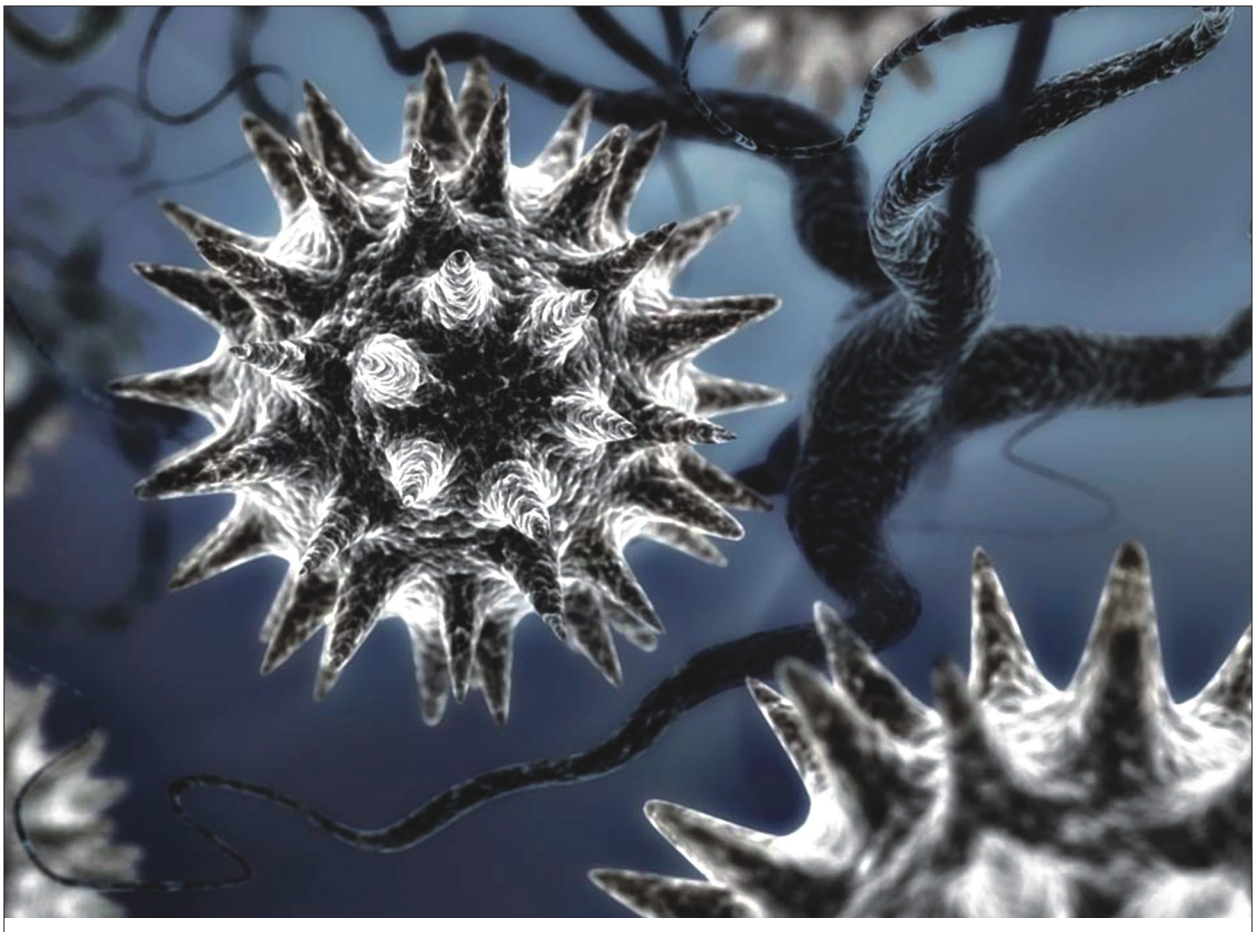

International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS International Journal of Virology

ISSN 1816-4900
DOI: 10.3923/ijv.2021.1.7

Research Article
Viral Investigation in the Mass Mortality Phenomenon Occurred During Summer Season in Cultured Tilapia Fish

