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Molecular characterisation and pathogenicity evaluation of *Aeromonas hydrophila* strains isolated from cultured tilapia *Oreochromis niloticus* in Egypt

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ABSTRACT

The objectives of the present study were to characterise *Aeromonas hydrophila* isolated from diseased tilapia *Oreochromis niloticus* cultured in Kafrelsheikh farms and to evaluate virulence and pathogenecity. About 200 adult fish exhibiting the onset of clinical signs were bacteriologically examined using *Aeromonas* isolation medium. The isolates were identified by 16S rRNA gene sequence analysis using species specific primers and then tested for ten virulence genes employing specific primers. Experimental challenge studies were also conducted to investigate the pathogenicity of the recovered *A. hydrophila* strains. Thirty five isolates were identified as *A. hydrophila* based on 16S rRNA gene sequence analysis, which were also positive for presence of four virulence genes (aerolysin, lipase, cytotonic heat stable enterotoxin and haemolysin). Results showed that majority of the examined strains carried one or more virulence genes.

Keywords: *Aeromonas hydrophila*, Molecular techniques, *Oreochromis niloticus*, Virulence genes

Introduction

Fish provides high quality cheap source of protein especially for the low income communities. Aquaculture is a fast growing animal production sector in Egypt. Intensification and stress of artificial environments results in the emergence of diseases. *Aeromonas hydrophila* is the most encountered fish pathogen in the freshwater habitats. *Aeromonas* infections producing septicaemia as well as ulcerative and haemorrhagic lesions in fish, leads to significant mortality in both wild as well as farmed freshwater and marine fish species that impact the economics of the aquaculture sector. Molecular diagnostic techniques provide an effective tool for fish pathogen detection. Aboyadak *et al.* (2015) used PCR technique to confirm that *A. hydrophila* was the main cause of disease outbreak in tilapia farms in Egypt with an incidence of 75% in Kafrelsheikh Governorate while Ashraf *et al.* (2017) studied the prevalence of haemolysin, cytolytic enterotoxin, aerolysin, cytotoxic enterotoxin and cytotonic heat stable enterotoxins in *A. hydrophila* and *A. caviae* isolated from Nile tilapia (*Oreochromis niloticus*) and catfish (*Clarias gariepinus*) obtained from local markets at Qalubia Governorate. A similar study was performed by Furmanek-Blaszk (2014) to determine the prevalence of five virulence genes *viz.,* aerolysin, haemolysin, elastase, cytolytic heat-labile enterotoxin and polar flagella from *A. hydrophila* strain isolated from the River Nile. Corresponding studies were recorded from different countries like Yang *et al.* (2018) who investigated the incidence and virulence features of *A. hydrophila* in grass carp in south China where the isolated strains were positive for aerolysin, elastase, hemolysin and lipase. Another study was conducted by Ruhil Hayati *et al.* (2015) to verify the presence of virulence genes in *A. hydrophila* isolated from farmed and wild *Oreochromis* spp., *Oreochromis mossambicus, Scortum barcoo, Puntius gonionotus, Leptobarbus hoevenii, Pangasius pangasius, Anabas testudineus, Clarias gariepinus* and *Cichlasoma* sp., sampled from Selangor, Malaysia concluding that *Aeromonas* infections revealed presence of different combinations of the virulence genes. Oliveira *et al.* (2012) studied the presence of aerolysin, hidrolipase, elastase and lipase genes in *A. hydrophila* isolates obtained from fish of the Sao Francisco River Valley in Brazil. The main objectives of the present study were to identify *A. hydrophila* strains from tilapia farms at Kafrelsheikh Governorate showing onset of *Aeromonas* septicemia and to screen for ten virulence genes using specific primers.

