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Molecular identification of some virulence genes among *Flavobacterium columnare* isolated from *Cyprinus carpio* (common carp)

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Abstract: Flavobacterium columnare is a significant pathogen of fresh water fishes. It possesses several virulance genes that augment its pathogencity. The objective of this study was to monitor the prevalence of three virulance associated genes, including glycosyltransferase (*gt*f), nitric oxide reductase (*nor*b) and thioredoxin (*tr*x) among *Flavobacterium columnare* isolated from Cyprinus carpio (common carp).
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1-Introduction

Columnaris is a common bacterial disease which affected at least 36 species of fresh water fish, caused high mortality in farmed Tilapia, specially hatcheries.

Although the pathogen *F.columnare* is ubiquitous in the freshwater environment and can cause catastrophic mortalities in both wild as well as domesticated species (**Morley and Lewis, 2010).**

The pathogenesis of Columnaris still remains largely unknown (**Declercq *et al.*, 2013**). It is suggested to be adhesion ability, especially to the gills, is of vital importance to infection. The adhesion ability of *F.columnare* is affected by various factors such as the concentration of nitrite, organic matter or temperature (**Decostere *et al.*, 2002**). Columnaris is associated with the environmental stress; the risk increases with higher temperature, higher feeding rates, and more organic loads as well as the increasing stocking densities (**Olivares-Fuster, 2010**).

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**).

Genetic variability of *F. columnare* species has been identified using different molecular markers (**Darwish and Ismaiel, 2005**). Restriction fragment length polymorphism analysis (RFLP) of the 16S rRNAgene divided *F. columnare* species into three genomovars, the coexistence of three main genomic groups within the species (genomovars I, II, and III) has been corroborated by DNA-DNA hybridization intergenic spacer region (ISR) sequencing (**Darwish and Ismaiel, 2005**), and single-strand conformation polymorphism (SSCP) analysis (**Olivares-Fuster et al., 2007**). To date, most of the genetic markers used for *F. columnare* strain typing have relied on ribosomal gene analysis.

Only a few genes from *F. columnare* have been described to date, and most known sequences from this pathogen correspond to ribosomal genes. Among the non-ribosomal sequences identified, the chondroitin AC lyase gene in *F. columnare* has been previously sequenced and characterized (**Xie et al., 2005**). Relation of chondroitin AC lyase activity to virulence in *F. columnare* has been recently reported (**Suomalainen et al., 2006**). Genes encoding outer membrane proteins, such as zinc metalloprotease and prolyloligopeptidase, have also been identified in a virulent *F. columnare* isolate (**Xie et al., 2004**). In addition, several proteases have been identified in *F. columnare*, but their specific roles in columnar is pathogenicity are still unknown (**Newton et al., 1997**).

So, this study aimed to identification and characterization of *gt*f, *nor*b, and *tr*x Genes in F.columnare isolated strain and estimate pathogenesis of isolated strain.

2- Materials and Methods:-

2.1- Sample collection:-

Total of one hundred and fourteen Cyprinus carpio fish. Diseased (n=83), apparent healthy (n=31), were randomly collected from different area throughout year (2018-2019), with different clinical signs. Where these fishes were transferred alive to Microbiological laboratory within 2 hrs. Samples were collected from skin lesion, gills, kidney, liver, and spleen for *F.columnare* isolation.

2.2- Clinical & post mortem examination of fish:-

Fish were examined clinically for any abnormal lesions according to **Noga (1996)** and **Austin and Austin (2007).** Naturally infected Cyprinus carpio were clinically examined and revealed skin lesion with ulceration and degeneration of underlying muscle fibers**.** The gill filaments showed presence of congestion, necrosis and covered with excessive mucus.

2.3-Bacteriological examination of samples:-

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. Plates were incubated at 25 °C for 3-5d. Yellow rhizoids, adherent to agar surface colonies with spreading margins were subcultured 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).** Phenotypic characterization of the isolate was also done following the protocol of **Bernardet (1989).** A loopful of 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.

2.4- pathogencity test:

It was done according to the methods described by **(Finlay and Falkaw1989).**

1- A total No. of 70 apparently healthy O. noliticus fish with average body weight (30-40 gm) were used for experimental infection with *Flavobacterium columner* isolated from Cyprinus carpio.

