E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2017; 5(1): 29-34

www.fisheriesjournal.com
Received: 07-11-2016
Accepted: 08-12-2016

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Diversity of enterobacteriaceae retrieved from diseased cultured *Oreochromis niloticus*

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Abstract
Enterobacteriaceae encompasses a group of fish pathogens that frequently occurred as water-borne infections and may be present in the tissues of apparently healthy fish and remain opportunistic in nature. Full clinical, bacteriological and phenotypic characterizations were done on two hundred of diseased *Oreochromis niloticus*, from some private farms from Beheira governorate during summer 2015. Prevalence% of the identified bacterial isolates and their incidence in the internal organs of the diseased fish were determined. Additionally, Antimicrobial sensitivity testing for the most prevalent isolates was also established using 11 antimicrobial discs. It was found that the infected fish have general septicemic signs like skin hemorrhages and ulcerations with noticeable focal hemorrhages and areas of necrosis in liver and congestion in gills and spleen. About 144 *Enterobacteriaceae* strains were retrieved from diseased fish. The identified strains were: *Citrobacter freundii, Edwardsiella tarda, Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Hafnia alvei, Klebsiella sp, Morganella morganii, Proteus vulgaris, Proteus mirabilis, Providencia sp, Salmonella sp, Serratia marcescens* and *Shigella sp*, with average prevalence% were 25.69, 10.42, 6.25, 11.81, 15.28, 4.17, 7.64, 4.86, 3.47, 1.39, 1.39, 1.39, 4.17 and 2.08 respectively. The results showed that the maximum number of isolates was claimed from liver (49.31%) and the lowest number of isolates was obtained from the kidney (13.19%). It was found that the bacterial isolates were markedly sensitive to Ciprofloxacin, gentamycin, Florfenicol, and Flumequine and moderately sensitive Doxycycline and less sensitive to Oxytetracycline, Erythromycin, and Ampicillin. The findings highlight the extreme diversity of *Enterobacteriaceae* that are potentially associated with fish diseases in Egypt.

Keywords: *Enterobacteriaceae,* antibiogram, phenotypic characterization

1. Introduction
Bacterial fish diseases were involved in serious warns to aquaculture systems in Egypt [1]. *Enterobacteriaceae*, can be termed as common water-borne fish bacterial infections, which may normally dwell in the tissues of apparently healthy fish, and the gastrointestinal tract of humans and animals [2, 3], and it causes hazardous outbreaks with mortalities whenever fish are exposed to stress like high temperature and poor water quality [4, 5]. They were integrating into producing diseases as primary pathogenic or opportunistic infections [6-8].
The occurrence of faecal coliforms in fish was defined as a marker for the pollution level of the environment, they live in [9], and *Shigella sp*, *Salmonella sp* and *E. coli* were the most repeatedly isolated [10].
Surveillance and recognition of *Enterobacteriaceae* from fish species were reviewed previously in *Nile tilapia*, *Mugil capito*, and *M. cephalus* in Egypt [11-13]. In wide range of cultured carps from India [14], and Eastern Mediterranean fish in Turkey [15]. The pervasive handling of antibiotics in the treatment strategies provoking an extensive distribution of resistant bacteria especially in the aquatic environment [16]. In this context, this study aims to examine the diversity of the *Enterobacteriaceae* encountered in disease problems in cultured Nile tilapia.

2. Material and Methods
2.1 Fish and sample collection
In our investigation, a total of two hundred (200) earthen pond cultured *O. niloticus* of different body weight averaged (170 ± 10 g), recently dead and in the moribund state, were collected from private fish farms at Beheira governorate, Egypt during an outbreak in summer 2015.

Fish were transported in ice box soon to the laboratory of the Department of Poultry and fish diseases, Faculty of veterinary medicine, Alexandria University as soon as possible. The collected fish were subjected to full clinical, postmortem (PM) lesions and bacteriological investigations.

2.2 Gross clinical and Postmortem (PM) examination

Clinical examination of diseased surveyed fish was accomplished to investigate any clinical abnormalities [17], while necropsy was done on recently dead and dying fish for uncovering the PM lesions [18].

