissue. (X) (DAB X 400).

Fig. (13). Normal cell line after 72 hrs post-incubation.

Fig. (14). Severe degree of CPE due to injection of mixed virus with titer (10^{-1}) typified by rounding, detached and dead cells with clear plaque formation.

Fig. (15). Gills of naturally infected silver carp showing marked swelling of a primary filament with goblet cells hyperplasia. The neighboring filament was necrotic. H, E. (X 250).

Fig. (16). Kidney of naturally infected common carp showing severe hyaline droplet degeneration of some convoluted tubules with high eosinophilic infiltration.

Fig. (17). Hepatopancreas of naturally infected common carp showing diffuse hydropic degeneration of the hepatocytes. H, E. (X 160).

Fig. (18): The result of RT-PCR of 6 positive sample through 2 % agarose gel and stained with ethidium bromide.

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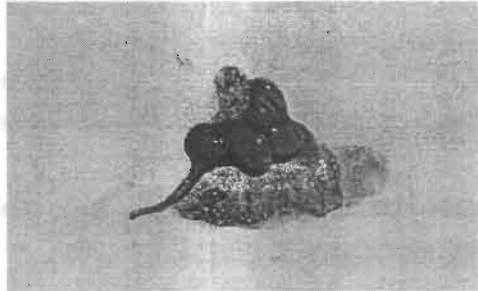

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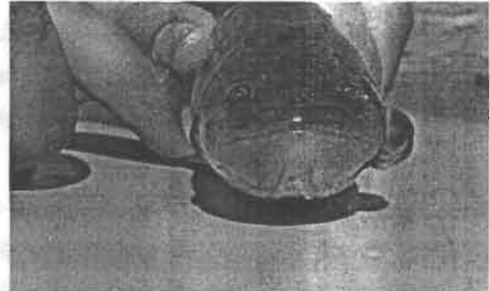

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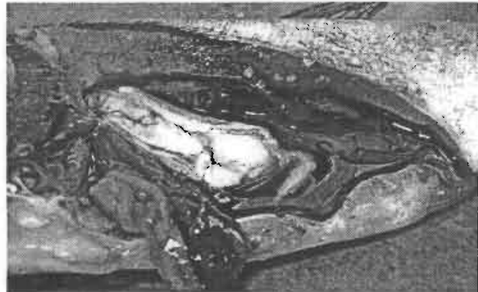

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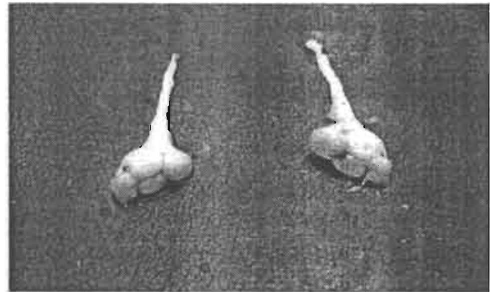

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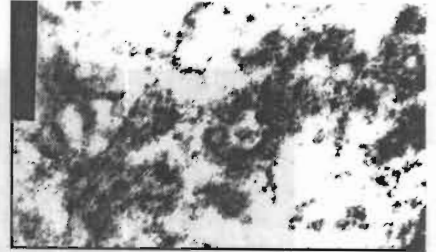

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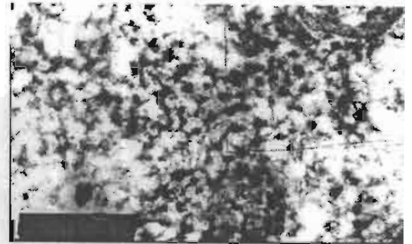

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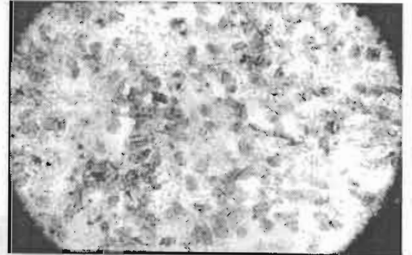