2- Each fish was injected by 1ml of 1.6 x 10^{7} conc. S/C injection and reared in tanks supplied with infected water with microorganism. Record visual sings on infected fish and mortality rates every day for 14days.

2.5- PCR application:-

2.5.1- DNA extraction:

DNA extraction had been done by 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 mastermix (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 (58°C for 40sec.) and extension (72°C for 30-40sec). A preheating step at 95°C for 2 minutes and a final extension step consisting of 7-10 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
Flavobacterim columnare 16S rRNA	GAAGGAGCTTGTTCCTTT	1000 bp	**Patra *et al.*, 2016**
	GCCTACTTGCGTAGTG		
Gtf	CCAACATTTGGAGGTAGCGG	678 bp	**Zhang and Arias, 2009**
	ACCTGCTAAACTGATGATGGTGG		
*nor*b	TGCACGGACATCTAGCATTTTG	341 bp	
	AAACATGGTAAAACGAACGATGAGG		
*Tr*x	TGGCATTAGCAATTACAGATGCTAC	254 bp	
	GATTTTTCAAAATGGGGAAGTAGTGG		

3- Result

3.1- Prevalence of *Flavobacterium columnare* among examined Cyprinus carpio.

Total of 114 fish were examined throughout the four seasons of the year (winter, spring, summer and autum). Results obtained that the percentage of fish from which *F.columnare* was isolated to the total of

examined fishes were 0.00%, 26%, 44.4%, and 37% in the four seasons respectively. **Table (2)**

Table (2): Prevalence of *Flavobacterium columnare* among examined Cyprinus carpio.

	No. of examined fish	No. of clinically diseased fish	No. of fish from which F.Columnare was isolated	%. of fish from which F.Columnare was isolated to the total of examined fish*
Winter	19	10	0	0%
Spring	23	18	6	26%
Summer	45	35	20	44.4%
Autumn	27	20	10	37%
Total	114	83	36	31.5%

***Percentage in relation to total number of examined fishes.**

3.2- Prevalence of *Flavobacterium columnare* among examined Cyprinus carpio isolated from different localities:

Total of 114 fish were collected from different localities (Abbassa fish hatchery, Bahr EL baker canal, Abbo- Humad fish marked in Sharkia Governorate. Results obtained that the percentage of fish from which F.columnare was isolated to the total of examined fishes were 40%, 20%, 33.3% respectively.

Table (3): Prevalence of *Flavobacterium columnare* among examined Cyprinus carpio isolated from different localities:-

	No. of examined fish	No. of clinically diseased fish	No. of fish from which F. Columnare was isolated	%. of fish from which F. columnare was isolated to the total of examined fish*
Abbassa fish hatchery	**50**	**40**	**20**	**40%**
Bahr EL baker	**40**	**25**	**8**	**20%**
fish Marked in Abbo- Humad	**24**	**18**	**8**	**33.3%**
TOTAL	**114**	**83**	**36**	**31.57**

***Percentage in relation to total number of examined fishes.**

3.3- Distribution of *Flavobacterium columnare* and their Prevalence in different tissues and organs of clinically infected Cyprinus carpio

The distribution of *F. columnare* obtained from carp (36 isolates), the pathogen was isolated from gills (10 isolates) by percentage 8.77% and skin lesion (21 isolates) by percentage 18.4%, the organism isolated only from the kidney (5 isolate) by percentage 4.4%. The organism was not isolated from liver and spleen. **Table (4).**

Table (4): Distribution of *Flavobacterium columnare* and their Prevalence in different tissues and organs of clinically infected Cyprinus carpio.

Organs	No. of samples	No. of *F. columnar* isolates	% of *F.columnar* isolates*
Skin	**114**	**21**	**18.4%**
Gills	**114**	**10**	**8.77%**
Liver	**114**	**0**	**0%**
Spleen	**114**	**0**	**0%**
Kidney	**114**	**5**	**4.4%**
Total	**570**	**36**	**6.3%**

***Percentage in relation to total number of examined fishes.**

3.4- pathogenicity test:

After 48h post infection experimentally infected fish show irregular ulcerative hemorrhagic area with coldness in the eye and fried fins, scale detachment.