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Abstract

Background and Objective: Nile tilapia is the main cultured species in Egypt. Mass mortalities in cultured freshwater fishes especially Nile tilapia assumed as an annual outbreak in last ten years during summer period. The aim of the present study was viral investigation of devastating mortality. **Materials and Methods:** Samples were collected from different semi-intensive polyculture fish farm. The average body weight of affected fish ranged from 200-300 g. The most common lesion by external examination was a red circle on one or both fish sides. The common postmortem lesion was hypertrophy with necrotic areas distributed all over the liver surface. The collected prepared samples were gone through three blind passages using an established cell line. **Results:** Cytopathic changes were showed in a cell that's include cells rounding, aggregation in the scattered area which was clear in between the 5th and 7th days post inoculation. Transmission electron microscopy visualization of cell line harvest revealed presence of virus-like particles, enveloped icosahedrons measuring 60-70 nm in diameter. **Conclusion:** It can be concluded that virus-like particles present in summer mortality outbreaks in Nile tilapia in Egypt may be one of the cause. Further studies are needed to identify the isolate and other causes of these outbreaks.

Key words: Tilapia, cytopathic, virus, mortality, polyculture fish and hypertrophy

Citation: Ammar, A.A., S.F. Sakr, A.M. Abdel Wahab, A.A. Ahmed, M.M. Eladwey and T.M. Nageeb, 2021. Viral investigation in the mass mortality phenomenon occurred during summer season in cultured tilapia fish. Int. J. Virol., 17: 1-7.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aquaculture has an important role in the development of many national economies, plays a key role in rural development and an important role in meeting high demand for aquatic animal production. The sharp increase in cost of animal protein have been focused the attention of government and fishermen toward the development of fish aquaculture. This is because its relative cheap protein source with good digestibility and high nutritive values one of the clear solutions of food problems for human beings[1].

The aquaculture industry in the world has been gradually developed as well as in Egypt, intensive fish farms, hatcheries and fish cages. The success in aquaculture industry depends on the selection of rearing and realizing the relationship between fishes, their environment and pathogens. Fish intensive culture is continually affected by environmental changes and bad management practices such as transportation, handling, crowding, therapeutic materials, fluctuating temperature and deterioration of chemistry. All of these factors will predispose the fishes to infectious diseases[2]. Nile tilapia is the main cultured fish in Egypt[3].

The disease has become a primary constraint to aquaculture growth and responsible for the severe impact on the economic development in many countries. Aquaculture losses directly threaten the livelihoods of whole communities through a reduction in food availability, loss of income and employment with all the associated social consequences as serious public health threat associated with commercially raised disease fish[4].

Mass mortalities in cultured freshwater fishes especially Nile tilapia (*Oreochomus niluticus*) assumed as an annual outbreak in last ten years during the period starting from May-August and even extending up to October in some governorate in Egypt. Mortalities from such disease could be routinely both in the wild and aquaculture ponds but they would not likely attract attention unless they reach 30 till 50% of total production leads to some farms to leave this sector[5].

Many diagnostic laboratories identified various pathogens representing different enzootic diseases for such fish mortalities. As Ahmed *et al.*[6] recorded mass mortality in cultured Nile tilapia *Oreochromis niloticus* in Kafr El-Sheikh Province, Egypt due to *Saprolegniosis*. Elsheshtawy *et al.*[7] cited investigation of Nile tilapia Summer Mortality revealed that the dominant bacteria were Gram-negative rods and identified as *Aeromonas* sp. Fathi *et al.*[8] recorded tilapia lake virus in Egypt in Nile tilapia affected by 'summer mortality' syndrome. Saad *et al.*[9], showed the main important economic diseases, *Saprolegniosis*, *Aeromonas*, parasitic and mycotoxins in Behera and Kafer El-Sheikh province.

The aim of this study was to study the viral investigation in summer mass mortality phenomenon of tilapia fish.

MATERIALS AND METHODS

Fish samples: A total of 100 live Nile tilapia fish, exhibiting septicemia signs, with the average weight ranging between 200 and 300 g were randomly collected from different semi-intensive polyculture fish farms with a history of mortalities in Kafr El-Sheikh, Husseiniya, El Behera and Port Said during summer season of 2017. The collected samples were transmitted in an icebox to a fine lab. of central laboratory for aquaculture research, Abbassa, Egypt. The samples were prepared for virus isolation and detection.

