Comparative pathogenecity of methicillin-resistant *Staphylococcus aureus* (MRSA) in Nile tilapia *(Oreochromis niloticus)* and *Tilapia zilli*.

Gaafar, A.Y.[1]*, Soliman, M. K.[2], Ellakany, H. F.[2], Affr, N. A.[3], Elbialy, A. K.[2], Mona S. Zaki [1], Younes, A. M.[1] and Abozahra, R.[4]

[1] Department of Hydrobiology, Veterinary Researches Division, National Research Center (NRC), Cairo, Egypt.
[2] Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, Egypt.
[3] Veterinary Services Authority, Kafr El-Sheikh Branch, Egypt.
[4] Pharmaceutical Microbiology Department, Faculty of Pharmacy, Damanhour University Egypt.
alkhateibyg@yahoo.com

Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) has been reported recently in cultured Nile tilapia *(Oreochromis niloticus*) causing remarkable morbidities and mortalities. This research is a descriptive Histopathological study of the infection caused by MRSA from natural outbreak in cultured Nile tilapia *(Oreochromis niloticus)*. Experimentally challenged *Oreochromis niloticus* and *Tilapia zilli* were also investigated for comparative pathology. Pathological changes in both naturally and experimentally infected fish were described mainly in the brain, kidney, spleen and gills. The alterations were ranging between minor degenerative changes to severe necrotic alterations. Pathogenicity test showed that there are less pathological changes in tissues of *Tilapia zilli* and the changes were more severe in Nile tilapia *(Oreochromis niloticus)*. It was concluded that, presence of *Tilapia zilli* in Nile tilapia ponds is not only a managemental fault in tilapia culture but also it could be a biosecurity breach for bacterial fish diseases, especially for the zoonotic MRSA strains.

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1. Introduction

Tilapias are the second most cultured fish, after carps**.** In the last decade, the 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 **(FAO, 2012)**.

Global Tilapia production is dominated by three species: Nile tilapia (*Oreochromis niloticus*), Mozambique tilapia *(Oreochromis mossambicus)* and Blue Tilapia *(Oreochromis aureus)* **(Rana, 1997).**

Fish losses due to diseases are now important problems that affect aquaculture venture and threaten the sustainability of the industry as a whole **(Lio-Po and Inui, 2010).** Outbreak of bacterial diseases in fish remains one of the most significant limiting factors affecting fish culture worldwide **(Zorrilla *et al.*, 2003).** Outbreaks of tilapia diseases have been observed to cause considerable financial and economical losses **(Bolivar *et al.*, 2001).**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a remarkable problem in human medicine because it has high resistance for all beta-lactam antimicrobials (penicillins, cephalosporins, carbapenems) **(Wulf and Voss, 2008**), **Duran *et al.* (2012)** stated that it also resists aminoglycosides, and macrolides rendering its infections hard-to-treat **.**

MRSA strains were first reported in the United Kingdom in 1961 **(Chambers, 2001)**, soon after the methicillin entered clinical use. Outbreaks of infections caused by MRSA were reported soon thereafter in Europe, and by the mid-1970s, MRSA had become a significant problem in the United States. MRSA is critical public health threats because it causes hospital-acquired infections that can be difficult and expensive to treat **(Kamal *et al.*, 2013)**. Many MRSA strains are susceptible only to vancomycin. Hence, there is also concern that MRSA may serve as a reservoir of organisms that may give rise to vancomycin-resistant strains that could not be killed with available antibiotics **(Fitzgerald *et al.*, 2001)**.

Publications stated that MRSA transmission has two main forms, hospital-acquired (HA) and community-acquired (CA). Although, HA MRSA infection is thoroughly investigated as the major form, CA MRSA presently represents an imminent hazard and may have severe consequences **(Calfee *et al.*, 2003**).

While MRSA has emerged in animals at a slower rate, this pathogen has now a significant concern in veterinary medicine **(Weese and van Duijkeren, 2010).**

Sub-inhibitory levels of antibiotics may be to blame for inducing resistance in commensal bacteria in farm animals. Antibiotic selective pressure may occur in environments such as waste water treatment systems, agricultural environments where antibiotics from veterinary or agricultural sources increase resistance **(Gaze *et al.*, 2008).**

The prevalence of MRSA isolation from hospitals, community, animals and their products has increased in different geographical locations **(Grema *et al.*, 2015)**.