Materials and methods

Sampling and bacteriological examination

Bacterial isolations were carried out from 200 nos. of *O. niloticus* showing clinical signs of skin haemorrhages, on tryptic soy agar (TSA) and subcultured until obtaining pure colonies and then tested on *Aeromonas* isolation medium. All bacterial cultures were incubated at 28°C for 24 h. The isolates were tested for biochemical characteristics using API20NE strips (Biomerieux©, France) and were also identified employing molecular methods by polymerase chain reaction (PCR). The primers used targeted a species-specific region of 16S rDNA (forward 5'-GGCCTTGCGCGATTGTATAT-3' and reverse 5'- GTGGCGGATCATCTTCTCAGA-3'), to identify *A. hydrophila* (Trakhna *et al.,* 2009).

DNA extraction and PCR amplification

DNA extraction was done by thermolysis after culturing the bacterial strains on brain heart infusion broth and incubated for 12 h in shaker incubator. One hundred microlitre of overnight bacterial culture was mixed with 400 µl of sterile distilled water, then transferred to heat block for 5 min at 95°C; followed by centrifugation at 15000 rpm for 2 min, at 4°C. The supernatant was used as DNA template which was stored at -20°C for further studies. Amplification of target genes using bacterial cell lysate as the source of template DNA, was performed in a thermal cycler. PCR amplifications were performed in a total volume of 25 µl, containing 0.125 µl ampli Taq gold DNA polymerase (5 U ml^{-1}); 2.5 µl 10 x PCR buffer; 2.5 µl dNTP mixture; 1 µl of each primer (Forward and Reverse) (20 pmol μl^{-1}) and 5 µl genomic DNA. Thirty PCR cycles were run under the following conditions; first step of denaturation at 94°C for 2 min, followed by 35 cycles of, denaturation at 94°C for 30 s, annealing for 30 s at 55.5°C and a final extension step at 72°C for 30 s. After the end of the cycles, one final extension step at 72°C for 10 min was added, according to Trakhna *et al.* (2009) to detect the *A. hydrophila* specific-16S rRNA gene. For the virulence genes examined, PCR conditions were performed under similar conditions according to references mentioned in Table 1.

Sequence analysis of the virulence genes

The positive virulence genes were subjected to DNA sequence analysis and aligned to the published sequences in the GenBank database of the National Center for Biotechnology Information (NCBI). The PCR products of the virulence genes detected were electrophoresed on 1% SeaKem® LE agarose gel (Lonza). The gel was placed on UV transilluminator; and the band (about 300 mg) was cut for extraction of DNA using fast gene gel PCR extraction kits according to Yousr *et al.* (2007).

Table 1. Primers used for detection of *A. hydrophila* virulence genes

Gene	Primers 5' → 3'	Size (Sequence length) (bp)	References
Hidrolipase (*Lip*)	F:AACCTGGTTCCGCTCAAGCCGTT R:TTGCTCGCCTCGGCCCAGCAGCT	65	Oliveira *et al.* (2012)
Elastase (*ahyB*)	F: ACACGGTCAAGGAGATCAAC R: CGCTGGTGTTGGCCAGCAGG	540	
Lipase (*pla/lip*)	F: ATCTTCTCCGACTGGTTCGG R: CCGTGCCAGGACTGGGTCTT	383 - 389	
Aerolysin (*aer*)	F: CCTATGGCCTGAGCGAGAAG R: CCAGTTCCAGTCCCACCACT	431 - 1987	
Cytotonic enterotoxin (*alt*)	F: TGACCCAGTCCTGG R: GGTGATCGATCACC	442	Hu *et al.* (2012)
Cytotoxic enterotoxin (*act*)	F: GAGAAGGTGACCACCAAGAACA R: AACTGACATCGGCCTTGAACTC	232	
Temperature sensitive protease (*eprCAI*)	F: GCTCGACGCCCAGCTCACC R: GGCTCACCGCATTGGATTCG	387	
Serine protease (*Ahp*)	F: ATTGGATCCCTGCCTA R: GCTAAGCTTGCATCCG	911	
Haemolysin (*hlyA*)	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGGG	392	
Cytotonic heat stable enterotoxin (*ast*)	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	331	Balsalobre *et al.* (2009)

