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EVALUATION OF VIRULENCE FACTORS IN ENVIRONMENTAL ISOLATES OF *VIBRIO* SPECIES

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Strains of Vibrio parahaemolyticus, Vibrio fluvialis *and* Vibrio mimicus *isolated from seafood and seawater were examined for characteristics related to infectivity, such as enzymatic activity and animal assays. All strains hydrolysed DNA, starch, gelatin and chitin. Variable results were obtained with the haemolysin, chondroitin, collagen, elastin and lecithin tests. Production of thermostable direct haemolysin by* V. parahaemolyticus *was detected in 7.1% strains derived from seafood and 2% from seawater. In the animal assays, strains of* V. fluvialis *showed positive results at skin PF (75%), mouse lethality (100%), but no fluid accumulation in the suckling mice model was noted. Concerning* V. mimicus, *results showed skin PF (100%), mouse lethality (100%) and fluid accumulation in suckling mice (66.6%).*

Key words: *Vibrio* – virulence factors – environmental vibrios – Kanagawa phenomenon

The epidemiological, ecological and taxonomic studies in the last 20 years, about the genus *Vibrio* from marine environment had shown twelve species as etiologic agents of a wide range of diarrhoeal and systemic diseases in man (Morris & Blake, 1985). These infections can be acquired by direct contact with natural aquatic environment or by consumption of seafood, where these microorganisms are widely distributed. Immunocompromised hosts or with pre existing liver or blood disorders are more suscetible (Janda et al., 1988).

Studies about this pathogenicity shows that microorganisms can produce several kinds of toxins including cell elongation factor (CEF), cell killing factor (CKF), permeability factor (PF), hemolysin and other enzymes, adhesins and a mouse lethal factor (Morris & Blake, 1985; Iwanaga & Ichinose, 1991). However, none of these factors has definitively been correlated with the pathogenicity.

The present study aimed to test the potential pathogenicity of *V. parahaemolyticus, V. fluvialis* and *V. mimicus* isolated from seafood and seawater from beaches in the city of Rio de Janeiro. Five kinds of tests were selected from the wide range available, namely enzymes assays, Kanagawa test to *V. parahaemolyticus,* mouse lethality test, permeability factor in guinea pig and intestinal fluid accumulation in suckling mice.

MATERIALS AND METHODS

Bacterial strains and growth conditions – Seventy eigth strains were used in this study. The species and sources are listed in Table I. All strains were kept in Buffered Nutrient Agar (Hofer, 1974).

Enzymes assays – The strains were grown on 5 ml of Brain Heart Infusion Broth (Difco) supplemented with 1 g% NaCl for 18 hr at 37 °C. The presence of amylase, chitinase, gelatinase, elastase, collagenase, chondroitinase, lecithinase, DNase and hemolysin were analysed by the methods described by West & Colwell (1984), Thorpe & Muller (1981) and Furniss et al. (1979). Strains of *V. parahaemolyticus* were also assayed to detect a beta hemolysis of human erythrocytes on Wagatsuma's Agar, a reaction known as the Kanagawa phenomenon (Furniss et al., 1979).

Mouse lethality test – Strains of *V. fluvialis* were grown for 24 hr at 37 °C (with shaking – 180 strokes/min) in Brain Heart Infusion

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TABLE I

Source of the bacterial strains

Species	Water	Fish	Crustaceans	Mollusks	Total
V. parahaemolyticus	49	14	7	–	70
V. fluvialis	4	–	–	1	5
V. mimicus	3	–	–	–	3
Total	56	14	7	1	78

Broth and 0,1 ml of culture were injected intraperitoneally into five mice weighting 25-34 g (Bowdre et al., 1981). An aerated culture (180 strokes/min. – 30 °C – 18 to 24 hr) in CAYE Medium (Spira & Cray, 1983) was used for the *V. mimicus,* and after centrifugation at 6000 g – 4 °C – 40 min, 1 ml of supernatant was injected subcutaneously into five mice weighting 25-34 g (Spira & Cray, 1983). In both cases the mortality was observed within 24 hr.

Growth conditions to skin PF and fluid accumulation assays – The strains of *V. fluvialis* and *V. mimicus* were grown in Brain Heart Infusion Broth with shaking (100 strokes/min. – 24 hr – 37 °C). After centrifugation (10000g – 40 min – 4 °C) the culture supernatant was filtered through a Millipore membrane (0.45 μm).

Permeability factor – According the recomendation by Oashi et al. (1972), three guinea pigs weighting 300 g of both sex were used. Filtered supernatant culture (0.1 ml) was injected intradermally, and twenty three hours later, the animals were injected intravenously with a solution (4% in 0.15 M NaCl) of Evans Blue dye at concentration 6 mg/100 g of body weight. After 1 hr, the animals were sacrificed by bleeding, the skin removed and the diameters of lesions measured. When the diameters exceeded 10 mm the reaction was taken as positive.

Fluid accumulation assay – Five suckling mice per sample were used in accordance to the method described by Takeda (1979). Mice were inoculated intragastrically by catheter. After 4 hr, the toxin activity was determined by a ratio between the weight of guts and the weight of remaining body. When the ratio exceeded 0.09 it was taken as positive.