In aquaculture, MRSA have been isolated for the first time from tilapia in Malaysia **(Atyah *et al.*, 2010)**. Also it was associated with mortalities and morbidities in cultured Nile tilapia in Northern Egypt **(Soliman *et al.*, 2014)**.

In this study, the precise histopathological investigations were identified from natural infection in Nile tilapia (*Oreochromis niloticus*) and experimentally infected Nile tilapia (*Oreochromis niloticus*) and *Tilapia zilli* using pathogenicity test.

2. Material and Methods

1. Fish:

Naturally infected histopathological specimens were obtained from previous study on Nile tilapia (*Oreochromis niloticus*) **(Soliman *et al.*, 2014)**. For experimental infection studies, a total number of 350 apparently healthy fishes: 250 *Oreochromis niloticus*, 100 *Tilapia zilli* were collected from private fish cages and fish farms. These fish were transported alive to the laboratory of the Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhur University, the average weight of fish were 30-50 gm.

Fish were kept in 160 liters glass aquaria. These aquaria were used for holding the experimental fish throughout the total period of the study, supplied with chlorine free tap water. The continuous aeration was maintained in each aquarium using an electric air pump. Water temperature was kept at 26 ± 1^{o}C. Fish were placed in aquaria and acclimatized for 2 weeks prior to the experiment.

The fish were fed on a commercial fish diet containing 25% crude protein. Feeding ratio was 1% of body weight. The daily amount of food was offered on two occasions over the day **(Eurell *et al.*, 1978)**.

2. Determination of lethal dose fifty (LD_{50}):

Staphylococcus aureus strain was kindly provided by Department of Poultry and Fish diseases, Faculty of veterinary medicine, Damanhur University. Which was isolated from naturally infected cultured Nile tilapia (*Oreochromis niloticus*) **(Soliman *et al.*, 2014)**.

Staphylococcus aureus was grown overnight in Tryptone Soya Broth (TSB) at 37 ^{o}C , then washed in PBS for 3 times by centrifugation at 4000 rpm and injected intra-peritoneally (I.P.) into healthy Nile tilapia *and Tilapia zilli*. Fish received a 50 µl intraperitoneal (I.P.) injection containing 1×10^{4}, 1×10^{5}, 1×10^{6}, 1×10^{7}, 1×10^{8} and 1×10^{9} *Staphylococcus aureus* CFU/fish. Control group received an I.P. injection of PBS. Fish were monitored for any signs of disease or mortality over a period of four weeks.

The intra-peritoneally injected fish were kept in well prepared glass aquaria, fed with 25% crude protein with feeding ratio of 1% of total biomass.

LD_{50} was determined according to **(Reed and Muench, 1938)** by the following equation:

$$\text{Proportional Distance (PD)} = \frac{\text{\% of mortality at dilution above 50\% - 50}}{\text{\% of mortality at dilution above 50\% - \% of mortality below 50\%}}$$

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

3. The pathogenicity test:

After calculation of LD_{50} of *Staphylococcus aureus* in Nile tilapia, 0.5 LD_{50} used for performing pathogenicity test in Nile tilapia and *Tilapia zilli* **(Table 1)**.

0.5 LD_{50} injected intraperitoneally (I.P.) into healthy 30 Nile tilapia and 30 *Tilapia zilli*. Every 5 hours one fish in each group was dissected out and the organs preserved in Davidson's fixative solution for 24 hours, then to ethanol 75%.

4. Histopathological examination:

From naturally infected fishes (Nile tilapia) and experimentally infected fishes (Nile tilapia and *Tilapia zilli*), after complete necropsy, fresh tissue specimens were collected from gills, liver, spleen, kidney, brain, gonads, skin and musculature and intestine in for histological examination. These specimens were rapidly fixed in Davidson's fixative solution for at 24 hours, then in ethanol 75% till processing. 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^{o} C). Paraffin blocks were prepared and cut 3 micrometer thick sections and stained with Hematoxylin and Eosine (H&E).