Median lethal dose (LD_{50}) of *A. hydrophila* strains in *O. niloticus*

A total number of 300 apparently healthy *O. niloticus,* were divided into six major groups for six inoculations (four strains containing virulence genes, one strain free from virulence genes and sterile saline solution as negative control), fifty fish per each group. Each group was divided into five subgroups, ten fish per each subgroup. The sixth group inoculated with sterile saline served as control. Twentyfour hour cultures of each *A. hydrophila* strain (confirmed by PCR amplification), on nutrient agar plates were used for the challenge studies. The colonies were picked and suspended in sterile saline, tenfold serial dilution was done and only the dilutions in the range of 10^6-10^9 were used for challenge experiments. Each fish group was intraperitonially injected with 0.5 ml of each bacterial dilution and observed for mortality one week post-inoculation. Freshly dead fish were subjected to histopathological studies. The LD_{50} of each *A. hydrophila* strain was calculated according to Reed and Muench (1938).

Histopathological examination

Tissue samples collected from the liver, kidney and spleen of freshly dead fish from the the challenge studies were fixed in neutral buffered formalin for histopathological investigations. The specimens were dehydrated and embedded in paraffin wax and sectioned at 3 µm, stained with haematoxylin and eosin (HE) and examined by light microscopy according to Bancroft and Gamble (2007).

Results and discussion

The fishes examined showed skin hemorrhages on external body surface characteristic of motile *Aeromonas* septicemia. Thirtyfive isolates of *A. hydrophila* were collected from diseased tilapia. The bacterial colonies were creamy-white, circular and convex having 2-3 mm in dia on TSA plates. Putative *Aeromonas* colonies grown on *Aeromonas* isolation medium with dark green center and light periphery were selected. Biochemical tests showed positive results for O-nitrophenyl-b-D-galactopyranoside, arginine dehydrolyase, lysine decarboxylase, citrate utilisation, indole production, gelatinase and Voges Proskauer. Acid was produced from mannitol. Besides, negative results were obtained for ornithine decarboxylase, H_2S production, urease and TDA (tryptophan-deaminase activity). No acid production recorded from inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and arabinose.

Molecular characterisation revealed that 35 isolates were positive to 16S rRNA gene (103 bp) (Fig. 1). Out of the ten virulence genes screened; four genes [(lipase

