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VIBRIOSIS IN SOME FISHES: MOLECULAR CHARACTERIZATION OF FISH PATHOGENIC VIBRIOS

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SUMMARY

Studies were undertaken to isolate and characterize vibrio species pathogenic to fish. Thirty six vibrio- like isolates were obtained from twenty one of 160 fish samples. Biochemically the collected isolates were identified as *Listonella anguillarum* biotype B and C, *Vibrio alginolyticus* and unidentified lactose-fermenting vibrio spp. Eight vibrio strains of different origins were selected and were grown onto tryptose soya agar with 5% sheep blood for preparation of outer membrane proteins (OMPs). The OMP profiles of the tested strains were separated using sodium dodecyl- sulphate polyacrylamide gel electrophoresis (SDS- PAGE). Dice index of similarity was determined with each strain. Analysis of OMPs showed that the local vibrio strains had 8-9 protein bands of molecular weight ranging from 19.7- 212.9 KDa and they contained three major protein bands of molecular weight 19.7, 36 and 80 KDa. Moreover, SDS - PAGE analysis of the local vibrio strains indicated OMP expression differences in protein between strains defined mutant and within the same species strains belonging to different biogroups possessed distinct electrophoretic banding proteins. In conclusion SDS-PAGE analysis of OMPs may be considered as sensitive, reliable and rapid diagnostic method to characterize vibrio isolates.

INTRODUCTION

Species of vibrio are the most potential virulent halophillus organisms found in marine environments causing vibriosis in salt and fresh water fishes. *Vibrio anguillarum* (*V. anguillarum*) was the first recorded species as fish pathogen

(Colwell and Grimes, 1984). It causes a terminal hemorrhagic septicemia in fish including high mortalities and great economic losses within aquculture (Thune et al., 1993 and Austin and Austin, 1999). *V. alginolyticus* was recorded from farmed seabream with high mortalities (Colorni et al., 1981) and other fishes (Tsai et al., 1990) moreover, it is implicated in severe vibriosis of marine aquaculture system (George et al., 2005). The bacteria usually utilize their external membranes to protect them from the damaging agents while allowing the selective uptake of nutrients (Achouak et al., 2001). The outer membrane of many gram negative organisms is the site of virulence factors including fimbrae, lipopolysaccharides and outer membrane proteins (Peterson and Quie, 1981). It consists predominately of phospholipids and proteins, either integral membrane proteins or lipoproteins, that constitutes about 50% of its mass (Koebnik et al., 2000). The outer membrane genes involved in heme uptake has been identified in *V. vulnificus* (Litwin and Byrne, 1998) and *V. anguillarum* (Mourino et al., 2004). The OMP profiles of 10 serotypes of V. anguillarum are antigenically similar to each other, although varying minor proteins, but had a major OMP, and for serotype 01 and 02 they also antigenically similar to the major OMP in other vibrio species (Simon et al., 1996 ; Suzuki et al., 1996 and Wang et al., 2002). Moreover, a new major OMP produced from different vibrio species by exposure to high concentration of bile salts (Aeckerberg et al., 2001 and Wang et al., 2003). The present work was planned to study the prevalence of vibriosis among the examined fishes; biochemical identification of the obtained isolates and molecular characterization of the isolated local vibrio strains based on OMP antigens profiles using SDA- PAGE and to find the relationship between the cell protein fingerprints and the biotypes of the recovered organisms.

MATERIAL AND METHODS

- Naturally infected fish:

A total of 160 clinically diseased fishes including 80 *Oreochromis niloticus* weighted 70 ± 5 g; 40 *Clarias gariepinus* weighted 200 ± 10g and 40 *Mugil cephalus* weighted 160 ± 10 g. These fishes were collected from their natural sources (private fish farms and El-Raih El-Tawfiki and its tributaries) during the period from April 2004- March 2005. The fishes were transported alive or freshly dead according to Brown and Gratzek (1980) and sent to the wet lab. at Fac. of Vet. Med. (Moshtohor). The collected fishes were examined clinically as described by Amlacker et al., (1970). Signs and lesions that observed were also recorded.

- Experimental fish:

Apparently healthy 15 *O. niloticus* weighted 50 ± 5 g obtained from a private farm were transported to the wet lab under all accurate method of transportation where they kept in well prepared aquaria. Three random samples from *O. niloticus* were used for bacteriological examination to ensure

their negativity to vibrio infection. The remaining fish were divided into 2 groups (6 fish for each) and placed in clean aquaria, each measured 1 x 0.4 x 0.5 m. supplied with dechlorinated tap water and sufficient areators. The fish received commercial pelleted food.