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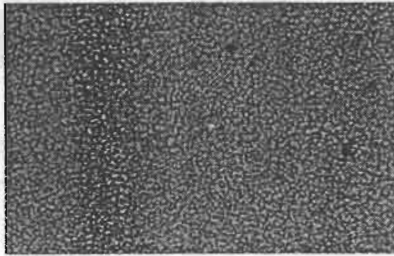

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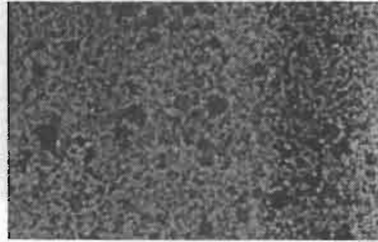

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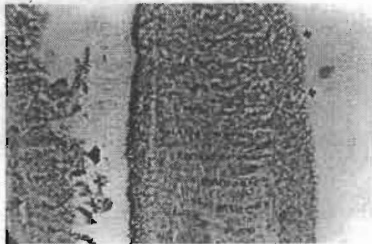

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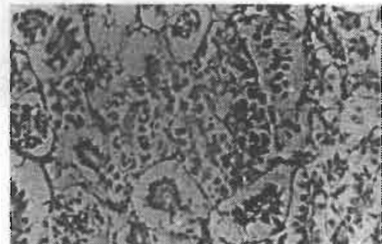

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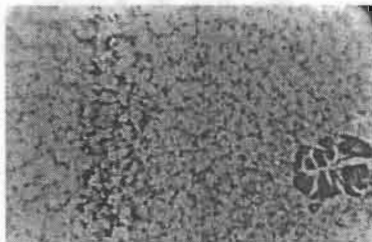

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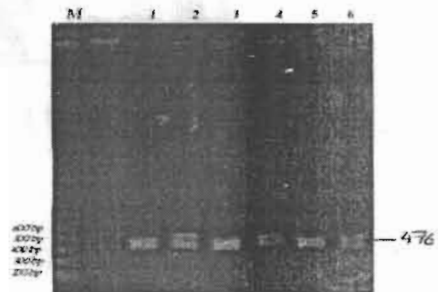

(18)

عزل وتمييز حمى الربيع الفيروسى من أسماك المبروك الفضى العادى فى منطقة كفر الشيخ

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فى هذه الدراسة تم عزل حمى الربيع الفيروسى من أسماك المبروك الفضى والعادى المستزرع فى مزارع خاصة فى منطقة كفر الشيخ تم تمييز شكل الفيروس من خلال الميكروسكوب الإلكترونى أيضاً باستخدام اختبار كيمياء الأنسجة المناعى مستخدمين بها أجسام مضادة للفيروس. أيضاً الأعراض الاكلينيكية والتى تتميز بالاحمرار فى منطقة البطن وحول الرأس والاحتقانات فى الأعضاء الداخلية وخصوصاً المثانة الهوائية .هذه النتائج الموضحة تدل على وجود الإصابة بالفيروس والتى كان لها الأثر الأكبر في حدوث النفوق بين أسماك المبروك.

أيضا تم تأكيد الاصابة من خلال الفحص بالميكروسكوب الالكترونى ، وكانت النتائج واضحة فى صورة شكل الرصاصة أو حدوة الحصان وهو الشكل المميز لحمى الربيع الفيروسى أو الشكل الحلقى المتشابك. أيضاً شكل السيتوبلازم والنواة الذى يتميز باللون الأحمر الداكن والذى يشير إلى وجود فيروسات من أنواع الـــ RNA .

هذه الدراسة أيضا توضح العدوى بحمى الربيع الفيروسى من أسماك المبروك الفضى والعادى المستزرع كأحد أنواع فيروسات الـــ RNA والتي تم تمييزها لأول مرة فى مصر فى منطقة كفر الشيخ .