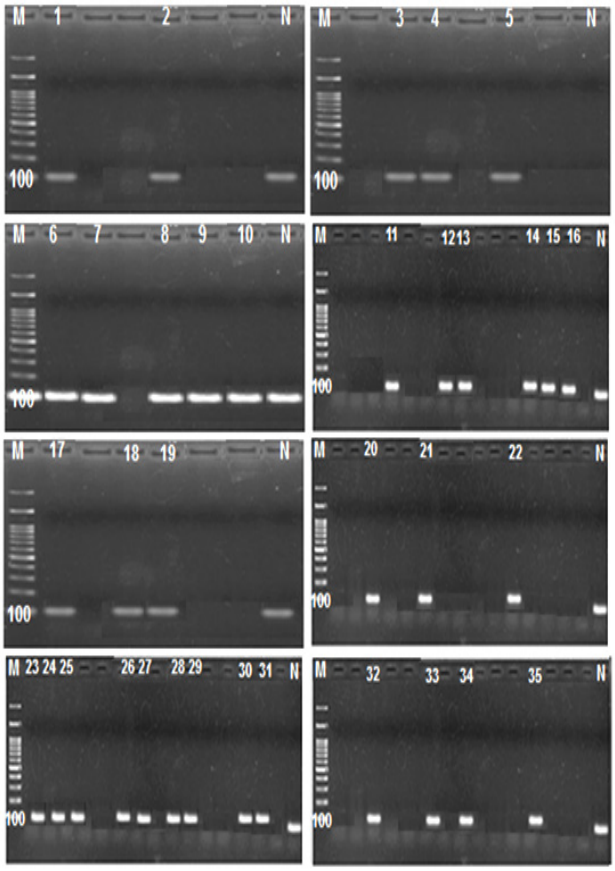

Fig. 1. Electrophoretic pattern of PCR products (103 bp) specific for *A. hydrophila* in 2% agarose gel stained with ethidium bromide
Lane M: 100 bp DNA ladder; Lane N: Negative control
Lane 1- 35: Positive *A. hydrophia*

(*pla/lip*), cytotonic heat stable enterotoxin (*ast*), aerolysin (*aer*) and haemolysin (*hlyA*)] were found in five isolates with an incidence of 24% of the strains. Three virulence genes *pla/lip, aer* and *ast* were present in seven isolates with an incidence of 33% of the strains. Two virulence genes *pla/lip* and *aer* occurred in six isolates with an incidence of 29% of the strains and one gene *pla/lip* located in three isolates with an incidence of 14% of the virulent strains. Incidence of each individual gene was 100% for lipase (*pla/lip*), 57% for cytotonic heat stable enterotoxin (*ast*), 24% for aerolysin (*aer*) and 86% for haemolysin (*hlyA*) from 21 strains out of 35 strains of *A. hydrophila* identified, while fourteen strains were free from any virulence genes. The prevalence of *A. hydrophila* virulence genes were distributed as follows: lipase (*pla/lip*), the most frequent virulence gene was detected in 21 strains, the cytotonic heat stable enterotoxin (*ast*) in 12 strains, aerolysin (*aer*) in 20 strains and

haemolysin (*hlyA*) in 5 strains (Fig. 2, 3, 4, 5). Depending on the frequency of the virulence genes in positive *A. hydrophila* strains, the genotypes carrying virulence genes represented 60.6%, while the genotypes free from virulence genes represented 39.4% (Table 2, 3).

DNA sequences of the virulence genes were compared with the published sequences in the NCBI GenBank database. Nucleotide sequences of the six virulence genes showed no differences and no mutations. Nucleotide sequence alignment of lipase gene (*pla/lip*) showed 98% identity, cytotonic heat stable enterotoxin (*ast*) showed 98% identity, aerolysin (*aer*) showed 98% identity and haemolysin gene (*hlyA*) showed 99% identity.

The LD_{50} experiment with strains containing four virulence genes (*pla/lip, ast, aer, hlyA*) caused 50% mortality within 12 h at doses of 1.8×10^6 and 1.6×10^6 cfu, while the other strains having three virulence genes (*pla/lip, ast* and *aer*) resulted in 50% mortality after 18 h at a dose of 1.5×10^6 cfu. The strains harbouring two virulence genes (*pla/lip and aer*) caused mortality after 24 h at dose of 1.2×10^6 cfu and the strain having only one virulence gene (*pla/lip*) caused mortality of the fish after 24 h at

