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STUDIES ON THE VIRULENCE OF *AEROMONAS HYDROPHILA* IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

(With 3 Tables)

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دراسات على ضراوة ميكروب الإيروموناس هيدروفيلا في البلطي النيلي

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تم عزل ١٢ عزله لميكروب الايروموناس هيدروفيلا من أسماك البلطي المريضة من مزارع الأسماك بكفر الشيخ وقد تم دراسة ضراوتها في أسماك البلطي النيلي. في هذه الدراسة وُجد أن هناك خمسة عزلات من المعزولات الضارية تشارك في مقاومتها للتأثير المميت لمصل الدم من أسماك البلطي . كما درست عدة عوامل أخرى مثل التلزن الذاتي في 0.2% من مادة الاكريفلافين وعدم الثبات بعد الغليان وكذلك انتاج طبقة S وانزيمات البروتياز والهيموليسين، وقد وجد أن هذه العوامل ليس لها علاقة بضراوة الميكروب. كما وأن التصنيف السيرولوجي أظهر أنه لا يمكن ضم جميع العزلات الضارية في مجموعة واحدة. من هذا نخلص الي ان المقاومة لمصل دم أسماك البلطي لها دلالة واضحة ويمكن اعتبارها مؤشر جيد لتصنيف ضراوة عزلات ميكروب الايروموناس هيدروفيلا.

SUMMARY

Twelve Aeromonas hydrophila isolates isolated from diseased fish in kafr El-Shiekh province were studied for their virulence in Nile Tilapia (*Oreochromis niloticus*). The most virulent five isolates used in this study shared a common resistance to the killing effect of Tilapia fish serum. Other factors such as lack of autoagglutiuation in 0.2% acriflavine, instability after boiling, production of an S-layer, proteases and haemolysins did not correlate well with virulence. In addition, serotyping could not group all the virulent isolates. On the other hand,

serum resistance proved to be a good indicator for screening virulence of *Aeromonas hydrophila* isolates.

Key Words: *Tilapia, Aeromonas, Hydrophlia.*

INTRODUCTION

Aeromonas hydrophila, an opportunistic pothogen associated with Motile Aeromonas Septicaemia, is an ubiquitous and heterogeneous organism which produce disease in fish under stress conditions or in concert with infection by other pathogens. *Aeromonas hydrophila* is frequently associated with diseases in fresh water fish such as Tilapia (Ahmed 1983 and Amin et al 1985). In warm water aquaculture, *A. hydrophila* is considered to be a major economic problem, but it is difficult to distinguish direct losses from those caused by secondary infections (Ruangapan et al 1986 and Austin and Austin 1987). Environmental stress factors such as overcrowding, low concentration of dissolved oxygen, high organic content in the water, industrial pollution, abrupt temperature changes, and physical injuries may also contribute to infections by *A. hydrophila* (Pippy and Hare 1969 and Shotts et al 1972).

The pathogenicity of *A. hydrophila* may be attributed to a variety of virulence factors such as [haemolysin (Kinya and Takagi, 1986), necrotizing toxin (Lallier et al., 1984), leucocidin (Caseliz, 1966) and cytotoxin (Lobountzov and Roudikov, 1968)] work in concert to contribute the overall virulence of this bacterium. Extracellular products (ECPs) including toxins of haemolysins, proteases and acetyl choline estrase (Ljungh and Wadstrom 1982; Leung and Stevenson 1988_a and Nieto et al 1991) appear to contribute to the establishment of *A. hydrophila* infection in fish. Other virulent determinants such as the presence of the S- layer (Dooley and Trust, 1988) and serum resistance (Mittal et al 1980; Janda et al 1984; Leung and Stevenson 1988_b) are also implicated in helping the bacteria to resist the attack by the host's non specific immunity such as serum and macrophage killing (Ingram, 1980) .

