Methods For Genetic Differentiation Of *Flavobacterium columnare* Isolated From Some Cultured Freshwater Fishes

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ABSTRACT

Flavobacterium columnare is an important economical pathogen that affects freshwater fishes. This study aimed to investigate the genetic variations among the isolated *F. columnare* from naturally infected *Clarias gariepinus, Oreochromis niloticus and Cyprinus carpio* using randomly amplified polymorphic DNA analysis (RAPD) PCR and Polyacrylamide gel electrophoresis (PAGE) in the presence of the sodium dodecylsulfate (SDS). The affected fishes with *F. columnare* suffered from hemorrhagic ulcer in the skin, severe fin rot especially at the caudal fin and respiratory manifestations. On ordal's selective media, the isolates showed characteristic yellowish rhizoids colonies. The isolated *F. columnare* from different fish species had no clear significant genetic variations. However, the total amount of the protein which is present in the cell wall of each isolate was completely different.

INTRODUCTION

Flavobacterium columnare, a Gram-negative gliding bacterium, is the causative agent of *Columnaris* disease. *F.columnare* is an important pathogen of freshwater fish, implicated in skin and gill disease *(1- 3). Columnaris* disease can occur as the primary disease in pond or tank raised *Channel catfish*, with mortalities as high as 50% *(4).* In salmonid species, the mortality rate is 34% *(5).*

The early signs of the *F. columnare* are frayed and ragged fins. The skin, gills, and fins of the fish show brown to yellow brown lesions. A characteristic lesion produced by *Columnaris* is a pale white band encircling the body, often similar to as the saddleback condition. Affected fish are anorexic and lethargic, tend to remain near the water surface, and may be flaring their operculum. Gill lesions may cause difficult respiration, and fish will die within 2–3 days post-infection without treatment *(6, 7).*

F. columnare isolates are isolated on ordal's selective media. The colonies obtained are yellow, flat, with irregular (rhizoid) edges and strongly adhered to the agar surface. All isolates are Gram-negative long rods *(2).*

There are great differences in the virulence of different *F. columnare* strains that are injected into catfish. The mortality rate varies from 100% to even 10% two weeks post injection *(8).* Among 17 isolates of *F. columnare*, Only 13 of the 17 isolates cause 100% mortality in the untreated cases of channel catfish within 48 hrs of infection .The other four isolates cause less mortality and dead fish only after 48 hrs post exposure *(9).* Differences in virulence among *F. columnare* isolates from different genomic groups are defined by random amplified polymorphism DNA (RAPD). However, no clear association is made to virulence with respect to RAPD group *(9).*

This study aimed to investigate the genetic variations among *F.columnare* isolates obtained from different fish species using RAPD- PCR and SDS-PAGE.

MATERIAL AND METHODS

1- Fish

Naturally infected *Clarias gariepinus, Oreochromis niloticus and Cyprinus carpio* were obtained from Abbassa Fish Farm at Sharkia province, Egypt.

2-Bacterial cultures and biochemical characterization

2-1-Media

Ordal's or Cytophaga agar medium and Cytophaga broth (like Ordal's medium without agar) were used for microbial isolation of the *F. columnare* from infected fishes *(10).*

2-2-Biochemical characterization

The biochemical preparation and culture charcters were examined according to the method described *(11)*.

3- Molecular analysis of *F.columnare* isolates by RAPD -PCR and SDS-PAGE

Strains of *F. columnare* previously isolated from *Clarias gariepinus, Oreochromis niloticus and Cyprinus carpio* were used for molecular analysis.

3-1.Identification of the *Flavobacterim columnare* isolates using PCR finger print technique (RAPD PCR):

DNA was extracted *(12)*. The primers sequences 10 – mer oligonucletuides HP25.1 Roth Random primer Kit D were used (Table 1) to detect the polymorphism among bacterial in the RAPD assay. Five min at 95°C for denaturation, followed by 45 cycles each one min at 95°C, one min at 40°C, and two min at 72°C. This was followed by a final extension of eight min at 72°C and an indefinite hold at 4°C..The amplified products were loaded into wells of a 2% agarose gel and subjected to electrophoresis for five hours at 36 volts. DNA bands visualized with five µg/ml ethidium bromide.