Bacterial isolation and identification :

Samples for bacteriological examination were taken from skin, liver, kidneys and intestine of sacrificed and dead fish and inoculated into thioglycolate semisolid agar and incubated at 37°C for 2-3 days. The growing cultures were examined by phase contrast microscope at 400 x at Animal Reproduction Research Institute- EL- Haram- Egypt. Culture characters on thioglycolate and thiosulfate citrate bile salts sucrose (TCBS) agar media were studied. Haemolytic activites on sheep blood agar were observed. The obtained isolates were identified by standard biochemical characterization testes as described by Baumann et al., (1984). For biotyping, the isolated strains were phenotypically identified using standard sets of biochemical tests as reported by Shewan and Vernon (1974) and Austin and Austin (1993).

Pathogencity assay:

- Bacterial inocula:

A selected strain of lactose-fermenting vibrio. was used. Thioglycolate broth culture was prepared containing approximately 10^8 CFU/ml using Macferland's opacity tubes.

Experimental design

This experiment was done to demonstrate the pathogenicity of the isolated lactose fermenting vibrio in *O. niloticus* because the obtained vibrio differ in some biochemical tests from the previously recorded fish pathogenic lactose fermenting vibrio (*V. vulnificus*). Each fish of the first group was inoculated via intraproteneal route with 0.1 ml of broth culture containing 10^8 CFU/ml. While those of the second group were inoculated intraprotenealy with 0.1 ml sterile broth per fish. Both the inoculated and control groups were kept under observation for 15 days post infection. Morbidity and mortality rates were recorded.

Molecular characterization :

- Bacterial strains:

A total of 8 strains of vibrios isolated from different fish species were examined. The selected isolates include 4 of *O. niloticus* origin (No. 2, 5, 7 and 8), 2 of *C. gariepinus* origin (No. 1 and 5) and 2 from *M. cephalus* (No. 3 and 4). All strains were stored at -70 °C in tryptic soya broth supplemented with 1% NaCl and 25% glycerol until their use (Tall et al., 2003).

- Preparation of outer membrane protein antigen (OMP):

The selected vibrio strains were grown on tryptic soya agar supplemented with 5% sheep blood and incubated overnight at 37°C and then suspended in 5 ml of brain heart infusion broth. Cells were

harvested by centrifugation at 8000 xg (at 4°C for 20 minute) and washed twice in phosphate buffered saline. The washed cells were suspended in 10 mL of 20 Tris buffer (1 M NaCl, 10 mM Tris hydrochloride, pH 7.4) and the cell suspension was sonicated for 60 seconds (Cidak- 1285, USA). The intact cells were removed by centrigugation at 6000 xg at 4°C for 30 minute. The membrane protein was shaken at room temperature for 30 minute. The preparation was centrifugated at 40000 xg for 30 minute at 4°C and the pellets washed three times in Tris buffer. Finally, the pellet (about 0.5 mL) was suspended in Tris buffer and maintained frozen at -70°C (Dubreuil et al., 1988).

Electrophoresis for outer membrane protein antigen :

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS- PAGE) of the OMP samples of eight vibrio isolates was performed as described by Laemmli (1970) using pre- stained high molecular weight standard marker (Blorad, USA). After electrophoresis, the gel was stained with silver stain and distained according to Hitchcock and Brown (1983). Dice index of similarity was determined with each isolate (Gostavo and Peter, 1998) and Dendrogram was then constructed (Advanced American Biotechnology, UPGMA ,USA).

RESULTS AND DISCUSSTION

Incidence of affection

Twenty-one of clinically diseased fishes were positive for vibrio isolation. The infected fish showing signs of skin ulcers, eroded fins, haemorrhages at the base of the fins ; diffuse haemorrhage over the body surface and around the anal opening (Fig. 1). Internally there were, muscle haemorrhages, congestion of internal organs, some had pale coloration of liver and enteritis while, others had necrosis in liver and kidney. These observations were nearly similar with those of El-Bouhy et al. (1990), Schäperclaus et al. (1992), Austin and Austin (1993), Actis et al. (1999) and Abbass et al. (2000).

As presented in table (1) the incidence of affection among the examined fish was 13.13%. The organism mainly recorded during autumn and spring. The higher incidence was observed for M. cephalus. This finding was higher than that recorded by Abbass et al. (2000) and lower than that reported by Abdel- Gabar et al. (1997). This observation may be attributed to species difference and seasonal variation.

