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Phenotypic and genotypic characterization of Antibiotic resistant strains of *Flavobacterium columnare* isolated from Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Flavobacterium columnare is an important pathogen affecting gills and skin of freshwater fishes causing columnaris disease. Two hundred diseased fish were randomly collected from different localities throughout year (2018-2019). *Flavobacterium columnare* (*F. columnare)* showed seasonal prevalence 0.00%, 10%, 40.00%, and 20.00% in winter, spring, summer and autumn respectively with total prevalence 35/200 (17.5%). *F. Columnare* was isolated in high percent from skin and gills and rare in internal organs. The invitro antibacterial sensitivity test was applied on 20 *F. columnare* isolated strains and revealed resistant to penicillin, cephalosporin, aminoglycoside, nitrofurans, polymyxin B and tetracycline. The objective of the study to monitor the prevalence of three antibiotics resistance genes (β-lactamase resistance genes (*bla*TEM, *bla* SHV) and tetracycline resistance gene *tet*A)among isolated strains and according to this study tested strains were resistant to multiple antibiotics and their genes were widely distributed. It could be concluded that, Phenotypic and genotypic characterization of antibiotic resistant strains of isolated *F. columnare* are similar.

1. INTRODUCTION

Columnaris disease is a branchial and dermal acute to chronic bacterial infection that affects at least 36 species of freshwater fishes (Shoemaker *et al.*, 2003). It caused high mortality in farmed tilapia, especially in hatcheries (Conroy and Conroy, 2008).

The etiological agent of "Columnaris disease" is *Flavobacterium columnare (F. Columnare).* It was first isolated by Davis in 1922 and named *Bacillus columnaris*. The organism was variously re-classified as *Chondrococcus columnaris* (Ordal and Rucker, 1944), in the order *Myxobacterales*, then *Cytophaga columnaris* (Garnjobst, 1945) then *Flavobacterium columnare* (Leadbetter, 1974) a gliding bacterial group in the order of cytophagales. Recently it has been renamed to *F. Columnare* (Bernardet *et al.*, 1996).

F. Columnare is a long, slender, non-flagellated Gram-negative rod, 0.3 to 0.7 µm wide x 3 to 10 µm long, which exhibits gliding motility on solid surfaces. Colonies on cytophaga agar are flat, yellow, rhizoid, strongly adherent, and spread across solid media surfaces forming irregular margins. The bacteria form columnar aggregates on infected tissue that are often referred to as "haystacks." The temperature range for growth of *F. Columnare* is reported to be between 37ºC to 40ºC with optimum 25ºC (Amend, 1982). Growth is strictly aerobic, and the bacterium is non-halophilic (Pacha and Porter, 1970). *F. columnare* can secret several extracellular enzymes and is endowed with the ability to degrade polysaccharide, destructing gills, muscle and skin (Bernardet and Bowman, 2006).

F. columnare is a phenotypically homogeneous species but harbors a large degree of genetic diversity. *F. Columnare* strains have 4 serotypes and one miscellaneous group (Anacker and Ordal, 1959), with genotypic variation between strains (Bernardet and Grinmont, 1989). There was a possibility of intra-species variation among strains of *F. Columnare* (Toyama *et al.*, 1996). For the control of this pathogenic bacterium, various type of chemical and drugs used in cultured ponds and in hatcheries. In the advance condition of disease outbreak various commonly known antibiotics also applied and after the long time use of these antibiotics, pathogenic bacterium becomes resistant against these antibiotics. Therefore, widespread use of antibiotics, especially in hatcheries and cultured pond as prophylactic and therapeutic agents to prevent the bacterial infection or load and also leads to the development of multiple drug resistances and causes mass mortality of cultured as well as wild fishes through bacterial infection.

So, this study aimed to study phenotypic and genotypic characterization for different antibacterial resistant strains of *F. Columnare*.

2. MATERIAL AND METHODS

2.1. Fish samples:

A total of two hundred Nile tilapia fish (Diseased (n=168) and apparent healthy (n=32)) were randomly collected from different localities throughout year (2018-2019). Fishes were transferred alive to Microbiological laboratory within 2 hrs.

2.2. Clinical & postmortem examination of fish
Fish were examined clinically for any abnormal lesions according to Noga (1996) and Austin and Austin (2007).

