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Subcellular degenerative changes in hepatopancreas and posterior kidney of *Streptococcus iniae* infected Nile tilapia using Transmission Electron Microscope

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ABSTRACT

Streptococcosis became one of the most dangerous bacterial diseases affects cultured marine and freshwater fish. In the present study *Streptococcus iniae* was isolated from diseased Nile tilapia (*Oreochromis niloticus*) with investigation of the clinical signs, post mortem lesions and focusing on the histopathological changes in liver and kidney using the light and Transmission Electron Microscope (TEM). Natural infected *O. niloticus* showed ascites, exophthalmia, presence of hemorrhagic patches all over the external body surface with inflammation and swelling of the anal opening. Post-mortem examination indicated presence of hepatomegaly, splenomegaly, congestion and swelling of posterior kidney. Four *Streptococcus iniae* isolates were recovered and identified using PCR from the examined fish. The $LD_{50\text{-}96h}$ of the recovered isolates in experimentally infected *Oreochromis niloticus* were ranged between 0.2 ml of $2.62 – 3 \times 10^7$ CFU/ml by intraperitoneal injection. Under the light microscopical examination, severe degenerative changes were present in the hepatopancreas including diffused hepatic cell vacuolation and necrosis, leukocytic infiltration, the posterior kidney tissue showed vacuolation of tubular epithelial cells, shrinkage of glomerular taught and increasing the Bowman's space. TEM examination of hepatopancreas revealed the presence of hepatocellular degeneration, increased vacuolation in the different areas of the cytoplasm, loss of endoplasmic reticulum contact in several areas. Kidney tissues showed numerous vacuolation, swollen of cuboidal epithelial cell with absence of brush border in the apical portion of the proximal convoluted tubules, nucleus and mitochondria were dispersed throughout the cytoplasm.

INTRODUCTION

Oreochromis niloticus is the second most important fish species in tropical and subtropical freshwater aquaculture (FAO, 2018), it also, considered the base of commercial fisheries in many African countries (Mohammed and Uraguchi, 2013). It tolerates different environmental conditions and feed on both formulated and natural feeds that makes it economically important (Adeyemi, 2009).

Streptococcus *iniae* was isolated for the first time from the subcutaneous abscesses of a captive Amazon freshwater dolphin (*Inia geoffrensis*) in 1976 in the United States by Peir and Madin (1976). Meanwhile, the first record of streptococci in fish was in Japan, from cultivated rainbow trout by Hoshina *et al*., (1958). Since then, many outbreaks have been reported in several other fish species (Inglis *et al*., 1993).

Streptococcus infection is a septicemic disease caused by many streptococcus species including *Streptococcus agalactiae*, *S. dysgalactiae* and *S. iniae* (Zamri-Saad *et al*., 2014). Many adverse environmental conditions considered predisposing to streptococcosis specially in susceptible fish species as low dissolved oxygen, low water quality, increase water temperature and high ammonia concentration (Francis-Floyd and Yanong, 2013).

Pop-eye disease is a synonym to fish streptococcosis, it defined as contagious disease causes high mortality among large sized fish with huge commercial loss in different countries as Australia, Italy, Japan, Korea, South Africa, Colombia, Indonesia and USA. (Austin and Austin, 2012; Anshary *et al*., 2014). The total losses induced by streptococcosis in farmed fish was estimated by 250 million USD in 2008 (Klesius *et al*., 2008). Acute form of streptococcal infection in *O. niloticus* has resulted in more than 50% mortalities during about 3 days to one week while the chronic form may extend for several weeks with low rate daily mortalities about one or two percent (Osman *et al*., 2017). The clinical signs of streptococcal infection are including hemorrhagic gills, hemorrhagic patches on the abdominal wall and clouding of the cornea with exophthalmia in infected *O. niloticus* in addition to the most prominent postmortem lesions were pale enlarged liver with hemorrhagic enlarged spleen and presence of thick ascetic fluid and sometimes tinged with blood in the abdominal cavity (Saleh *et al*., 2017b).