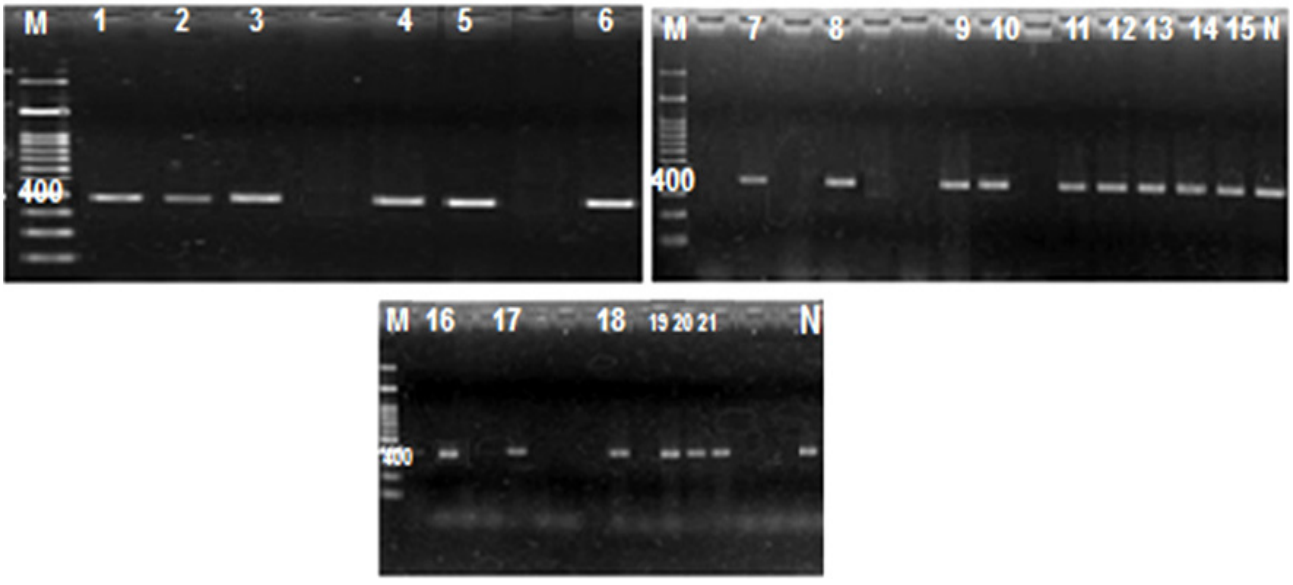

Fig. 2. Ethidium bromide stained agarose gel of PCR products representing amplification of 383-389 bp amplicon of the Lipase (*pla/lip*) gene in *A. hydrophila*
Lane M : 100 bp DNA ladder; Lane N : Negative control; Lane 1-21 : Positive *pla/lip*

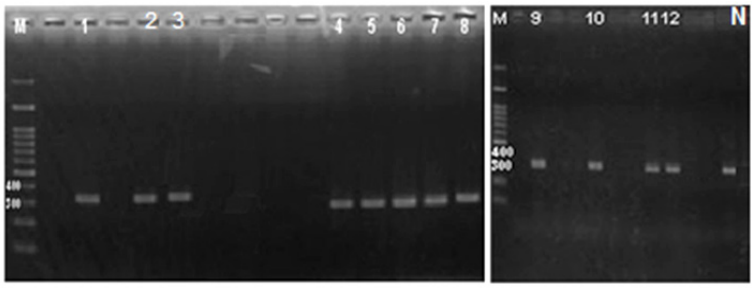

Fig. 3. Ethidium bromide stained agarose gel of PCR products representing amplification of 328 bp amplicon of the cytotonic heat stable enterotoxin (*ast*) gene in *A. hydrophila*
Lane M : 100 bp DNA ladder; Lane N : Negative control; Lane 1- 12 : Positive *ast*