2.3. Bacteriological examination
Samples from skin lesion, gills, kidney, liver, and spleen were taken under aseptic condition for *F. Columnare* isolation. The isolation of *Flavobacterium* sp. was performed on Selective Cytophaga Agar (SCA) (Farmer, 2004) supplemented with neomycin and polymyxin B to suppress sensitive bacteria and select only bacteria with low nutrient requirement. The plates were incubated at 25 °C for 3-5d. The positive plates were sub-cultured for purification. Based on culture character, phenotypic characterization of the isolate was done following the protocol of Bernardet (1989). A loopful from each pure culture was inoculated on two tubes of semisolid cytophaga agar medium, one of them was used as a stock culture, and the other one was used for further studies.
Yellow rhizoids, adherent to agar surface colonies with spreading margins were sub-cultured and Gram stained to check purity. The isolate was identified as *F. columnare* on the basis of growth in the presence of neomycin and polymyxin B, presence of flexirubin-type pigment, chondroitinase production, Congo red binding and production of a diffusible gelatin-degrading enzyme (Griffin, 1992).

2.4. Antibacterial susceptibility test:
The antimicrobial sensitivity test for twenty *F. columnare* isolates were performed by disc diffusion test according to Bauer *et al.*, (1966) and interpreted according to NCCLS/CLSI (1975), using different antibiotic disc (amoxicillin, amoxicillin\/ clavulanic acid, amikacin, cefixime, ciprofloxacin, novobiocin, neomycin, norfloxacin, nitrofurantion, poly mixin B, and tetracycline).

2.5. Antibacterial resistant genes assay:
2.5.1. DNA extraction
DNA extraction had been done following manufacturer's instructions of QIAamp DNA mini kit.

2.5.2. PCR amplification
Universal primers of the genes were used Table (1).

2.5.3. Preparation of PCR Master Mix:
It was performed according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit.

2.5.4. Cycling conditions of the primers during PCR:
The PCR conditions were 35 cycles of Primary denaturation (94°C for 5 minute), Secondary denaturation (94°C for 30sec.), annealing (54°C for 40sec.) and extension (72°C for 10 minutes). A preheating step at 95°C for 2 minutes and a final extension step consisting of 10 minutes at 72°C were also carried out.

2.5.5. Agarose gel electrophoreses (Sambrook *et al.*, 1989) with modification:
PCR products were electrophorized using 1.5% Agarose gel using Gel casting apparatus (Biometra). The gel was photographed by a gel documentation system and the data analyzed through computer software.

Table 1 Oligonucleotide primers sequences.

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
Flavobacterium columnare 16S rRNA	GAAGGAGCTTGTTCCTTT GCCTACTTGCGTAGTG	1000 bp	Patra *et al.*, 2016
*bla*TEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	Colom *et al.*, 2003
*bla*SHV	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392 bp	
*Tet*A(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576 bp	Randall *et al.*, 2004

3. RESULTS

3.1. Prevalence of *Flavobacterium columnare* among the examined Nile tilapia:
Total of 200 fish were examined throughout the four seasons of the year from different localities (Abbassa fish hatchery, El-qantaruh, Bahr EL baker canal, Abo- Humad fish marked and Ismailia canal). The total prevalence of *F. Columnare* among the examined fish were; 8.33%, 26.67%, 23.33%, 25% and 12.5 % from Abbassa fish hatchery, El-qantaruh, Bahr EL baker canal, Abo- Humad fish marked and Ismailia canal respectively (Table 2).

3.2. Seasonal prevalence of *Flavobacterium columnare* among examined Nile tilapia:
Total of 200 fish were examined throughout the four seasons of the year (winter, spring, summer and autumn). Results obtained that the seasonal percentage of *F. Columnare* isolated to the total number of examined fish throughout the year were 0.00%, 10%, 40.00%, and 20.00% in winter, spring, summer and autumn respectively (Table 3).

3. 3. Distribution of *Flavobacterium columnare* and their prevalence in different tissues and organs of Nile tilapia:
The total isolates of *F. columnare* from Nile tilapia was 73 isolates, the pathogen was isolated from gills (28 isolates) with percentage 14% and skin lesion (30 isolates) with percentage 15%. The organism isolated only from the kidney (5 isolate) with percentage 2.5%. The organism isolated from liver (4 isolates) with percentage 2%, and from spleen (6 isolates) with percentage 3% (Table 4).

Table 2 Prevalence of *Flavobacterium columnare* among examined Nile tilapia

	No. of examined fish	No. of clinically diseased fish	No. of positive samples for F. Columnare	%. of positive samples for F. Columnare *
Abbassa fish hatchery	60	44	5	8.33
El-quntaruh	30	25	8	26.67
Bahr EL baker	30	28	7	23.33
Abo- Humad fish Market	40	36	10	25
Ismailia canal	40	35	5	12.5
Total	200	168	35	17.50

*Percentage in relation to total number of examined fish.

