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PREVALENCE OF *AEROMONAS HYDROPHILA* IN *OREOCHROMIS NILOTICUS* FARMS IN KAFREL-SHEIKH GOVERNORATE

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ABSTRACT

One hundred and Fifty six samples of Nile tilapia (Oreochromis niloticus), (53 samples from diseased and 103 samples from apparently healthy), were collected from different farms in different locality in Kafr El-sheikh Governorate,the samples were analysed for the prevalence of Aeromonas hydrophila over a period of nine months from first of December 2007 to end of Agusts 2008. and for their anti-microbial resistance using the disk diffusion method. Moreover the bacteria were tested for their pathogen city for fish and hemolytic activity,

The prevalence of Aeromonas hydrophila among Oreochromis niloticus were 6(35.3%).There was a clear seasonality, as one isolates (1.3%) were recovered in the winter months while five isolates (6.4%) recovered in summer.

Despite the high level of homogeneity observed in strains of Aeromonas hydrophila in biochemical patterns, they displayed different degrees of virulence for Tilapia fish; were 16.7% strong virulent, 50% virulent ,16.7% weak virulent and 16.7% avirulent by subcutaneous injection of Aeromonas hydrophila isolates .

All strains of Aeromonas hydrophila showed hemolytic activity on blood agar except one avirulant strains showing non hemolytic activity. There is slight correlation was found between hemolytic activity and degree of virulence of the strains,also the isolates were tested against 13 antimicrobial agents and the result were , all the isolates were found 100% resistant to Penicillin, Streptomycin, Polymyxin B,95% for Ampicillin and 70% for Ciprofloxacin.All strains 100% sensitive to Amikacin, Gentamicin, Nalidixic acid, Kanamycin, Neomycin, Tetracycline, and 90%Chloramphenicol.

INTRODUCTION

Aeromonas hydrophila, is a Gram-negative, rod shaped, mainly motile, facultative anaerobic, oxidase positive and glucose fermenting bacteria ***(Nordmann and Poirel, 2002).*** Some strains of ***Aeromonas hydrophila*** are capable of causing septicemia in fish and amphibians as well as extraintestinal and deep wound infections, gastroenteritis, cellulitis, meningitis, bacterimia, soft tissue infections, peritonitis, bronchopulmonary infections, and infection in humans with compromised immune systems ***(Ahmed 1983; Amin et al.,1985; Janda 1991; Chang et al., 1997).*** Furthermore, is frequently associated with disease in fresh water fish such as Tilapia ***(Ahmed 1983 and Amin et al.1985)***. In warm water aquaculture, ***Aeromonas hydrophila*** is considered to be a major economic problems, but it is difficult to distinguish direct losses from those caused by secondary infection ***(Ruangapan et al 1986 and Austin and Austin 1987)***. Aeromonds are common contaminants in a wide spectrum of foods namely fishes, raw and cooked meat, poultry, vegetables,milk and milk products ***(Agarwal et al.,2000; Bachhil et al.2002).*** These foods play an important role in the dissemination of the potentially pathogenic ***Aeromonas*** to humans.

Aeromonas hydrophila is able to survive and multiply at low temperatures in a variety of food products stored between –2 and 10 C such as beef, roast beef and pork ***(Krovacek et al., 1992; Mano et al., 2000)***, and can produce virulence factors even at these low temperatures ***(Mateos et al., 1993; Merino et al.,1995).***

The pathogenicity of the organism is associated with the liberation of virulence factors and cell associated endotoxin. Virulence factors include the production of exotoxins (cytotoxin or enterotoxin) and α- B-hemolysins and ability to bind and to invade epithelial cells ***(Krovacek et al (1994).***

The main objective of this study was carried out to throw light on the following:

1- Prevalence of the ***Aeromonas hydrophila*** isolated from *Oreochromis niloticus* and seasonal occurance.

2- Correlation between the hemolytic activity and pathogenicity for fish of the isolated strains.