Table 1. RAPD-PCR primers

Primer	Sequencing (5'-3')
1	ACC GCG AAG G
2	GGA CCC AAC C
3	ACC TGA ACG G
4	CTT CCC CAA G
5	GGT CTA CAC C

3-2-Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Whole-Cell Proteins

Protein sample of 10-15 µg proteins were denatured for 5 minutes at 100°c in sample buffer (1 volume of protein sample : 2 volume of sample buffer) . Appropriate amounts of proteins sample and control molecular weight standard (15µl) were carefully injected at the bottom of the wells of the gel slabs. The slab was placed in the electrophoretic chamber (E-C Apparatus Corporation St. Petersburg, Florida - USA). Power supply (Bio Rad, Germany) with constant voltage was 30 V used to apply until the samples migrated through the stacking gel, which takes about 20 minutes, then the voltage was raised to 60 V until the tracking Bromophenol blue dye was reached to the end of the gel. The polyacrylamide gel was stained (1% SDS, 50 mM Tris-HCl, pH 6.8, 1% 2-mercaptoethanol, 10% glycerol)for 2 hours at room temperature *(13)*.

RESULTS AND DISCUSSION

Naturally infected fishes with *Flavobacterium columnare* were appeared lethargy, off-food and loss of balance with respiratory manifestations. Fin rot was observed specially the caudal fins were frayed and severe destructed. Skin lesions appeared as whitish, yellowish or yellowish white mucoid exudates with red hemorrhages at the peripheries. In more advanced case, ulcerative hemorrhagic areas at the body and gill cover were observed with complete loss of caudal and dorsal fins (Fig.1). Similar results were previously reported *(3, 14)*.

F. columnare isolates were isolated on ordal's selective media. The results were demonstrated in Table 2, reported that, they gave the characteristics yellowish rhizoids colonies. These results are agreed with that previously recorded *(15)* which showed that that *F.columnare* was differentiated from other yellow pigmented gram negative aquatic bacteria by: (1) the ability to grow in the presence of neomycin sulfate and polymyxin B (2) Colonies on cytophaga agar plates typically rhizoids and pigmented pale yellow. Also the results revealed that the biochemical characteristics of *F.columnare* were the ability to liquefy the gelatin, catalase test was positive, inability to reduce nitrate, and vogues proskour was negative, inability to produce indole, inability to ferment glucose, lactose, sucrose, mannitol and arabinose and variable effects with cytochrome oxidase test. The same results were previously obtained *(16-18)*.

The results showed that primer 1, 2, 3, and 4 have nearly the same pattern for the 3 isolates of *F. columnare* (Fig. 2, 3) while the primer 5 showed different patterns among the 3 isolates of *F. columnare* (Fig. 4) . These results indicate that there are no clear significant genetic variations among the 3 isolates of *F.columnare*. These results may be attributed to the isolated *F.columnare* from *Clarias gariepinus, Oreochromis niloticus and Cyprinus carpio* were from the same water source and the infection will spread among different fish species in the polyculture system. RAPD-PCR results showed that significant genetic variations among different *F. columnare* strains from diverse fish species ***(9, 19-21).***

The results demonstrated in Fig. 5 showed that there were great variations between the levels of protein in the cell wall of the isolated *F. columnare* from different fish species. These results may be due to the type of the fish species would influence the protein amount and percentage in the cell wall of the *F.columnare*. These results are concomitant with that previously recorded study ***(22)*** which showed that Gel electrophoresis method has been used both singly and in combination with biochemical and phenotypic characteristics to classify and identify different species of bacteria.

Conclusion: The isolated *F. columnare* from different fish species had no clear significant genetic variations but the total amount of the protein which is present in the cell wall of every isolate was completely different.

