

ISOLATION AND IDENTIFICATION OF FLEXIBACTER COLUMNARIS IN CULTURED FISH IN EGYPT

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ABSTRACT

The present study was carried-out on naturally infected fish with *Flexibacter columnaris*. The number of fish examined were 100 fish collected from different farms of *O. niloticus*, *Common carp*, *Clarias lazera* in Behera, Kafr El-Sheikh and Alexandria Governorates. The clinical signs and Post mortem (P.M.) lesions includes erosions of jaws with congestion all over the body, haemorrhage and inflammation with darkening of body colour and enlarged, congested spleen and kidney. Trails for isolation of *Flexibacter columnaris* from different organs were made and we successfully isolate 4 strains grow on specific media (Ordal's) with yellow colonies, Gram negative short rods, non capsulated from (*O. niloticus* and *Common carp*) only. Biochemically it oxidase, ureas and indole negative, while, positive with glucose and maltose. Due to the morphological and biochemical characters of the isolates we identified it as *Flexibacter columnaris* especially the presence of rod like shape. The isolates were highly sensitive to antibiotics especially, Neomycin, Pencillin and Erythromycin.

INTRODUCTION

Fish supplies about 30 % of the total animal protein in the diet of Asian populations, 20% in Africa and 10% in Latin America (FAO, 1996).

One of the main factors affecting fish production and efficiency is the fish diseases and especially that result from bacterial diseases. Particular attention has been directed to *Flexibacter columnaris* which is a Gram-negative bacterium, that induces a systematic infection in fishes known as columnaris disease (Gibello et al., 2004). With all these previously mentioned points of view, the aim of this study, is the determination of the following: Incidence of *Flexibacter columnaris* among different fish species and losses resulted from infection of the fish by *Flexibacter columnaris*. The bacteriological characters of isolated *Flexibacter columnaris*. In vitro sensitivity test of the isolated *Flexibacter columnaris* against the common used antibiotics.

MATERIALS AND METHODS

Naturally infected fish : A total number of 100 fish samples of different Spp. with different signs of systemic infection were collected from different areas in both Beheira and Kafr El-Sheikh Governorates. The fish samples were classified as shown in Table (1).

Table (1) : Classifications of fish samples among different fish species and their localities :

| Number of fish samples | Fish species | Place of sample |
|------------------------|-------------------------------------|--|
| 50 | <i>Oreochromis niloticus</i> | Behera, Kafr El-Sheikh and Alexandria Governorates |
| 30 | <i>Common Carp</i> | |
| 20 | <i>Clarcis lazera</i> | |

2. Media used for bacterial isolation :

Ordal,s media for *Flexibacter columnaris* (Oxoid, 1984) with formula of :

| | |
|---|--------|
| Bacto Tryptone | 2 gm |
| Yeast-extract | 2gm |
| Tween 80 | 10 ml |
| Sodium chloride (NaCl) | 5 gm |
| Calcium chloride (hydrated) CaCl ₂ -2H ₂ O ₂ | 0.1 gm |
| Bromo-thymol blue | 0.03gm |
| Agar | 15gm |
| Dist. H ₂ O up to | 1000ml |

All ingredients were mixed and dissolved by heating, pH was adjusted to 7.4 and sterilized by autoclaving at 121 C for 15 min., after cooling to 50 C, 10.0 ml filter sterilized sucrose (0.5 g/ml) were added.

II-Methods :

1-Clinical and macroscopic examination: Both dead and sacrificed fish either naturally or experimentally infected were examined according to **Amlacher (1970)** .

2-Bacteriological examination:- The outer surface of naturally infected and apparently healthy fish were disinfected with ethyl alcohol (70 %). Fish was opened, and the internal organs were exposed and then samples were taken from internal organs (**Amlacher, 1970**).

A.Primary isolation of *Flexibacter columnaris*:- Well-developed isolated colonies were transferred by sterile loop for purity and identification by make recultivation of isolated bacteria on Shotts-Waltman media (Specific media for isolation of *Flexibacter columnaris*) and incubated at 20 – 25 C for 24 hrs. **Waltman and Shotts (1984)**.

Biochemical identification according to (Horne and Barnes, 1999):-

2.B-Identification and biochemical characterization of the isolates:-

Identification of the isolates was carried-out by determining their morphological, cultural and biochemical characters according to the criteria of **Morrison et al. (1981)**.

2.B.1-Identification of the isolates:-

Colonies of Gram negative rods which gave negative cytochrome oxidase reaction were selected for further examination.

2.B.2-Morphological examination:-

Stained smears from suspected colonies showed Gram negative slightly straight rods or short rods of about 3.0 – 1.0 X 2.0 µm in size, non capsulated and non-sporulated. Representative colonies (which shows typical colonial appearance and morphological characters) transferred and stabbed into agar slants, nutrient broth and to semi-solid agar medium and incubated at 25 C for 24 hr. for further biochemical identification.

2.B.4-Sensitivity test of the isolated *Flexibacter columnaris* to various antibiotics:-

These tests were done according to the method described by **Busch (1982)**.