Transmission Electron Microscopy is an advanced viewing method that helps in the study of tissue microstructures, It is also, a highly useful equipment for studying and examining the structure of cells and studying the interaction between bacteria and host cells and visualization of the detail that is not presented by light microscopy (Graham and Orenstein, 2007; Saleh *et al*., 2017a).

The purpose of the present work is isolation and molecular identification of streptococci from diseased farms, determine their pathogenicity for *Oreochromis niloticus* and describe the histopathological changes affecting hepatopancreas and posterior kidney of experimentally infected fish with exploring the subcellular ultra-structures changes using TEM.

MATERIALS AND METHODS

Fish Samples: Thirty fish samples ranged between 80 – 250 g in body weight were collected from 3 infected Nile tilapia farms located at Elhox, Metoubes district, Kafrelsheikh governorate during July 2018. Each sample was packed in a separate clean plastic bag after washing with sterile phosphate buffer saline to decrease the contamination, samples were immediately stored in ice box just after collection then transported to fish diseases lab, National Institute of Oceanography and Fisheries, Alexandria.

Clinical examination: The clinical examination was performed according to the method described by Conroy and Hermann, (1981).

Post mortem examination: The post mortem examination was performed according to the method described by Austin and Austin, (2007).

Isolation and identification of the causative agent:

Tissue samples were taken from different organs as spleen, hepatopancreas and posterior kidney then were homogenized in one sample which is representative to the sampled fish according to the method described by Aboyadak *et al*., (2016 a). Primary cultivation was done by inoculating tryptic soy broth (TSB) from each sample followed by incubation for 48 hours at 37^{o}C. A loopful from each (TSB,

Oxoid, UK) tube was streaked on Tryptic soy agar (TSA), brain heart infusion agar (BHI) and blood agar plates then the streaked plates was incubated for 48 hours at 37 ^{o}C.

Preliminary identification streptococci:

Gram staining technique was done according to Black and Black, (2015), for preliminary identification of the recovered isolates and Gram positive isolates was further subjected to oxidase test and catalase as biochemical test for detection of streptococcus ones according to Cruickshank *et al*., (1982).

Definitive identification *Streptococcus iniae* isolates using PCR:

Genomic DNA extraction:

Presumptive bacterial isolates were cultivated overnight on tryptic soy broth, DNA extraction was done according to method described by Shambrook *et al*., (1989).

PCR mixture:

The PCR reaction was performed in a total volume of 25 µl, each consists 12.5 µl Master Mix [HotStarTaq Master Mix (Qiagen, Hilden, Germany), which contains 2.5 units of DNA polymerase per reaction, 200 µ. Mol. of each deoxynucleotide triphosphate (dATP, dGTP, dCTP and dTTP), 1× PCR buffer (with 1.5 mm-MgCl2)] + 1.25 µl of each oligonucleotide primer (20 pmol/µl) + 5 µl of extracted bacterial DNA + 12.5 µl of nuclease free double deionized distilled water.

Thermal cycler programing:

The Specific primer used in DNA amplification for *S.iniae* was listed in Table (1). DNA amplification were performed according to methods described by Zlotkin *et al*. (1998) for detection of specific sequences in the 16S rRNA. Amplification was carried out in Peltier thermal cycler Model: MG 96+ enzyme® USA, , briefly each cycle starts with initial denaturation for 5 min at 94 °C followed by 30 cycle of denaturation for 30 sec at 94 °C after that annealing at 50 °C for 30 sec then extension at 72 °C for 30 sec and ended with a final extension step at 72 °C, for 10 min.

PCR products assay:

Amplified PCR products were analyzed by electrophoresis using 1.5-2 % (W/V) purified agar Oxoid™, UK) dissolved in Tris borate EDTA buffer supplemented with ethidium bromide at a concentration of 0.5 µg/ml as described by Lee *et al*., (2012). 5 µl of PCR product mixed with 5X Gel loading dye was loaded to agar gel in Tris borate EDTA buffer (0.89 M tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0), a 100 bp DNA ladder **(**Intron Biotechnology, Inc, USA**)** was used as a molecular weight marker. Gels was run at 100 volt for 60-90 min then visualized by UV transilluminator (Winpact Scientific, USA).