Table (1): Experimental design of LD_{50} test of *Oreochromis niloticus* and *Tilapia zilli* against *Staphylococcus aureus*.

Group	Injection Dose	Fish species	Number of fish
1	Control (Injected I.P. with PBS)	*Oreochromis niloticus*	15
		Tilapia zilli	15
2	1×10^4 CFU/fish	*Oreochromis niloticus*	15
		Tilapia zilli	15
3	1×10^5 CFU/fish	*Oreochromis niloticus*	15
		Tilapia zilli	15
4	1×10^6 CFU/fish	*Oreochromis niloticus*	15
		Tilapia zilli	15
5	1×10^7 CFU/fish	*Oreochromis niloticus*	15
		Tilapia zilli	15
6	1×10^8 CFU/fish	*Oreochromis niloticus*	15
		Tilapia zilli	15
7	1×10^9 CFU/fish	*Oreochromis niloticus*	15
		Tilapia zilli	15

3. Results

1. Experimental infection studies:

Determination of lethal dose 50 (LD_{50}):

Challenge experiments using six different doses of *Staphylococcus aureus* by I.P. injection caused no mortality or morbidity in *Tilapia zilli* over a period of four weeks. Only one fish died at the highest dose (10^9 CFU/fish) 6 days post-injection. But *S. aureus* caused mortalities in Nile tilapia as shown in **table (2)**.

Table (2): LD_{50} testing of *Oreochromis niloticus* against intra-peritoneal injection of *Staphylococcus aureus*.

Inoculums	Bacterial dose (CFU/fish)	Fish died/injected	mortality %	LD_{50}
S. aurues	1×10^6	5/15	33.3	$10^{7.165}$
S. aurues	1×10^7	8/15	53.3	
S. aurues	1×10^8	9/15	60	
S. aurues	1×10^9	11/15	73.3	
Control	Injected I.P. with PBS	0/15	0	

Bacterial colonies with similar morphological characteristics to *Staphylococcus aureus* were recovered on mannitol salt agar plates from the kidney of the only fish that died. After 28 days post-infection, *Staphylococcus aureus* was isolation from the inoculated *Tilapia zilli*. The identification of the bacterial colonies was confirmed by PCR and sequencing with mecA gene primers (sequence homology 100%) **(Soliman *et al.*, 2014)**.

Calculation of LD_{50}:

$$PD = \frac{53.3-50}{53.3-33.3} = 0.165$$

Log LD_{50}=0.165+7= 7.165

$LD_{50}=10^{7.165}$

The pathogenicity test:

After 60 hours of injection mortality % of Nile tilapia was 100 % while in *Tilapia zilli* no mortality was observed after 75 hours post injection.

2. Histopathological results:

Naturally infected *O. niloticus* specimens:

Brain showed severe congestion in the main cerebral and meningeal blood vessels, perivascular cuffing around cerebral blood vessels due to proliferation of satellite cells, and gliosis especially nearby some areas of white matter vacuolation. Also there was abundance of mononuclear cells inside main blood vessels (Fig.1a).

Hepatopancreas showed many circulatory disturbances, such as congestion and hemorrhages, mononuclear cell infiltrations were abundant usually nearby areas of hepatocellular degeneration and necrosis, also activation of melanomacrophage centers was noticeable (Fig.1d,f).

Spleen showed depletion of both white and red pulp.

Posterior kidney showed tubule-glomerular degeneration and necrosis, areas of interstitial necrosis infiltrated usually with mononuclear cells and hemorrhages, and activation of melanomacrophage centers (Fig.1b,e).

Gills showed hyperplasia at the base of secondary gill lamellae with the presence of congestion in lamellar blood vessels, with mild separation of the epithelium from underlying tissue of the secondary gill lamellae (Fig.1c).

Comparative histopathology results:

Comparative histopathological picture is summarized in **table (3)**.

Table (3): Comparative histopathological findings after experimental infection of *Oreochromis niloticus* and *Tilapia zilli* by *Staphylococcus aureus*.

