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# LABORATORY EVALUATION ON PATHOGENIC POTENTIALITIES OF *VIBRIO FURNISSII*

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*Sixteen strains of* Vibrio furnissii *recovered from 16 Brazilian patients with diarrhea were screened for virulence-associated factors. All strains were non-invasive, non-fimbriated, and did not produce either enterotoxins or cholera-like toxin. In contrast, most were hemolytic on blood agar and their broth-culture supernatants damaged HeLa cell monolayers. These cytolysins, as accepted for other enteropathogenic members of the family Vibrionaceae, might be determinants of pathogenicity in* V. furnissii*-mediated enteritis.*

Key words: *Vibrio furnissii* – *Vibrio* pathogenicity – diarrhea – cytolysin

*Vibrio furnissii,* formerly classified as the aerogenic bio-group of *V. fluvialis* (Lee et al., 1981), was recognized as a new species within the genus *Vibrio* in 1983 (Brenner et al., 1983). Unlike *V. fluvialis,* established as a genuine enteropathogen and linked to more than 500 cases of gastroenteritis in Bangladesh (Huq et al., 1980), *V. furnissii* has been regarded as a free living bacterium, ubiquitous in marine environments and animal stools (Lee et al., 1981). Despite this, *V. furnissii* has also been associated with human gastroenteritis (CDC, 1969; Magalhães et al., 1990) and implicated as a cause of fatal diarrhea in piglets (Nacescu et al., 1986).

Because the diarrheagenic role of *V. furnissii* remains ill defined (Janda et al., 1988; West, 1989; Kelly et al., 1991), we decided to investigate whether Brazilian clinical isolates of *V. furnissii* have virulence-associated factors that could make them potential enteropathogens.

## MATERIALS AND METHODS

*Bacterial strains* – We studied sixteen strains of *V. furnissii* recovered from 3250 human diarrheal stools screened for *Vibrio* at a clinical laboratory in Recife, Brazil, between 1990 and 1991 (Magalhães et al., 1992). All strains were confirmed biochemically as *V. furnissii* by standard procedures (Lee et al., 1981; Brenner et al., 1983; Baumann et al., 1984). Working cultures of each isolate were maintaincd at –70 °C in defibrinatcd rabbit blood and revived on sheep blood agar (SBA) when desired.

*Fimbriae* – Fimbrial appendages were searched with a transmission electron microscope. Bacterial cells were cultured on CFA agar (Evans et al., 1977) for 20 hr at 37 °C and suspended in phosphate buffered saline (PBS). One drop of the bacterial suspension was applied to carbon-coated collodium grid screens for 1 min. Grids were negatively stained with 2% (wt/vol) uranyl acetate for 2 min and examined in a Jeol 100 CX electron microscope.

*Hemagglutination* – The capability to agglutinate erythrocytes of human, sheep, rabbit, and mouse was verified by a rapid test (Evans et al., 1977).

*Plasmids* – Plasmid DNA extraction was done by the method of Birnboim & Doly (1979) and analyzed by electrophoresis of cell lysates through 0.7% agarose gels. Plasmids were subsequently stained with ethidium bromide and visualized with UV illumination.

*HeLa cell assays* – Adherence to HeLa cells was tested according to the one step 3-hr in-

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cubation assay (Cravioto et al., 1979). Briefly, HeLa cells were grown to 50% confluence "leopard spot fashion" on circular cover slips (Bellco Glass, Inc., Vineland, NJ.) in Eagle's minimal essential medium (MEM; Nissui Pharmaceutical Co., Tokyo) supplemented with 10 % (vol/vol) fetal calf serum (Sigma Chemical Co., St. Louis), 0.15 % (Wt/Vol) $Na_2$ $HCO_3$, and with gentamicin (10 μg/ml). Monolayers were washed with PBS; fresh medium with or without 0.5 % d-mannose was added. Duplicate coverslips in a 24-multiwell tissue culture plate were inoculated with 5 X $10^6$ colony forming units of the bacterial culture in the logarithimic growth. Plates were incubated for 3 hr at 37 °C in 5% $CO_2$. Coverslips were washed ten times with PBS, fixed with methanol, stained with 10% Giemsa solution for 30 min, and examined by light microscope. *Escherichia coli* O111:H2, an enteropathogenic strain exhibiting a localized pattern of adherence, was the positive control.

