Benha Veterinary Medical Journal 37 (2019) 144-148

Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

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Original Paper

Bacteriological studies on Aeromonas and pseudomonas species in Nile tilapia (*Oreochromis niloticus*)

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ARTICLE INFO

Keywords

Aeromonas
Bacteriological
Fish
Pseudomonas
Sensitivity tests.

Received 23/08/2019
Accepted 03/11/2019
Available On-Line 12/05/2020

ABSTRACT

The present study was conducted on 250 diseased Nile tilapia (*Oreochromis niloticus*) of various sizes collected from different fish markets at Kaliobia Governorate, Egypt, during the period from May 2017 to January 2019 for inspection of Aeromonas and Pseudomonas strains. Samples were collected from apparently pathognomonic lesions in muscle, liver, kidney, intestine and spleen for bacteriological examination. The results revealed that, 161 Aeromonas and Pseudomonas species; 118 Aeromonas (73.3%) and 43 Pseudomonas species (26.7%) were isolated mostly from 50 muscle lesion samples followed by 41 liver, 35 kidney, 32 intestine and 3 spleen lesion samples. Aeromonas strains were highly sensitive to meropenem followed by ciprofloxacin, norfloxacin, gentamycin and florphenicol. Meanwhile, they were highly resistant for ampicillin and methicillin followed by oxacillin, penicillin-G, amoxicillin, cefotaxime, oxytetracycline and streptomycin. In addition, Pseudomonas strains were highly sensitive to meropenem followed by gentamycin, norfloxacin, ciprofloxacin and florphenicol. In contrast, they were highly resistant for ampicillin, methicillin and penicillin-G followed by amoxicillin, oxacillin and cefotaxime.

1. INTRODUCTION

Fish diseases due to bacterial infections mainly, *Aeromonas* and *Pseudomonas* species were considered the main causes of diseases including ulcerative syndrome leading to high mortalities also can be a problem for human consumers and high economic losses in Egypt (Abdel-Hadi *et al*., 2008; Shayo *et al*., 2012 and Hanna *et al*., 2014).

Members of the *Aeromonas* and *Pseudomonas*, are Gram-negative rods, either straight or curved facultative anaerobes, catalase-positive and most are motile by polar flagella. Their nutritional requirements are very simple and most grow on common laboratory media. They are widespread in freshwater, sewage, soil and their numbers rise with the amount of organic matter present (Markey *et al.,* 2013). Aeromonads produce extracellular enzymes (haemolysins, lipases, proteases, β-lactamases, amylases, chitinases and nucleases) involved in their ecology, survival and pathogenicity (Stratev *et al*., 2015). In addition, the pathogenicity of *A. hydrophila* strains have been linked to some virulence factors produced by them including structural features associated with adhesion, cell invasion, resistance to phagocytosis as well as extracellular factors such as aerolysin, a pore-forming toxin, which is cytolytic and enterotoxin genie (Chopra and Houston 1999; Rabaan *et al.,* 2001).

Pseudomonas (*Ps.*) *aeruginosa* and *Ps. fluorescens* are considered problematic pathogens as they possess cell-associated virulence factors such as (pili, flagella, lipopolysaccharide and alginate/biofilm). They also produces a number of extracellular products such as protein exotoxin A, proteases, type III secretion system exoenzymes, rhamnolipid, haemolysin with lecithinase activity; elastase (*las* B and *las* A), siderophores (pyochelin, pyocyanin, and pyoverdine by *Ps. aeruginosa* and thioquinolobactin by *Ps. fluorescens*) and phospholipase C. (Mavrodi *et al*., 2001; Markey *et al.,* 2013). These virulence factors play a role in disease pathogenesis.

As *Aeromonas* and *Pseudomonas* are considered one of the most important fish pathogens and can be a problem for human consumers too, the present study was conducted to throw light over their infection in freshwater fish, Nile-tilapia (*O. niloticus*).

2. MATERIAL AND METHODS

2.1. Samples collection:

250 diseased Nile tilapia (*O. niloticus*) of various sizes were collected from different fish markets at Kaliobia Governorate, Egypt, during the period from May 2017 to January 2019 for bacteriological examination of *Aeromonas* and *Pseudomonas*

2.2. Clinical and postmortem examinations

It was performed using the method described by Schaperdaus *et al*. (1992).