Pathogenicity test:

It was conducted for determining the lethal dose fifty (LD_{50}) and confirming pathogenicity of the recovered *Streptococcus iniae* isolated for *Oreochromis niloticus,* pathogenicity test was performed according to the method described by El-Bahar *et al*., (2019). Briefly, 260 apparently healthy *O. niloticus* with average body weight 100 ± 10 g were randomly divided into 5 groups using 26 glass aquaria (10 fish per aquarium), each with 80 litter capacity. Each aquarium was filled with chlorine free tape water with 20% daily change. Continuous aeration and filtration was performed using specific aerator and filter, temperature was maintained at 26 ± 2 sing electrical heater, fish were acclimatized for 10 days. During the experiment fish were daily feed at a rate of 3% of body weight with commercial floating fish ration containing 25 % protein. Fish in group (1, two aquarium) were inoculated with 0.2

ml sterile normal saline and kept as control negative, each of other groups (2-5) were divided to 3 subgroups (a & b & c, in duplicates) subgroup a were intraperitoneal-inoculated with 0.2 ml using (3×10^7) CFU, subgroup b were inoculated with (3×10^6) CFU and subgroup c with (3×10^5) CFU from the challenged strain. Fish were observed daily for 7 days for appearance of clinical signs and mortalities, dead fish were considered only when *Streptococcus iniae* was re-isolated as and confirmed, the $LD_{50\text{-}96\ h.}$ was calculated according to Dias *et al*. (2016).

Histopathological examination:

Tissue specimens from hepatopancreas and posterior kidney were taken freshly from the experimentally infected fish. Tissue specimens were trimmed and fixed in 10% neutral buffered formalin solution for 48h. Then, the fixed specimens were processed through the conventional paraffin embedding technique (Culling, 1983). Thin tissue sections (5 µm) were prepared and stained with haematoxylin and eosin (H&E), slides were examined using Optika microscope with digital camera (Optika, Italy).

Histopathological examination of tissues using Transmission Electron Microscope TEM:

Hepatopancrease and posterior kidney tissues of experimentally infected *Oreochromis niloticus* were prepared according to the method described by Graham and Orenstein (2007). Briefly, Small pieces of fresh specimens were fixed immediately in 4% formaldehyde, 1% glutaraldehyde (4F1G) in phosphate buffer solution (PH 7.2) at 40 °C for 3 hours, then, specimens were postfixes in 2% Osmium Tetroxide (OsO4) at 4^oC for 2 hours. Sample were washed in the buffer and dehydrated at 4^oC through a graded series of ethanol, then were embedded in resin to polymerize. Samples then were cut into ultra-thin sections about 90 nm in with ultra-microtome. Staining was done by uranyl acetate for 15 min then by lead citrate for another 2 min. Scanning of tissue sections were performed using high tension (80,000 KV), and (2000 X) magnification powers using Tecnai Spirit-Orbit Scientific, Transmission Electron Microscope (TEM), FEI Company, USA.

RESULTS AND DISCUSSION

The recorded clinical signs for both naturally and experimentally infected fish were identical including hemorrhages at fin bases, fin and tail erosions, inflammation and redness of the abdominal wall and around the anal opening (Figs. 1, a & b). Exophthalmia, corneal opacity and ascites were observed on a few number of naturally infected fish, some severely affected fish were swims at water surface in a circular movement pattern. The recorded clinical signs were in harmony with the findings mentioned by (Nguyen *et al*., 2001; Suanyuk *et al*., 2010; Figueiredo *et al*., 2012; Hossain *et al*., 2014; Rahmatullah *et al*., 2017), they recorded presence of high mortality, darkened skin, exophthalmia, lethargy and erratic swimming behavior with the presence of hemorrhages over the external body for both *Streptococcus iniae* naturally and experimentally infected Nile tilapia, monosex tilapia, red hybrid tilapia and Japanese flounder (*Paralichthys olivaceus*). Karsidani *et al*. (2010) also, mentioned the same findings during streptococcosis outbreak that hits farmed rainbow trout as bilateral exophthalmia, ascites as well as anal prolapse with hemorrhagic patches on the external surface of the affected fish. The recorded clinical findings were mostly induced by the invading bacteria and their circulated toxins during septicemia, which result in hemorrhages, ulcers, exophthalmia and corneal opacity are a direct sign indicating eye infection, the altered swimming