3- The resistance of isolated strains against different antibiotics.

MATERIALS AND METHODS

2.1. Bacterial strains and growing conditions:

Six strains of presumptive ***Aeromonas hydrophila*** were isolated from 156 ,*Oreochromis niloticus* collected from different farms in Kafr El-sheikh Governorate during a period of nine months from first of December 2007 to end of august 2008.Bacteria were isolated by inoculating plates of R-S agar ***(Shotts and Rimler1973)*** from fish skin, gills, liver, spleen and kidney then incubating the cultures at 28_C for 24 h. Yellow colonies were then selected and identified to the genus Aeromonas**.** Suspected colonies were picked up and streaked onto the surface of Starch Ampicillin Agar (SAA), 5% sheep blood agar, nutrient agar, and Tripticase Soy Agar (TSA) plates and Triple Sugar Iron (TSI) slant. All plates incubated at 28 C for 24 hour. The isolated bacteria was identified by culture morphology, Gram-stain and biochemically according to **(*Popoff, 1984, Palumbo et. al., 1985, Glunder and Siegman, 1989 and Bisgaard et al., 1995*).** The colonies that showed typical reaction in TSI and positive for cytochrom oxidase test, oxidation and fermentation reaction of glucose and catalase test were confirmed as ***Aeromonas hydrophila***.

2-2-Determination of hemolytic activity:

Carried out according to ***Gerhardt et al.,(1981)***.The strains were tested for beta-hemolytic activity on Columbia agar base (Oxoid) supplemented with 5% sheep erythrocytes with incubation at 28°C for 48 h. The presence of a clear colorless zone surrounding the colonies indicated B-hemolytic activity

2-4-Pathogenicity assay:

Carried out according to ***Kozin´ska et al.(2002).*** Nile tilapia fish of 30-35 gm/fish were obtained from El-Zawia fish farm at KafrEl -shiekh and free from disease, and transported to the laboratory in plastic buckets. The fish were maintained in 100-l aquaria with aerated fresh water at 30+- 2C for 2 weeks and fed commercial pellets in order to adapt to laboratory conditions. All fish were injected subcutaneously with 0.1 ml of a suspension containing $600x10^6$ bacterial cells in phosphate-buffered saline(PBS). Ten fish were injected per strain, and ten other fish, were injected with 0.2ml of sterile PBS as controls negative.The fish were then replaced into the aquaria and held under the same conditions as before injection. The morbidity and mortality of the fish were monitored daily for 7 d and the virulence level of the strains estimated and classified into four grouped as follows:- more than six fish with disease symptoms and mortality of five to 10 fish (strongly virulent); more than six fish with disease symptoms and mortality of one to four fish(virulent); more than four fish with disease symptoms and without mortality (weakly virulent) and one to four fish with slight pathological signs and without mortality or neither pathological signs nor mortality noted (avirulent).Recently dead fish and any survivors after 7 days were tested bacteriologically for the presence of injected bacterium in damaged fish tissues.

2-5- Antimicrobial susceptibility:

a- Culture Media:

Mueller-Hinton agar (Oxoid): This medium was used for the disk diffusion test. It produces large and clear zone of inhibition when sensitive organisms are in contact with susceptible antibiotic.

b-Antibiotic sensitivity disks: (Oxoid)

The antibiotics and concentration ranges tested were showed as following table:

Antimicrobial agent	Disc potency	Symbol
Amikacin	30ug	A
Nalidixic acid	30ug	ND
Ampicillin	10ug	Amp.
Ciprofloxacin	5ug	
Chloramphenicol	30ug	C
Streptomycin	10ug	S
Gentamicin	10ug	Gn
Kanamycin	30ug	K
Neomycin	30ug	N.
Penicillin	10ug	P.
PolymyxinB	300U	Pol.B
Tetracycline	30ug	T.
Trimethoprim sulfamethoxazol	25ug	TXM

c- Methods:

The resistance of all strains to different antimicrobial agents was determined by the disk diffusion method (Becton Dikinson, Microbiology Systems, MD, USA) according to ***FineGold and Martin (1982)***. The degree of sensitivity was determined and interpretation of their sensitivity were done according to ***Oxoid Manual (1982) and Koneman et al., (1983)***.