Bacterial isolation and identification

A total of 36 suspected vibrio isolates were obtained on thioglycolate media incubated at 37°C for 2-3 days and some of them showing swarming

over the surface of agar plates. On TCBS agar plates characteristic colonies were observed and their color was yellow or green according to the species of vibrio. All the collected isolates produced haemolysis on 5% sheep blood agar supplemented with 1.5% NaCl. As shown in table (2) all the collected isolates had typical biochemical characteristics of vibrios and typable *L. anguillarum*; *V. alginolyticus* and lactose-fermenting vibrio species. These results were similar with those recorded by Baumann et al. (1984) and Austin and Austin (1993). Swarming of the *V. alginotyticus* on solid media is attributed to presence of lateral flagellae (Baumann et al., 1984). Biotypes of *L. anguillarum* recorded among the obtained isolates were B and C based on their reaction toward indole reaction and production of acid from mannitol and sucrose. These findings came in accordance with that recorded by Shaaban et al. (1995) and Abbass et al. (2000). Moreover, *V. alginolyticus* recorded in the present study from *M. cephalus, O. niloticus* and *C. gariepinus*. This finding was partially in agreement with those of Burke and Rodgers (1981) who isolated *V. alginolyticus* from *M. cephalus*. In the same respect George et al. (2005) isolated *V. alginolyticus* from farm-reared and wild shrimps. Of further interst was the finding that two strains are sucrose negative this observation was supported by Farmer et al. (1985) who found 1% *V. parahaemolyticus* and 10-15% of *V. vulnificus* were sucrose positive. While, the lactose-fermenting vibrios isolated from *O. niloticus* in this work nearly similar with those observed by Tison et al., (1982) and Davidson and Oliver (1986) who isolated unidentified lactose-fermenting vibrio species from marine fish but, the obtained isolates some what differ in biochemical characters from *V. vulnificus* biogroup 2 that causing septicemia in fish described by Austin and Austin (1993).

Table (1): Prevalence of vibrio infection among the examined fish species and the number of isolated strains

Fish species	No. of examined fish	No of infected fish	% of infection	Number of isolated strains
O. niloticus	80	10	12.5	16
Cl. gariepinus	40	5	12.5	11
M. cephalus	40	6	15	9
Total	160	21	13.13	36

Table (2): Differential characteristics of locally isolated vibrio species.

Characteristics	*L. anguilarum*	*V. alginotyticus*	Lactose-ferminting Vibrio species
No. of strain tested	22	10	4
Swarming on media	-	+	-
Catalase	+	+	+
Cytochrome oxidase	+	+	+
Citrate	+	+	-
Methyle red	-	+	+
Voges proskauer	±	+	+
H_2S (TSI)	- **	+	+
Indole	±	+	-
Sucrose	±	+	-
D- Mannitol	±	+	-
Growth in 1% glycine	-	±	-
Acid from:			
Glycerol	+	+	±
Glucose	+	+	+
Lactose	-	-	+
Maltose	+	+	+
Growth in % NaCl			
3.5	+	+	+
6	- **	+	
8.5	-	±	-
Gas from glucose	-	-	-
Growth at :			
25 °C	+	+	+
37 °C	+	+	+
40°C	-	+	-
43°C	-	-	-
Color of colonies on TCBS	Yellow/green	Yellow *	Green
Haemolysis on blood agar	+	+	+

* two strain has green colonies on TCBS agar.
** one strain was positive.

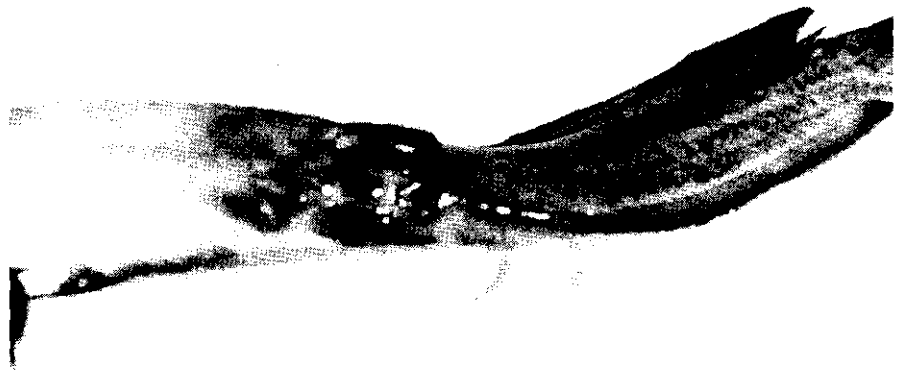

Fig. (1): *C. gariepinus* naturally infected with *V. anguillarum* showing severe haemorrhages in the skin, fins and around the anal opening.

