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SOME MICROBIOLOGICAL INVESTIGATIONS ON *AEROMONAS HYDROPHILA* GROUP IN *OREOCHROMIS NILOTICUS* AND *CLARIAS LAZERA* IN ASSIUT GOVERNORATE

(With 6 tables)

By

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بعض الفحوص الميكروبيولوجية على مجموعة ميكروبات الايروموناس هيدروفيلا فى أسماك البلطى النيلى وقرموط اللازيرا فى محافظة أسيوط

ألفونس فخرى بسطاوروس ، آمال أحمد محمد

تضمنت هذه الدراسة فحص عدد ١٥٠ سمكة من أسماك الماء العذبة بواقع عدد [١٠٠ من أسماك البلطى النيلى + ٥٠ من أسماك القرموط اللازيرا] جمعت عشوائيا من أسواق السمك المتعددة بأسيوط وذلك لاستبيان العدد الكلى لمجموعة ميكروبات الايروموناس هيدروفيلا باتباع وسيلة الانتشار السطحى , ولقد تبين من الدراسة أن ٤٦% , ١٨ % من أسماك البلطى النيلى و القرموط اللازيرا تحتوى على ميكروبات مجموعة الايروموناس هيدروفيلا بمتوسط ٣,٤ × ١٠^{٤} / جم , ٤,١ × ١٠^{٣} / جم من وزن الجسم على التوالى وكانت الدراسة تهدف أيضا الى عزل ميكروبات هذه المجموعة بطريقة الاغناء والاخصاب ثم الزرع على مستنبتات خاصة بهذه الميكروبات وقد تم عزل ٨٠ عترة ميزت الى مستوى الرتبة كما يلى :- ٥١ عترة ايروموناس هيدروفيلا , ١٧ عترة ايروموناس سوبريا , ١٢ عترة ايروموناس كافى ولقد تمت دراسة الخواص الممرضة والنشاط الانزيمى للمعزولات وباجراء اختبارات الحساسية بالمضادات الحيوية على هذه المعزولات اتضح انها حساسة بفاعلية ١٠٠% لكل من النيومايسين وحامض النالديكسيك وغير حساسة بنسبة ١٠٠% للتتراسيكلين والامبيسلين والكوليستين سلفات, هذا وقد تمت دراسة الاهمية الصحية لهذه الميكروبات وكذلك الاشتراطات الصحية الواجب توافرها لدرء خطر هذه الميكروبات على صحة المستهلك.

SUMMARY

150 freshwater fishes including 100 (*O. niloticus)* and 50 (*C. lazera)* were collected from different fish markets of various sanitation levels at Assiut. The samples were examined for enumeration of *Aeromonas hydrophila* group. The obtained results pointed out that 46 % and 18 % of the examined *O. niloticus* and *C. lazera* fishes were positive for *Aeromonas hydrophila* microorganisms with an average count of 3.4×10^4 /g / fish and 4.1×10^3 /g / fish using surface spread plate technique respectively. Eighty *Aeromonas hydrophila* strains isolated in this study were characterized according to species level as follow: 51 *Aeromonas hydrophila*, 17 *Aeromonas sobria* and 12 as *Aeromonas caviae*. All strains were examined for their ability to produce virulence factors and enzymes. Concerning the antibiotic sensitivity of the isolated strains, they were sensitive to Neomycin and Nalidixic acid with an activity of 100 %. On the other hand, they showed a high degree of resistance to Tetracyclin, Colistin sulphate and Ampicillin. The public health impotance as well as recommended sanitary measures were discussed.

Key Words: *Aeromonas Hydrophila Group, Oreochromis Niloticus, Clarias Lazera*

INTRODUCTION

Genus *Aeromonas* has been calssified with family *Vibrionaceae* (Baumann and Schubert, 1984), but more recently Colwell et al, (1986) have proposed a new family, the *Aeromonadaceae.*

A. hydrophila microorganisms could be readily isolated in considerable numbers from fishes (Pin et al 1994) and fish products (Gobat and Jemmi, 1993). Furthermore, *Aeromonas hydrophila* is considered to be the principle cause of bacterial haemorrhagic septicemia in freshwater fish (Ahmed, 1982).

The genus *Aeromonas* consists of ten named species (Koneman et al, 1994). The most important three motile species in human are *A.hydrophila*, *A.sobria* and *A.caviae* (Brooks et al, 1995). Many laboratories continue to group all motile aeromonads in the general category, *A.hydrophila* group or complex (Hickman-Brenner et al, 1987).