After 72h post infection sever fin rot in the caudal fin with complete loss of scales in surrounding area with increase area of sloughing of the muscle in abdominal area with yellowish coloration of surrounding area**.**

Re-isolation of all inoculated bacterial isolates was obtained from dead and sacrificed fish. Moreover, the results of the culture and biochemical characteristics of the re-isolated bacterial isolates

revealed the same morpho-chemical characteristics of the inoculated bacterial isolates.

Mortality rate was recorded every day for 14 days T**able (5).**

Table (5): Mortality Patterns 14 Days Post S/C Injection of fish With Virulent Strains of *Flavobacterium columner*.

Fish group	Injected strain	Fish No.	No. of diseased fish 14 day post-injection														Dead fish		Survived fish	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	No.	%	No.	%
Group1	Strain1	10	-	1	-	-	2	-	-	1	1	-	-	-	-	-	5	50	5	50
Group2	Strain2	10	-	-	1	1	-	-	-	1	-	1	-	-	-	-	4	40	6	60
Group3	Strain3	10	1	-	-	1	-	-	-	1	1	-	-	-	-	-	4	40	6	60
Group4	Strain4	10	-	2	1	-	1	1	-	1	-	1	-	-	-	-	7	70	3	30
Group5	Strain5	10	1	-	-	3	1	-	-	-	1	1	-	-	-	-	7	70	3	30
Group6	Strain6	10	-	-	1	1	1	-	-	1	1	-	-	-	-	-	5	50	5	50
control	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	100

3.5- Molecular confirmation of isolated Flavobacterium columnare.

3.5.1- Molecular identification of isolated strain:

Six isolated strains were molecular identified with specific primer of *Flavobacterium columnare*, according to the result, there are five isolates give positive with amplicon size 1000bp.

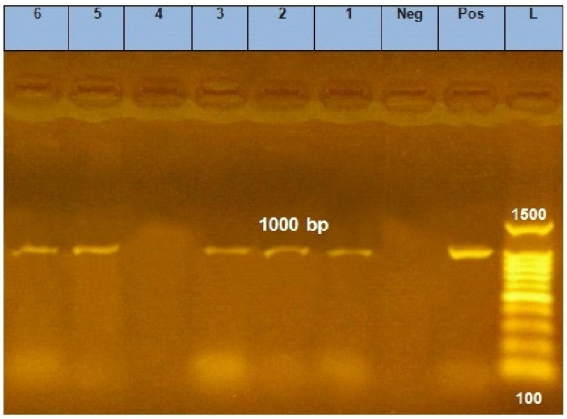

Fig. (1): Agarose gel electrophoresis of PCR amplification products of 16 SRNA of *Flavobacterium columnare*.
Lane L:100 bp ladder as molecular size DNA marker.
Lane Pos.: Control positive of *Flavobacterium columnare gen*.
Lane Neg.: Control negative.
Lanes 1,2,3,5 and 6: positive *Flavobacterium columnare strain.*

3.5.2- Molecular identification of virulence genes of isolated *Flavobacterium columnare* strain:

According to this study tested strains have two virulance genes (*nor*b, *gt*f), strain 2,3 have *gt*f gen with amplicone size 575bp and *nor*b gen with amplicone size 341bp.

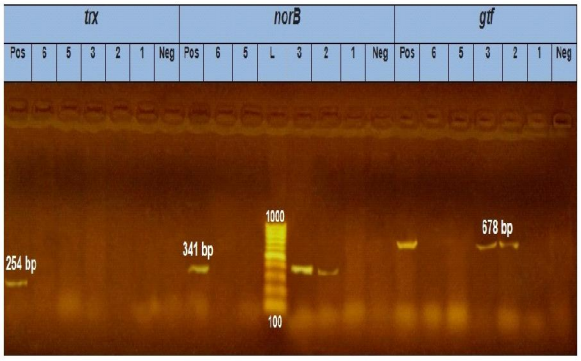

Fig. (2): Agarose gel electrophoresis of multiplex PCR of *gt*f, *nor*b and *tr*x, as virulance genes of *Flavobacterium columnare*.
Lane L:100 bp ladder as molecular size DNA marker.
Lane Pos.: Control positive of *gen nor*b, *gt*f, *tr*x.
Lane Neg.: Control negative.
Lanes 1,2,3,5 and 6: positive *Flavobacterium columnare strain.*

4- Discussion

The present study was carried out on naturally & experimentally Cyprinus carpio fishes infected with *Flavobacterium columnare* to investigate the clinical signs & postmortem finding, isolate the causative agent and study the virulence of microorganism.