Clinical investigation: Clinical examination of affected fish was done out as described by Amlacher[10] determine the clinical alteration on the skin, scales, eyes, abdomen, tail and fins. Also, fish behavior was examined before sampling.

Postmortem examination: The post mortem was done on living and freshly dead fishes to examine all internal organs including gills, spleen, kidney, liver and intestine for detection the abnormalities. The examination was done according to Amlacher[10].

Semi-thin sections examination for determined the target samples: Fixation is accomplished by perfusion of the whole fish with alcohol formalin followed by immersion of pieces to be examined in neutralized osmium tetroxide. The embedding medium is a mixture of equal parts of n-butyl and ethyl methacrylate polymerized by ultra-violet light. Sectioning is done by means of a glass knife on an International ultra-thin sectioning microtome set at 0.1 μ. The sections are floated on warm water to spread, then placed on carbon coated grids, dried and put into toluene to dissolve the plastic[11].

Sample preparation: The brains and viscera (kidneys, livers, spleens and hearts) of the suspected fish were removed aseptically, pooled and manually homogenized with nine volumes of Hanks' balanced salt solution. The homogenates were centrifuged at 1700 g for 10 min at 4°C to pellet cellular debris. The supernatant solution was further clarified by two rounds of centrifugation at 5000 g for 10 min at 4°C followed by two rounds of centrifugation at 13000 g for 10 min at 4°C[12].

Establishment of primary tissue culture of Nile tilapia: Healthy *Oerochromus niloticus* (50-60 g in weight) were collected from local fish farms of Central Lab. for Aquaculture

Research, Abbassa and Agriculture Research Center (CLAR). The fish were anaesthetized by adding 2 mL of the clove oil solution (9 parts ethanol+1 part clove oil) in 5 L of water containing fish. The disinfection of surface of the fish was carried out by wiping the fish in 70% (v/v) ethanol. Liver tissues were removed aseptically and washed four times in phosphate buffer saline containing antibiotics (500 IU mL^{-1} penicillin, 500 µg mL^{-1}. streptomycin and 2.5 µg mL^{-1} amphotericin B) before preparing explants. Liver tissues were minced into small pieces (approximately 1 mm^3 in size) and washed four times in Modified Eagle's Minimum Essential Medium (EMEM) with Earl's balanced salt solution and L. glutamine medium (Sigma, cat. No. M. 4642, St. Louis, USA)[13].

Subculture and maintenance: At when cells reaching to 95% confluence The cells were harvested with 0.25% trypsin-EDTA (trypsin 0.25%, EDTA 0.02%) solution (Gibco, Carlsbad, CA, USA) and seeded at 1:3 ratio and maintained in the complete L-15 medium with 15% FBS and antibiotics. After 3rd subculture, the concentration of FBS in medium was reduced to 10% (L-15-10) and antibiotics and antimycotic were reduced to the normal concentrations of 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.25 µg mL^{-1} amphotericin B[14].

Trials of virus isolation on established cell line: Stoked cells in 50 mL disposable plastic prescription flasks were examined for quality and confluent monolayer sheet. The cells were trypsinized and incubated at 28°C for 10 min then shacked well to obtain separated individual cells. The growth media was added to the cell suspension, then distributed into 24 well-flat bottom tissue culture plates by 500 µL of cells suspension. The plates were sealed with pressure sensitive adhesive cellulose tape and incubated at 28°C for 24 hrs. After examination the plates under the inverted microscope for the formation of about 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 28°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 per well). The tissue culture plates were incubated at 28°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 product. The harvest was prepared for 3 successive blind passages by the same way[15].