Strains that showed adherence were tested for entry into HeLa cells, using previously described methods (Vesikari et al., 1982; Small et al., 1987). To do this, we used the same system cell-bacteria as that used in the adherence assay, except that coverslips were put within 1 dram shell vials. After addition of the bacterial inoculum, the vials were centrifuged at 2,000 rpm for 10 min and incubated at 37 °C in 5% $CO_2$ for 90 min. Thereafter, the monolayers were washed with PBS and covered with MEM containing gentamicin (50 μg/ml) and lysozyme (300 μg/ml). After incubation for 3 hr, the monolayers were again washed to remove the gentamicin. The test was assessed as recommended before (Janda et al., 1991). As positive control, a recent clinical isolate of *Shigella flexneri,* carrying a functional plasmid of 120 MDa, was included.

*Toxin assays* – Bacterial strains were cultured in 10 ml of BHI in 100 ml Erlenmeyer flasks, with shaking at 37 °C for 18 hr. Cultures were centrifuged at 16.000 rpm for 20 min, the supernatant was removed, filtered, and stored at -70°C until tested.

To detect cytotoxin, twofold dilutions of the supernatants in MEM were incubated with monolayers of HeLa cells grown to subconfluence in 96 wells microtiter plates. After 21 hr, the monolayers were washed with PBS, fixed, Giemsa stained, and examined for signs of cytotoxicity or cell death.

Enterotoxin assay was carried out employing the suckling mouse model (Dean et al., 1972). An average ratio of the intestinal weight to remaining carcass weight of at least 0.09 was considered as a positive response.

The ability of *V. furnissii* to produce cholera-like toxin was investigated by using the Vet-RPLA latex agglutination test (Denka Seiken Co., Tokyo) as recommended by the manufacturers.

*Hemolytic activity* – Hemolysin production was evaluated streaking bacterial cultures on sheep blood agar. Plates were incubated at 37 °C for 24 hr and examined; strains producing a clear zone surrounding the colonies (beta-hemolysis) were considered positive. Extracellular hemolysin were estimated essentially as described by Wall et al. (1984).

## RESULTS

Cultures of *V. furnissii* neither displayed fimbriae nor were able to agglutinate erythrocytes. On the other hand, bacterial cells of all strains showed a long and single polar flagellum.

Two strains harbored plasmids. One carried a plasmid of 2.5 MDa and the other a plasmid of 34 MDa, but no virulence mark could be ascribed to them.

Of 16 strains tested, only 4 (25%) showed mannose-resistant adherence to HeLa cells. If compared with the enteroadherent *E. coli* control strain, *V. furnissii* presented a very smaller number of bacteria bound to the cell monolayer (Fig. 1). None of the adherent *V. furnissii* strains were able to invade HeLa cells.

Except the supernatant of the strain H-155, which was non-toxigenic and non-hemolytic, all the other filtrates of broth cultures were toxic for HeLa cells. Concerning the magnitude of cytotoxicity, we observed a large variation among the various strains examined. The grade of toxicity was independent of the titer of hemolysin (Table). After coincubation, monolayers were detached, and the reminiscent cells became compact with a very poor stained or acidophilic cytoplasm and a dark or granular nucleus; usually, only the nucleus were distinguished (Fig. 2).