Time of sampling	*O. niloticus*	*T. zilli*
After 10 hours	*	- Hepatopancreas showed mild vacuolation
After 20 hours	- Hepatopancreas showed mild cloudy swelling with normal structure of pancreatic acinar cells. - Spleen showed beginning of activation of MMCs and high storage of blood inside the spleen not distributed yet to the peripheral circulation.	- Spleen showed normal structure with minor congestion. - Hepatopancreas showed vacuolation with some congestion especially in sinusoidal spaces.
After 30 hours	- Hepatocytes showed mild cloudy swelling and mild degenerative changes occurring in pancreatic acinar cells. - Gills showed beginning of hyperplasia and congestion with mild separation of the epithelium from underlying tissue of the secondary gill lamellae.	-
After 35 hours	-	- Spleen showed mild congestion. - Hepatopancreas showed congestion with some vacuolation.
After 40 hours	- Spleen showed discrete vacuolations in white pulp due to initial mobilization of white blood cells to peripheral circulation with dispersed (not aggregated) MMCs. - Gills showed congestion and hyperplasia at the base of secondary gill lamellae - Brain showed congestion of main brain blood vessels, gliosis and perivascular cuffing.	- Posterior kidney showed normal structure of kidney with some congestion and degenerative changes in renal interstitial tissue (Fig.3a).
After 45 hours	- Brain showed congestion of main brain and meningeal blood vessels, gliosis, perivascular cuffing and high appendance of mononuclear cells inside blood vessels lumina (Fig.2a). - Spleen showed congestion and depletion of white pulp due to mobilization of white blood cells to peripheral circulation with activation of MMCs (Fig.2b). - Posterior kidney showed congestion and glomerular necrosis and activation of MMCs (Fig.2c).	-
After 50 hours	- Spleen showed white pulp depletion, severe congestion and hemorrhages with activation of MMCs. - Posterior kidney showed congestion and glomerular and interstitial necrosis and activation of MMCs. - Hepatopancreas showed congestion and discrete areas of hepatocellular degeneration and necrosis (Fig.2d).	- Hepatopancreas showed congestion with some vacuolation (Fig.3c). - Spleen showed some congestion with activation of MMCs (Fig.3d).
After 55 hours	- Hepatopancreas showed abundance of resident macrophage in sinusoidal spaces. - Gills showed congestion and hyperplasia at the base of secondary gill lamellae and telangiectasis.	-
After 60 hours	- Hepatopancreas showed severe congestion, vacuolation and cloudy swelling of hepatocytes (Fig.2e). - Posterior kidney showed severe hemorrhages and tubule-glomerular and interstitial necrosis (Fig.2f).	- Hepatopancreas showed mild vacuolation and sinusoidal congestion with congestion of main blood vessels.
After 70 hours	**	- Hepatopancreas showed vacuolation and severe congestion of main blood vessels, with mild activation of MMCs (Fig.3e).
After 75 hours	**	- Hepatopancreas showed diffuse vacuolation of hepatocytes and severe congestion of main blood vessels, with mild activation of MMCs. - Spleen showed severe congestion of main blood vessels and mild activation of MMCs (Fig.3f).

- No new findings, * Normal tissue structure, ** All experimental fish were died, MMCs: melanomacrophage centers.