behavior may be associated with brain invasion which was also recorded by Pretto-Giordano *et al*., (2010). Baiano and Barnes (2009), recorded development of meningoencephalitis in *Streptococcus iniae* affected fish with highly mortality that often between 30-50% of stocked fish.

Internal signs found during dissection of both naturally infected as well as challenged fish samples showed presence of thick yellow ascetic fluid in the abdominal cavity, enlargement, congestion and inflammation of hepatopancreas, posterior kidney and spleen (Figure 1, c), these findings were in accordance with the observations recorded by (Nguyen *et al*., 2001; Salvador *et al*., 2005; Abuseliana *et al*., 2011). Circulating pathogenic bacteria and their extracellular toxic products are a potent inflammatory inducers that give rise to congestion and petechial hemorrhages involving the affected organs.

Under oil immersion lens 4 isolates appeared as Gram positive spherical cocci ranged between 0.75 – 0.94 µm in diameter, arranged in long or short chains, other researches as (Lau *et al*., 2003; Russo *et al*., 2006) have similar description.

Culture characteristics of the recovered *Streptococcus iniae* isolated on brain heart infusion agar indicated the growth of small non pigmented colonies about 1 mm in diameter, while on tryptic soy agar grown colonies were translucent to slightly opaque, round, convex, whitish and ranged between 1.25-1.75 mm in diameter. On blood agar they are producing a small whitish to translucent colonies that were surrounded by a zone of beta heamolysis, the tested isolates were catalase and oxidase negative. The morphological and culture characteristics also, the biochemical tests (catalase and oxidase) indicated presence of *Streptococcus iniae*, in harmony with this results, (Lau *et al*., 2003; Russo *et al*., 2006; Shin *et al*., 2006; Nho *et al*., 2009).

Table 1: Primers used for DNA amplification of *Streptococcus iniae* isolates

Primers	Oligonucleotide Sequence (5'-3')	Target Gene	Amplified Fragment
F: Sin-1b	CTAGAGTACACATGTAGCTAAG	16S rRNA	300 bp
R: Sin-2b	GGATTTTCCACTCCCATTAC		

All of the recovered four streptococcus isolates were subjected to PCR analysis using specific primers targeting the 16S rRNA gene of *S. iniae*, the results indicated that all of the tested isolates were *S. iniae* by their characteristic bands at 300 bp (Figure 1, d), many researches as (Zlotkin *et al*., 1998; Al-Harbi, 2011; Colorni *et al*., 2002; Zhou *et al*.,2008) described the same findings that proves the current work results.

Out of the thirty examined diseased fish samples, 4 isolates were recovered and confirmed as *Streptococcus iniae* that representing 13.3 %, which was higher than that recorded by Shoemaker *et al*., (2001) in tilapia (3.81%) but relatively lower than the findings of Saleh *et al*. (2017a), they isolated *Streptococcus iniae* from (20.2 %) of tested samples, this difference could be attributed to difference in the sampling time and the studied area.

The pathogenicity test results and the calculated $LD_{50\text{-}96h}$ of the recovered *S. iniae* isolates were 0.2 ml of 2.69×10^7, 2.62×10^7, 3×10^7 and 2.7×10^7 CFU / ml for isolates 1, 2, 3 and 4 respectively (Table 2), this results was different from that recorded by (Aboyadak *et al*., 2016b; Saleh *et al*., 2017b; Rahmatullah *et al*., 2017)

this can be explained by the difference in virulence of isolates together with the difference in challenged fish weight.