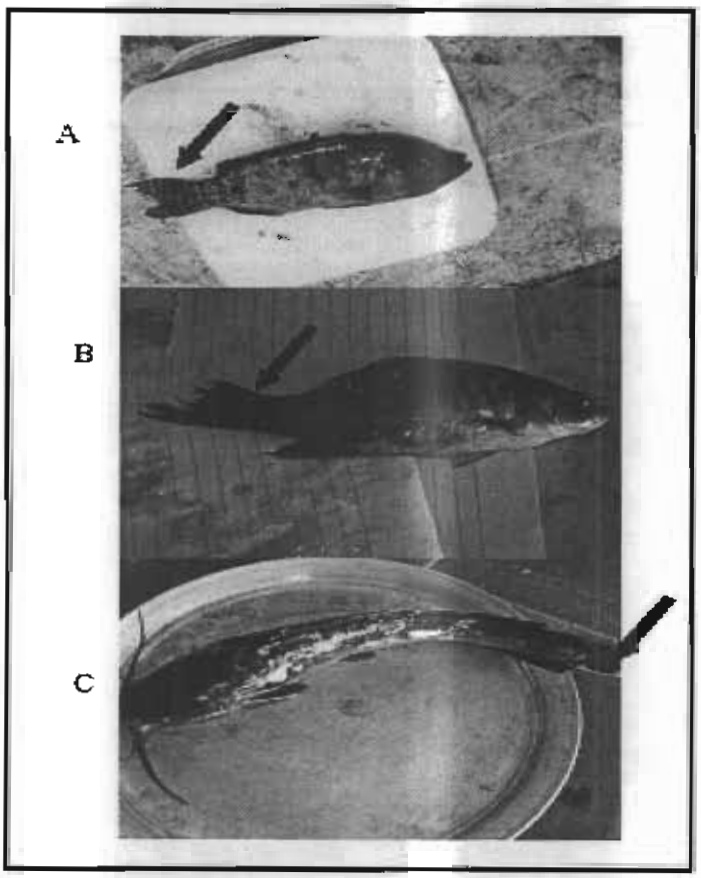

Fig. 1. Naturally infected *O. niloticus* (A), *C.carpio* (B), and *C. gariepinus* (C) from which *F. columnare* were isolated. They showed severe fin rot especially at the caudal fin, loss of scales and severe emaciation.

Table 2. Cultural and biochemical properties of *Flavobacterium columnare* isolated from naturally infected fish

Item	Result
Growth on ordinary media.	-ve
Growth on cytophagal agar media in the presence of Neomycin sulfate and Polymyxin B, Motility.	+ve
Pigment	Yellow pigment colonies
Colonies characters	Yellowish rhizoids colonies
Gram stain	- ve bacilli
Morphology	long rods bacilli
Cytochrome oxidase	±ve
Fermentation of glucose, lactose, sucrose ,mannitol, arabinose, Indole production, Voges proskauer, Nitrate reduction	-ve
Gelatin liquefaction, Catalase test	+ve

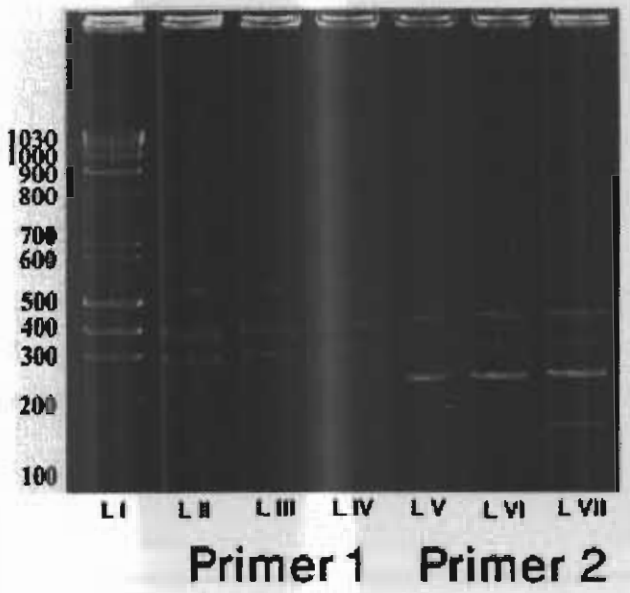

Fig. 2. Agarose gel of PCR products amplified from *F. columnare* isolates using Primer 1, 2 Lane I 100:1030bp DNA Ladder. Primer1 (Lane II ,III, LV) . Primer 2 (Lane V, VI, VII) . *F. columnare* isolated from *C. gariepinus* (L II , and LV) , *F. columnare* isolated from *O.niloticus (LIII,VI) . and F. columnare* isolated from *C.carpio* (L IV,VII)