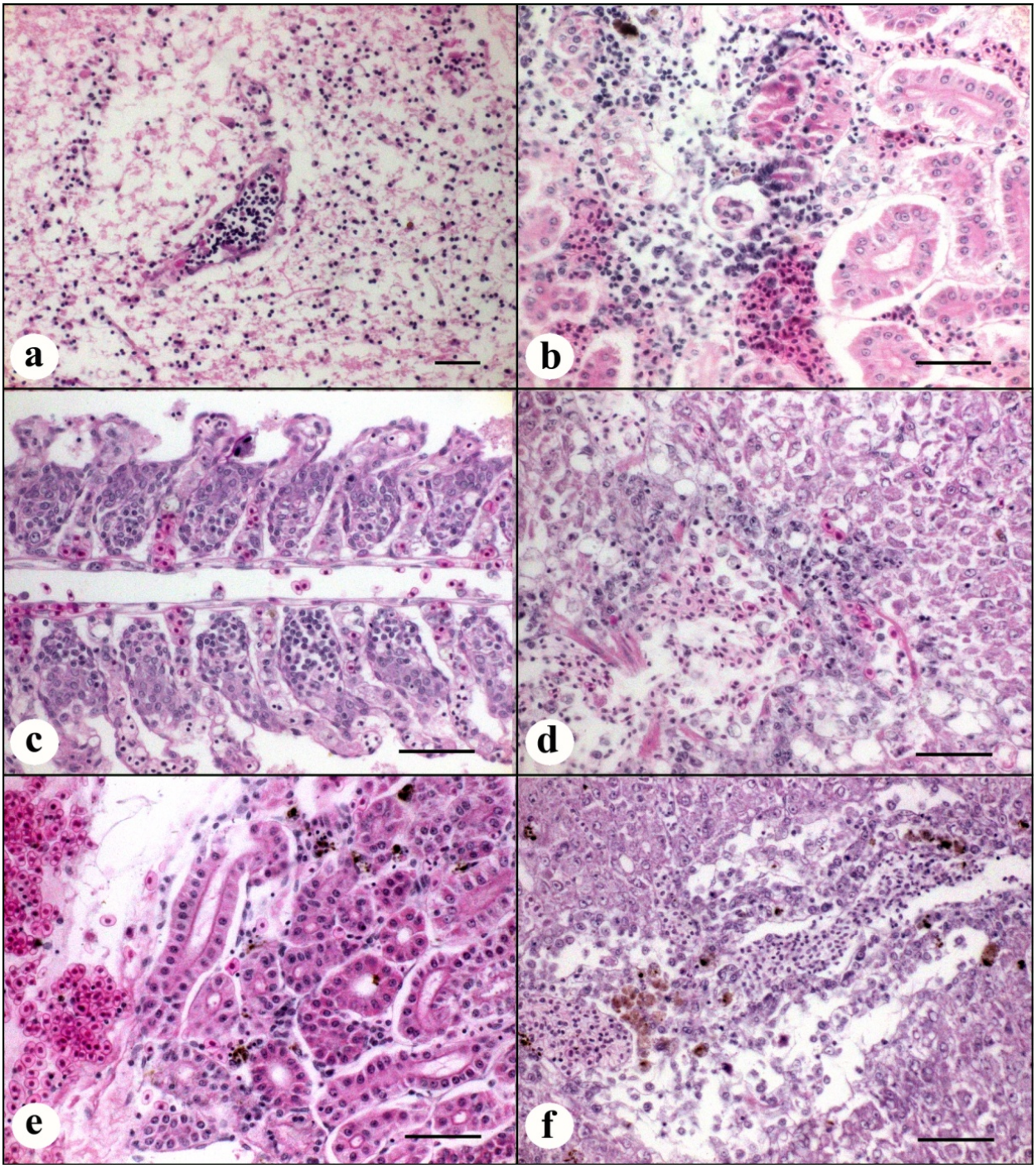

Figure (1): Nile Tilapia (*Oreochromis niloticus*) naturally infected with Methicillin-resistant *Staphylococcus aureus* (MSRA): (a) brain showing congestion with the presence of mononuclear cells (lymphocytes) inside the blood vessels. (b) Posterior kidney showing interstitial necrosis with presence of mononuclear cell infiltration associated with hemorrhages and some tubular necrosis. (c) gills showing hyperplasia at the base of secondary gill lamellae with the presence of severe congestion in central blood vessels and separation of the epithelium from underlying tissue in the secondary gill lamellae also mononuclear cell infiltration in the proliferated spongial cells. (d) Hepatopancreas showing presence of mononuclear cell infiltration in severely necrosed tissue area. (e) Posterior kidney showing hemorrhages and activation of melanomacrophage center. (f) Hepatopancreas showing mononuclear cell infiltration in necrotic area and activation of melanomacrophage centers. Hematoxylin & Eosin stain **(Bar = 50 µm)**.