2.3. Bacteriological examination

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2.3.1. Sampling:
Under complete aseptic conditions 445 samples were collected from apparently pathognomonic lesions in muscle, liver, kidney, intestine and spleen by a number of 139, 118, 99, 70 and 19, respectively.

2.3.2. Isolation and identification of suspected Aeromonas and Pseudomonas species:
The surface of lesions was smeared by hot spatula, then a sterilized loopful and inoculated onto Tryptone soya broth and incubated aerobically at 28 ºC for 18 hours. A loopful from incubated Tryptone soya broth was streaked onto Tryptone soya agar and MacConkey's agar plates and incubated for 18 hours at 28 ºC. The pale colonies on MacConkey's agar and yellowish or creamy in color colonies on Tryptone soya agar were picked up and the following tests (Oxidase test and Catalase test) were performed.
The purified colonies were morphologically identified by Gram stain and biochemical tests (Quinn et al., 2002; Austin and Austin, 2007; Markey et al., 2013). The suspected colonies (that gave Oxidase +ve and Catalase +ve) were taken and cultivated on the following media: Aeromonas base agar; Rimler- Shotts agar (R.S.), Eosin methylene blue agar (EMB), Brilliant green agar, XLD agar, Pseudomonas agar and Pseudomonas Cetrimide agar then incubated for another 18 hours at 25 ºC and 28 ºC. Suspected colonies were kept in Semi-solid nutrient agar. Moreover, the *in vitro* sensitivity test was done ~~on~~ for each isolated bacteria to study its anti-microbial sensitivity according to Koneman *et al.* (1997).

3. RESULTS

The results of bacteriological examination of examined fish and in- vitro sensitivity tests for isolated strains were tabulated in tables (1-5).
The clinical examination of the studied fish showed irregular redness all over the fish body especially at the ventral part of abdomen, base of the fins, loss of fin membrane and sometimes loss of fin rays with grey patches at the tip of them (fins rot), and around the anal opening. Others showed eye cloudiness, detached scales, skin ulceration and abdominal distention. Internally, abdominal dropsy with reddish ascetic exudates and congestion of internal organs were observed. Congested intestine that sometimes filled with yellow mucous like materials was noticed. The infected fish showed abdominal dropsy with reddish ascetic exudates, liver paleness and enlarged in some fish and congested with gray patches in other fishes; congested kidneys; congested and enlarged spleen and congested intestine that sometimes filled with yellow exudates like materials in some fish.
The recovered Aeromonas isolates in the present study were Gram-negative, straight rods with round end, non-capsulated, non-sporulated. Moreover, they showed yellow to creamy colonies on Tryptone soya agar, pale colonies, and become pink on MacConkey's agar media. On Rimler-Shotts medium (R.S) they produced yellow convex colonies; on Aeromonas agar they gave green colonies darker in center than emerging. Only *A. hydrophila* strains gave violet to metallic green sheen colonies on EMB media due to lactose utilization, they gave yellow colonies on Thiosulphate-citrate-bile-sucrose (T.C.B.S) agar due to fermentation of sucrose.
Biochemical identification showed that all 118 isolates had characteristic biochemical reaction to be Aeromonas species, 102 *A. hydrophila* and 16 *A. caviae* where, all *A. hydrophila* isolates were positive for oxidase test, catalase test, indole test, Voges-Proskauer, citrate utilization, lysine decarboxylase with H_2s production, fermented glucose, mannitol, sucrose and lactose, but they were negative for methyl red, and urease tests. Meanwhile, all *A. caviae* were positive for oxidase test, catalase test, methyl red test, citrate utilization, lysine decarboxylase without H_2S production, fermented glucose and mannitol, but they were negative for sucrose and lactose fermentation, Voges-Proskauer and urease tests.
Pseudomonas isolates are Gram-negative, straight or slightly curved rods. They grew well and showed large, flat, spreading and irregular colonies with greenish-blue coloration on the culture with a characteristic fruity, grape-like odour of aminoacetophenone (*Ps. aeruginosa*) and yellowish colonies (*Ps. fluorescens*) on nutrient agar. Large, pale colonies on MacConkey's agar (unable to utilize lactose) with greenish-blue pigment superimposed. Red colonies on Brilliant green agar, indicative of an alkaline reaction. Red colonies on XLD agar, no H_2S and no fermentation of sucrose and lactose sugars. Yellowish green colonies *Ps. fluorescens* and bluish green colonies *Ps. aeruginosa* on Pseudomonas agar. Small and smooth with blue-green pigmented colonies, *Ps. aeruginosa* on Pseudomonas Cetrimide agar.
Biochemical identification showed that all 43 isolates had characteristic biochemical reaction to be Pseudomonas species, 29 *Ps. aeruginosa* and 14 *Ps. fluorescens* where, all *Ps. aeruginosa* isolates were positive for oxidase test, catalase test, citrate utilization, urease test, lysine decarboxylase without H_2S production, fermented glucose and mannitol but they were negative for sucrose and lactose fermentation, indole, Voges-Proskauer and methyl red tests. Meanwhile, all *Ps. fluorescens* were positive for oxidase test, catalase test, citrate utilization, urease test, lysine decarboxylase without H_2S production, fermented mannitol but they were negative for glucose, sucrose and lactose fermentation, indole, Voges-Proskauer and methyl red tests .