Pathogencity Test:

In the present study pathogenicity assay demonstrated that the tested strain of lactose fermenting vibrio was virulent for the inoculated fish. All inoculated fish showing signs of vibriosis as haemorrhages all every body minute ulcer over the skin and detached scales. The morbidity rate among them was 100%. Moreover, the mortality rate reached 66.66%. These observations were nearly similar with those observed by Austin and Austin (1993) who reported that *V. vulnificus* (Lactose fermenting vibrios) infection in eel induced haemorrhagic condition characterized by redness on the body and the mortality rate was 80% in experimentally infected fish.

Molecular characterization

The selected vibrio strains subjected to molecular diagnosis were classified according to biochemical identification into 3 strains (No1, 4 and 7) as *V. alginolyticus*, 4 strains as *L. anguillarum* biotype C (No 2, 3) and biotype B (No 5 and 6) and unidentified lactose -fermenting vibrio (No 8).

As shown in Fig. (2) and table (3) the SDS-PAGE pattern of the outer membrane protein had several protein bands of different molecular weights ranging from 212.9-19.7 KDa. However, SDS- PAGE analysis revealed that the vibrios OMP complexes are composed of 8-9 protein bands showing major protein bands with molecular weight of 80 (8 strains); 19.7 (6 strains); 36 (5 strains); 66 and 106 (4 strains) and 102, 111.2 and 114.1 KDa (3 strains) and several minor protein bands were observed. The OMP profiles of the local vibrio strains showed three major protein bands of molecular weight 19.7, 36 and 80 KDa were detected in the majority of the strains. In the same respect, Simon et al. (1996) found that the OMP profiles of 10 serotypes of *V. anguillarum*, although varying the minor proteins, contained a major OMP of 35 - 42 KDa. Moreover, Tall et al.

(2003) revealed that SDS-PAGE analysis of *V. fluvialis* OMP complexes possessed more than eight protein with four major protein bands of molecular weight of 43, 30, 20 and 14 KDa while, OMP complexes of V. tubliashie contain a single major protein band of 40 KDa and OMP complexes isolated from *V. vulnificus* possessed two major protein bands of 35 and 40 KDa. The differences in the minor bands among the examined strains of the same vibrio species may be used in differentiation between them. Therefore the difference in minor bands can help in preparing diagnostic kits to differentiate between different strains. In the present study, OMPs pattern of local strains showed a major antigenic protein bands of molecular weight 80 KDa. This result

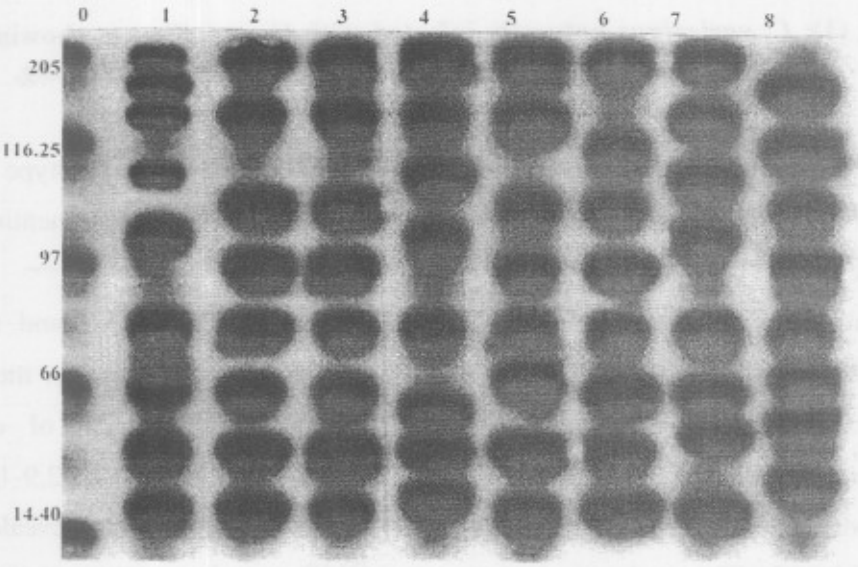

Fig. (2) : SDS- PAGE profiles of OMPs isolated from fish pathogenic vibrios. Lanes 1-8 represent tested strains and lane O is the protein marker.