In recent years, *A.hydrophila* group received increasing attention as an agent of foodborne diarrhoeal disease in healthy people (Varnam and Evans, 1991). Foods including fish may play an important role in the etiology of human *A.hydrophila* gastroenteritis outbreaks. Molero et al, (1989) found that *A.hydrophila* was recovered in two cases out of eight cases of acute gastroenteritis, in all cases fish or shellfish had been ingested outside the patient's homes. On the otherhand, *A.hydrophila* produces a number of potential virulence factors including cytotoxins, haemolysins and caseinases (Paniagua et al, 1990 and Mateos et al, 1992).

Reviewing the available literature concering antimicrobial sensitivity of *A.hydrophila* groups. It is shown that the organisms have high frequency of resistance to several antibiotics, this may be related to many years of exposure to drugs and their improper application (Ansary et al, 1992 and Khater et al, 1997).

Due to the public health significance of *A.hydrophila* group and little information regarding its incidence in freshwater fishes. The present work was undertaken to study, the presence of *A.hydrophila* group in some freshwater fishes (*O. niloticus* and *C. lazera*), the biochemical reactions of the isolates, the ability of the isolated strains to produce virulence factors as well as the antibiotic sensitivity of isolated stains.

MATERIAL and METHODS

A total of 150 freshwater fishes (100 *O. niloticus* and 50 *C. lazera)*, collected during summer and winter seasons from local fish markets at Assiut City were investigated. The fish samples were transferred to the laboratory with a minimum of delay where they were subjected to bacteriological examination. Samples from skin, flesh, liver and kidneys were taken under aseptic conditions from the collected fishes were used for isolation and identification of the *A. hydrophila* group organisms after disinfection by flaming.

Experimental procedures:-

(1) Determination of Aeromonas organisms count:

Ten grams from each organ , namely skin , flesh, liver and kidney of each fish , were transferred aseptically to a sterile blender jar to which 90 ml of sterile peptone water 0.1% were added to provide a dilution of 10^{-1} . The blender was operated to give 3000 r.p.m. for not more than 2.5 minutes, then the mixture was allowed to stand for 15 minutes at 19 ±

1 °c in winter and 30 ± 1 °c in summer. The contents of the jar were mixed by shaking before ten fold serial dilution were prepared up to 10^{-6} using sterile peptone water. The count of *Aeromonas* organisms was determined by using surface spread plate technique and carried out on the aforementioned dilutions as recommended by Palumbo, et al, (1985), using MacConkey Manitol Ampicillin agar. The number of suspected colonies which showed red colour in countable plates were enumerated as Aeromonas organisms.

(2) Isolation of Aeromonas spp.:

I) Enrichment procedure:

20 g of each sample (skin, flesh and organs) were aseptically transferred to 180 ml of Trypticase Soy broth containg 10 µg Ampicillin / ml and blended for 2 mintues, incubated at 28 ± 1 °c for 24 hrs.

II - Isolation and identification techniques:

After incubation of enrichment broth, 0.1 ml of each was streaked on the surface of MacConkey Mannitol Ampicillin medium and incubated at 28 ± 1 °c for 24 hrs (Ahmed et al, 1991). Loopfuls from suspected colonies which showing typical red pigment were picked up onto nutrient agar slants and incubated at 28 ± 1 ° c for 24 hrs for further identification. The isolates were identified morphologically and biochemically according to the methods described by Koneman et al, (1994). Acriflavine agglutination test and stability after boiling were conducted to detect the virulence of isolated strains as reported by Miltal et al, (1980). The identified strains were evaluated for the haemolytic activity of 5 % sheep blood agar and gelatinase production on agar with 15 % gelatin (Rogulska et al, 1994), while DN-ase production was evaluated by streaking on DN-ase agar medium (Palumbo et al, 1985).

(3) Antimicrobial susceptibility testing:

All strains obtained in this study were tested for antimicrobial susceptiblity by dick diffusion method as described by Finegold and Martin (1982), using the following antibiotics: Tetracycline (30 µg), Colistin sulphate (10 µg), Erythromycin (15µg), Rimactan (Rifampicin) (30 µg), Kanamycin (30 µg), Streptomycin (30 µg), Neomycin (30 µg), Nalidixic acid (30 µg), Norfloxacin nicotinate (4 µg), Polymyxin-B (300 u), Ampicillin (10 µg), and Trimthoprim - Sulfamthaxazol (1.25 + 23.75 µg).