RESULTS

Table (1): Prevalence of ***A. hydrophila*** from apparently healthy and diseased *Orechromas nilotucus.*

Healthy status	Total of examined samples	Total bacterial isolates	*Aeromonas hydrophila*	Other bacteria
Diseased	53	9	4(44.4%)	5(55.6%)
Apparently healthy	103	8	2(25%)	6(75%)
Total	156	17	6(35.3%)	11(64.7%)

Table(2): rate of isolation of ***A.hydrophila*** from skin and internal organ of diseased and apparently healthy Tilapia fishs.

Organ	Diseased fish (4)	Apparently healthy fish (2)
	No (%)	No (%)
Skin	2(50)	1(50)
Gills	1(33.3)	0(0)
Spleen Heart	0(0)	1(50)
Kidney	1(16.7)	0(0)
Liver	0(0)	0(0)
	0(0)	0(0)
Total	**4(100)**	**2(100)**

Table (3): Incidence of ***A.hydrophila*** in relation to season.

season	Total collected samples	No of isolated strains
Summer	78	5(6.4%)
Winter	78	1(1.3%)
Total	**156**	**6(3.8%)**

Table (4): Correlation of degrees of virulence with hemolytic activity in six strains of ***A. hydrophila.***

Isolate No	%	Degrees of virulence	Hemolytic activity On Sheep RBCs
1	16.7%	Strong virulent	++ +
2	50%	Virulent	++
3			++
4			++
5	16.7%	Weak virulent	+
6	16.7%	A virulent	-

+ positive for haemolytic activity - Negative for hemolytic activity

Pathogenicity for fish:

There was no death or sign of disease in fish injected with sterile PBS. (Table 3) presents the virulence level of six ***Aeromonas hydrophila*** strains tested in this study. Five strains were classified as strongly virulent, Virulent and weak virulent for fish. These strains caused motile aeromonad septicaemia with intensive external signs including hemorrhages, necrosis and ulcers. Between 10 and 80% of the fish died during the experiment, depending on the infection strain used. One strain were avirulant for fish and cause no mortality and signs of disease. There were differences in the pathogenicity among individual strains of ***Aeromonas.hydrophila***. Strongly virulent, virulent, weak virulent and these avirulent strains differed in degree of haemolytic activity.

Table (5): Antibiotic resistance of ***Aeromonas hydrophila*** isolates.

Antibiotic	*Aeromonas*.hydrophila	
	Resistance %	Sensitivity %
Ampicillin	87.3	16.7
Penicillin	100	0
ciprofloxacin	66.7	33.3
Amikacin	0	100
Streptomycin	100	0
Gentamicin	0	100
Nalidixic acid	0	100
Kanamycin	0	100
Neomycin	0	100
Tetracycline	0	100
Chloramphenicol	16.7	83.3
Polymyxin B	100	0
Trimethoprim–sulfamethoxazol	50	50

The resistance patterns obtained with the 6 ***Aeromonas hydrophila*** strains against 13 antibiotics are shown in (Table 4). All strains showed 100% resistance to penicillin, Polymyxin B and Streptomycin In addition, the lowest resistances encountered were 83.3% to Ampicillin, 66.7% to Ciprofloxacin, while the rest were under 50%. In contrast, all the strains were susceptible to Amikacin, Gentamicin, Nalidixic acid, Kanamycin, Neomycin and Tetracycline.