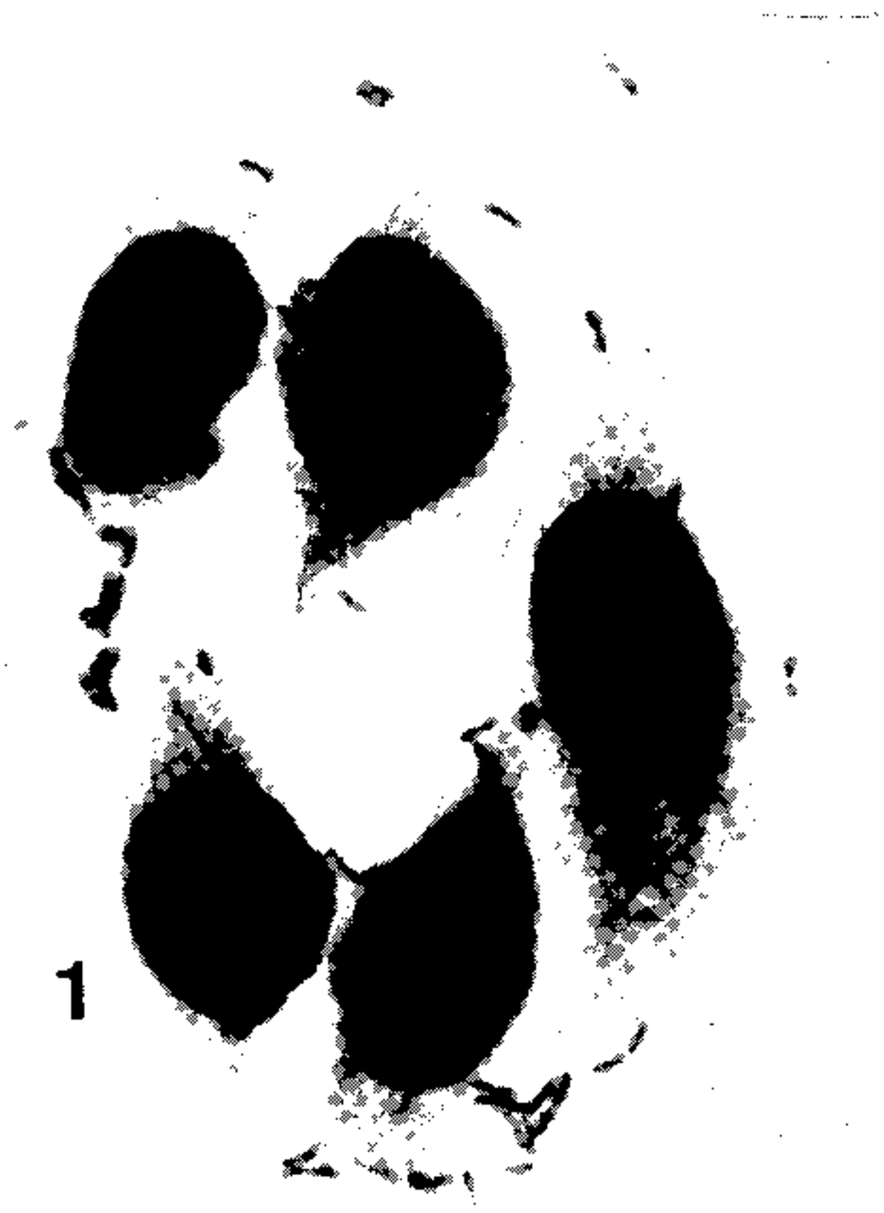


Fig. 1: HeLa cells infected with *Vibrio furnissii* H-6648 for 3hr.