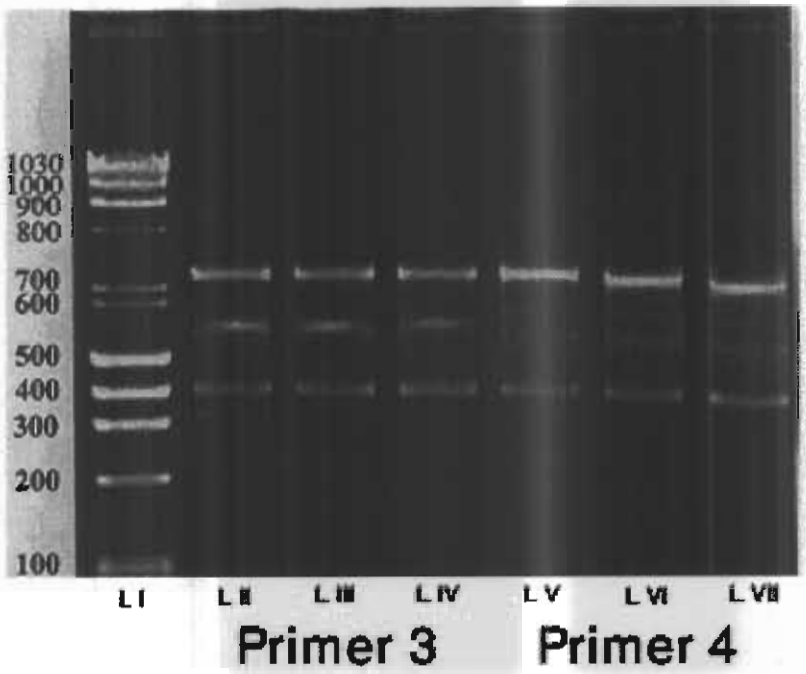

Fig. 3. Agarose gel of PCR products amplified from *F. columnare* isolates using Primer 3, 4. Lane I 100:1030bp DNA Ladder. Primer3 (Lane II, III, LV) . Primer 4 (Lane V, VI, VII). *F. columnare* isolated from *C. gariepinus* (L II , and LV) , *F. columnare* isolated from *O.niloticus (LIII,VI) F. columnare* isolated from *C.carpio* (L IV,VII) .

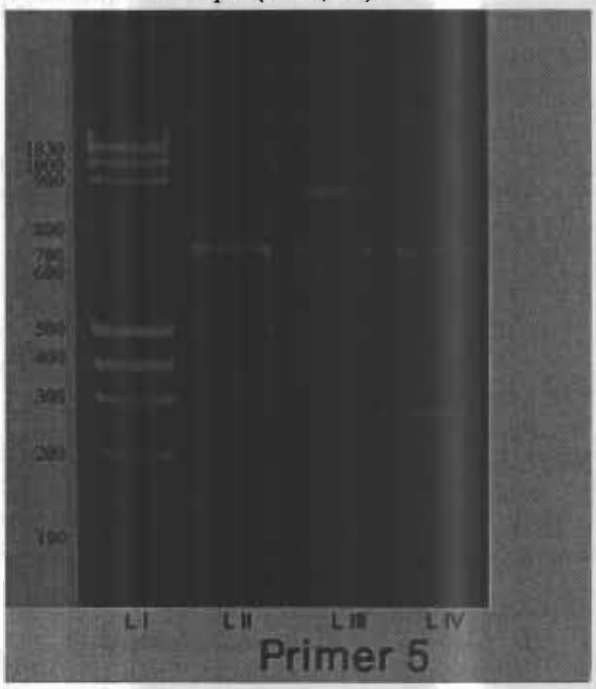

Fig. 4. Agarose gel of PCR products amplified from *F. columnare* isolates using Primer 5 Lane I 100:1030bp DNA Ladder. Primer5 (Lane II ,III, LV). *F. columnare* isolated from *C. gariepinus* (L II) , *F. columnare* isolated from *O.niloticus (LIII)* . *F. columnare* isolated from *C.carpio* (L IV,)