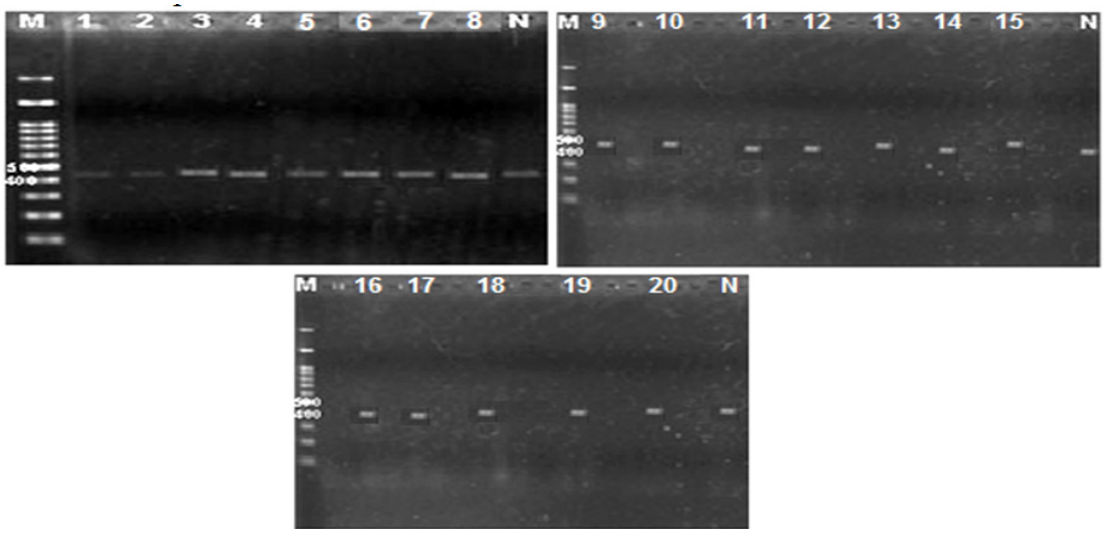

Fig. 4. Ethidium bromide stained agarose gel of PCR products representing amplification of 431 bp amplicon of the Aerolysin (*aer*) gene in *A. hydrophila*
Lane M : 100 bp DNA ladder; Lane N : Negative control; Lane 1- 20 : Positive *aer*

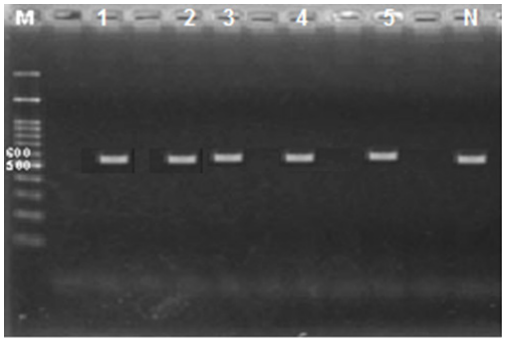

Fig. 5. Ethidium bromide stained agarose gel of PCR products representing amplification of 592 bp amplicon of the haemolysin (*hlyA*) gene in *A. hydrophila*
Lane M :100 bp DNA ladder; Lane N : Negative control
Lane 1- 5 :Positive *hlyA*

Table 2. List of virulence genes present in *A. hydrophila* strains

Gene	No. of isolates	Occurrence (%) in isolated strains	Occurrence (%) in virulent strains
Lipase (*pla/lip*)	21	60%	100%
Cytotonic heat stable enterotoxin (*ast*)	12	34%	57%
Haemolysin (*hlyA*)	5	14%	24%
Aerolysin (*aer*)	18	51%	86%

Table 3. Occurrence and combination of virulence genes in *A. hydrophila* strains

Gene	No. of isolates	Occurrence (%) in isolated strains	Occurrence (%) in virulent strains
(*pla/lip*), (*ast*), (*aer*), (*hlyA*)	5	14%	24%
(*pla/lip*), (*ast*), (*aer*)	7	20%	33%
(*pla/lip*), (*aer*)	6	17%	29%
(*pla/lip*)	3	10%	14%

cell concentration of 1×10^6 cfu, while the virulence genes negative strain caused 50% mortality after 36 h at cell concentrations of 1.8×10^6 cfu. The four strains of *A. hydrophila* carrying virulence genes, caused mortality of all fish groups within 48 h at 10^6 cfu while, the strain free from virulence genes caused mortality of all fish groups at 78 h at 10^6 cfu. This may be attributed to the presence of other virulence genes, rather than the tested genes, indeed, the variance of the primers used and the pathogen properties as well. Generally the clinical signs of fish infected with virulence genes negative strains were limited to ascites, poor appetite, pale gills and sudden death. The experiments revealed that, the dose 10^6 cfu caused 50% mortalities in fish within 12-24 h. Pachanawan *et al.* (2008) reported LD_{50} value of 3.4×10^6 cfu ml^{-1} in *O. niloticus,* for *A. hydrophila* strains carrying four virulence genes. In contrary,