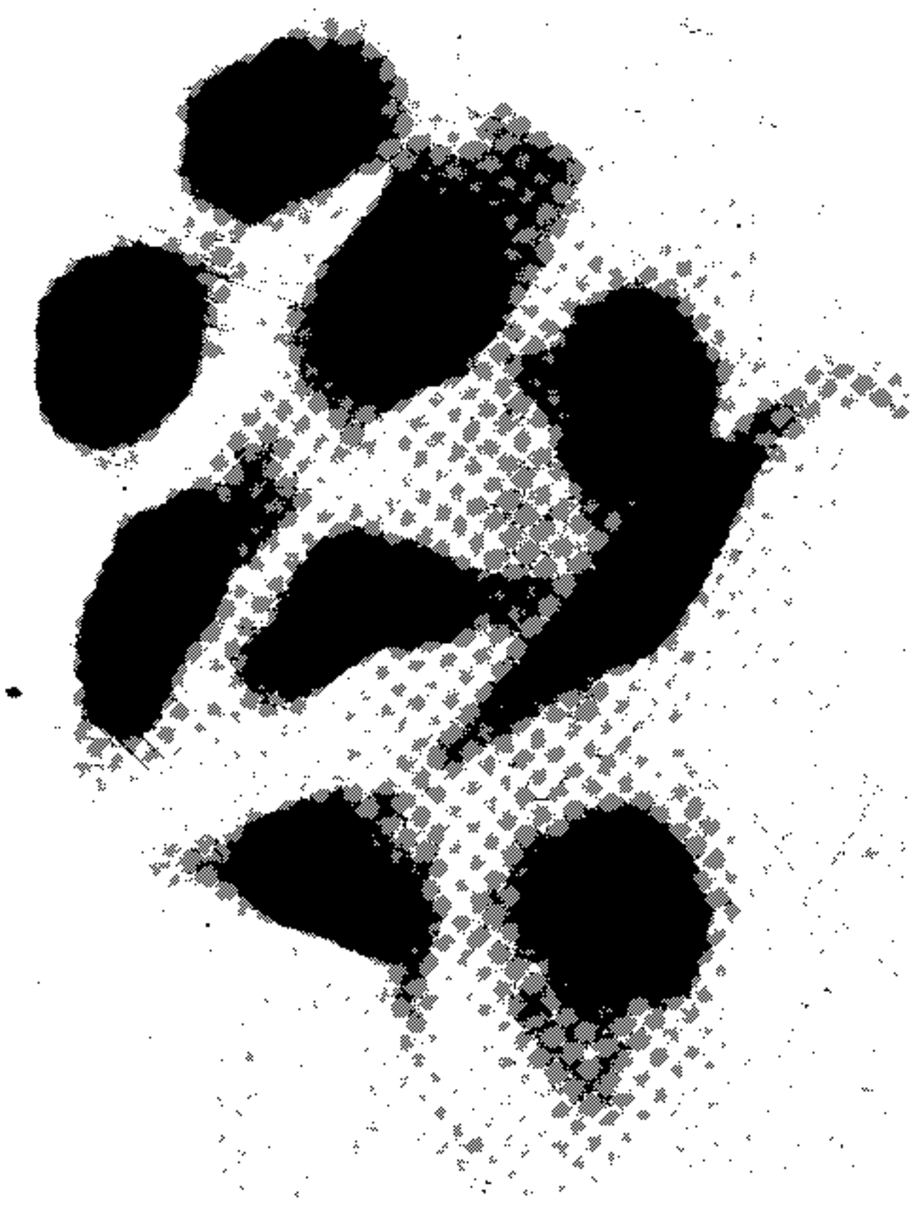


Fig. 2: monolayer treated for 21 hr with the broth-culture filtrate of the same strain. Giemsa stain. Magnification: X900.

No strain of *V. furnissii* induced accumulation of secreted fluids into the intestinal tract of suckling mice, or was able to produce cholera-like toxins.

Fourteen (87.5 %) strains of *V. furnissii* produced beta hemolysis on sheep blood agar. Of these, however, only six strains furnished broth-culture supernatants with hemolytic activity (Table).