Electron microscopy (EM) examination: Glass slider and grids were cleaned using 70% ethyl alcohol and distilled water several times to remove any contamination. The sodium chloride films were thermally evaporated from rock salt crystals into the glass slide. High transparent, homogeneous, mechanical and thermally stable carbon films were evaporated on the top of sodium chloride films. The grids now, have carbon coded side and shinny side. The harvested from the cell lines was centrifuged at 3000 rpm 5 min, one drop from the supernatant from each harvest took and put on clean glass slide. The grids were picked by using thin forcipes and put the carbon coded side on the drop for 1 min. The grids put on clean filter paper for removing the excess solution (the shinny side facing the filter paper) and left for 20 min till complete dryness. Drops from 2% uranyl acetate stain put on clean grooved waxy plate and the grids put on the stain for 3 min (the carbon coded side facing the stain). The grids put on labeled clean filter paper for removing the excess stain (the shinny side facing the filter paper) and left for 1 hr till complete dryness. Then fixed on the electron microscope holder and examined by transmission electron microscope[16].

RESULTS

Felid signs: The average body weight of affected fish ranged from 200-300 g. External examination of affected fish showed pale in color while, others were had hemorrhagic areas on the body surfaces. The most common lesion was red circle on one or both fish sides (Fig. 1a-b).

Internal abnormality: The post mortem was done on living and freshly dead fishes to examine all internal organs including gills, spleen, kidney, liver and intestine for detection the abnormalities.

The common symptom in the affected fish was the liver which showed hypertrophy with necrotic areas distributed all over liver surface (Fig. 2a-b).

Histo-pathological investigation: A technique is described for obtaining thin sections of fish tissue help in the investigation the predilection tissues for virus diagnosis. Muscles, liver and kidney were the main organs of investigation. The muscles were showed lacerated muscle fiber in many places of musculature (Fig. 3), multiple necrotic foci in the liver (Fig. 4) and many degenerated areas in the kidney (Fig. 5).

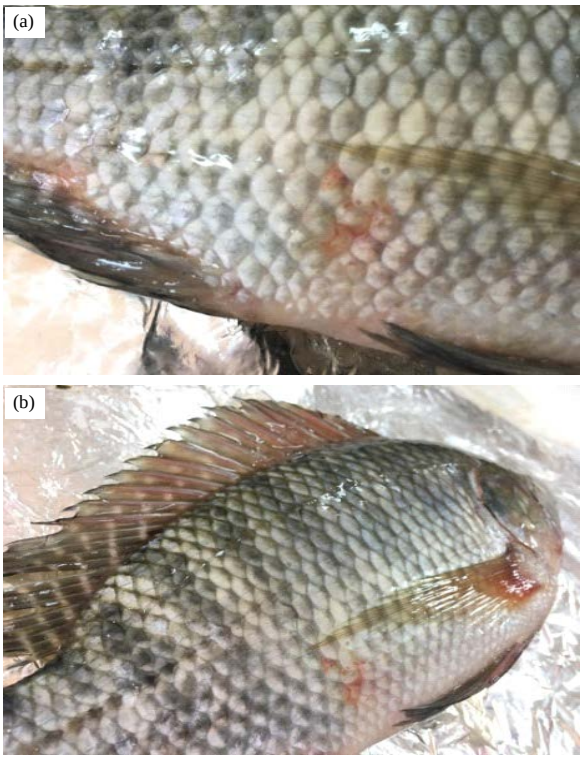

Fig. 1(a-b): Red circle on fish sides

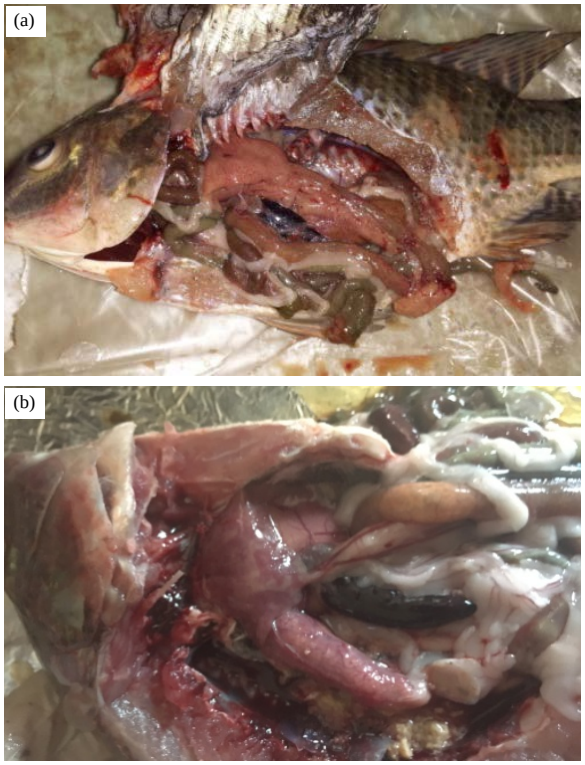

Fig. 2(a-b): Liver showed hypertrophy with necrotic areas distributed all over surface