In this work, 12 *A. hydrophila* isolates were isolated from diseased *O.niloticus* fish from Kafr El-Shiekh farms. Several virulence

factors were used to correlate with the LD_{50} values for the isolates. Moreover, serum resistance for screening the virulence of *A. hydrophila* isolates were evaluated.

MATERIALS and METHODS

Bacterial isolates and media:

All the 12 isolates were isolated from fish and confirmed to A.hydrophila by using a standard biochemical diagnostic kit (Microbact 24 E System, Adelaide, South Australia) and their identitis were further confirmed according to the criteria used by Enany (1983) and Popoff (1984). Bacteria were grown on Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) at 25°c. Stock cultures were maintained at -70°c in TSB containing 15% glycerol.

For protease assay, cultures were grown at 25°c on brain heart infusion agar (BHIA) according to Allan and Stevenson (1981). Haemolysins were detected using 5% (v/v) heparinized Tilapia red blood cells in TSA base medium according to Branden and Janda (1987). The extent of haemolysin and protease production was estimated by the diameter of the clear zone after 24 and 48 h.

For detection of the S-layer, *A. hydrophila* isolates were spotted on TSA medium supplemented with 30 and 150 *ug/ml* Congo Red. Red colonies were obtained by the bacteria produced an S-layer while those does not produced S-layer gave white colonies according to Ishiguro et al (1985).

Buffers:

Bacterial cultures were washed and re-suspended in phosphate buffered saline (PBS) containing 123.2 mM Na Cl, 10.4 mM Na_2HPo_4 and 3.2 mM $kH_2 P0_4$. pH was adjusted at 7.2, according to Hetrick (1983).

Antiserum preparation and bacterial agglutination:

Preparation of formalin-killed whole cell antigens was done according to song et al (1976). Bacteria grown on TSB, washed with PBS several times and killed with 0.4% formalin PBS solution. Antisera

was prepared in O.niloticus by injection of fish intraperintoneally twice at 0 and 4 weeks with 0.1ml PBS containing $1x10^7$ cells and the fish were bled 2 weeks after the last injection. Agglutination titre was performed according to the method of Roberson (1990).

Surface characteristics:

Agglutination in acriflavine (0.2%) was done on glass slide using colonies of 18h on TSA culture. For precipitation after boiling, *A. hydrophila* were grown on brain heart infusion broth (BHI) for 18 h at 25°c. Stability of the cells was verified after boiling for 1h. (Mittal et.al., 1980).

Fish:

Nile Tilapia (*Oreochromis niloticus*) weighing approximately 20 ±5g obtained from a fish farm and maintained in well areated water aquaria 100 L capcity at 25 - 28°c for 7 days prior to the experiment. The fish were fed on commercial pellets at 5% wet body weight/day supplied from El-Zawia fish farm at Kafr El-Shiekh.

The LD_{50} values were calculated according to the method of Reed and Munech (1938)

Survival assay in o.niloticus serum:

Blood was collected from the caudal vein of fish, then the serum was separated. Bacteria grown on TSB at 25°c for 24 h were collected by centrifugation (at 3000 r.p.m for 5 min.) and washed three times with PBS. The bacterial suspension was mixed with the serum to give a final serum concentration of 50%. Bacterial number was adjusted to about $5x10^7$ cells/ml (Macfrarland No.5). The tubes were incubated at 25°c. Samples (0.1 ml.) were taken at 1,3 and 5 h for serial dilutions and plate counts on TSA. As a control, serum was inactivated by heating at 47°c for 30 minutes (Sakai 1981).

To evaluate the killing effect of the fresh serum of fish, the viability of *A. hydrophila* cultures was routinely measured before and after the 2h incubation period. A ratio was calculated to represent the survival ability of *A. hydrophila* in fish serum. The values were the average of duplicate samples.

RESULTS

Characterization of 12 isolates of *A. hydrophila* isolated from diseased fish, were based on virulence factors such as surface characteristics, production of proteases, haemolysins, S-layer, serum sensitivity and LD_{50} values in fish were Summerized in table (1) .