Table 2: Pathogenicity test results and calculated ($LD_{50\text{-}96h}$)

Group	Subgroup	S. *iniae* isolate	No. of inoculated fish	No. of dead fish at 96h	$LD_{50\text{-}96h}$
1	-	-	20	0	0
	a		20	14	
2	b	SI1	20	5	2.69×10^7
	c		20	0	
	a		20	12	
3	b	SI2	20	4	2.62×10^7
	c		20	1	
	a		20	10	
4	b	SI3	20	3	3×10^7
	c		20	0	
	a		20	11	
5	b	SI4	20	5	2.7×10^7
	c		20	0	

SI1, SI2, SI3 & SI4: are *Streptococcus iniae* isolate number 1, 2, 3 & 4.

The histopathological examination results indicated the presence of several degenerative changes in the investigated tissues. Histopathological examination of hepatopancreas shows the presence of obvious diffused hepatic cell vacuolation and necrosis. Other pathological lesions including leukocytic infiltration, nuclear fragmentation and hemorrhages were also dominant (Figs. 2, a - d), many other researchers as Aboyadak *et al*., (2016b), recorded similar lesions in *Oreochromis niloticus* experimentally infected with *Streptococcus iniae*. Regarding the posterior kidney tissue, vacuolation of tubular epithelial cells, shrinkage of glomerular taught and increasing Bowman's space, with hemorrhage, glomerular taught hypertrophy and narrowing of Bowman's space were clear in most of examined samples (Figs. 3, e & f). These findings indicating the severity of streptococcal infection on posterior kidney tissue, Ali *et al*. (2018), recorded the involvement of bacterial virulence factors and toxins in tissue destruction of infected host. In harmony with this study, Dewi *et al*. (2015) recorded numerous pathological lesions in liver and kidney of Nile tilapia infected with *Streptococcus iniae* and Liath *et al*. (2017), found marked congestion and lymphocytic infiltration in kidney, liver, and spleen, they also recorded hemorrhage and thrombosis in the glomeruli and tubules along with atrophy in hematopoietic tissue in kidney and liver of hybrid tilapia infected with *Streptococcus agalactiae*, they also, found some samples with congested hepatic sinusoids and portal blood vessels, thrombosis in portal blood vessel, and vacuolar (fatty) degeneration of hepatocytes. Parenchymatous organs (liver and kidney) are the most severely affected organs during septicemia because of their high blood supply and so become affected by the circulating microbial toxins.

Changes in ultra and micro structures of hepatopancreas and posterior kidney tissues of fish challenged with *S. iniae* that were determined by TEM augments the histopathological examination results. TEM investigation of the hepatopancreas revealed marked vacuolation in the cytoplasm of hepatocytes which indicate the presence of diffused degenerative changes. Different cell organelles and components were present in the cytoplasm as glycogen, lysosome, oil droplets and some mitochondria which appear to be separated and degenerated, while, others were enlarged in size. Spherical shape nucleus was easily visualized and was ranged between 4 µm to 5 µm in diameter. Endoplasmic reticulum lost its contact in several

areas (Figs. 3, a & b). Abdelhamed *et al*. (2017) described nearly similar results during TEM study of hepatopancreas and kidney of channel catfish infected with *Aeromonas hydrophila*. Scanning of the posterior kidney tissues showed the presence of numerous vacuolation in the cuboidal epithelial cell lining the apical portion of proximal convoluted tubules with absence of its brush border. Lysosomes were observed above the oval nucleus, nucleus and mitochondria were dispersed throughout the cell cytoplasm also mitochondria fragmentation was observed. Some autophagic lysosomes were present which indicate the proliferation of lysosomes. The capillary channel was filled with erythrocytes which indicated presence of congestion (Figs. 3, c & d).

CONCLUSION

In conclusion *Streptococcus iniae* considered one of most important Gram positive bacteria affecting cultured *Oreochromis niloticus* in Egypt. This work documents cellular and subcellular degenerative changes affecting hepatopancreas and posterior kidney of experimentally infected *Oreochromis niloticus* with virulent *Streptococcus iniae* isolates by both histopathological examination and transmission electron microscope investigation.