RESULTS

The results are tabulated in tables 1, 2, 3, 4, 5 and 6

DISCUSSION

The summarized results in table (1) verify that 46 (46 %) of *O.niloticus* were contaminated with Aeromonas species in numbers varied from 2×10^3 /g / fish to 15×10^4/g / fish with an average count of 3.4×10^4 / g / fish. while 9 (18 %) of *C. lazera* fish samples proved to be contaminted with *Aeromonas* spp. The maximum count was 7.2×10^3 /g / fish, the minimum count was 1.2×10^2 / g/ fish, with an average count of 4.1×10^3 / g / fish. Therefore, one can assume that the Aeromonas spp. existed in high percentage and count in *O.niloticus* than *C. lazera* as Aeromonas microorganisms are normal inhabitants of the intestinal tract of *O. niloticus* (Akelah, 1978). In this study, the incidence of *A. hydrophila* group in fishes was found to be 36.67 % (Table 1). The present result is nearly similar to those reported by Pin et al, (1994) who found that 40 % of fishes were contaminated with *A.hydrophila* group.

In this study, 46 (46 %) of *O. niloticus* fish samples were contaminated with *A.hydrophila* microorganisms (Table, 1). Such results were significantly high as compared with those reported by Ahmed (1982) who found that 15 % of *O.niloticus* were contaminated with *A. hydrophila*. On the other hand, Abou-El-Gheit, et al, (1995) reported higher incidence 63.64 %. The successful isolation and identification of the same microorganisms were recorded by some investigators from the different extra - intestinal organs such as liver, spleen and kidneys as well as from the deep skin lesions of fishes. (Thune et al, 1986 and Sakai et al, 1993).

Concering *Clarias lazera* fishes, the organisms were isolated from 9 (18%) of samples (Table 1).

A.hydrophila group is wide spread in the water environment, it has been isolated from water of rivers (Mateos et al, 1992) and also from *Mormyrus kannume*, cultured carp and Ornamental fishes (Ahmed et al, 1991; Samia et al, 1996 and Khater et al, 1997), as well as, from retail food of animal origin such as fish and sea foods (Palumbo et al, 1989). On the other hand, Gobat and Jemmi (1993), found that some ready-to-eat fish products such as hot and cold smoked fish (10.9-14.3%) and

graved salmon (10.5 %) had a relatively high percentage of positive samples for the presence of mesophilic Aeromonas species.

A.hydrophila microorganisms amounting 80 strains were isolated from the examined fish samples. These strains included 71 (88.75 %) from *O. niloticus* and 9 (11.75 %) from *C.lazera* samples. Fifty one strains (63.75 %) were identified as *A. hydrophila*, 17 (21.25 %) as *A. sobria* and only 12 (15 %) out of the 80 strains were identified as *A. caviae* (Table 2). These findings agree to a certain extent, with that reported by Gobat and Jemmi (1993). On the other hand, it disagree with that reported by Pin et al, (1994) who failed to isolate *A.Caviae* from 80 food samples including fish.

In this study, the incidence of motile Aeromonas species among fish samples denoted the seasonal occurrence of the organisms with a peak of incidence during summer season followed by winter (Table, 3). These results supported those of Meyer (1970); Tysset et al, (1970); Rippy and Cabelli (1980); Faisal et al, (1989) and Samia et al, (1996) who got more or less the same results and attributed these pattern of incidences to the high growth and mutiplication of *A.hydrophila* with the decrease of oxygen content of water during summer which inturn, make the fishes more susceptible to infection.

The allocation of Aeromonas strains to species based on the method of Koneman et al, (1994) showed that all the 80 strains isolated gave the typical biochemical reactions (table, 5). A finding that simulate those reported by (Palumbo et al, 1989).

All of the 80 strains were tested for their ability to produce haemolysin. 35 (68.63 %) of 51 *A.hydrophila* strains, 11 (64.70 %) of the 17 *Aeromonas sobria* strains but none of the 12 *A. caviae* strains lysed the sheep erythrocytes with a variable halo diameter between 0.5 and 2 mm (Table, 4). Varnam and Evans (1991) reported that a number of phenotypic characters have been proposed as markers of enteropathogenicity of Aeromonas species and added that the most important of these is haemolysis. Concerning gelatinase and DN-ase production, the majority of the 80 Aeromonas strains had DN-ase and gelatinase activities (Table, 4). The present result is somewhat similar to those reported by Palumbo et al (1989) and Paniague et al, (1990).