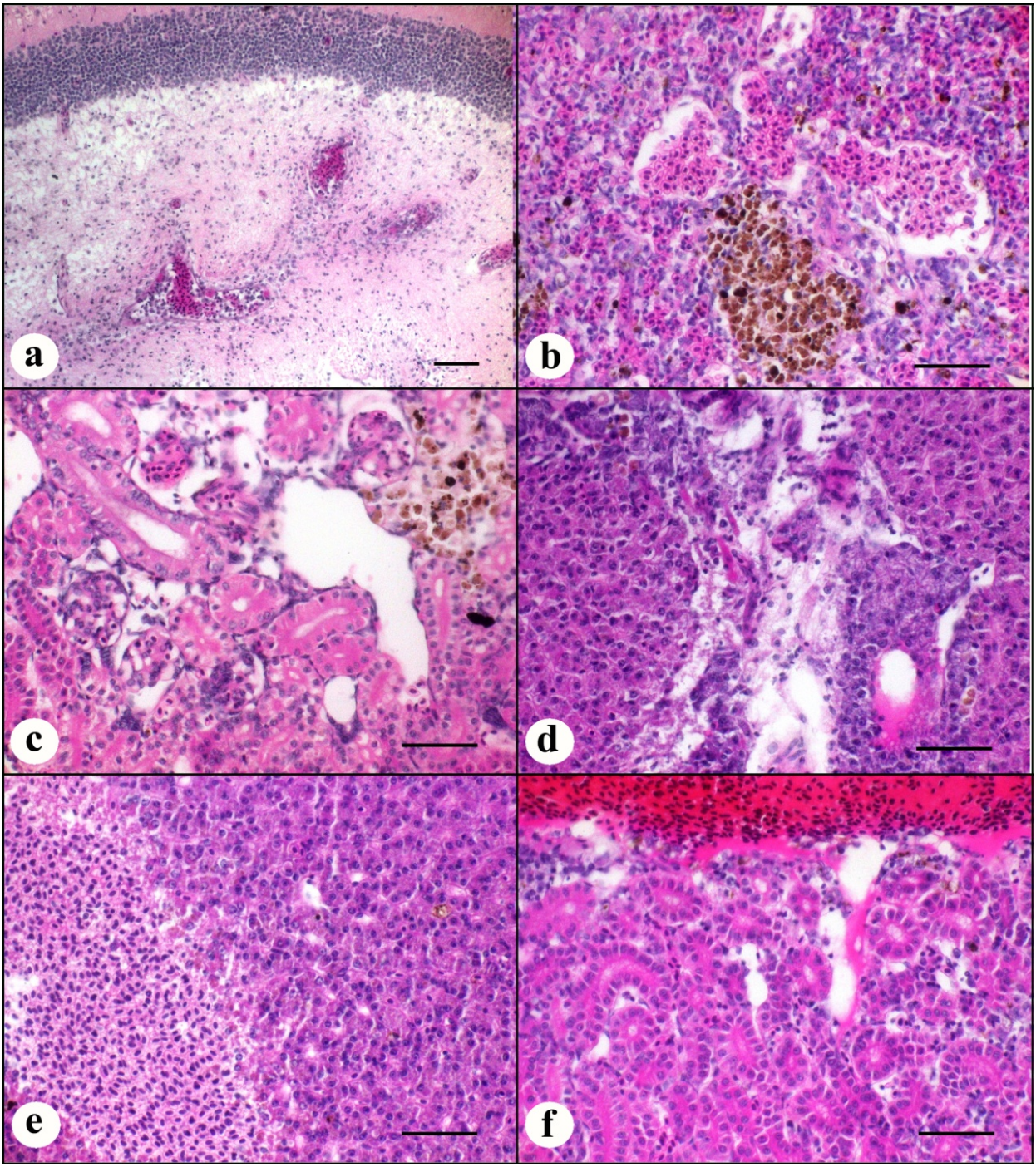

Figure (2): Nile Tilapia (*Oreochromis niloticus*) experimentally infected with Methicillin-resistant *Staphylococcus aureus* (MSRA): (a) brain after 45 hours of injection showing congestion of main cerebral blood vessels and gliosis (perivascular cuffing). (b) Spleen after 45 hours of injection showing congestion of spleen and activation of melanomacrophage centers with some vacuolation inside the spleen. (c) Posterior kidney after 45 hours of injection showing activation of melanomacrophage centers and some degenerative changes in glomerulai. (d) Hepatopancreas after 50 hours of injection showing the presence of hepatocellular necrosis. (e): Hepatopancreas after 60 hours of injection showing severe hemorrhages inside necrotic area with cloudy swelling of hepatocytes. (f) Posterior kidney after 60 hours of injection showing severe hemorrhages. Hematoxylin & Eosin stain (**Bar = 50 µm)**.