DISCUSSION

Biochemical identification of ***Aeromonas.hydrophila*** demonstrated that the prevalence of ***Aeromonas hydrophila*** among *Oreochromis niloticus* were (35.3%), these result simulate the result obtained by **Habib and *Chowdhury (2001)*** who isolated ***A.hydrophila*** in percent 33.3%. previous

studies isolate ***Aeromonas hydrophila*** from *Oreochromis niloticus* with higher incidence 46% ***(Bastawros and Amal 1999)*** and 94% ***(Maria et al.2004).*** It has been suggested that the fish either harbours the pathogen at the time of capture or become infected during transport ***(Wiklund and Dalsgaard, 1998).*** With regard to the seasonal incidence of isolated ***A.hydrophila***, 5(6.4%) isolates were recovered during summer season, while only 1(1.3%) isolates were recovered during winter (Table 3). In contrast ***Topic popovic et al.(2000)*** isolated ***A.hydroophila*** in winter and not isolated it in summer season Also our result lower than recorded by ***(Samia et al.,1996 and Cipriano et al.,2001)*** they isolated ***A.hydrophila*** with incidence 14(77.7%) during summer season and 4(22.2%) during winter . The higher incidence in summer than winter in our country may be attributed to high temperature of water during summer which decreases oxygen solubility in water,so low oxygen content of water resulting in stress on fish and increase multiplication of ***A.hydrophila***, which in turn make the fish susceptible to infection..

Hemolysin production is one of the properties associated with gastrointestinal infections caused by aeromonads. Results of this study show that the majority of the isolates were able to elaborate this virulence factor. In our study, 5 of the 6 (83.3%) ***Aeromona hydrophila*** strains found in the present study were able to produce β-hemolytic activity. Previous studies reported 200 of the 240 (83.3%) ***Aeromona hydrophila*** strains were able to produce β-hemolysis ***(Burke et al. 1983; Araujo et al. 1991; Asmat, and Gires, 2002).*** Other scientist found 57% of the ***Aeromonas hydrophila*** showed hemolytic activity on agar plate ***(Chua Kek Heng,et al. 2005).*** From our result ,no higher activity was

found in the less virulent strains (table 4) as reported by ***(Lallier et al. 1984).*** Others authors, mentioned that some association between ***Aeromonas hydrophila***, phenotypic profile and specific disease signs was observed. ***(Kozin´ska et al.,2002).***

In most pathology studies, motile aeromonads isolated from fish have been assigned to ***Aeromonas hydrophila (Santos et al. 1988; Del Corral et al. 1990; Kozin´ska 1996). Aeromonas hydrophila*** has been recovered from diseased fish ***(Huys et al. 1996).*** All ***Aeromonas*** strains (motile) were isolated from diseased fish and, after experimental infection, caused the disease in fish. Earlier reports indicated that ***Aeromonas hydrophila*** strains can be pathogenic for carp ***(Kozin´ska 1996)*** as well as for other fish species ***(Santos et al.1988; Toranzo et al. 1989).*** It is important in pathology studies to differentiate the pathogenic from the non-pathogenic strains. The results of our study showed that the hemolytic activity of some ***Aeromonas hydrophila*** differ in the of degree hemolysis, exept one strains has hemolytic activity and non pathogenic for fish (avirulant) (table 4).It was found in previous studies that pathogenic strains of motile aeromonads differ from non-pathogenic strains in their degree of hemolytic activity, ***(Leung et al. 1994; Kozin ´ska 1996; Sopin´ska et al. 1997).***

Susceptibility Patterns of ***Aeromonas hydrophila*** to antimicrobial agents have varied,. In vitro susceptibility of the ***Aeromonas hydrophila*** isolates to a variety of antibiotics shown in (Table 5). Most strains were 100% resistant to Penicillin, Streptomycin, Polymyxin B, 95%to Ampicillin, and 70% to Ciprofloxacin. The strains also showed 100% sensitivity to