Information derived from table (5) revealed that 34 (66.67 %) out of 51 strains of *A.hydrophila* did not agglutinate in acriflavine and settled down after boiling, these were the highly virulence strains. The autoagglutination of cells of Aeromonas after boiling has been correlated with pathogenicity to mice and this phenomenon has also been proposed

as a virulence marker (Varnam and Evans, 1991). The remaining strains 17 (33.33 %) were agglutinated in acriflavine and did not settle down aftter boiling (Table, 5). Nearly similar results were reported by Ahmed et al, (1991) who found that 30 % of *A. hydrophila* strains were agglutinating in acriflavine and did not settle down after boiling.

The antibiogram study conducted on the isolated strains showed that Nalidixic acid and Neomycin were the most effective antibiotic against the strains of *A.hydrophila* group at a rate of 100% (Table 6). Nearly similar results were reported by Molero et al, (1989) and Abou El-Gheit et al, (1995) who found that all strains of *A.hydrophila* were sensitive to Nalidixic acid and Aminoglycosides. On the other hand, a high degree of resistant (100%) of *A. hydrophila* strains were detected in the present work against Tetracycline, Colistin Sulphate and Ampicillin out of 12 tested antibiotics (Table 6). These findings agree to a certain extent with those reported by Abou El-Gheit et al, (1995) and Khater et al, (1997). A contradictory results were reported by Molero et al, (1989) and Dixon and Issvoran (1992) who found that all and 29 % of the 2 and 42 isolates of *Aeromonas spp.* isolated from patients suffering from acute gastroenteritis and Ornamental fish samples, were sensitive to tetracycline respectively.

In conclusion, the information given by the achieved results revealed that *Aeromonas spp.* existed in the examined fishes in varying percentages. There is a risk associated with consuming raw fish. The risk can be avoided by only consuming thoroughly cooked fish, red meat and sea foods. In addition, good food handling practices in the home including washing hands thoroughly after handling raw fish reduce the risk of illness. On the other hand, preventing contamination of ready-to-eat fish and sea foods by sorting and handling these products separately and do not permit cooked fish and sea foods to contact equipment that may have been contaminated by raw fish and sea foods. Finally, thoroughly and properly clean and sanitize all equipments, food utensils and contact surfaces.

The presence of significant levels of virulent strains of *A.hydrophila* in fish in this study i.e. *Aeromonas spp.* which did not agglutinate in acrifalvine and settled down after boiling and also had haemolytic activity indicates that fishes may play a significant role in the epidemiology of Aeromonas associated gastroentritis.

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Table 1: Total *Aeromonas hydrophila* microorganisms count per gram of examined fishes.

Species	No.of samples Examined	Positive Samples	Count / g of fish		
			Min.	Max.	Average
O. niloticus	100	46 (46%)	2×10^3	15×10^4	3.4×10^4
C. lazera	50	9 (18%)	1.2×10^2	7.2×10^3	4.1×10^3
Total	150	55 (36.67%)			

Table 2: Distribution of *Aeromonas hydrophila* group in examined fish samples.

Fish species	No. of isolated Strains	*Aeromonas hydrophila* group		
		Aeromonas hydrophila	*Aeromonas Sobria*	*Aeromonas caviae*
O.niloticus	71 (88.75 %)	46 (64.79 %)	14 (19.72%)	11 (15.49%)
C. lazera	9 (11.25%)	5 (55.56 %)	3 (33.33%)	1(11.11%)
Total	80	51 (63.75%)	17 (21.25 %)	12 (15 %)

Table 3: The incidence of motile Aeromonas species in fish samples examined during summer and winter seasons

Fish species	No. Of Examined Samples	No. of +ve Samples	*Aeromonas hydrophila* group					
			A. hydrophila		*A.sobria*		*A.caviae*	
			No. of isolates in					
			summer	Winter	Summer	winter	summer	Winter
O.niloticus	100	46	35	11	8	6	7	4
C.lazera	50	9	4	1	3	0	1	0

Table 4: Haemolysin, DNase and Gelatinase activities of Aeromonas species isolated from examined fish samples

Aeromonas Species	No. of examined Isolates			Haemolysin Positive Strains	DNase Positive Strains	Gelatinase Positive Strains
	O. niloticus	*C. lazera*	Total			
Aeromonas Hydrophila	46	5	51	35(68.63%)	48(94.12%)	48(94.12%)
Aeromonas Sobria	14	3	17	11 (64.71%)	15(88.24%)	11 (64.71%)
Aeromonas Caviae	11	1	12	0	9 (75%)	7 (58.33%)

Table 5: Differentiation of Aeromonas species isolated from fish samples.