RESULTS

1-Results of clinical signs of naturally infected fish:

The clinical signs of fish which proved to be positive for *Flexibacter columnaris*, were in the form of erosion of jaws with congestion all over the body (Fig. 1). These symptoms, however did not appear in all cases. Haemorrhages on the body surfaces and operculum with inflamed vent or darkening of skin as well as bilateral exophthalmia and some fish showed eye opacity were also observed (Fig, 2 and 3). Post-mortem examinations revealed the presence of enlarged dark spleen, enlarged and congested kidneys, while liver was pale or congested with congestion of all internal organs plus

presence of the bloody fluid in the abdomen. (Fig. 4).

2-Results of isolation and identification of *Flexibacter columnaris*:-

A-Identification of the isolate:-

Attempts to isolate *Flexibacter columnaris* from different organs (liver, spleen, kidney, gills and intestine) gave 4 isolates that grew on Shotts-waltman medium, which gave yellow colonies (from *O. niloticus* and *Common carp*) while catfish gave -ve results. Stained smears from suspected colonies showed Gram-negative short rods to coccobacillary, non sporulated and non capsulated.

3-Results of biochemical tests:- The biochemical characterization of the isolates are summarized in table (5). From the table, all isolates of *Flexibacter columnaris* gave negative reactions to oxidase, urea hydrolysis, indole, phenylalanine deaminase, lactose and xylose fermentation, while gave positive reaction for catalase, glucose, maltose, and hydrolysis to tween 80.

4-Results of sensitivity test:- The results of sensitivity test explained in Table (6) indicated that the *Flexibacter columnaris* was highly susceptible to erythromycin, neomycin, penicillin and streptomycin, and of intermediate sensitivity to kanamycin and norfloxacin and of moderate sensitivity to chloramphenicol, colistin and flumoxone. But *Flexibacter columnaris* was resistant to ampicilline.

5.1.Results of the presumptive test:-

The most virulent *Flexibacter columnaris* strain which gave rapid

and severe clinical, P.M lesions and presumptive test was used for infection high mortalities (80 % , 4/5) in case of in the different experimental work.

Tables and Figures :

Table (5): Biochemical characteristics of *Flexibacter columnaris* isolates.

| No. of <i>F. columnaris</i> isolates | Morphology and colour of colony on Shotts Waltman media | Catalase | Cytochrome oxidase | Lipase: Tween 80 | Urease | Glucose | Lactose | Maltose | Mannitol | Sucrose | Citrate | Indole production | Methyl red | Nitrate reduction |
|--------------------------------------|--|----------|--------------------|------------------|--------|---------|---------|---------|----------|---------|---------|-------------------|------------|-------------------|
| I | Yellow, short rod, cocci, bacilli, non sporulated and non capsulated | + | - | + | - | + | - | + | + | - | + | - | + | + |
| II | | + | - | + | - | + | - | + | + | - | + | - | + | + |
| III | | + | - | + | - | + | - | + | + | - | + | - | + | + |
| IV | | + | - | + | - | + | - | + | + | - | + | - | + | + |

Table (6) : Results of sensitivity test .

| <i>F. columnaris</i> Isolates | Antibiotic | Conc. Of AB | Diameter of zone of inhibition | Susceptibility | |
|-------------------------------|-----------------|-------------|--------------------------------|----------------|-------|
| | | | | R | S |
| I | Ampicilline | 10 µg | 9 mm | R | -ve |
| | Chloramphenicol | 30 µg | 14 mm | I | +ve |
| | Colistin | 10 µg | 9 mm | I | +ve |
| | Flumoquine | 15 µg | 12 mm | I | +ve |
| | Erythromycin | 15 µg | 20 mm | S | +++ve |
| | Kanamycin | 30 µg | 14 mm | I | ++Ve |
| | Neomycin | 30 µg | 17 mm | S | +++ve |
| | Penicillin | 10 µg | 24 mm | S | +++ve |
| | Streptomycin | 10 µg | 16 mm | S | +++ve |
| | Norfloxacin | 30 µg | 16 mm | I | ++ve |
| II | Ampicilline | 10 µg | 10 mm | R | -ve |
| | Chloramphenicol | 30 µg | 14 mm | I | +ve |
| | Colistin | 10 µg | 11 mm | I | +ve |
| | Flumoquine | 15 µg | 12 mm | I | +ve |
| | Erythromycin | 15 µg | 19 mm | S | +++ve |
| | Kanamycin | 30 µg | 14 mm | I | ++ve |
| | Neomycin | 30 µg | 17 mm | S | +++ve |
| | Penicillin | 10 µg | 22 mm | S | +++ve |
| | Streptomycin | 10 µg | 16 mm | S | +++ve |