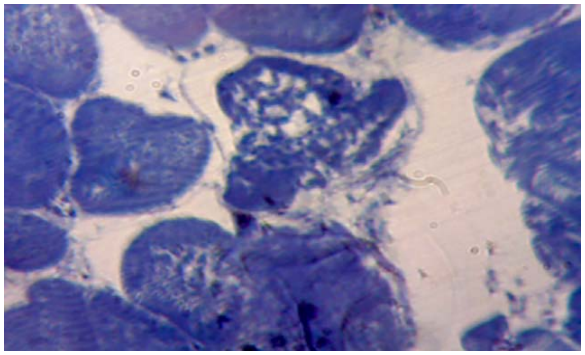

Fig. 3: Muscles showed lacerated muscle fiber

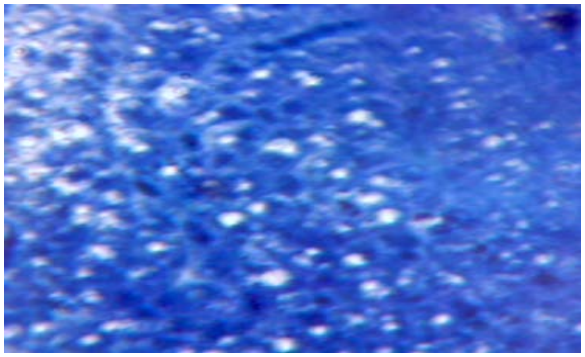

Fig. 4: Multiple necrotic foci in the liver

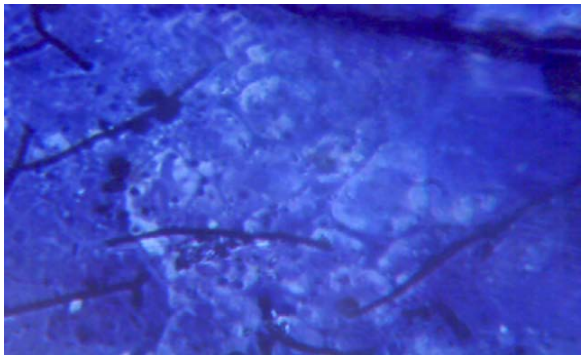

Fig. 5: Many degenerated areas in the kidney

Primary tissue culture formation: The hepatocytes showed typical sequence of events observed in most fish liver cells. Initially, single cell suspensions seeded uniformly in primary tissue culture plates were distributed uniformly and thereafter, some cells began to clump-forming small aggregates, which were loosely held together within the first day in culture. The observations of attached monolayer cells spreading were made after the third day and most cells, except cell in aggregates, were visibly spread with membrane invaginations forming spindle-like morphologies by the seventh day (Fig. 6a-b).