Amikacin, Gentamicin, Nalidixic acid, Kanamycin and Neomycin, our results are in contrast with data reported by other authors for clinical strains***(Morita et al.,1994;Ko et al., 1996).***found that strains of ***Aeromonas hydrophila*** isolated from different geographical areas were100%susceptible to ampicillin, polymyxinB. Strains of ***Aeromonas hydrophia*** isolated from rivers ***(Gon˜i-Urriza et al., 2000)*** showed 59% resistance against nalidixic acid while we encountered no resistance at all (0%). In addition, these authors found 14% resistance to tetracycline and 1% to gentamicin against 0% for each them, in this work strains of ***Aeromonas hydrophila*** were100% sensitivity to amikacin, ciprofloxacin, chloramphenicol, gentamicin, and kanamycin was found in a study that included a broad number of clinical and environmental *strains* ***(Mascher et al., 1988; Soliman 1988; Molero et al.,1989; Sohair and Eman 2002),*** in contrast to the resistance to ciprofloxacin found here. Also in a previous study analyzing 43 clinical strains of ***Aeromona hydrophila***, and 100% of them were susceptible to gentamicin and 100% of them to ciprofloxacin ***(Vila et al., 2002).*** Our results show that the drugs with the best antimicrobial effect against ***Aeromonas hydrophila,*** in agreement with data reported by other authors ***(Ko et al., 1996;Ka¨mpfer et al.,1999; Vila et al., 2002).*** Therefore, these drugs could be the treatment of choice for extraintestinal infections, and also for patients with chronic diarrhoea caused by Aeromonas.

In conclusion, From the present study the hemolytic activity can differentiate between pathogenic and non pathogenic strains but is insufficient to determine their pathogen city for fish and the rate of infection with ***A.hydrophila*** in the summer higher than winter.

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مدى انتشار ميكروب الايرومونس هيدروفيلا فى مزارع اسماك البلطى النيلي فى محافظة كفر الشيخ

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تم تجميع 156عينة من اسماك البلطي النيلي (53من الأسماك المريضة '103من الأسماك السليمة ظاهريا) من المزارع المختلفة بمحافظة كفر الشيخ تم تحليل هذه العينات لمعرفة مدى انتشار ميكروب الايروموناس هيدروفيلا على مدي 9 شهور(موسم الشتاء والصيف) وذلك من بداية ديسمبر 2007 حتي نهاية أغسطس 2008 ومعرفة مدى مقاومة هذه المعزولات للمضادات الحيوية وأيضا مدى ضراوته لأسماك البلطي النيلي بالإضافة معرفة العلاقة بين درجه التحلل الدموي وضراوة الميكروب.

وقد أوضحت هذه الدراسة أن ميكروب الايروموناس هيدروفيلا ينتشر بنسبة 35.3% قى اسماك البلطي النيلي في مزارع كفر الشيخ كما وجد أن نسبة انتشارها فى موسم الشتاء 1(1.3%) آم في موسم الصيف 5(6.4%).

وعلى الرغم من التشابه الكبيرفى الاختبارات الكميائية لميكروب الايروموناس الا ان هناك اختلاف كبير فى ضراوة الميكروب حيث وجد ان هذة المعزولات منها 16.7ا %، شديد الضراوة 50%، 16.7ا % ضعيف الضراوة،و 16.7ا % غير ضاري. كم أوضحت الدراسة أن هذه المعزولات له تأثير على تحلل الدم ماعدا معزوله واحدة ليس لهل تأثير على التحلل الدموي وغير ضارية .وتبين أن هناك علاقة بين ضراوة الميكروب والتحلل الدموي على الأجار.

كما اختبرت المعزولات ضد 13 مضاد حيوي ووجد أن مقاومة الميكروب كانت بنسبة 100 % للبنسلين، الاستر بتوميسين،البولى مكسين بي، 95% للامبسيلين ،70% للسيبروفلوكساسين وكانت جميع المعزولات حساسة بنسبة 100%اميكاسين، الجينتاميسين النالدكسك اسد والكانا ميسين، النيوميسين ، التتراسيكلين وبنسبة 90%للكلورمفنيكول.