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Escherichia fergusonii: A New Emerging Bacterial Disease of Farmed Nile Tilapia (*Oreochromis niloticus*)

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Abstract: Tilapia is one of the most important cultured freshwater fish in Egypt. In June (2013) an unidentified bacterial disease outbreak occurred in earthen ponds raised Nile tilapia (*Oreochromis niloticus*). The API 20E test identified the strain as sorbitol-negative and positive for ADH, ornithine decarboxylase and amygdalin and provided the code 5144113, corresponding to *E. fergusonii*. The clinical signs of diseased fish showed emaciation, focal reddening of the skin at the base of the fins, exophthalmia with inflammation of periorbital area. Postmortem lesions showed enlargement of gallbladder and spleen with discolored hepatopancreas and congested posterior kidney. Lethal Dose fifty (LD_{50}) was performed for experimental infection studies. Histopathological examination of naturally as well as experimentally infected specimens revealed pathological lesions in the hepatopancreas, posterior kidney, spleen, myocardium, skin and gills. The lesions ranged from circulatory, degenerative changes and mononuclear cells infiltrations to necrotic changes in the corresponding organs. As a conclusion, this study was the first evidence that *E. fergusonii* can infect fish especially farmed tilapia, causing considerable mortality and morbidity, in heavily manured earthen ponds. *E. fergusonii* causes significant pathological lesions in tilapia that are comparable to other susceptible terrestrial animals. Due to its rising zoonotic importance, *E. fergusonii* infection to fish should be seriously considered as public health hazard.

Key words: *Escherichia fergusonii* · Nile tilapia · *Oreochromis niloticus* · API · Histopathology

INTRODUCTION

Tilapia is one of the most important cultured freshwater fish it comes after carps as the second most important. In the last decade, the world production of farmed tilapia has shown a tremendous increase jumping from 1,303,310 metric tons in 2001 to 3,497,391 metric tons in 2010 [1].

Great economic losses are caused by fish diseases [2]. Outbreak of bacterial diseases in fish remains one of the most significant limiting factors affecting fish culture worldwide [3].

Escherichia fergusonii is an emerging animal and human pathogen that was named in honor of the American microbiologist William H. Ferguson [4]. *E. fergusonii* strains are gram-negative rods, oxidase negative, catalase positive, nonspore-forming and generally motile.

They are facultative anaerobes, have a diameter around 0.8–1.5 mm, lengths from 2 to 5 mm and are peritrichous flagellated. The results of DNA hybridization experiments showed the closest relatives to this new species were *E. coli* - *Shigella* spp., which are up to 64% related. It can be differentiated from *E. coli* by being sorbitol and lactose negative but adonitol, amygdalin and cellobiose fermentation positive [4-7]. The complete genome of the multidrug resistant (resistant to 22 antibiotics) *E. fergusonii* ECD-227 strain is known and was compared to that of the nonpathogenic human fecal strain *E. fergusonii* ATCC35469 [8].

Many studies have demonstrated its ability to cause disease in both humans and animals. *E. fergusonii* has been isolated from people with conditions such as wound infection, urinary tract infections, bacteremia, enteric diseases, pancreatic carcinoma, endophtalmitis and pleuritis [5, 9-13].

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In animals, *E. fergusonii* has been isolated from pigs, sheep, cattle, goats, horses, reindeer, ostriches, turkeys and chickens displaying symptoms of salmonellosis-like infections, including scour as well as mastitis, meningitis, abortion and septicemia [6, 7, 12, 14-17].

E. fergusonii isolates were obtained from the microbial populations of an up-flow anaerobic sludge blanket reactor, used for treating wastewater from the gelatin industry [18]. Steele, Brown *et al.* [19] isolated zoonotic strains from seabirds.

Due to its uprising zoonotic importance, *E. fergusonii* gained recent interest by researchers, so it was surprising to isolate it from classical bacterial disease outbreak in farmed tilapia. This study provided the first report to isolate *E. fergusonii* from a fish pond in North Egypt. This isolate caused disease and mortality in fish and surviving fishes may be a reservoir for further infection.

