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Analysis of some virulence factors of *Vibrio vulnificus* isolated from Rio de Janeiro, Brazil

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SUMMARY

Twenty strains of *V. vulnificus* isolated from the environment were investigated for characteristics related to their infectivity such as colonial morphology, enzymatic activity and animal assays. The presence of DNase, chitinase, amylase, lecithinase and gelatinase was observed in 100% of the strains, haemolytic activity was absent, and variable results were obtained in elastase, collagenase and chondroitinase. In the animal assays, 70% of the strains were lethal to adult mice, while 45% caused fluid accumulation in suckling mice. Although all strains had opaque colonies, only 3 of the 20 had the three enzymes elastase, collagenase and gelatinase, and only one of these was virulent in animal assays.

INTRODUCTION

Vibrio vulnificus is the most invasive species of the genus *Vibrio*, with three different clinical syndromes [1]. The first involves progressive infection with a few diarrhoeal symptoms and is characterized by a rapid outset of fulminating septicaemia followed by the appearance of cutaneous lesions with 50% mortality. Patients with hepatic dysfunctions or other syndromes involving iron metabolism appear to be most susceptible to this type of infection [2]. The second, represented by cellulitis, results from the direct contact of wounds or skin lesions with sea ecosystem constituents in both apparently healthy and debilitated patients, and sometimes progresses into septicaemia [1, 3]. The third form, seldom found, causes acute self-limited diarrhoea with a low mortality rate [4].

Studies on the pathogenicity of this microorganism demonstrate that, in strains isolated from clinical cases, there is a strict relationship between the presence of a polysaccharide capsule-like structure and virulence [5]. Other factors have also been reported including the presence of some siderophore-like proteins in the membrane [6], the production of enzymes that damage the surrounding tissues [7, 8], and various toxins acting like invasive factors in acute infections [1, 3]. An interesting point is that some factors were detected in environmental strains as well as in strains of human origin [9].

In this paper, biochemical characteristics as well as potential virulence factors have been investigated in strains of *V. vulnificus* isolated from sea water and oysters from the coast of the state of Rio de Janeiro.

MATERIALS AND METHODS

Samples

Two types of samples were used. The first (15 strains) was isolated from sea water from the coast of Rio de Janeiro. The second (5 strains) was from oysters from natural breeding areas found in the Sepetiba Bay (Rio de Janeiro State). The cultures were maintained at room temperature in buffered nutrient agar with 1% NaCl [10].

Biochemical characteristics

The biochemical tests were performed as previously described [11, 12]. Colonial morphology was examined on nutrient agar (Difco) and brain heart infusion agar (Difco), using the criteria of Simpson and co-workers [5].

Enzymatic assays

Published techniques [12–14] were used in the investigation of amylase, gelatinase, elastase, collagenase, chondroitinase, lecithinase, DNase and haemolysin.

Animal assay

Each strain was inoculated into five adult and five suckling mice. Adult animals, weighing approximately 20g each, were inoculated intraperitoneally with 0·1 ml of an 18 h culture which had been incubated at 37 °C (about 10^8 cells/ml) in BHI Broth (Merck), supplemented with additional 2·5% NaCl, and mortality observed until 18 h after inoculation [15].

Suckling mice (3–5 days old) received 0·1 ml of supernatant from an 18 h growth in heart infusion broth (Difco), obtained after centrifugation at 10000 g at 4 °C for 30 min, with 0·01 g% of Evans Blue. Mice were inoculated intragastrically by catheter. After 4 h the toxin activity was determined by a radio between the weight of guts and the remaining body [15].

RESULTS

The biochemical characteristics of *V. vulnificus* isolated from sea water and oysters were in accordance with those reported in the literature [12, 17].

When cultures were inoculated on to nutrient agar and BHI agar we observed either mixtures of opaque and translucent colonies, or opaque colonies into both media with an excellent contrast and sharp differentiation between the colony types (Table 1).

All 20 strains produced DNase, chitinase, amylase, lecithinase and gelatinase, but no haemolytic activity of sheep erythrocytes was seen. Variable results were obtained for elastase, collagenase and chondroitinase (Table 2).

The animal assays (Table 3) showed that 70% of the strains were lethal for adult mice, while 45% caused intestinal fluid accumulation in suckling mice.

Only one strain had enzyme profile D (with the production of collagenase, gelatinase and elastase), and was pathogenic to both groups of mice.