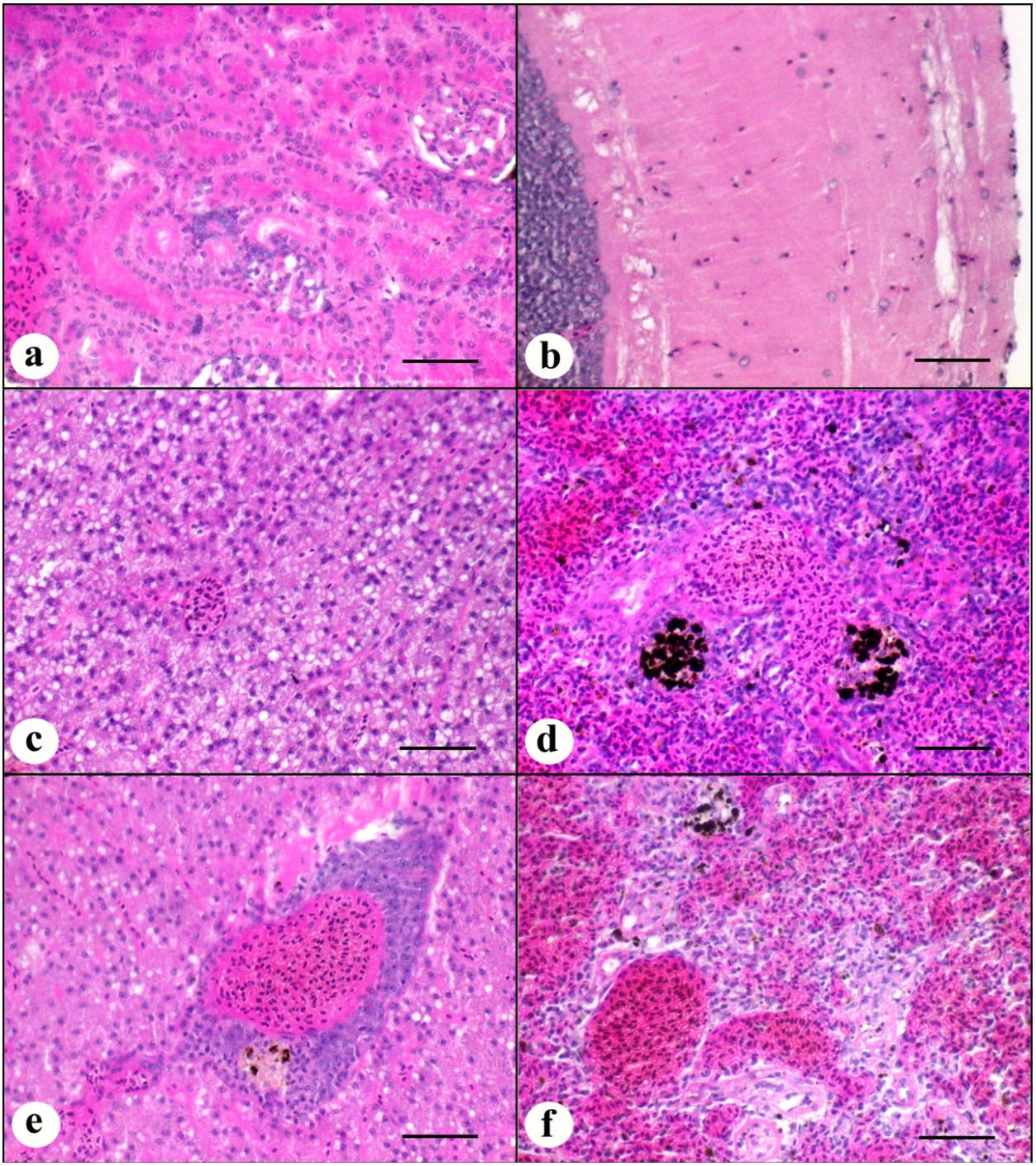

Figure (3): *Tilapia zilli* experimentally infected with Methicillin-resistant *Staphylococcus aureus* (MSRA): (a) Posterior kidney after 40 hours of injection showing normal structure of kidney with some congestion in interstitial renal tissue. (b) Brain after 50 hours of injection showing normal structure of brain. (c) Hepatopancreas after 50 hours of injection showing congestion and mild vacuolation. (d): Spleen after 50 hours of injection showing some congestion with activation of melanomacrophage centers. (e): Hepatopancreas after 70 hours of injection showing beginning of vacuolation and severe congestion of main blood vessels with mild activation of melanomacrophage centers. (f): Spleen after 75 hours of injection showing severe congestion of main blood vessels and mild activation of melanomacrophage centers. Hematoxylin & Eosin stain **(Bar = 50 µm)**.

4. Discussion

Nile Tilapia is the main cultured fish species in Egypt, in the last decade it faced many seasonal bacterial disease outbreaks, due to increasing stress magnitude, such as culture intensification, increasing environmental pollution due to high organic loads and agricultural discharges.

New emerging bacterial fish diseases, which were uncommon to infect tilapia, rose as a new threat for its aquaculture, this may be attributed to close contact of aquaculture environment with animal and/or human wastes. Bacterial pathogens such as Methicillin-resistant *Staphylococcus aureus* (MRSA) stated by **Atyah *et al.* (2010)** in Malaysia and **Soliman *et al.* (2014)** in Egypt, and also *Escherichia fergosonii* in Egypt (data under publication).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a remarkable problem in human medicine **(Wulf and Voss, 2008 and Duran *et al.*, 2012)** and its new attitude as fish pathogen obtained high zoonotic consideration**.**