Out of 12, eight isolates showed identical biochemical patterns (isolates No. 1,6,7,8,9,10,11and 12). However, other phenotypic characters could be used to distinguish these isolates from one another. Isolates No.1 and 6 were different in respect to their serum sensitivity, LD_{50} values and stability after boiling. Isolate No.8 was similar to No.6 in serum sensitivity and production of protease and haemolysins. However, isolate no.8 had a higher LD_{50} value than isolate No.6 Isolates No.8,9,10,11 and 12 were different in serum sensitivity and production of an S-layer . Therefore, none of the 12 isolates were identical.

Determination of the virulence of *A.hydrophila* was carried out on Nile Tilapia (*Oreochromis niloticus*). Five isolates out of the 12 were classified as virulent (No. 1,2,3,4 and 5). The LD_{50} values were 10^{6} , $10^{6.4}$, $10^{4.8}$, $10^{6.6}$ and $10^{5.2}$ respectivly . Isolates No.6 and 7 were classified as weakly virulent and their LD_{50} values were $10^{7.2}$ and $10^{7.4}$. The remainder of isolates (No. 8,9,10,11 and 12) were considered as avirulent isolates and their LD_{50} values were $> 10^{8}$, $> 10^{8}$, $>10^{7.7}$, $>10^{8}$ and $> 10^{8}$ respectively.

All the virulent isolates were serum resistant. Three out of five of the virulent isolates were precipitated after boiling and two out of five did not agglutinate in acriflavine. All the virulent isolates possessed S-layer and produced a large amounts of proteases and haemolysins (table 2). However, the production of S-layer, proteases and haemolysins also occurred at a high frequency (40 %) in the avirulent group.

Serotyping was also used to group the 12 isolates of *A. hydrophila*. The antibodies were raised against the formalin-killed whole cells of four virulent strains (No. 1,2,3 and 4), the weakly virulent isolate (No. 6) and one avirulent isolate (No. 8). The antibody titers were displayed in table (3).

DISCUSSION

Serum resistance has been suggested to be an important factor for virulence in *A. hydrophila* (Mittal et.al 1980, Janda et at 1984, Leung and Stevenson 1988_b). The ability of *A. hydrophila* to survive and grow in host serum would play a major role in systemic infection.

The results displayed in table (1) showed that all five virulent isolates were resistant to the bactericidal effect of serum.

On the other hand, one to two logs of the population of the weakly virulent and avirulent isolates were killed by the serum after 2 h. All isolates, bactericidal effect of serum was almost inhibited after inactivation the serum pre-heated at 47°c for 30 min. Since the serum-killing factor was proved to be heat-labile, it was believed that this factor could be linked with complement as suggested by Sakai (1981).

A relation between cell surface characteristics and virulence was reported by Mittal et al (1980); who suggested that the virulence of *A. hydrophila* isolated from fish could be easily assessed by the acriflavin test (cells do not agglutinate in 0.2% acriflavine) followed by testing their instability after boiling.

In this study, all the virulent isolates were serum resistant (table2). Only 60% of the virulent isolates (three out of five) precipitated after boiling and 40% (two out of five) were negative in agglutination in acriflavine. Similar results were obtained by Song et.al (1976).

All the virulent isolates possessed S-layers and produced large amount of protease and haemolysins. This agree with that reported by Branden and Janda (1987) for different Aeromonas spp. However, the production of S-layer, proteases and haemolysins also occured at a high frequency (40 %) in case of avirulent group (table 2). This suggests that the surface characteristics, the presence of S-layer, and production of proteases and haemolysins are not reliable indicators for determination of the virulence of *A. hydrophila.*

The results in this work, showed that all the virulent isolates (five out of five, 100%) were serum resistant (table 2) and only 20% of the a virulent isolates (one out of five, 20%) were serum resistant. The serum

resistance property found to be the most reliable factor in distinguishing virulent and avirulent isolates of *A. hydrophila.* This agree with rhe results of Ruangapan et al (1986) and Roberson (1990).