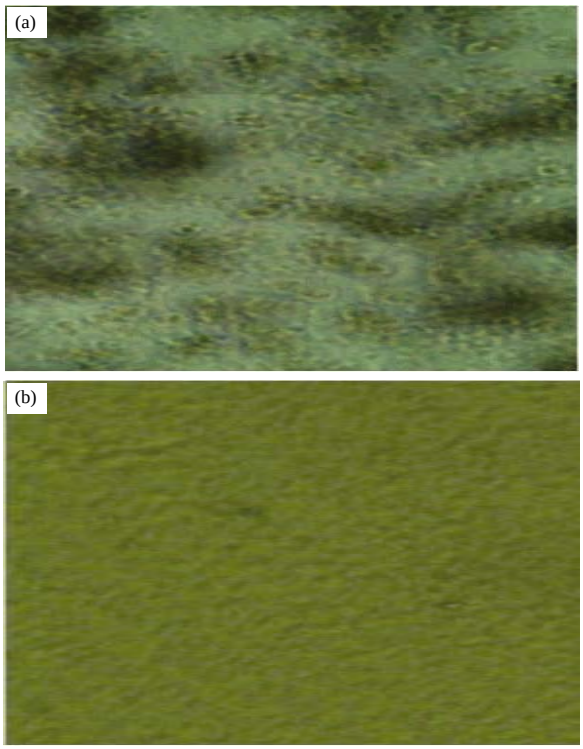

Fig. 6(a-b): Cell aggregate formed monolayer sheet

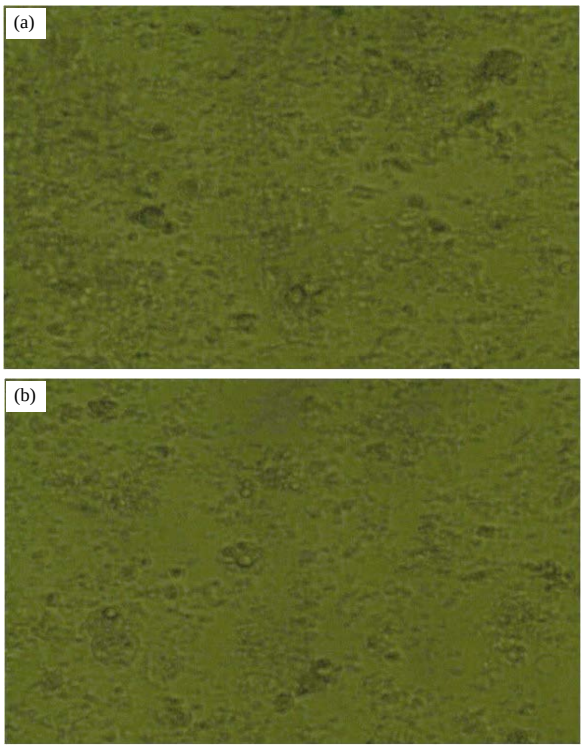

Fig. 7(a-b): Cells rounding, aggregation in scattered area of the monolayer followed by cellular darkness and clusters formation

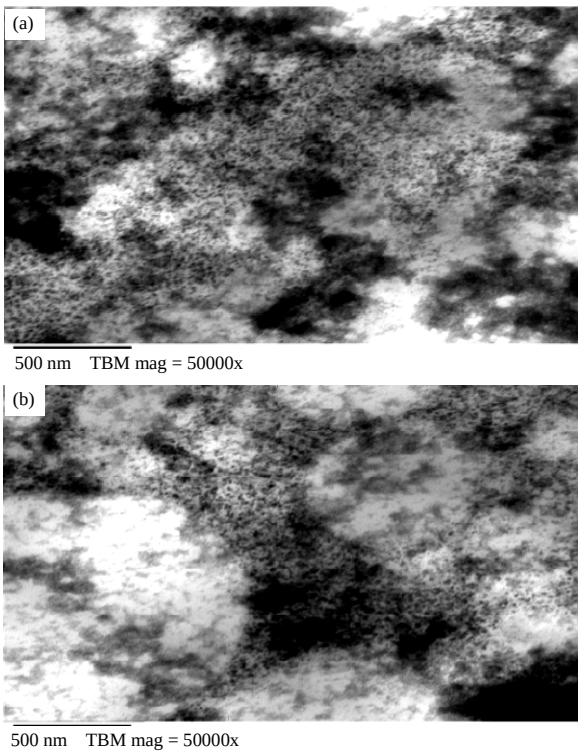

Fig. 8(a-b): Virus-like particles, enveloped icosahedrons measuring 60-70 nm in diameter

Tissue culture rearing: With regular subculture the morphologies of the hepatocytes in monolayer exhibited extended membrane elongations with the entire cell structures spread out. Aggregation of hepatocytes was observed with loosely organized cell-cell clumps, which could be dislodged easily into single cell suspension. However, the cell aggregates observed after 3rd-7th subculturing which were more stable and relatively harder to dislodge into single cell suspension.

Cell line inoculation: The collected prepared samples were gone through three blind passages using tissue culture plates. The inoculated plates were examined daily for recording the cytopathic changes (CPE) in cells. Out of 100 inoculated samples 25 samples from Husseiniya and Kafr El-Sheikh showed CPE that's include cells rounding, aggregation in scattered area of the monolayer followed by cellular darkness and clusters formation. The CPE was clear in between the 5th and 7th days post inoculation (Fig. 7a-b).