The isolates of *Flavobacterium columnare* were typical in morphological characters and started to grow on cytophage agar medium with adding neomycin and polymyxin B, at 25°c within 3-5 days, forming pale yellow, rhizoid and tightly adherent mucoid colonies. *Flavobacterium columnare* colonies glided or swarmed on cytophage agar and had irregular edges. However, during storage and subculture, colonies changed to less rhizoid more discrete colony leading to a complete loss of the colony morphology. This result agree with **Taysser (2015)**, **Nagwa (2019)** who recorded that low nutrient

media required for growing of *Flavobacterium columnare* with adding neomycin and polymyxin B to media to be selective for isolation.

Regarding to microscopic appearance of *F.columnare*, the investigation revealed that, microorganism is gram negative, long rods and scattered in arrangement. This result agree with **Abd El-Rahman (1996), Verma *et al.*, (2015).**

Regarding to biochemical character of isolated organism, this study revealed that, isolates were positive for catalase, gelatin liquefaction, MR, VP test but negative for sugar fermentation with variable effect with oxidase test. This result similar to those recorded by **Verma *et al.*, (2015).**

All of the previous results were agree with **Griffin (1992),** who mentioned that, five characteristics that differentiates *Flexibacter columnare* from other morphologically similar yellow pigmented Gram negative aquatic bacteria. These characteristics are (1) the ability to grow in the presence of neomycin sulfate and polymyxin B; (2) colonial morphology on agar plates typically has a rhizoid pattern and yellow pigmentation; (3) production of a diffusible, gelatin-degrading enzyme; (4) binding of aqueous congo red dye to the surface of the suspect colony; and (5) production of a diffusible chondroitin sulfate A degrading enzyme. The bacteria form characteristic haystack-like columnar aggregates in cytological wet mounts of infected tissue.

Regarding to this result, *F. columnare* isolated from common carp in percent of 31.57% according to total examined fishes. This result was higher than that recorded by **El- Talaway (2008) and Taysser (2015)** who recorded that, prevalence of columnaris disease among naturally infected clarias garepinus was 22.9 and 25 % respectively. **Kumar *et al.*, (2012)** isolated *F.columnare from* 60 diseased African catfish showed symptoms for Columnaris disease with epithelial infection causing necrotic gill or skin and fins erosion in percent 40%.

Regarding to prevalence of *F.columnare* among examined fishes through four seasons of the year, the result revealed percentage of fish from which *F.columnare* was isolated to the total of examined fish in winter, spring, summer and autumn season were 0.00%, 26%, 44.4%, and 37% in the four seasons respectively. **Table (2).**

These result was agree with **El- Gamal (2000), El-Talaway (2008), 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 worm water is clear so columnaris disease called worm water disease. **Suomalainen *et al.*, (2005)** recorded that, the mortality and severity of infection are temperature dependant and out break occur at farms in sequence throughout the warmest summer months. When water temperature rise above 20°c.

According to this result, *F.columnare* was isolated in high percent from skin, fins and gills and rare in internal organs. This result agree with **El Gamal (2000), El- Talaway (2008) and Nagwa (2019)**, who isolated F.columnare from skin, gills and kidney only. But **Kumar *et al.*, (1986)** who succeeded to isolate *F.columnare* from skin lesion and kidney of affected fish. And also **Hawke and Thune (1992)** recorded that, channel cat fish 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*. And disagree with **Hatai and Hoshina (1971)** reported that *Flexibacter columnaris* couldn't multiply in the internal organs but only in tissue where the bacteria can survive under aerobic environmental conditions like in gill and skin.

Failure of isolation of *F.columnare* from internal organs could be due to production of diffusible necrotizing substance or photolytic material by microorganism that leading to pathological alternation in internal organ although the organism is not present. **Abd-Elaziz (1988) and Plumb (1994)**.