2.3 Bacteriological examinations

2.3.1 Bacterial culture and colonial purification

The fish surfaces were swapped with 70% ethyl alcohol for surface disinfection and then inocula were taken from the liver, Kidney, spleen, and heart under the complete aseptic condition to be cultured on Tryptic soya broth and agar (Difco, Detroit, MI, USA) and incubated at 25 ^{0}C for 24 - 48 hrs. After the incubation period, a single colony from each suspected isolate was picked up and re-streaked on a new plate of MacConkey's agar (Oxoid) and re-incubated at the same conditions. Pure colonies were further streaked onto a selective medium for the *Enterobacteriaceae* known as KIA (Klingler Iron Agar) Medium. When they have been grown, loopful of each pure culture was streaked onto a nutrient agar slope or semisolid agar medium to be used as a stock culture for further biochemical and phenotypic identification.

2.3.2 Biochemical identification and Phenotypic characterization

The criteria used for identification of the isolates are based on colonial characteristics (colony morphology and arrangement) and gram staining of the microorganisms [19]. Bacterial isolates were presumptively identified using conventional biochemical tests including catalase with 3% hydrogen peroxide solution, cytochrome oxidase with oxidase strips (Remel), motility in a motility test medium (BD Biosciences, MA), citrate utilization using Simmons's citrate (Remel), sugar utilization using triple sugar iron (TSI, Remel), oxidation/fermentation of glucose using of basal media with glucose as the sole carbohydrate source (BD Biosciences), and esculin hydrolysis using bile esculin agar (Remel).

Obtained isolates were selected for further phenotypic confirmatory tests using an automated phenotypic microbiology identification system utilizing growth-based technology through accommodating colorimetric reagent cards that are incubated and interpreted automatically (VITEK2 Compact System, BioMerieux, France).

2.3.3 Prevalence and Incidence of the bacterial isolates

Prevalence%, as well as the incidence of the bacterial isolates gotten from the internal organs of diseased *O. niloticus*, was encountered.

2.3.4 Antibiogram (Antimicrobial sensitivity) testing

The antimicrobial discs were obtained from (Oxoid, England). The graduated rule to 0.5 mm was used for reading the diameter of the zones of inhibition twice at right angles. Antibiotic discs used for Antimicrobial susceptibility test were; Ciprofloxacin (CIP) (5 µg), Cefotaxime (CTX) (30 mg), Gentamycin (CN) (10 µg), Florfenicol (FFC) (30 mg), Norfloxacin (NOR) (10 mg), Doxycycline (DO) (30 µg), Flumequine (UB) (30 µg), Oxytetracycline (OT) (30 µg), Ampicillin (AMP) (10 µg), Sulfamethoxazole/Trimethoprim (SXT) (23.7+1.25 mg), and Erythromycin (E) (15 µg). Susceptibility to several antibiotics was determined using the disc diffusion technique [20]. The inoculum was prepared in 0.85% saline, and turbidity was adjusted to 0.5 McFarland's standard (approximately 2 x 10^8 CFU / ml). Petri dishes of nutrient agar supplemented with 2% NaCl were streaked with 1 ml of the prepared inoculum, then put the different antimicrobials discs and incubated at 25 ^{o}C for 24-48 hours. The diameter of the zones of inhibition (the point at which no growth is visible) was read twice at right angles with a ruler graduated to 0.5 mm.

3. Results and Discussion

3.1 Clinical signs and PM lesions of diseased fish

The examined diseased Nile tilapia showed generalized septicemic signs (Plate 1), whereas, they have large hemorrhagic ulcers over the whole side musculature of the body (Fig: 1) and have erythematic hemorrhages at the mouth, opercula, dorsum and at the base of pectoral and pelvic fins (Fig: 2), and. The PM lesions were noticeable focal hemorrhages and areas of necrosis in liver and congestion in gills and spleen (Fig: 3), (arrows). These may be nearly similar to that recorded by Noor El- Deen *et al*. [1].

Plate 1: Diseased Nile tilapia showing erythematic hemorrhages at mouth, opercula, dorsum and at the base of pectoral and pelvic fins (**Fig: 2**), and large hemorrhagic ulcers over the whole side musculature of the body (**Fig: 1**).

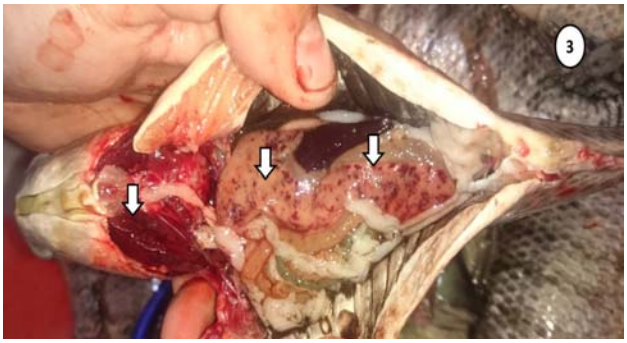

Fig: 3: Diseased Nile tilapia has noticeable focal hemorrhages and areas of necrosis in liver and congestion in gills and spleen (arrows).