Characteristic	*Aeromonas Hydrophila* No. of strains (51)	*Aeromonas sobria* No. of strains (17)	*Aeromonas Caviae* No. of strains (12)
Oxidase	+	+	+
Esculin hydrolysis	+	-	+
Motility	+	+	+
Indol	+	+	+
Voges-proskauer	+	+	-
Gas from glucose	+	+	-
Acid from:			
L-Arabinose	+	V	+
Sucrose	+	+	+
Mannitol	+	+	+
Inositol	-	-	-
Acriflavine Agglutination test And appearance after boiling	**34(66.67 %) / 17(33.33%)*	N. D	N. D
Growth in peptone 1% with 0% Na Cl	+	+	+
With 7% NaCl	-	-	-
With 11 % NaCl	-	-	-

List of abbreviation

+ , 90 % or more of strains positive.
- , 90 % or more of strains negative.
v , 11 % - 89 % of strains positive.
N. D, Not done.

* 34 (66.67 %) ——— ***the numerator gives the number of strains which did not agglutinate in***

17 (33.33 %) *acriflavine and settled down after boiling.*
The denominator represents the number of strains which agglutinated in acriflavine and did not settle down after boiling.

Table (6) : In vitro antimicrobial drug sensitivity of *Aeromonas hydrophila* group.

Contet / disc	*Aeromonas hydrophila*				*Aeromonas sobria*				*Aeromonas caviae*			
	No. of strains (51)				No. of strains (17)				No. of strains (12)			
	No. of sensitive isolates	*% of sensitivity*	*No. of resistant isolates*	*% of resistance*	*No. of sensitive isolates*	*% of sensitivity*	*No. of resistant isolates*	*% of resistance*	*No. of sensitive isolates*	*% of sensitivity*	*No. of resistant isolates*	*% of resistance*
Tetracycline (30 ug)	*0*	*0.00*	*51*	*100*	*0*	*0.00*	*17*	*100*	*0*	*0.00*	*12*	*100*
Colistin- sulphate (10 ug)	*0*	*0.00*	*51*	*100*	*0*	*0.00*	*17*	*100*	*0*	*0.00*	*12*	*100*
Trimthoprim " sulfamthoxazol " (1.25 + 23.75 ug)	*45*	*88.24*	*6*	*11.76*	*11*	*64.71*	*6*	*35.29*	*8*	*66.67*	*4*	*33.33*
Erythromycin (15 ug)	*3*	*2.88*	*48*	*94.12*	*1*	*5.88*	*16*	*94.12*	*2*	*16.67*	*10*	*83.33*
Rimactan " Rifampicin (30 ug)	*35*	*68.63*	*16*	*31.37*	*7*	*41.17*	*10*	*58.82*	*8*	*66.67*	*4*	*33.33*
Kanamycin (30 ug)	*41*	*80.39*	*10*	*19.61*	*11*	*64.71*	*6*	*35.29*	*10*	*83.33*	*2*	*16.67*
Streptomycin (30 ug)	*36*	*70.58*	*15*	*29.42*	*9*	*52.94*	*8*	*47.06*	*7*	*58.33*	*5*	*41.67*
Neomycin (30 ug)	*51*	*100*	*0*	*0.00*	*17*	*100*	*0*	*0.00*	*12*	*100*	*0*	*0.00*
Nalidixic acid (30 ug)	*51*	*100*	*0*	*0.00*	*17*	*100*	*0*	*0.00*	*12*	*100*	*0*	*0.00*
Norfloxacin Nicotinate (4 ug)	*13*	*25.49*	*38*	*74.51*	*3*	*17.65*	*14*	*82.35*	*2*	*16.67*	*10*	*83.33*
Polymyxin-B (300 U)	*5*	*9.80*	*46*	*90.20*	*4*	*23.53*	*13*	*76.47*	*1*	*8.33*	*11*	*91.67*
Ampicillin (10 ug)	*0*	*0.00*	*51*	*100*	*0*	*0.00*	*17*	*100*	*0*	*0.00*	*12*	*100*