MATERIAL AND METHODS

Fish: Fifteen naturally infected Nile Tilapia (*Oreochromis niloticus*) weighing 250 - 300 gm, were collected from disease outbreak in June (2013) from an earthen ponds in Kafr El-Sheikh Governorate in Northern Egypt (31°24'29.0"N 30°54'18.4"E). Externally they showed emaciation, focal reddening of the skin in the base of the fins, exophthalmia with inflammation of periorbital area. Internally they showed enlargement of gallbladder, enlarged spleen, discolored hepatopancreas and inflamed posterior kidney (Fig. 1). The fish specimens were collected after 2 weeks of the disease commencement, they transferred live to the laboratory for clinical examination, tissue specimen collection and bacterial isolation.

For experimental studies, a total number of 130 apparently healthy Nile tilapia (*Oreochromis niloticus*) fishes, with average weight of 30-50 gm, they were transported to the laboratory and kept in 60 liters glass aquaria. These aquaria were used for holding the experimental fish throughout the whole period of the study, supplied with chlorine free tap water. The continuous aeration was maintained in each aquarium. Water temperature was kept at 26±1°C. Fish were placed in aquaria and acclimatized for 2 weeks before commencing the experiments. They were maintained on a commercial diet containing 25% crude protein, with feeding ratio 1% of total biomass as maintenance ration [20].

Isolation and Identification of the Causative Agent: Samples from posterior kidney and hepatopancreas were inoculated onto Tryptic Soy Broth (Oxoid, UK) at 25°C for 48 hrs. A loop was streaked onto MacConkey agar plates then incubated at 25°C for 48 hours. Identification of all isolates was done by cultural, morphological and biochemical characters through using API-20E (biomereux, France) according to manufacturer instructions. Mycological and virological examinations also were done.

Determination of 96 Hours Lethal Dose Fifty (LD_{50}): After identifying the isolated bacteria, *Escherichia fergusonii* was grown overnight in Tryptone Soya Broth (TSB) at 25°C, then washed in PBS for 3 times after centrifugation at 4000 rpm and injected intraperitoneally (IP) into healthy Nile tilapia. Ten Fish / group were injected 50 µl dose containing 1×10^4, 1×10^5, 1×10^6, 1×10^7, $1\times10^8$and 1×10^9 CFU/fish respectively. Control group was injected by PBS only. Fish were monitored for any signs of disease or mortality for 96 hours post injection. The injected fish were kept in well aerated glass aquaria and feeding was stopped during the challenge.

LD_{50} was determined according to [21] by the following equation:

$$\text{Proportional distance(PD)}=\frac{\%\,of\ molarity\ at\ dilution\ above\ 50\%-50}{\%\,of\ molarity\ at\ dilution\ above\ 50\%-\%\,of\ molarity\ below\ 50\%}$$

Then LD_{50}= (PD × dilution factor) + log dilution above 50%.

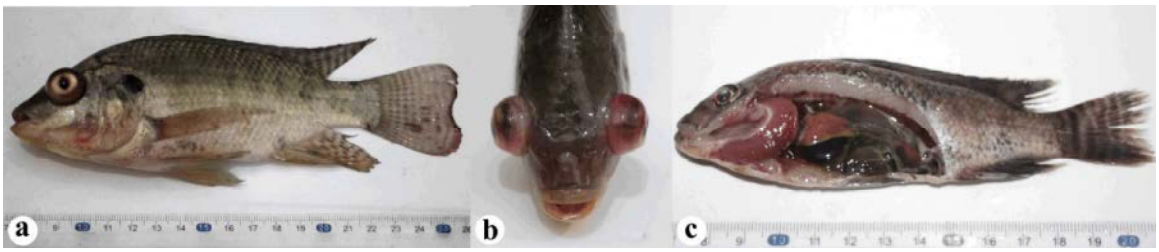

Fig 1: Nile tilapia (*Oreochromis niloticus*) naturally infected with *Escherichia fergusonii* showing exophthalmia with inflammation of periorbital area (a, b), enlargement of gallbladder, discolored hepatopancreas and inflamed posterior kidney (c).