Table 1 Prevalence of positive samples for Aeromonas and Pseudomonas species isolation among examined 250 Nile tilapia *(O. niloticus)* fish

No. of examined lesion samples	No. of Positive samples	Positive percentage		No. of single pure culture				No. of mixed culture	
		%*	%**	Aeromonas spp.		Pseudomonas spp.			
				No.	%***	No.	%***	No.	%***
445	128	51.2	28.8	85	66.4	10	7.8	33	25.8

*Percentage in relation to number of examined fish samples (250). **Percentage in relation to no. of lesion samples (445). ***Percentage in relation to no. of positive samples (128)

Table 2 Prevalence and distribution of Aeromonas strains isolated from examined lesion samples

Aeromonas species lesion samples	A. hydrophila			A. caviae			Total	
	No.	%*	%**	No.	%*	%**	No.	%**
Muscle	29	28.4	24.6	7	43.8	5.9	36	30.5
Liver	25	24.5	21.2	4	25.0	3.4	29	24.6
Kidney	23	22.6	19.5	3	18.7	2.5	26	22.0
Intestine	22	21.6	18.6	2	12.5	1.7	24	20.3
Spleen	3	2.9	2.5	0	0.0	0.0	3	2.5
Total	102	100.0	86.4	16	100.0	13.6	118	

*Percentage in relation to number of each Aeromonas species isolated (102 and 16). **Percentage in relation to total number of isolated Aeromonas species (118)

Table 3. Prevalence and distribution of Pseudomonas species isolated from examined samples

Pseudomonas species	Ps. aeruginosa			Ps. fluorescens			Total	
lesion samples	No.	%*	%**	No.	%*	%**	No.	%**
Muscle	11	37.9	25.6	3	21.4	7.0	14	32.6
Liver	7	24.1	16.3	5	35.7	11.6	12	27.9
Kidney	5	17.2	11.6	4	28.6	9.3	9	20.9
Intestine	6	20.7	13.9	2	14.3	4.7	8	18.6
Spleen	0	0.0	0.0	0	0.0	0.0	0	0.0
Total	29		67.4	14		32.6	43	100.0

*Percentage in relation to number of each Pseudomonas species isolated (29 and 14).
**Percentage in relation to total number of isolated Pseudomonas species (43)

Table 4 In-Vitro anti-microbial sensitivity test for isolated Aeromonas strains

Antimicrobial agents		Disk concentrations	Sensitive		Intermediate		Resistant		AA
			No.	%	No.	%	No.	%	
Amoxicillin	AMX/25	25µg	6	5.1	11	9.3	101	85.6	R
Ampicillin	AM10	10 µg	0	0.0	2	1.7	116	98.3	R
Cefotaxime	CTX/30	30 µg	5	4.2	12	10.2	101	85.6	R
Ciprofloxacin	CIP/5	5 µg	96	81.4	15	12.7	7	5.9	S
Florphenicol	FFC/30	30 µg	90	76.3	15	12.7	13	11.0	S
Gentamicin	CN/10	10 µg	95	80.5	14	11.9	9	7.6	S
Meropenem	MEM	10 µg	98	83.0	16	13.6	4	3.4	S
Methicillin	ME5	5 µg	2	1.7	0	0.0	116	98.3	R
Norfloxacin	NOR/10	10 µg	96	81.4	13	11.0	9	7.6	S
Oxacillin	OX1	1 µg	0	0.0	3	2.5	115	97.5	R
Oxytetracycline	T/30	30 µg	5	4.2	15	12.7	98	83.1	R
Penicillin-G	P10	10 u	0	0.0	3	2.5	115	97.5	R
Streptomycin	S/10	10 µg	4	3.4	17	14.4	97	82.2	R
Trimethoprim/ Sulphamethoxazol	SXT/25	(1.25/23.75) µg	17	14.4	63	53.4	38	32.2	IS