These signs may be appointed to the virulence factors of the pathogenic enteric bacteria, which have virulence-associated factors like somatic antigens, adhesins, lipopolysaccharides

(Lipid-A), colicins, and siderophores [21]. Many of these factors are able to pierce the epithelial layers of the intestinal mucous cells and facilitate the scenario of pathogenicity. Extra-cellular protein is also involved [22]. Additionally, hemolysin and leukotoxins were also combined with the disease process [23, 24].

3.2 Results of bacteriological examinations

Full morphological, biochemical and phenotypic identification results of the recovered bacterial isolates from diseased Nile tilapia were determined as in Table (1 &2).

Table 1: Morphological and traditional biochemical characteristics of the bacterial isolates retrieved from diseased Nile tilapia.

Parameters	Isolates								
	I	II	III	IV	V	VI	VII	VIII	IX
Grams reaction	-	-	-	-	-	-	-	-	-
Morphology	Rods	Rods	Rods	Small rods	Straight rods	Rods	Coccobacilli	Rods	Small rods
Motility	-	+	+	+	-	+	+	+	+
Catalase test	+	-	-	+	+	+	+	+	+
Citrate test	+	-	+	+	+	+	+	+	-
Oxidase test	-	-	-	+	-	-	-	+	-
Indole test	-	+	-	-	+	-	-	-	-
Urease activity	+	+	+	-	-	-	+	-	+
Methyl Red	+	+	+	+	-	+	+	+	+
Voges Proskauer	+	-	-	-	-	-	-	-	-
Growth on KIA (Klingler Iron Agar) Medium:-									
Slope	Y	Y	R	R	Y	Y	Y	R	R
Butt	Y	Y	Y	R	Y	Y	Y	R	Y
Hydrogen Sulphide (H2S)	-	-	+	-	-	+	+	-	+
Gas production	+	+	+	-	+	+	+	-	+
Sugar fermentation test:									
Glucose	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G
Lactose	A	A	A	-	A	A/G	A	-	-
Sucrose	A	A	A	A/G	A	A/G	A	A/G	A
Mannitol	A	A	A	-	A	A	N/A	-	-
Maltose	A	A	A	-	A	A/G	N/A	-	-

Keys: N/A = Not applicable, - = No growth, + = Growth, A/G = Acid production and gas production, A = Acid production only and no gas production, Y = Yellow (Acid reaction), R = Red-pink (Alkaline reaction).

The recovered bacterial isolates suspected to be as following; I = *Klebsiella pneumoniae*, II = *Enterobacter spp*, III = *Citrobacter freundii*, IV = *Pseudomonas aeruginosa*, V = *Escherichia coli*, VI = *Salmonella spp*, VII = *Serratia marcescens*, VIII = *Pseudomonas spp*, IX = *Proteus vulgaris.*

Table 2: Further biochemical characteristics of primary and secondary differentiating tests members of *Enterobacteriaceae* retrieved from diseased Nile tilapia.

Bacterial species	Primary Differentiating Tests				Secondary Differentiating Tests				
	H_2S Prod	Lac. Ferm.	Motility	Indole Prod	VP	Methyl Red	Sim. Citrate	Lysine Decarb	Orn. Decarb
C. freundii	+	+/-	+	-	-	+	+	-	-
E. tarda	+	-	+	+	-	+	-	+	+
Entero. aerogenes	-	+(w/gas)	+	-	+	-	+	+	+
Entero. cloacae	-	+(w/gas)	+	-	+	-	+	-	+
Esch. coli	-	+(w/gas)	+	+	-	+	-	+/-	+/-
Hafnia alvei	-	-	[+]	-	[+]b	+	-	+	+
Klebsiella sp.	-	+(w/gas)	-	+/-	+/-	-	+	+	-
M. morganii	-	-	+	+	-	+	-	-	+
Prot. vulgaris	+	-	+	+	-	+	-	-	-
Prot. mirabilis	+	-	+	-	[+]b	+	+/-	-	+
Providencia sp.	-	-	+	+	+/-	+	+	-	-
Salmonella sp.	+	-	+	-	-	+	+	+	+
S. marcescens	-	-	+	-	+	-	+	+	+
Shigella sp.	-	-	-	+/-	+	+	-	-	+/-

Isolation of some highly pathogenic bacteria like *Salmonella sp., E. coli, Klebsiella sp, Citrobacter sp,* and *Proteus sp*, from diseased fish, gives an indication of fecal pollution of the fish water [3, 25].