Li *et al.* (2011) reported LD_{50} at 10^3-10^5 cfu ml^{-1} and Viji *et al.* (2011) recorded LD_{50} at 10^4-10^5 cfu for *A. hydrophila* in zebra fish and gold fish respectively. The strain free from virulence genes caused 50% mortalities at a concentration of 1.8 x 10^6 cfu in 36 h. The control group showed no mortalities. Haemorrahges on the external body surface may be attributed to the haemolytic effect of the exotoxins; haemolysin (α-haemolysins) and aerolysin (β-haemolysins). Both haemolysins induce pore-formation in the cell membrane of the RBCs, leading to haemolysis (Zhang *et al.,* 2000; Singh *et al.,* 2008; Singh *et al.,* 2010; Hidalgo and Figueras, 2013). Lipase, alter the structure of the cytoplasmic membrane of the host tissue cells and help colonisation of *A. hydrophila* on the host tissues causing necrosis (Oliveira *et al.,* 2012), as well as digest the cellular components of the host tissue cells (Zang *et al.,* 2000). The cytotonic heat stable enterotoxin increase the vascular permeability of the gut causing detachment of the intestinal mucosa. Presence of aerolysin in pathogenic *A. hydrophila* infections may help in diagnosis, prevention and control of the spread of disease and mortalities in aquaculture (Singh *et al.,* 2010; Al-Maleky *et al.,* 2011).

Aboyadak *et al.* (2015) investigated tilapia farms in Kafrelsheikh governorate and isolated 12 *Aeromonas* isolates (identified by PCR using *Aeromonas* sp. primer; amplicon 953 bp) and confirmed them as *A. hydrophila* using *A. hydrophila* specific 16S rRNA gene primer (amplicon 103 bp) whereas in the current study 35 *A. hydrophila* strains were identified using *A. hydrophila* specific 16S rRNA primers out of which 21 strains were characterised as carrying one or more virulence genes using specific primers. Ashraf *et al.* (2017) studied 225 diseased fish samples that included 125 Nile tilapia *(O. niloticus)* and 100 catfish (*Clarias gariepinus*), collected from different fish markets at Qalubia Governorate for detection of *Aeromonas* species and associated virulence genes and reported that aerolysin (*aer*) gene was detected in 9 out of 10 *A. hydrophila* strains and in 3 out of 6 *A. caviae;* haemolysin (*hly*) gene in 7 out of 10 *A. hydrophila* and in 2 out of 6 *A. caviae; A. hydrophila* cytolytic enterotoxin (*Ahcytoen*) gene in 6 out of 10 *A. hydrophila* and in 1 out of 6 *A. caviae;* cytotoxic enterotoxin (*act*) gene in 6 out of 10 *A. hydrophila* and in 3 out of 6 *A. caviae* and cytotonic enterotoxins - heat-stable (*ast*) gene in 7 out of 10 *A. hydrophila* and in 3 out of 6 *A. caviae* strains. In the present study, *pla/lip* was detected in 21 isolates out of 21 *A. hydrophila; ast* gene in 12 out of 21 *A. hydrophila, aer* gene in 18 out of 21 *A. hydrophila* and *hlyA* gene in 5 out of 21 *A. hydrophila.* Ruhil Hayati *et al.* (2015) tested the presence of 10 virulence genes in *A. hydrophila* isolated from diseased freshwater fish and reported that all strains contain *lip, exu* and *ser* genes whereas none of the strains possessed *ela, alt* and *fla* genes. Other genes *viz., ast, act, aer* and *hlyA* were present in 30%, 30%, 76% and 95% of the strains, respectively. In the present study, four virulent genes *viz.*, lipase, cytotonic heat stable enterotoxin, haemolysin and aerolysin were detected out of the ten virulence genes screened. Yang *et al.* (2018) performed molecular characterisation of *A. hydrophila* from grass carp and their results showed that the incidence of aerolysin, elastase, hemolysin and lipase genes were 71.43, 46.03, 0.00 and 55.56%, respectively but in the present study the incidence of the genes was 100% for lipase, 57% for cytotonic heat stable enterotoxin, 24% for hemolysin and 86% for aerolysin.