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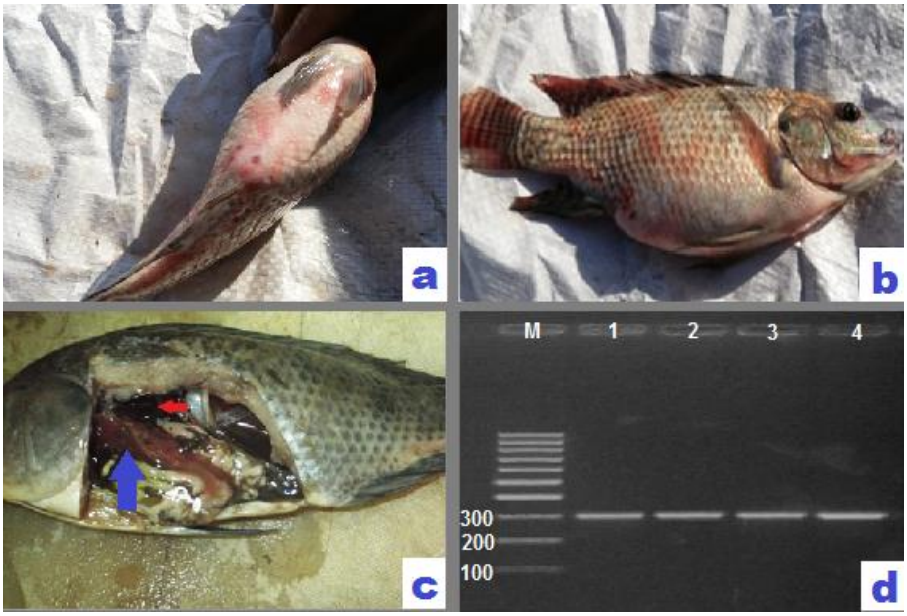

Fig. 1: a) Naturally infected *Oreochromis niloticus* showed hemorrhagic patched on fins, abdominal wall and with inflamed congested anal opening. b) Naturally infected *Oreochromis niloticus* showed exophthalmia, ascites and heamorrhagic patches on the caudal peduncle. c) Experimentally infected *Oreochromis niloticus* showed enlarged spleen (splenomegaly) red arrow and congested enlarged hepatopancreas (blue arrow). d) 1.5 % agar gel electrophoresis of the PCR products showing the characteristic bands at 300 bp of the 4 recovered *S. iniae* isolates, M: 100 bp: DNA marker.