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| | | | | | |
|-----|-----------------|-------|---------|---|-------|
| | Norfloxacin | 30 µg | 15 mm | I | ++ve |
| III | Ampicilline | 10 µg | 10 mm | R | -ve |
| | Chloramphenicol | 30 µg | 14 mm | I | +ve |
| | Colistin | 10 µg | 10 mm | I | +ve |
| | Flumoquine | 15 µg | 12.5 mm | I | +ve |
| | Erythromycin | 15 µg | 19 mm | S | +++ve |
| | Kanamycin | 30 µg | 14 mm | I | ++ve |
| | Neomycin | 30 µg | 16.5 mm | S | +++ve |
| | Penicillin | 10 µg | 22 mm | S | +++ve |
| | Streptomycin | 10 µg | 15 mm | S | +++ve |
| | Norfloxacin | 30 µg | 15 mm | I | ++ve |
| IV | Ampicilline | 10 µg | 10.5 mm | R | -ve |
| | Chloramphenicol | 30 µg | 13 mm | I | +ve |
| | Colistin | 10 µg | 11 mm | I | +ve |
| | Flumoquine | 15 µg | 13 mm | I | +ve |
| | Erythromycin | 15 µg | 18 mm | S | +++ve |
| | Kanamycin | 30 µg | 15 mm | I | ++ve |
| | Neomycin | 30 µg | 15 mm | S | +++ve |
| | Penicillin | 10 µg | 23 mm | S | +++ve |
| | Streptomycin | 10 µg | 14 mm | S | +++ve |
| | Norfloxacin | 30 µg | 16 mm | I | ++ve |

S= High Susceptibility I = Intermediate sensitivity R = Resistant

DISCUSSION

Bacterial diseases are responsible for mortality in both wild and cultured fish (**Roberts, 1989**). The effect of bacteria varies from that of primary pathogen to that, the secondary invader in the presence of other disease agents, they may also serve as a stress factors and predispose fish to other diseases (**Badran and Eissa, 1991**).

Therefore, the present study was carried-out on naturally infected fish with *F. columnaris* to investigate the clinical signs and P.M. lesions and isolation and identification of the

causative agent . Also studying the sensitivity of some antibiotics to *F. columnaris* as well as histological changes. The number of fish examined were 100 fish collected from different farms of *O. niloticus*, *Common carp*, *Clarias lazera* in Behera, Kafr El-Sheikh and alexandria Governorates. The clinical signs and P.M. lesions includes erosion of jaws with congestion around mouth , haemorrhage and inflammation with darkening of body colour and enlarged, congested spleen and kidney. There was paleness of the liver with congestion of all internal organs.

These symptoms attributed mainly to bacterial infection. (**Oraic et al., 2002**).

Trails for isolation of *F. columnaris* from different organs were made and we successfully isolate 4 strains grow on specific media (Shotts-Waltman Medium with yellow colonies, Gram negative short rods, non capsulated from *O. niloticus* and *Common carp* only. Biochemically it oxidase, ureas and indole negative, while, positive with glucose and maltose. Due to the morphological and biochemical characters of the isolates we identified it as *F. columnaris* especially the presence of 8 flagella which appeared in EM. Results of biochemical tests were similar to these reported by other investigators including (**Danley et al., 1999**).

The isolates were highly sensitive to antibiotics especially, Neomycin, Pencillin and Erythromycin.

Dalsgaard and Madsen (2000) indicated that flumiquine, tetracycline, sulphamerazine, oxytetracycline, tribissen, chloramphenicol, gentamycin, streptomycin and tetramycin, and oxolinic acid are the most effective compounds against all isolates of *F. columnaris*.

Also, **Bakal et al. (2004)**, they found that all strains of *F. columnaris* were sensitive to neomycin, gentamicin tetracycline, chloramphenicol, cotrimoxazole, nalidixic acid, flumequine, enrofloxacin, carbencillin and gentamicin, florfenicol, sulphadimethoxine and ornetoprim. Presumptive test was made by injecting the 4 isolates of bacteria in *Oreochromis niloticus*, *Common carp* and *Monosex tilapias*. The strain which gave high, rapid, severe, clinical signs and P.M. lesions with

high mortality about 80 % was taken for further experimental work. (**Fernandez et al., 2003**).

This study concluded that *F. columnaris* diseases are one of the most important bacterial diseases among cultured fish species. The susceptibility to infection with increasing poor environmental conditions and increasing the bacterial virulence. *F. columnaris* mainly isolated on Shotts-Waltman media and identified biochemically through oxidase, urease, maltose and glucose fermentation test and Electron microscope for demonstration of peritrichous flagellation. Penicillin, Neomycin, Erythromycin are proved to be drug of choice for treatment of *F. columnaris* infection.

F. columnaris may have immunosuppressive effect in case of acute and chronic infection that it makes liver damage and lymphoid depletion of spleen.

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Legend of figures :

Fig. (1): ***Oreochromis niloticus*** naturally infected with ***F. columnaris*** showing redness of abdomen, isthmus and around the mouth.

Fig. (2): ***Oreochromis niloticus*** naturally infected with ***F. columnaris*** showing haemorrhages under the operculum and pectoral fin.

Fig. (3): ***Oreochromis niloticus*** naturally infected with ***F. columnaris*** showing haemorrhagic patches of abdomen with eye opacity.

Fig. (4): ***Oreochromis niloticus*** naturally infected with ***F. columnaris*** showing congestion of the liver, kidney and gills with bloody fluid in abdomen.



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