In conclusion, a correlation between serum resistance and virulence was proved, therefore, this work propose the use of serum for screening virulence of *A. hydrophila* in fish diagnostic laboratories may be useful.

Moreovere, further works by using large numbers form A.hydrophila isolats, from different sources are baddly needed to screening the virulence.

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Table (1) : Characterization of 12 isolates of *A . hydrophila* .

	Isolate No.	PPT after boiling	PPT in acriflavine	Protease production	Haemolysin Production	S-layer production	Serum * surviral ability	L D_{50} in Tilapia
Avirulent isolate weakly virulent virulent isolates	1	+	+	+++	++	+	1.5 ± 0.10	10^{6}
	2	-	+	+++	++	+	2.2 ± 0.1	$10^{6.4}$
	3	+	+	+++	++	+	2.9 ± 0.2	$10^{4.8}$
	4	+	-	+++	++	+	2.8 ± 0.2	$10^{6.6}$
	5	-	-	+	++	+	4.7 ± 0.2	$10^{5.2}$
	6	-	-	+++	++	-	0.037 ± 0.001	$10^{7.4}$
	7	-	+	+++	++	+	0.0095± 0.0005	$10^{7.2}$
	8	-	-	+++	++	-	0.0062 ± 0.0001	$> 10^{8}$
	9	-	+	+++	++	-	0.000017 ± 0.000001	$>10^{8}$
	10	-	+	++	+	-	2.2 ± 0.1	$> 10^{7.7}$
	11	-	-	++	+	+	0.0008± 0.0002	$> 10^{8}$
	12	-	+	+	-	+	0.0088 ± 0.0004	$> 10^{8}$

* Serum survival ability was calculated by dividing the viable bacterial population after the serum treatment by the initial population before serum treatment.The survival values are calculated as means ± standard deviation of duplicate samples. A value greater than one indicates serum resistance, while a value less than one indicates serum sensitivity .

Table (2) : Characterization of virulent, weakly virulent and avirulant isolates of *A .hydrophila* .

Test	Virulent isolates		Weakly virulent		Avirulent isolates	
	No.	%	No.	%	No.	%
1 - No agglutination in acriflavine (0.2%)	2/5	40	1/2	50	3/5	60
2 - Instability after boiling	3/5	60	0/2	0	0/5	0
3 - Serum resistance	5/5	100	0/2	0	1/5	20
4 - Prescnce of S-layer	5/5	100	1/2	50	2/5	40
5 - Production of proteases(+ + +)	4/5	80	2/2	100	2/5	40
6 - Production of haemolysins(+ +)	5/5	100	2/2	100	2/5	40

Table (3) : Bacterial agglutination using antiserum prepared against formalin - killed whole cells of *A . hydrophila* .

Antigen	Virulent isolates				Weakly virulent isolate NO. 6	Avirulent isolate No. 8
	No. 1	No. 2	No. 3	No. 4		
1	1/256	1/256	1/256	1/128	1/32	1/2
2	1/128	1/256	1/32	1/32	1/2	1/4
3	1/32	1/64	1/256	1/16	1/8	1/2
4	1/64	1/128	1/128	1/256	1/8	1/2
5	1/4	1/32	1/2	1/64	1/4	1/32
6	1/2	1/2	1/2	1/2	1/32	1/16
7	1/8	1/4	1/8	1/32	1/8	1/16
8	1/64	1/128	1/32	1/16	1/4	1/8
9	1/64	1/16	1/8	1/8	1/4	1/4
10	1/32	1/32	1/16	1/32	1/8	1/16
11	1/8	1/8	1/8	1/16	1/8	1/8
12	1/4	1/4	1/2	1/8	1/4	1/2

* No autoagglutination between bacterial isolates and slaine .