Cell line harvest examination: Transmission electron microscopy visualization of centrifuged cell line harvest revealed presence of virus-like particles, enveloped icosahedrons measuring 60-70 nm in diameter (Fig. 8a-b).

DISCUSSION

There have been numerous recent out breaks of tilapia diseases in Japan, Taiwan, Thailand and other Asian countries. However, cultured Egyptian tilapia has faced higher mortality rates particularly during summer months in recent years. Several attempts have been made to identify the causative agents of these outbreaks[17]. The present study was carried out to investigate viral causative agent of fish mortalities in tilapia farms in Kafr El-Sheikh, Husseiniya, El Behera and Port Said, Egypt.

The observed clinical signs of infected Nile tilapia resample to the general signs of septicemic diseases similar to those reported by Dahdouh *et al.*[18]. These gross lesions including pale fish, loss of orientation, exophthalmia, heavy mucous secretion on fish surfaces, cornel opacity and hemorrhagic areas around the mouth, vent and margins of the pectoral fins. The most common lesion was red circle on one or both fish sides (Fig. 1).

Moreover, the postmortem examination revealed that the affected Nile tilapia agreement with the findings of Omar *et al.*[19] which was enlarged of internal organs especially liver, spleen and kidneys. The most signs were enlarged liver with necrotic area (Fig. 2).

The histopathology examination of infected lesions is distinctive and can be used as guide for specific sample collection via it can be known the virus tropism and so, used as diagnostic support for the virus isolation. Semi-thin sections examination of muscle, liver and kidney were the main organs of investigation. The muscles were showed lacerated fiber in many places of musculature tissue (Fig. 3), multiple necrotic foci in the liver (Fig. 4) and many degenerated areas in the kidney (Fig. 5). So that, samples from kidneys, livers and muscles were the main target samples that were manually homogenized, centrifuged, clarified and prepared for virus isolation[12].

The development of cell lines would be valuable for isolation of the virus in any disease outbreaks in fish and also for studying species-specific responses of the viruses at the cellular level. Primary hepatocytes culture was developed from the liver of the Nile tilapia. Many cell lines have been established from freshwater, marine and brackish water finfish around the world. Several continuous cell lines have been developed using the expellant procedure, which has many advantages over the use of cell suspensions speed, ease and maintenance of cell interactions and the avoidance of enzymatic digestion which can damage the cell surface but, it was difficult to get tissue fragments to adhere with the plate surface[13].

A continuous cell line, from the tilapia liver was successfully established. The cells, similar to other tropical teleost derived cell lines, proliferated at 28°C dependent on FBS concentration. The cells initiated similar to fibroblastic reticular cells and exhibited rounded attached with each other similar that was recorded by Wen *et al.*[20]. The collected prepared samples were gone three blind passages using the established cell line plates. The inoculated plates were showed the cytopathic changes in cells that's include cells rounding, aggregation in scattered area of the monolayer followed by cellular darkness and clusters formation (Fig. 7).

Electron microscopes have a greater resolving power than a light-powered optical microscope, because electrons have wavelengths about 100,000 times shorter than visible light and can achieve better than 50 pm resolution and magnifications of up to about 10,000,000 x, whereas ordinary, non-confocal light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x. Electron microscopes are used to observe a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals and crystals[21].

Basic isolation and preliminary identification methods were followed for the identification of the causative agent of the mortalities. Transmission electron microscopy visualization of centrifuged cell line harvest was used for this propose which revealed presence of virus-like particles, enveloped icosahedrons measuring 60-70 nm in diameter (Fig. 8) that was decided presence of virus infection in affected fish specially in that samples collected from Husseiniya and Kafr El-Sheikh.

SIGNIFICANCE STATEMENT

The virus may be considered responsible about the summer mortality of Nile tilapia in Egypt. Further studies are needed to identify the isolate and other causes of these outbreaks.

This study discovers virus in summer mortality of Nile tilapia in Egypt that can be beneficial for the researcher to uncover the critical areas of that phenomenon that many researchers were not able to explore it.

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