The Pathogenicity Test: After calculation of LD_{50} of *E. fergusonii*, I.P. injection of 0.5 LD_{50} was used for the challenge in Nile tilapia.

Half LD_{50}; 0.1 mL/fish (about0.5×10^6 CFU); was injected intraperitoneally (IP) into healthy 40 Nile tilapia, while 20 fish were injected PBS as negative control. Clinical symptoms and mortality were first monitored hourly for first 12 hours and thereafter every day until the 5th day. Moribund fish were collected instantly from each group, dissected out and the organs preserved in Davidson's fixative for 24 hours, then kept until processing in 70% ethanol.

Histopathological Examination: From naturally and experimentally infected fishes, after complete necropsy, fresh tissue specimens were collected from gills, liver, spleen, kidney, heart and skin for histopathological examination. The fixed specimens were processed through the conventional paraffin embedding technique (dehydration through ascending grades of ethanol, clearing in xylol and embedding in paraffin wax at 60°C). Paraffin blocks were prepared and cut into 3 mμ thick sections and stained with Hematoxylin and Eosin (H&E) [22].

RESULTS

Identification of Isolates: Media revealed Escherichia coli like organism with pink colonies which were oxidase negative. The API 20E test identified strain as sorbitol-negative and for ADH, ornithine decarboxylase and amygdalin which provided the code 5144113, corresponding to *E. fergusonii*.

Mycological and virological isolation from the fish internal organs were negative.

Determination of 96 Hours Lethal Dose Fifty (LD_{50}): Challenge experiments using six different doses of *E. fergusonii* by I.P. injection caused mortalities in Nile tilapia as shown in Table (1).

Calculation of LD_{50}:

$$PD = \frac{60 - 50}{60 - 30} = 0.333$$

$LD_{50} = 10^{7.165}$
$\text{Log } LD_{50} = 0.333+6 = 6.333$
$LD_{50} = 1X\ 10^{6.333}$

Histopathological Results:

Naturally Infected *O. Niloticus*: Hepatopancreas showed severe congestion of main hepatic blood vessels and sinusoidal spaces, multifocal necrotic areas with fibrosis and mononuclear cell infiltrations (Fig. 2). Posterior kidney showed severe hemorrhages with glomerulo-tubular necrosis, also some areas of interstitial necrosis with mononuclear cell infiltrations were evident, with activation of melanomacrophage centers. Spleen showed multifocal necrotic areas with depletion of white pulp. Myocardium showed few focal necrotic areas with focal mononuclear cell infiltrations. Exophthalmic Peri-occular area showed congestion, edema fibrosis and mononuclear cell infiltrations. Gills showed hyperplasia at the base and the tips, with eosinophilic granular cell infiltration with separation of the epithelial lining of the secondary gill lamellae (Fig. 2).

Inflamed skin areas showed congestion, hemorrhages and mononuclear cell infiltrations.

Experimentally Infected Fish: Posterior kidney after 50 hours of injection showed interstitial necrosis with mononuclear cell infiltrations, hemorrhages and glomerulo-tubular degeneration turns into severe necrosis after 70 hours, also activation of melanomacrophage centers were common (Fig. 3). Hepatopancreas after 50 hours of injection showed diffuse hepatocytic and pancreatic acinar degeneration turns into necrosis after 65 hours.

Spleen after 70 hours of injection showed multifocal necrotic areas especially at the ellipsoid area with remarkable depletion of both white and red pulp. Gills after

Table 1: LD_{50} testing of *Oreochromis niloticus* against intra-peritoneal injection of *Escherichia fergusonii*

Inoculums	Injection(CFU/fish)	Fish died/injected	mortality%	LD_{50}
E. fergusonii	1×10^4	1/10	10	
E. fergusonii	1×10^5	3/10	30	
E. fergusonii	1×10^6	6/10	60	10^6
E. fergusonii	1×10^7	8/10	80	
E. fergusonii	1×10^8	9/10	90	
E. fergusonii	1×10^9	10/10	100	
Control	Injected with PBS	0/10	0	