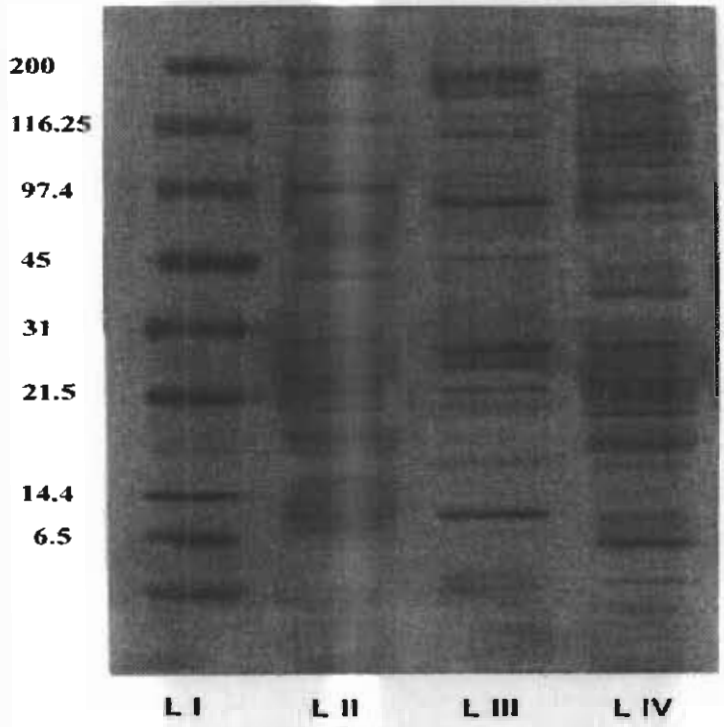

Fig. 5. SDS-PAGE of *F. columnare* isolates Lane I 6.5-200 KD marker. Lane II *F. columnare* isolated from *C. gariepinus*. Lane III *F. columnare* isolated from *O.niloticus*. Lane IV F. columnare isolated from *C.carpio*.

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الملخص العربي

طرق التفريق الجيني لميكروب الفلافوبكتريم كولمنار المعزول من بعض أسماك المياه العذبة المستزرعة

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الفلافوبكتريم كولمنار من الميكروبات المهمه ذات التاثير الاقتصادي علي اسماك المياه العذبه . الهدف من هذه الدراسه هو تحديد الفرق الجيني الكلي لميكروب الفلافوبكتريم كولمنار المعزول من اسماك القرموط النيلي والبلطي النيلي والمبروك الشائع . وتم استخدام اختبار البلمره المتسلسل العشوائي تحديد نسبه البروتين في غلاف الخليه لكل نوع من الفلافوبكتريم كولمنار المعزول من الاسماك المختلفه .

وكانت النتائج كما يلي:- 1- علامات المرض علي الاسماك المصابه متمثله في وجود نزيف علي الجلد مع تقرحات وايضا وجود تاكل عنيف في الزعانف خاصه الزعنفه الزيليه وو وجود اعراضات تنفسيه. 2- تم عزل الميكروب علي وسط الاوردلاذ . وكانت النتيجه هي ظهور مستعمرات دائريه متعرجه صفراء اللون . وتم عمل اختبارات كميائيه للتاكد من البكتريا المعزوله. وتم اخذ الفلافوبكتريم كولمنار من كل فصيله اسماك . 3-كانت النتائج تدل علي عدم وجود اختلاف ما بين التركيب الجيني لميكروب الفلافوبكتريم كولمنار من الاسماك المختلفه ولكن كان هناك اختلاف واضح مابين نسبه البروتين الموجود في غلاف كل كل ميكروب . لذا من الممكن انتشار مرض الكولمنارز مابين الانواع المختلفه من الاسماك عند حدوث العدوي .