Table 1. *Colony morphology in relation to the culture media*

Culture media		
Nutrient agar	Heart infusion agar	Number of strains
O*	O	7
T–O	T–O	6
T–O	O	5
O	T–O	2
Total		20

* O, opaque colonies; T–O, opaque and translucent colonies.

Table 2. *Distribution of the strains according to enzymatic profile*

	Enzymatic profile			
Enzymes	A ($n = 7$)	B ($n = 7$)	C ($n = 3$)	D ($n = 3$)
Amylase	+	+	+	+
DNase	+	+	+	+
Gelatinase	+	+	+	+
Lecithinase	+	+	+	+
Chitinase	+	+	+	+
Elastase	−	+	−	+
Collagenase	−	−	−	+
Chondroitinase	−	−	+	−
Haemolysin	−	−	−	−

Table 3. *Correlation between enzymatic profile and tests for biological activity*

Enzymatic profile*	Percentage distribution of animal assays			
	AM + SM + †	AM + SM −	AM − SM +	AM − SM-
A/C	25	15	5	5
B	10	15	—	10
D	5	—	—	10
Total	40	30	5	25

* Enzymatic profile, Table 3.
† AM, lethal for adult mice; SM, fluid accumulation in suckling mice.

DISCUSSION

Ecological studies show that *V. vulnificus*, like all the other species of the genus *Vibrio*, is a normal constituent of the marine environment [18]. Its occurrence is determined by environmental factors such as salinity and temperature [8, 19]. It is found in high frequency in sea water and sea food, which contrasts with the small number of clinical cases reported worldwide. This indicates a large variation in the pattern of virulence among the strains; this is similar to *V. parahaemolyticus*, in which only 1% of the strains found in the aquatic environment is pathogenic for man [3].

Patients with pre-existing liver or blood disorders, or a history of alcohol abuse,

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are more susceptible to infection with *V. vulnificus*, which results in elevated serum iron levels; the resistance of *V. vulnificus* to killing by human serum is mediated by the presence of excess iron [2].

The evaluations carried out by West [1] indicated that strains of *V. vulnificus* are more resistant to lysis by complement than are *V. parahaemolyticus* and *V. cholerae*, and that during invasion it can activate both the classic and alternate pathways [2].

Another characteristic that confers resistance to the bactericidal effects of human serum is the polysaccharide capsule, recognized by Simpson and co-workers [5] in strains isolated from clinical and environmental material. In the laboratory, the presence of capsule is recognized by colonial morphology as capsulated strains form opaque colonies; translucent colonies result when this structure has been lost. This phenomenon was observed in all strains in both culture media; moreover the preservation of the strains for periods from 1 to 4 years in buffered nutrient agar did not alter this characteristic. This result is in contrast to that observed by Simpson and colleagues [5], who reported the transformation of opaque colonies into translucent ones in more than 60% of samples kept in maintenance medium.

The invasive capacity and resulting tissue damage suggest the role of toxins or components of enzymatic nature (e.g. collagenase, gelatinase, elastase), or the action of compounds with cytolytic and proteolytic activities. In our strains we found four enzymatic profiles with variation in elastase, collagenase and chondroitinase. The presence of elastase was detected in 50% of strains, collagenase in 15% and chondroitinase in 15%; in only 15% of strains were both collagenase and elastase detected.

However, there was an inverse relationship between pathogenicity for mice and production of elastase and collagenase, although every strain presented opaque colonies that suggested the presence of a polysaccharide capsule.

The mortality among our animals (70%) was greater than that reported by Kaysner and co-workers [8], of 60% mortality within 72 h, and less than that reported by Tilson and Kelly [9] in an analysis of 29 environmental strains, of which 25 were pathogenic (86%). This difference may be a reflection of the fact that their experiments were carried out immediately after isolation of the microorganism, and that observation of the animals was prolonged for 24 h instead of the 18 h period adopted by us.

Virulence has also been ascribed to the production of enterotoxin, types LT or ST, observed by the accumulation of intestinal fluid in suckling mice, usually detected in the O 1 and non-O 1 strains of *Vibrio cholerae*, *V. fluvialis* and *V. hollisae*. In *V. vulnificus* the activity of these substances depends on the presence of a bacterial envelope. We obtained extremely variable results when using this model (Table 3), but these are in agreement with those obtained by Bowdre and colleagues [15]. It may be that these findings are related to the existence of other toxic substances or enzymatic factors.

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