RESULTS

All strains hydrolysed DNA, gelatin, chitin and starch. Variation in the enzymatic activities in each species is observed in Table II. The enzymatic profile of *V. parahaemolyticus* shows similar features among strains from seawater and seafood. The Kanagawa pheno-

TABLE II

Results of biological activity of *Vibrio* strains

Enzymatic profile					*V. parahaemolyticus*		*V. fluvialis*		*V. mimicus*
CL	CN	EL	HE	LE[a]	Seawater n = 49	Seafood n = 21	Seawater n = 4	Seafood n = 1	Seawater n = 3
+	+	–	–	+	24 (48,9)[b]	14 (66,6)	–	–	–
+	+	–	–	–	13 (26,5)	1 (4,7)	–	–	1 (33,3)
+	+	+	–	+	8 (16,3)	3 (14,3)	1 (25,0)	–	–
+	+	+	–	–	2 (4,4)	–	–	–	–
+	+	+	+	+	1 (2,0)	2 (9,5)	–	–	–
+	+	–	+	+	1 (2,0)	1 (4,7)	–	–	–
–	+	–	+	+	–	–	–	1 (100,0)	–
–	+	+	–	+	–	–	2 (50,0)	–	–
–	–	–	–	+	–	–	1 (25,0)	–	–
–	+	+	+	–	–	–	–	–	2 (66,6)

a: CL – collagenase; CN – chondroitinase; EL – elastase; HE – hemolysin; LE – lecithinase.
b: number of strains positive (% of strains positive).

men was observed in five strains (7.1%) from seafood (three from crustaceans and two from fish) and one (2%) from seawater.

The animal assays presented at Table III reveal that all seawater strains of *V. fluvialis* were lethal for adult mice and 75% were positive in the permeability factor in guinea pig. None of the assays determined intestinal fluid accumulation in suckling mice. The seafood strain were negative for all animal assays.

TABLE III

Pathogenicity tests of *Vibrio fluvialis* and *V. mimicus*

Test	*V. fluvials*		*V. mimicus*
	Seawater	Seafood	Seawater
Skin PF	3/4[a]	0/1	3/3
Mouse lethality	4/4	0/1	3/3
Fluid accumulation	0/4	0/1	2/3

a: number of strains exhibiting the indicated character/ number of strains tested.

All *V. mimicus* strains presented positive results in skin PF, mouse lethality test and 66.6% determined intestinal fluid accumulation in suckling mice.

DISCUSSION

The occurrence of *Vibrio* sp. in the marine environment and marine products has been reported (Janda et al., 1988). Among all species, twelve have been associated with human diseases like gastroenteritis, wound or ear infection and septicemic syndromes, and seven have been described as pathogens of fish (Colwell, 1984).

The study of the pathogenic mechanisms has showed the presence of toxins and several components of enzymatic nature and compounds with cytolitic and proteolytic activities but little is known about specific pathogenic role. In this work, the enzymatic activities have been analysed and all the strains showed variable results (ten enzymatyc profiles – Table II).

Unique amongst the pathogenic vibrios is the correlation between pathogenicity of *V. parahaemolyticus* and beta hemolysis of human erythrocytes in a Wagatsuma Agar, known as Kanagawa phenomen. This microorganism in Japan, where seafood makes up a large part of the diet, is recognized as a major cause of diarrhoeal disease (Adiss et al., 1989). Ubiquitous in coastal waters, if present in low number in undercooked or contaminated seafood this organism can rapidly proliferate at temperatures of 25-44 °C (Aiso, 1967). In this study, the high percentage of strains with Kanagawa phenomenon (9.1%) similar to those detected by Sarkar et al. (1987) in environmental strains shows the relevance when compared with 1% described in the world literature (Hofer & Silva, 1984; West, 1989).

In the same way variable results are observed in *V. fluvialis* and *V. mimicus*. The former is associated with sporadic or outbreak diarrhea and *V. mimicus*, until recently considered to be biochemical variants of *V. cholerae*, have been isolated from a variety of clinical disorders associated with exposure to aquatic environment and gastroenteritis after consumption of raw seafood (West, 1989). Some studies have provide indirect evidence that enzymes and toxins may play important role in the pathogenesis of these diseases.

In the present study the *V. mimicus* strains examined showed toxic activity at the skin PF assay in guinea pig (100%), mouse lethality test (100%) and fluid accumulation in suckling mice (66.6%), strongly suggesting the potential to cause human gastroenteritis. This is in accordance with data obtained by Nishibuchi & Seidler (1983), Spira & Cray (1983) and Chowdhury et al. (1987) in the clinical and environmental strains.

Production of Shiga-like toxin is recognized in *V. fluvialis*, although the same toxin is also detected at 01 and non 01 *V. cholerae, V. parahaemolyticus* and *Aeromonas hydrophila*. The significance of different factors detected in these species remains obscure. We found that *V. fluvialis* strains isolated from marine environment expressed toxic activity at the skin PF assay in guinea pig and in mouse lethality test. It shows that the strains had virulence properties, but futher studies need to be carried out to elucidate this virulence role since this strains were not able to produce fluid accumulation in suckling mice, different from the results obtained by Nishibuchi & Seidler (1983).

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