Regarding to pathogencity of isolated *Flavobacterium columnare* which were injected s/c in fish. The mortality rate in six groups of fish indicated that isolated strain numbers 4, 5 were the most virulent isolated with mortality rate 70% while, strain number 1, 6 were moderate virulent with mortality rate 50% but isolated strain numbers2, 3 were low virulent with mortality rate 40% within 14 days post injection. These results indicate that there was wide variation in virulence among *Flavobacterium columnare* isolates, similar finding were reported by **Amend (1982)** who mentioned that virulence of *F. columner* was classified into four categories: high, moderate, intermediated and low virulence.

Also, these results agree with **Mona Moustafa (1994)** who mentioned that there was a degree of virulence between the strains of *F.columner*. it also mentioned that highly virulent strain of *F.columnare* could be caused 40 – 100 % mortalities of tilapia fish within 18hr and 6 days respectively, while moderately virulence strain caused 20 – 60 % mortality within 24hr and 4 days respectively. The less virulent strains caused 20% mortality within 7 days.

Also **Decostere et al., (2002)** mentioned that adherence capacity of *F.columnare* to fish tissue was strongly related to the virulence of *F.columnare*. **Darwish *et al.*, (2004)** reported that the difference in virulence were known to exist between *F.columare* strains, producing variance in mortality.

This study demonstrated a Polymerase chain reaction (PCR) method with species-specific primers that uses the AC lyase gene to distinguish *Flavobacterium columnare* isolates from other closely related yellow pigmented bacteria, and this technique was found to be more sensitive for the detection of Flavobacterium columnare infection in fish than the standard culture techniques because Flavobacterium columnare is often overgrown by the fast-growing bacteria such as Pseudomonas spp. and others (**Tiirola *et al.*, 2002).**

Regarding the molecular identification of F.columnare by PCR produced unique and clear bands corresponded to the 1000bp by internal fragments of 16SrRNA gene confirmed without doubt that the isolated strain were F.columnare, these results were similar to those reported by **Darwish *et al.*, (2004), Verma *et al.*, (2015), Nagwa (2019).**

F. columnare has been reported to be a morphological, serological and genomically diverse species reported by **Darwish and Ismaiel, (2005).**

According to **Bernardet and Grimont, (1989),** as some *F. columnare* strains shared only a 78% similarity by DNA-DNA hybridization. Genomovar ascription has become routine for *F.columnare* strain characterization **(Olivares-Fuster *et al.*, 2007).** However, beyond ribosomal variability, no study has been conducted to investigate the intra-species variation of *F.columnare* at the single gene level, mainly due to the lack of genetic information available for this species.

In the current study *gtf*, *norb*, *trx* have been identified and characterized in isolated strains of *F. columnare*. These genes presented a high similarity to homologous genes within the *Flavobacterium* genus, showing the highest similarity with *F. johnsoniae* gene sequences. Although strains within the same genomovar showed identical gene sequences, there was a 4 to 7% nucleotide sequence variation observed between genomovars for each gene. The variability found at the nucleotide level was also translated to the amino acid level. **Lee *et al.*, (1998)** suggested that single amino acid substitutions could change the biological activity of proteins. **Zhang Y, Arias CR (2009)** founded that there are some non conserved amino acid substitutions between the genomovar I ALG-00-530 and the genomovar II ATCC 49512 in Gtf, and NorB protein sequences. Therefore, the activity of these proteins could differ between *F. columnare* genomovars. *Gtf*, *norb*, and *trx* genes characterized in the present study have been described as virulence factors in other bacterial species (**Almiron *et al.*, 2001; Narimatsu *et al.*, 2004; Bjur *et al.*, 2006; Loisel-Meyer *et al.*, 2006**). Our expression data showed that *trx* genes are not expressed after 24 h growth following inoculation into fresh cytophage broth culture medium. Agreed with **Zhang Y, Arias CR (2009)** founded that *norb*, and *trx* genes are weakly or not expressed when cells reached the end of the log phase but they were strongly expressed during the first few hours following inoculation into fresh Shieh broth culture medium. In conclusion, our data confirmed the genomic diversity of *F. columnare* at the single gene level. Nucleotide sequences of *gtf*, *norb,* and *trx* of *F. columnare* differed between genomovars I and II. Multiple gene expression patterns existed both between and within genomovars.

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