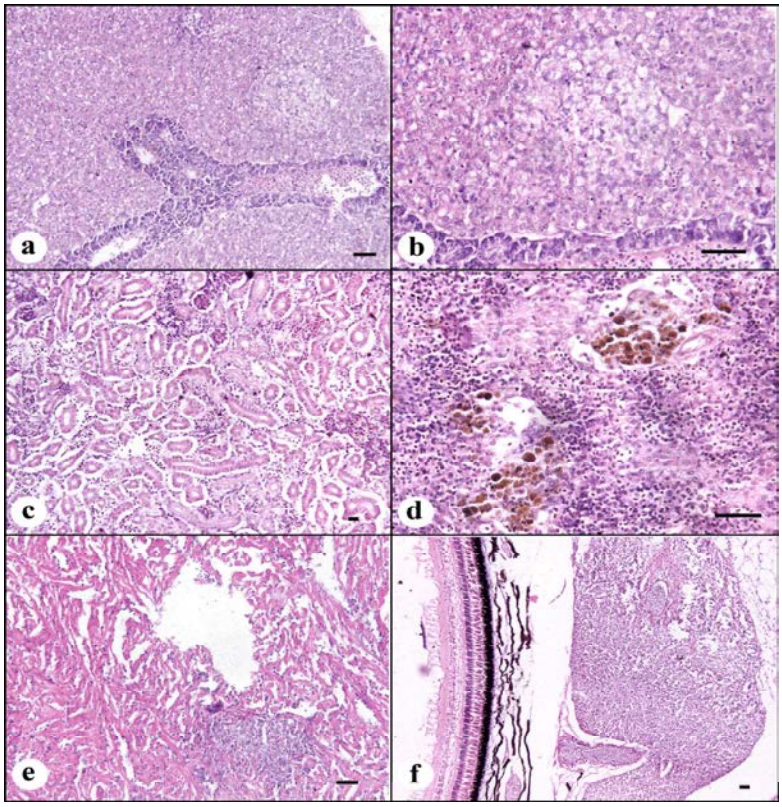

Fig 2: Nile tilapia (*Oreochromis niloticus*) naturally infected with *Escherichia fergusonii* (a) Hepatopancreas showing multifocal necrotic areas with congestion of main hepatic blood vessels(b) Higher magnification of previous picture (c) Posterior kidney showing some areas of interstitial necrosis with mononuclear cell infiltrations (d). Spleen showing multifocal necrotic areas with depletion of white pulp (e), Myocardium showing focal necrotic areas with focal mononuclear cell infiltrations. (f) and Postooccular area showing congestion, edema and mononuclear cell infiltrations. Hematoxylin & Eosin, (Bar = 50 µm).

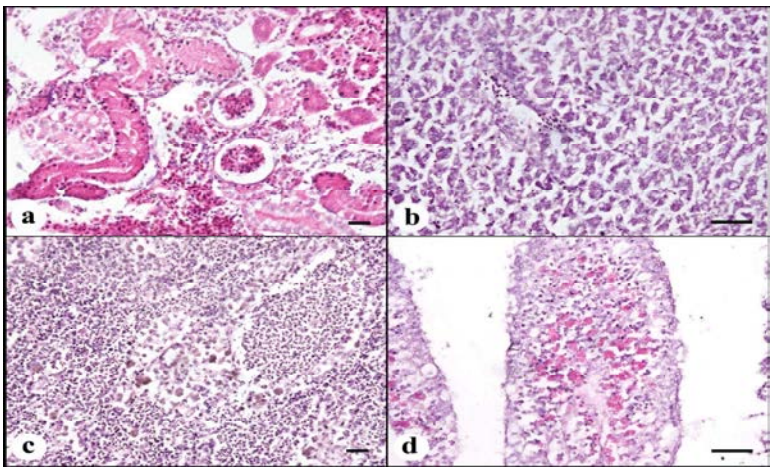

Fig 3: Nile tilapia (*Oreochromis niloticus*) experimentally infected with *Escherichia fergusonii* (a) Posterior kidney after 50 hours of injection showing severe interstitial necrosis with mononuclear cell infiltrations, hemorrhages and moderate tubular necrosis. (b) Hepatopancreas after 70 hours of injection showing diffuse hepatocytic necrosis. (c) Spleen after 70 hours of injection showing depletion of white and red pulp and necrosis at ellipsoid area. (d) Gills after 50 hours of injection showing mononuclear and eosinophilic granular cell infiltration of hyperplasic tips of secondary gill lamellae. Hematoxylin& Eosin, (Bar = 50 µm).