No.: Number of isolates. AA: Antibiogram activity. %: Percentage in relation to total number of isolates (118)

Table 5 In-Vitro anti-microbial Sensitivity test for isolated Pseudomonas strains

Antimicrobial agents		Disk concentrations	Sensitive		Intermediate		Resistant		AA
			No.	%	No.	%	No.	%	
Amoxicillin	AMX/25	25µg	1	2.3	3	7.0	39	90.7	R
Ampicillin	AM10	10 µg	0	0.0	3	7.0	40	93.0	R
Cefotaxime	CTX/30	30 µg	3	7.0	4	9.3	36	83.7	R
Ciprofloxacin	CIP/5	5 µg	31	72.1	8	18.6	4	9.3	S
Florphenicol	FFC/30	30 µg	29	67.4	8	18.6	6	14.0	S
Gentamicin	CN/10	10 µg	33	76.7	7	16.3	3	7.0	S
Meropenem	MEM	10 µg	35	81.4	6	14.0	2	4.6	S
Methicillin	ME5	5 µg	3	7.0	0	0.0	40	93.0	R
Norfloxacin	NOR/10	10 µg	32	74.4	8	18.6	3	7.0	S
Oxacillin	OX1	1 µg	1	2.3	3	7.0	39	90.7	R
Oxytetracycline	T/30	30 µg	5	11.6	28	65.1	10	23.3	IS
Penicillin-G	P10	10 u	0	0.0	3	7.0	40	93.0	R
Streptomycin	S/10	10 µg	4	9.3	27	62.8	12	27.9	IS
Trimethoprim/ Sulphamethoxazol	SXT/25	(1.25/23.75) µg	5	11.6	30	69.8	8	18.6	IS

No.: Number of isolates. %: Percentage in relation to total number of isolates (43). AA: Antibiogram activity

4. DISCUSSION

The results of clinical and postmortem examinations of studied fish were similar to that reported by Loch and Faisal (2010), Kumar and Ramulu (2013), Hanna *et al.* (2014), Noor El- Deen *et al.* (2014), and Abd El Tawab *et al.* (2016). The prevalence of Aeromonas and Pseudomonas species isolation (Table 1) revealed that, 128 out of 250 examined fish (51.2%) were positive for their isolation, where 95 (74.2%) were positive with pure single cultures, 85 Aeromonas spp. (66.4%) and 10 Pseudomonas spp. (7.8%) as well as 33 (25.8%) mixed ones. These results came in accordance with that obtained by El- Dien *et al.* (2010), Shayo *et al.* (2012), Abd El Tawab *et al.* (2016) and Abd El Tawab *et al.* (2017). The results of bacteriological examination of examined fish lesion samples revealed that, a total of 161 Aeromonas and Pseudomonas species, 118 Aeromonas and 43 Pseudomonas species were isolated from muscle, liver, kidney, intestine and spleen lesion samples with numbers of 50, 41, 35, 32 and 3, respectively. Nearly similar results were recorded by El-Hady and Samy (2011), Hanna *et al*. (2014), Ibrahim- Lamis (2015) and Maarouf *et al*. (2017). Regarding to Aeromonas species, the recorded

results in (Table 2) cleared that, 118 (73.3 %) Aeromonas species were isolated from examined samples, 102 *A. hydrophila* strains (86.4%) and 16 *A. caviae* strains (13.6%). They were isolated mostly from muscle lesion samples 36 (30.5 %) (*A. hydrophila* 24.6% and *A. caviae* 5.9%), followed by liver samples 29 (24.6%) (*A. hydrophila* 21.2% and *A. caviae* 3.4%), then kidney samples 26 (22.0%) (*A. hydrophila* 19.5% and *A. caviae* 2.5%), intestine 24 (20.3 %) (*A. hydrophila* 18.6% and *A. caviae* 1.7%) and spleen samples 3 (2.5 %) that was *A. hydrophila* only. These results agree with those of Mohamed *et al*. (2006), Mahdy (2007), Ibrahim- Lamis (2015), Abd El Tawab *et al*. (2017) and Sayed (2017).