3.3 Prevalence of bacterial isolates in naturally examined diseased fish

Table 3: Showing the prevalence of bacterial isolates in the examined naturally infected fish

Fish species	No. of examined fish	No. of infected	Percentage (%)
O. niloticus	200	130	65

The prevalence of bacterial isolates in the different examined naturally infected fish is illustrated in table (3), whereas, it was found that (130) naturally examined fish out of (200) were found to be infected with different types of bacteria with an average prevalence of (65%).

Additionally, about 144 *Enterobacteriaceae* strains were recovered from diseased fish (Table 4). The identified strains were: *Citrobacter freundii, Edwardsiella tarda, Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Hafnia alvei, Klebsiella sp, Morganella morganii, Proteus vulgaris, Proteus mirabilis, Providencia sp, Salmonella sp, Serratia marcescens* and *Shigella sp*, with average prevalence% were 25.69, 10.42, 6.25, 11.81, 15.28, 4.17, 7.64, 4.86, 3.47, 1.39, 1.39, 1.39, 4.17 and 2.08 respectively.

Table 4: Prevalence percentage (%) of members of *Enterobacteriaceae* recovered from diseased Nile tilapia.

Identified bacterial isolates	No. of isolates	Prevalence%
Citrobacter freundii	37	25.69
Edwardsiella tarda	15	10.42
Enterobacter aerogenes	9	6.25
Enterobacter cloacae	17	11.81
Escherichia coli	22	15.28
Hafnia alvei	6	4.17
Klebsiella sp.	11	7.64
Morganella morganii	7	4.86
Proteus vulgaris	5	3.47
Proteus mirabilis	2	1.39
Providencia sp.	2	1.39
Salmonella sp.	2	1.39
Serratia marcescens	6	4.17
Shigella sp.	3	2.08
Total number of isolates	144	

3.4 The incidence of the identified bacterial isolates from the internal organs of the naturally infected fish

The incidence of bacterial isolates retrieved from internal organs of naturally infected fish was illustrated in table (5). The results showed that the highest number of isolates was recovered from liver (49.31%) and the lowest number of isolates was obtained from the kidney (13.19%).

Table 5: Incidence percentage (%) of members of *Enterobacteriaceae* retrieved from the internal organs of diseased Nile tilapia.

organs / Bacterial isolates	Liver		Kidney		Heart		Spleen	
Citrobacter freundii	20		4		5		8	
Edwardsiella tarda	6		2		1		6	
Enterobacter aerogenes	4		2		2		1	
Enterobacter cloacae	12		2		1		2	
Escherichia coli	11		2		3		6	
Hafnia alvei	2		1		2		1	
Klebsiella sp.	4		2		2		3	
Morganella morganii	3		1		1		2	
Proteus vulgaris	2		1		1		1	
Proteus mirabilis	1		-		1		-	
Providencia sp.	1		-		1		-	
Salmonella sp.	1		-		1		-	
Serratia marcescens	3		1		1		1	
Shigella sp.	1		1		-		1	
Total	No.	%	No.	%	No.	%	No.	%
	71	49.31	19	13.19	22	15.28	32	22.22

Members of *Enterobacteriaceae* were established to be worldwide distributed and normally inhabit fish tissues. They are considered to be a part of the normal intestinal flora of humans and other animals, while others were retrieved in water or soil, or and plants.

Several reports on their prevalence% among diseased fish were demonstrated. Coliforms from Nile tilapia inhabiting Lake Nasser constitute nearly about 43% of skin or gill samples, and all raw fish flesh samples [26]. Also, *Proteus sp*, *Citrobacter sp,* and *Providencia sp*, retrieved from the gills and intestines of marketable fishes of the Volga and Caspian Seas, and half of them were detected in the fish kidneys, spleen, and liver [27]. Furthermore, the prevalence% of total *Enterobacteriaceae* was (52.5%), including (39.7%) *Shigella* sp, (14.3%) *Salmonella typhimurium*, (11.1%) *S. typhi*, (6.3%) *S. enteritidis*, (25.4%) *Escherichia coli*, (1.6%) for *Proteus sp* and *Enterobacter aerogenes* respectively [28].