50 hours of injection showed, separation of the epithelial lining of the secondary gill lamellae, with hyperplasia at the base and the tips, with mononuclear and eosinophilic granular cell infiltration (Fig. 3).

DISCUSSION

E. fergusonii can be distinguished from *E. coli* by being sorbitol and lactose negative but adonitol, amygdalin and cellobiose fermentation positive. *E. fergusonii* ferments L-arabinose, L-rhamnose, maltose, trehalose, cellobiose, D-xylose and D-arabitol. Fermentation of D-glucose occurs with gas production [4].

Forgetta *et al.* [8] provided the first genome sequence of a multidrug resistant and pathogenic *E. fergusonii* Strain ECD-227 isolated from a broiler chicken. This isolate caused disease and 18–30% mortality in one-day old chicks, with the possiblity that surviving chicks may be a reservoir for further infection.

Colonization of the poultry gut by potential pathogenic bacteria such as *E. fergusonii* could result in the contamination of the environment and food chain. Therefore, the potential of *E. fergusonii* to become an important animal and possible emerging opportunistic zoonotic pathogen raises its importance in food safety and public health [7, 15]. Simmon's study showed that *E. fergusonii* is widespread in broilers from the Fraser Valley of British Columbia (Canada). This suggests that *E. fergusonii* may be a normal member of the broiler microflora, like its closest genetic relative, *E. coli*. As for *E. coli*, some strains of *E. fergusonii* can be pathogenic due to the presence of virulence factors.

Several virulence factors, including the presence of a heat-labile toxin on a plasmid, are involved in the pathogenesis of *E. fergusonii* and *E. fergusonii* isolate was found to be resistant to numerous antibiotics [8].

The description of histopathological lesions due to *E. fergusonii* in animals is very rare; there is no description in fish so far. Mostly in terrestrial animals the lesions are confined to gastro-intestinal tract causing diarrhea as main symptom.

In ostrich, the cecal mucosa showed locally extensive areas of hemorrhages and fibrino-necrotic typhlitis with a white-yellowish material covering the mucosal surface. Multiple serosalpetequial hemorrhages and fibrinous peritonitis were present. Histologic examination revealed an intense mononuclear infiltration in the lamina propria and submucosa of the cecum and extensive superficial necrosis associated with fibrin and serocellular deposits [16]. In chicken, Liver showed dilated and congested central vein, liver revealed portal tract congested vessels; there were cytoplasmic vacuolization of hepatocytes and focal area of hepatic necrosis infiltrated by leucocytic cells. Submucosal mononuclear cells infiltration [23], these results also were comparable to some extent to those obtained in naturally and experimentally infected tilapia.

In horses it's reported to cause severe, acute, fibrinous endocarditis, Isolation of the bacteria in moderate to high amounts from the intestine, the tricuspid valve and the liver [12] which was similar to some extent to the observation in naturally infected tilapia. While in cattle respiratory system was affected as severe focally extensive fibrino-necrotic pneumonia [24].

These comparative results revealed some similarity in tissue pathology between these terrestrial animals and our experimental fish. Also the affected animals give some idea about the host that harbors the bacteria which can be considered as the predicted source of infection to the fish. The affected farm used animal and poultry manure for fertilization of tilapia grow out ponds, so this may be a possible source of infection in our case.

In conclusion, this study was the first evidence that *E. fergusonii* can infect fish especially farmed tilapia, causing considerable mortality and morbidity, in heavily manured earthen ponds. *E. fergusonii* causes significant pathological lesions in tilapia that are comparable to other susceptible terrestrial animals. Due to its rising zoonotic importance, *E. fergusonii* infection to fish should be seriously considered as public health hazard.

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