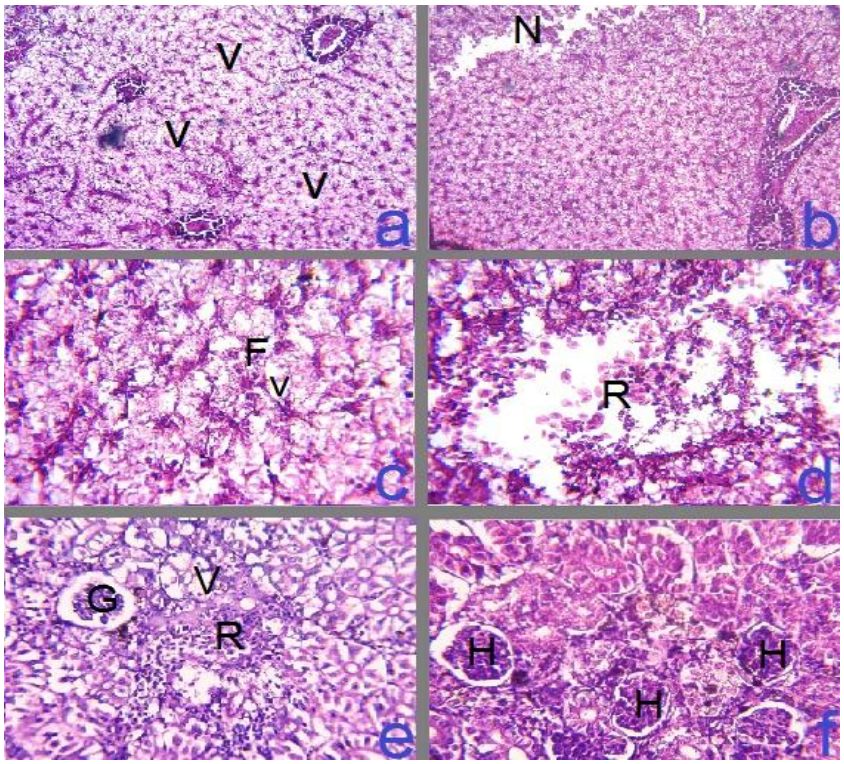

Fig. 2: a & b) hepatopancreas of *Oreochromis niloticus* experimentally infected with *Streptococcus iniae* with obvious diffused hepatic cell vacuolation (V) and necrosis with leukocytic infiltration (N), H & E, X = 100. c & d) hepatopancreas of *Oreochromis niloticus* experimentally infected with *Streptococcus iniae* with Nuclear fragmentation (F), hepatic cell vaculation (V), necrosis (N), hemorrhage represented by extravasation of RBCs (R), H & E, X = 400. e & f) Posterior kidney of experimentally infected fish showing vacuolation of tubular epithelial cells (V), shrinkage of glomerular *taught* and increasing Bowman's space (G), with hemorrhage (R), glomerular *taught* hypertrophy and narrowing of Bowman's space (H), H & E, X = 400.

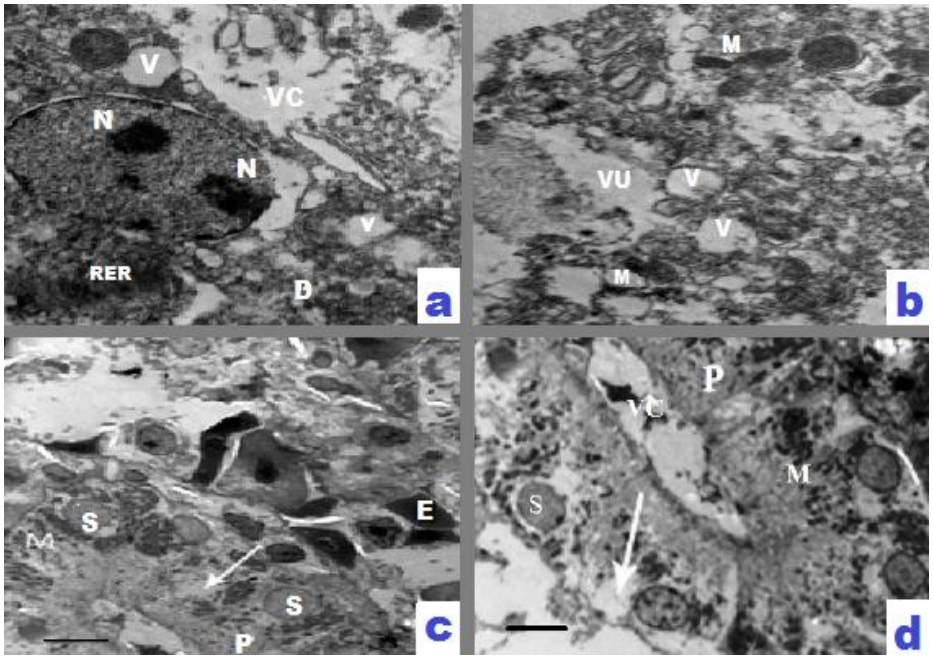

Fig. 3: a & b) Transmission electron micrograph experimentally infected *Oreochromis niloticus* hepatopancreas showing vacuolation of cytoplasm (V), degeneration and separation of cells (D) and fragmentation of mitochondria (M). Nucleus (N), Rough Endoplasmic Reticulum (RER), enlarged mitochondria (M) and increased vacuolation (VC), Magnification power (X) = 2000. c & d) Transmission electron micrograph of experimentally infected *Oreochromis niloticus* posterior kidney showing swollen epithelial cell (P), vacuolation of cytoplasm (arrow), some areas of increased vacuolation (VC), mitochondria (M), lysosomes (S) and erythrocytes (E), Magnification power (X) = 2000.