Aly *et al*. [11] mentioned that the prevalence% of total *Enterobacteriaceae* in diseased Nile tilapia was 44.1%, and the identified bacteria were *C. freundii* (26.74%), *E. coli* (10.46%), *Yersinia intermediate* (10.46%) and *Enterobacter cloacae* (2.32%). Also, Hassan *et al*. [12] that the prevalence in Nile tilapia was 92.5%, and the identified bacteria were; *E. coli, Salmonella arizonae, C. braakii, Enterobacter sakazakii, C. freundii, Raoultella ornithinolytica, Enterobacter cloacae, Klebsiella ozaenae* and *Proteus vulgaris* were 27%,

21.6%,19%,10.8%,8.1%,5.4%, 2.7%, 2.7% and 2.7% respectively. While they also mention that the prevalence in Mugil capito was 70%, and the identified bacteria were *Escherichia coli, Enterobacter cloacae, Salmonella arizonae, Klebsiella pneumoniae, Citrobacter braakii* and *Proteus mirabilis* isolation were 42.8%, 21.4%, 14.2%, 7.2%, 7.2 and 7.2% respectively.

Additionally, *E. coli* and *Ps. aeruginosa* contributed the most (*L. rohita* - 55.5%; *C. carpio* - 52.06%; *C. mrigala* - 40.18% and *C. catla* - 38.0%) with the values ranging from 38% (Catla) to 55.50% (Rohu) [14]. From Mediterranean fish in Turkey, Matyar [15] demonstrated *Enterobacter cloacae* (49.5%) and *Klebsiella oxytoca* (15.1%). These differences may be accredited to several factors like fish species, age, season, and type of culture.

3.5 Results of Antibiogram testing of the bacterial isolates:-

Antimicrobial susceptibility tests against different pooled isolates of *C. freundii, Ed. tarda and E. coli* were illustrated in table (6). It was found that these bacterial isolates were markedly sensitive to Ciprofloxacin, gentamycin, Florfenicol, and Flumequine and moderately sensitive Doxycycline and less sensitive to Oxytetracycline, Erythromycin, and Ampicillin.

Table (6): The diameters of the inhibition zone of Antibiogram testing for *C. freundii, Ed. tarda* and *E. coli* isolate retrieved from diseased fish.

Antimicrobial discs	Disc Code	Concentration	Inhibition zone (Cm)		
			C. freundii	*Ed. tarda*	*E. coli*
Ampicillin	AMP	(10 µg)	1.1	1.2	1.0
Cefotaxime	CXT	(30 mg)	2.1	2.5	1.9
Ciprofloxacin	CIP	(5 µg)	3.7	3.6	3.8
Doxycycline	DO	(30 µg)	2.8	2.5	3.0
Erythromycin	E	(15 µg)	1.6	2.0	2.2
Florfenicol	FFC	(30 mg)	3.5	3.2	3.0
Flumequine	UB	(30 µg)	3.4	3.2	2.9
Gentamycin	CN	(10 µg)	3.3	3.5	3.2
Norfloxacin	NOR	(10 mg)	2.7	2.6	2.4
Oxytetracycline	OT	(30 µg)	1.3	1.4	1.2
Sulfamethoxazole-Trimethoprim	SXT	(23.7+1.25 mg)	2.0	2.1	2.3

Our results were in line with the findings of Jawahar [29] and Karimi [30], whose findings were similar with bacterial human pathogens highly sensitive to ciprofloxacin, gentamicin, and chloramphenicol.

The relatively high resistance to ampicillin is in agreement with the findings by Vaseeharan *et al*. [31] and Karimi [30] who found that about 93.4% of gram-negative bacteria were isolated from fish, were resistant to ampicillin, also the findings of Newaj *et al*. [2] of predominance resistance to ampicillin of 90.2%. Similarly, Matyar [15] mentioned that there was a high incidence of resistance to ampicillin (66.7%). Developing antibiotic-resistant bacteria to be widespread, possibly will be accredited to several factors; Extensive use and misuse of several antibiotics in [32], commensal bacteria, and resistance genes developed [33], Also, McIntosh *et al*. [34] demonstrated the incidence of R-plasmids in antibiotic resistant Aeromonas species from Atlantic salmon. The study of Rhodes *et al*. [35] supported evidence that related tetracycline-resistance encoding plasmids have been transferred between different Aeromonas species and *E. coli* and between the hospital and aquaculture environments in distinct geographical locations.

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