Histological sections of the liver tissue showed areas of coagulative necrosis as well as accumulation of melanomacrophages (Fig. 6, 7). The spleen showed lymphoid depletion (Fig. 8), and kidney sections revealed congestion, necrosis, interstitial nephritis and lymphocytic infiltration (Fig. 9).

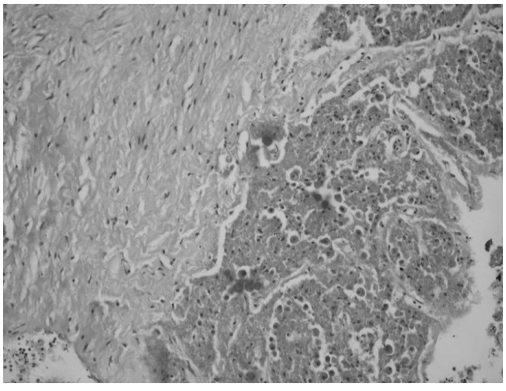

Fig. 6. Photomicrograph of histological section of liver tissue showing coagulative necrosis

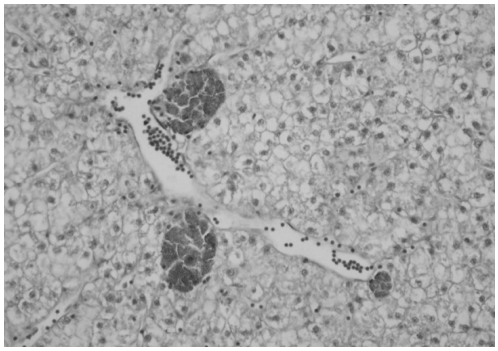

Fig. 7. Histological section of liver showing melanomacrophage centers

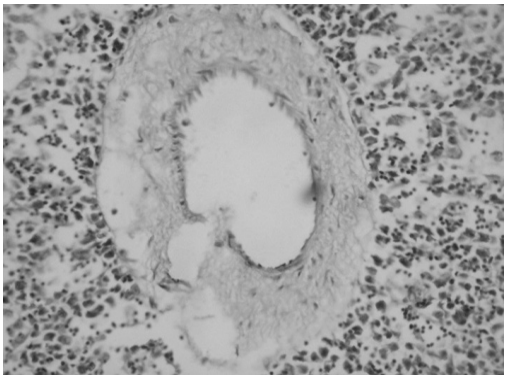

Fig. 8. Photomicrograph section spleen showing lymphoid depletion

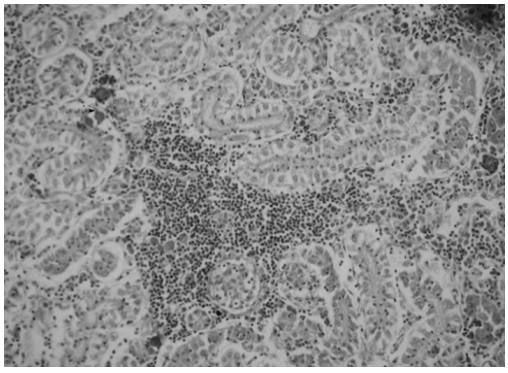

Fig. 9. Histological section of kidney showing congestion, necrosis, interstitial nephritis and lymphocytic infiltration

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