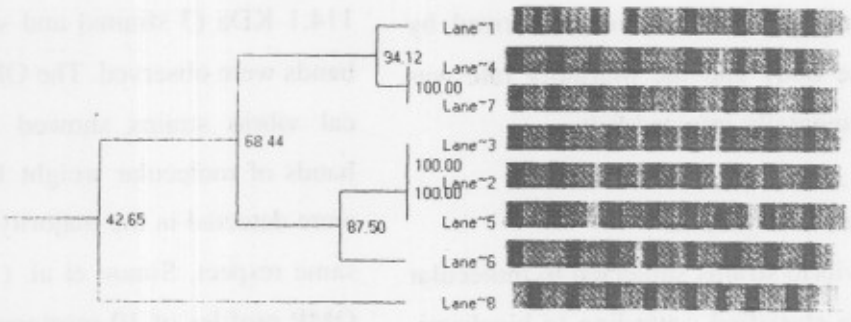

Fig (3) : Electrophoretic migration patterns and similarity dendrograms of vibrio strains isolated from fish. Lanes 1-8 represent the tested strain.

Table (3): Comparison between number and molecular weight of relative bands produced by SDS- PAGE analysis of OMP of the local vibrio strains.

Molecular/ weight (KDa)	Marker 0	Local vibrio strains								
		1	2	3	4	5	6	7	8	No. of reactive bands
212.9					+					1
210.9							+			1
209.9								+		1
206.9			+			+				2
205.9		+		+						1
205	+									1
172.1									+	1
169.7		+								1
145.9								+		1
144.5						+				1
141.1		+	+		+					3
140.4				+						1
120.2									+	1
117.4							+			1
116.3	+									
111.2		+			+			+		3
107			+	+						1
106						+	+		+	4
102		+			+			+		3
97.9									+	1
97.2						+	+			2
97.1			+	+						2
97	+									
80		+	+	+	+	+	+	+	+	8
70.1						+				1
69.2									+	1
66	+		+	+			+	+		4
62.1		+								1
57.5					+					1
48.9									+	1
42.3								+		1
37.8						+				1
36		+	+	+	+		+			5
26.4									+	1
23.1					+					1
19.7		+	+	+		+	+	+		6
14.4	+									
Total	5	9	8	8	8	8	8	8	8	65

was supported by Biosca et al. (1993), who found a major protein band (36 KDa) in the majority of *V. vulnificus* biotype 1 and 2 also, observed that immunoblotting with antisera to whole cells of selected strains of biotype 1 and 2 showed strong antigenic response to outer membrane proteins 66, 60, 48, 46 and 44 KDa these were common to all strain examined. In addition, Davey et al. (1998) and Wang et al. (2002) demonstrated antigenically related outer membrane proteins among vibrio species.

Regarding the species, the present study revealed that, the OMP profiles of *L. anguillarum* possessed major OMP bands of molecular weight 19.7 and 80 KDa while, *V. alginolyticus* contained major OMP bands of molecular weight 80, 102 and 111.2 KDa. These findings were supported by Biosca et al. (1993), Biswas and Chakrabarti (1994) and Wang et al. (2003) they revealed that the major OMP for *V. vulnificua* biotype 2 was 36 KDa, for *V. parahaemolyticus* was 55 KDa and for *V. anguillarum* was 38 KDa respectively. The difference in major OMPs band may be attributed to the difference in culture condition and vibrio species (Davey et al., 1998).

The great difference on the size of the genome among different strains attributed to accumulation of multiple fragments of similar size in the same position in the gel that are intensively stained and to the protocol of electrophoretic conditions hampering the comparison of data at various laboratories (Farber, 1996 and Wassenaar and Newell, 2000). Moreover as presented in Fig. (3), cluster analysis of the OMP pattern of the identified vibrio strains on the basis of similarity coefficient values and average linkage method, they were grouped into five types this finding agreed with Saitou and Nei (1987) and Tall et al., (2003). In conclusion, different vibrio species had been isolated from diseased fish. Each vibrio strain had a distinct protein profile which can be used to differentiate between them and SDS-PAGE analysis of OMP may be considered as sensitive, reliable and rapid diagnostic method to characterize vibrio isolates.

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