Table 3 Seasonal prevalence of *Flavobacterium columnare* among examined *Nile tilapia:*

	No. of examined fish	No. of clinically diseased fish	No. of positive samples for F. Columnare	%. of positive samples for F. Columnare *
Winter	50	30	0	0
Spring	50	43	5	10
Summer	50	50	20	40
Autumn	50	45	10	20
Total	200	168	35	17.5

*percentage in relation to total number of examined fish.

Table 4 Distribution of *Flavobacterium columnare* and their prevalence in different tissues and organs in clinically infected *Nile tilapia*

Organs	No. of sample	No. of *F. Columnar*e isolates	% of *F. Columnar*e isolates
Skin	200	30	15
Gills	200	28	14
Liver	200	4	2
Spleen	200	6	3
Kidney	200	5	2.5
Total	1000	73	7.3

3.4. Antibacterial sensitivity test against isolated strains of Flavobacterium columnare;

Twenty tested isolates of *F. Columnare* were resistant to Amoxicillin (20/20), Amoxicillin\ clavulanic acid (20/20), cefixime (18/20), Neomycin (20/20), Nitrofurantoin (18/20), Polymyxin B (20/20), Tetracycline (20/20) (Table 5)

Table 5 Antimicrobial resistance of *Flavobacterium columnare:*

Antibiotic class	Antibiotic agents	Zone	Result
penicillin	Amoxicillin (25 *mg*)	8	20/20
	Amoxicillin/clavulanic acid (30 *mg*)	6	20/20
Cephalosporin	Cefixime (5 *mg*)	6	18/20
Aminoglycoside	Neomycin (30 *mg*)	10	20/20
Nitrofurans	Nitrofurantoin (30 *mg*)	8	18/20
Poly peptides	Polymyxin B (300 *mg*)	6	20/20
Tetracyclines	Tetracycline (30 *mg*)	8	20/20

3.5. Molecular identification of antibiotics resistance genes of isolated *Flavobacterium columnare* strains:

The result showed that examined F. Columnare strains were positive for presence β-lactamase resistance genes (*bla* TEM, *bla* SHV) and tetracycline resistance gene *tet*A with amplicon size 516bp, 392bp and 576bp for *bla*TEM, *bla*SHV and *tet*A respectively. According to this study tested strains were resistant to multiple antibiotics and their genes were widely distributed (Fig. 1)

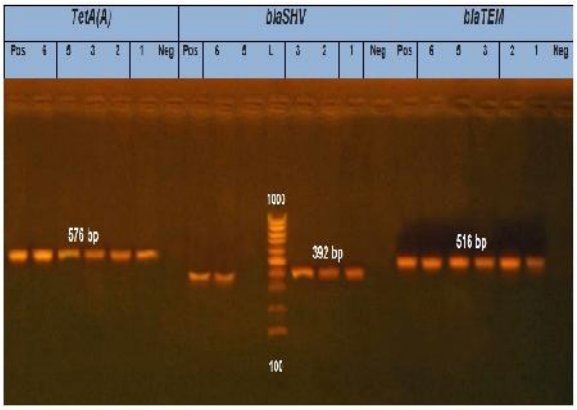

Fig. 1 Agarose gel electrophoresis of multiplex PCR of *bla*TEM (516bp), *bla*SHV(392bp) and *tet*A(576bp) as antibiotics resistant genes of *Flavobacterium columnare* strain

4. DISCUSSION

Columnaris is a common bacterial disease which affects at least 36 species of freshwater fish, caused high mortality in farmed Tilapia, especially hatcheries.

The total prevalence of columnaris disease among the total examined naturally infected Nile tilapia was 17.5 % table (2). Nearly similar El-Gamal (2000) recorded that the prevalence of *F. Columnare* among naturally infected tilapia was 17.8%.

This result was less than that recorded by Yasser (2011), who recorded prevalence rate of *F. Columnare* among naturally infected Nile tilapia 21.8%. And, Taysser (2015) who isolated columnaris disease from tilapia in a percent 22.5%, but, Nagwa (2019) isolated *F. columnare* in a percent 42.8%. In addition, our result was higher than that recorded by Kamal (2013) who recorded that the prevalence of *F. Columnare* among wild and cultured *O.niloticus* was 4.16% and 2.5% respectively.