In Northern Egypt 2014[s] outbreak, the authors noticed that the only affected species in the ponds was Nile Tilapia, while the unwanted cohabitate species; *Tilapia zilli* was not harmed by MRSA. Further investigations were needed to elucidate this observation.

Histopathological examination of naturally infected specimens of Nile tilapia revealed classical septicemic picture. The brain also showed histopathological changes denoting that MRSA has the ability to penetrate the blood-brain barrier in fish exactly like terrestrial animal **(Sheen *et al.*, 2010)**. Hepatopancreas and posterior kidney showed many degenerative changes may be attributed to the bacterial exotoxins **(Spaulding *et al.*, 2013)**. Spleen showed depletion of both white and red pulp may be due to cellular mobilization to inflammatory sites to combat the proliferating bacteria.

Pathogenicity testing of both Nile tilapia and *Tilapia zilli* with half LD_{50} of Nile tilapia revealed serious histopathological findings and mortality in Nile tilapia while in *Tilapia zilli* histopathological findings were moderate or mild. This observation indicated that *Tilapia zilli* is more tolerant to MRSA infection than Nile tilapia, as a cohabitate in Nile tilapia ponds, the speculation that *Tilapia zilli* can be carrier state or a future source of infection in Nile tilapia aquaculture premises can be true.

It can be concluded that, presence of *Tilapia zilli* in Nile tilapia ponds is not only a managemental fault in tilapia culture but also it could be a biosecurity breach for bacterial fish diseases, especially for the zoonotic MRSA strains.

Corresponding author:
Dr. Alkhateib Y. Gaafar
Department of Hydrobiology, Veterinary Researches Division, National Research Center (NRC), Cairo, Egypt.
Email: alkhateibyg@yahoo.com.

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