Meanwhile, disagreed with others who recorded lower incidence, El-Dien *et al.* (2010) and Noor El- Deen *et al.* (2014). Meanwhile, for Pseudomonas species, the obtained results (Table 3) revealed that, 43 Pseudomonas species were isolated from examined samples, 29 *Ps. aeruginosa* strains (67.4%) and 14 *Ps. fluorescens* strains (32.6%). They were mostly isolated from muscle lesion samples 14 (32.6%) (*Ps. aeruginosa* 25.6% and *Ps. fluorescens* 7.0%) followed by liver samples 12 (27.9%) (*Ps. aeruginosa* 16.3% and *Ps. fluorescens* 11.6%) then kidney samples 9 (20.9%) (*Ps. aeruginosa* 11.6% and *Ps. fluorescens* 9.3% %) and 8 intestine lesion samples (18.6 %) (*Ps. aeruginosa* 13.9% and *Ps. fluorescens* 4.7%) but both Pseudomonas species failed to be isolated from spleen samples. Nearly similar results were recorded by Eissa *et al*. (2010), Khalil *et al*. (2010), El-Hady and Samy (2011), Hanna *et al*. (2014) and Abd El Tawab *et al*. (2016).

The morphological characteristics of the colonies, Gram staining and the biochemical profile of both Aeromonas and Pseudomonas species isolated such as the fermentation of certain sugars or enzymatic reaction as protease, lipase and extracellular pigmentation production were similar to those previously reported (Jayavignesh *et al.*, 2011; Markey *et al*., 2013; Panda *et al*., 2013; Hanna *et al*., 2014; Abdel-Haq-Fatma El- Zahraa, 2018).

The results of *in vitro* sensitivity tests for 118 isolated Aeromonas strains (Table 4) revealed that they were highly sensitive to meropenem followed by ciprofloxacin, norfloxacin, gentamycin and florphenicol. Meanwhile, they were intermediate sensitive to trimethoprim/ sulphamethoxazol. Moreover, they were highly resistant for ampicillin and methicillin followed by oxacillin, penicillin-G, amoxicillin, cefotaxime, oxytetracycline and streptomycin. Nearly similar results were recorded by Kore *et al.* (2014), Ibrahim- Lamis (2015), Didugu *et al.* (2016) and Abd El Tawab *et al*. (2017). In addition, the results of *in vitro* sensitivity tests for 43 isolated Pseudomonas strains (Table 5) appeared that, they were highly sensitive to meropenem followed by gentamycin, norfloxacin, ciprofloxacin and florphenicol. Meanwhile, they were intermediate sensitive to trimethoprim/sulphamethoxazol; oxytetracycline and streptomycin. In contrast, they were highly resistant for ampicillin, methicillin and penicillin-G followed by amoxicillin, oxacillin and cefotaxime. Nearly similar results were recorded by El-Hady and Samy (2011), Hanna *et al*. (2014), Roy *et al*. (2014), Abd El Tawab *et al*. (2016) and Abdel-Haq-Fatma El-Zahraa (2018). The recorded results are of serious concern as these drugs, especially β-lactam antibiotics, are still considered the most recommended for the treatment of bacterial infections in fish, animals and human. However, their efficiency has greatly deteriorated due to the production of β-lactamases by resistant bacterial strains.

5. CONCULOSION

It could be concluded that, Aeromonas and Pseudomonas species specially, *A. hydrophila*, *A. caviae*, *Ps. aeruginosa* and *Ps. fluorescens* strains are important pathogens causes ulcer type disease with ulcerative and septicemic syndrome among fish, they are sensitive to meropenem, ciprofloxacin, norfloxacin, gentamycin and florphenicol, can be used for treatment of these cases.

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