Regarding prevalence of *F. Columnare* among examined fish in winter, spring, summer and autumn were 0.00%, 10%, 40.00%, and 20.00% respectively (Table 3) These result was in agreement with El- Gamal (2000), El-Talaway (2008), Peselis (2011), Taysser (2015), who concluded that incidence of columnaris disease increase when water temperature arise above 16°C typically during summer. The relationship between increase *F. Columnare* infection and warm water is clear so columnaris disease called worm water disease. Suomalainen *et al.* (2005) recorded that the mortality and severity of infection are temperature dependent and outbreak occur at farms in sequence throughout the warmest summer months when water temperature rise above 20°c.

F. Columnare was isolated in high percent from skin, fins and gills and rare in internal organs as in table (4). This result agree with El- Gamal (2000), El- Talaway (2008) and Nagwa (2019) who isolated F. Columnare from skin, gills and kidney only. Also Hawke and Thune (1992) recorded that, channel catfish may have systemic *F. Columnare* infection without external lesion; internally may be swelling of posterior kidney. Also Plumb (1994) mentioned that columnaris disease in catfish, in some instances became systemic with few pathological changes occurring in the visceral organs, whether not the bacteria isolated from the internal organs are taxonomically *F. columnaris* is not clear, but they may be isolated from the kidneys of more than 50% of catfish necropsied with

epidermal *F. columnaris*. In contary Hatai and Hoshina (1971) reported that *Flavobacterium columnare* couldn't multiply in the internal organs but only in tissue where the bacteria can survive under aerobic environmental conditions as in gill and skin.

In case of extensive necrotic areas of dermis without gross internal pathology due to columnaris disease infections, the death of fish is due to loss of electrolytes and proteins through the open lesion and in case of extensive damage of gill occur due to asphyxiation and loss of plasma electrolytes (Snieszko and Axelrod 1980). Eissa (1994) reported that Post-mortem examination of Nile Tilapianaturally infected with *Flavobacterium columnare* revealed no internal lesions. Tripathi *et al.,* (2005) isolated F.columnar from skin lesion of infected fish only.

The *invitro* antibacterial sensitivity was applied for the isolated *F. Columnare* in order to choose the most effective antibiotic to be used for controlling the bacterial infection. The present finding showed that *F. Columnare* strains were resistant to penicillin, cephalosporin, aminoglycoside, nitrofurans, poly peptides, tetracycline. These results agree with El-Gamal (2000), who reported that *F. Columnare* isolates were resistant to tetracycline, erythromycin. Also, Kubilay *et al.* (2008) and Yasser (2011) mentioned that *F. Columnare* isolates were resistant to penicillin, neomycin. However, Hesami *et al.*, (2010) reported that all strains of *F. Columnare* were susceptible to ampicillin, erythromycin, streptomycin, tetracycline, trimethoprim-sulphate but displayed acquired resistance to neomycin and polymyxin. Nagwa (2019) reported that strains of *F. Columnare* were susceptible to tetracycline, nalidixic acid, trimethoprim, erythromycin, streptomycin, doxycycline and highly resistant to neomycin. The incidence of multiple drug resistance by Flavobacterium columnare have been reported by Kumar *et al.,* (2012). Fabian *et al.* (2019). This antibiotics resistance could be attributed to mutation and frequent use of drugs for diseases prevention in fish farming (Ogbonne *et al.*, 2018). In this study, isolates displayed acquired resistance towards combinations of amoxicillin/clavulanic acid. This result agrees with Darwish *et al.,* (2008) who noted acquired resistance in American *F. columnare* channel catfish isolates for ormetoprim/sulfadimethoxine.

In this study, the isolates displayed acquired resistance towards tetracycline. Tetracycline is one of the most commonly used antibiotics worldwide for the treatment of bacterial fish diseases. This result agrees with Declercq *et al.,* (2013) who reported that 10 isolates of *F. Columnare* displayed acquired resistance towards oxytetracycline. In contrary Suomalainen *et al.* (2006) reported that no resistance for *F. columnare* against oxytetracycline.

Nitrofuran is not allowed for use in aquaculture. The present study shows acquired resistance of *F. columnare* isolates towards nitrofuran this result agree with Declercq et al. (2013).

The isolated strains were tested for presence of antibiotics resistance genes by using multiplex PCR with specific primer. These genes were β-lactamase resistance genes (*bla*TEM,*bla* SHV) and tetracycline resistance gene *tet*A. According to the study isolated strains have three resistance genes. (*bla* TEM (516bp), *bla* SHV(392bp) and *tet*A (576bp). As in fig. (1), the findings of this study suggest that isolated strains were resistant to multiple antibiotics and their genes were widely distributed

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