TABLE

Hemolytic and cytotoxic activities of 16 clinical isolates of *Vibrio furnissii*

| Strains | Hemolysis on blood agar | Supernatants[a] | |
|---|---|---|---|
| | | Hemolysin | Cytotoxin |
| H-19 | + | - | 1/8 |
| H-074 | – | 0 | 1/4 |
| H-121 | + | 0 | 1/2 |
| H-155 | – | 0 | 0 |
| H-433 | + | 1/8 | 1/4 |
| H-461 | + | 0 | 1/4 |
| H-980 | + | 0 | 1/8 |
| H-2210 | + | 1/16 | 1/4 |
| H-4057 | + | 0 | 1/32 |
| H-4594 | + | 1/4 | 1/4 |
| H-4671 | + | 0 | 1/2 |
| H-6546 | + | 1/32 | 1/32 |
| H-6648 | + | 0 | 1/16 |
| H-7420 | + | 1/4 | 1/2 |
| H-7777 | + | 0 | 1/8 |
| H-8961 | + | 1/2 | 1/4 |

*a*: titer.

## DISCUSSION

*Vibrio furnissii* shares with *V. fluvialis* many ecological, biochemical, and virulence-associated attributes. Although genetically different (Brenner et al., 1983), some bacterial taxonomists (Baumann et al., 1984) resist to regard *V. furnissii* as an independent species. In fact, sometimes it is laborious to separate both organisms, specially when the anaerogenic, esculin, and salicin-negative strains are considered.

Despite the likeness with *V. fluvialis,* the role of *V. furnissii* in human diarrheal disease is still controversial. Perhaps earlier reports (Huq et al., 1980; Lee et al., 1981), only linking the anaerogenic biogroup of *V. fluvialis* to gastroenteritis, have been responsible for the widespread concept that *V. furnissii* is a free living organism. In northeastern Brazil, how-

ever, *V. furnissii* has frequently been associated with diarrhea (Magalhães et al., 1990, 1992). During a recent survey carried out in Recife, before the cholera epidemic, it is listed as second only to *V. parahaemolyticus* among *Vibrio*-linked enteritis (Magalhães et al., 1992).

*Vibrio furnissii,* as happen with other enteropathogenic species of *Vibrio,* exhibited a polar flagellum and produced cytolysin. Flagellum is an important attribute of virulence in several bacteria (Yancey et al., 1987; Liu et al., 1988; Eaton et al., 1992). Nonetheless, whether that appendage has some role in the pathogenesis of *V. furnissii* enteric infections remains to be determined.

Similar to the environmental strains of *V. furnissii* (Chikahira & Hamada, 1988), culture supernatants of our clinical isolates did not produce either cholera-like toxin or enterotoxin; whereas, they were highly cytolytic. In fact, the most striking extracellular property, associated with pathogenicity, displayed by many culture supernatant fluids of *V. furnissii* was their ability to lyse erythrocytes and their lethal effects on epithelial cells. Hemolysins have been considered as involved in the enteropathogenicity of *V. metschinikovii* (Miyake et al., 1988) and *Aeromonas hydrophila* (Thelestam & Ljungh, 1981; Stelma et al., 1986), bacterial species taxonomically closed to *V. furnissii.*

Albeit most of our strains were hemolytic and toxic for HeLa cells, distinguishing hemolysin activity from cytotoxicity was somewhat hard. In both cases the end response was the same, that is detachment of the cell monolayer. As have previously been shown with respect to *V. fluvialis* (Lockwood et al., 1982) hemolysins and true cytotoxins might simultaneously be present in broth-culture supernatants. Indeed, many filtrates of broth-cultures of *V. furnissii,* although non hemolytic, were toxic for HeLa cells, meaning that hemolysin and cytotoxin might be independent cytolysins or, alternatively, that Hela cells are more susceptible to the hemolysins of *V. furnissii* than the mouse erythrocytes. Nevertheless, the observation that the cytotoxic effect of many strains just was observed after 6 hr of contact between HeLa cells and supernatants was a strong evidence that besides hemolysin a true cytotoxin would also be present in the supernatants of broth-culture of *V. furnissii.*

Although case control studies are still needed before we decided with confidence the real importance of this organism in diarrheal illness, cytolysins, damaing epithelial cells, may be a major factor of virulence in *V. furnissii*-mediated enteritis.

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