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Characterization of *Vibrio cholerae* isolated from the aquatic basins of the State of Pernambuco, Brazil

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Summary Through a continuous bacteriological monitoring programme carried out by the Health Secretariat of the State of Pernambuco, Brazil, two isolates of *Vibrio cholerae* O1 El Tor Ogawa were discovered in an endemic area in 2001, during a cholera inactive period, along with six *V. cholerae* non-O1/non-O139 strains and two *Aeromonas veronii* biovar *sobria* strains showing an unusual characteristic of agglutination with O1 antiserum. Between that time and 2005, eight other O1 isolates were found. The virulence genes present in the *V. cholerae* differed among strains, with only three O1 strains harboring the *ctx*A gene. The O1 and some non-O1/non-O139 strains displayed identical patterns of amplification of the 16S-23S intergenic spacer region. RAPD of the 10 *V. cholerae* O1 strains, with the two primers used, revealed heterogeneity. The presence of *V. cholerae* carrying virulence genes in the aquatic basins examined confirms that they constitute a vibrio reservoir during a cholera inactive period, thus strengthening the argument for a continuous monitoring programme and preventative measures for cholera, mainly in the areas where the supply of drinking water is deficient.

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1. Introduction

In 1991, cholera re-entered Brazil after a century of absence (Guthmann, 1995; Hofer, 1993), and by 2001, 168 598 human cases had been registered. Of this total, 166 357 occurred in the northeast region, with 31 524 in the State of Pernambuco (MS-Brasil, 2002a). During the entire epidemic period, *Vibrio cholerae* was found in river waters in the affected areas (Colaço et al., 1998). No case was reported in the whole country in 2002 and 2003. However, 21 cases were registered in the State of Pernambuco in 2004, and five in 2005 (WHO, 2005, 2006).

A programme for bacteriological monitoring of aquatic basins, particularly those where *V. cholerae* O1 was isolated during the epidemic period, was established by the Health Secretariat of the State of Pernambuco. Many of these basins receive untreated sewage, favoring the propagation of enteropathogens because they also supply water for the local populations (MS-Brasil, 2002b). The monitoring programme was maintained during the post-epidemic period when no human cases were reported.

Vibrio cholerae lives naturally in riverine, brackish and estuarine ecosystems, and *V. cholerae* non-O1/non-O139 is frequently isolated from aquatic environments. *Vibrio cholerae* O1 is seldom isolated from aquatic ecosystems in the interepidemic periods of the disease, or it may be found in the non-toxigenic form (Faruque and Nair, 2002); it can persist in a 'viable but non-culturable' form (Binsztein et al., 2004) or in the form of biofilms (Faruque et al., 2006). It is known that virulence genes are found in environmental strains of *V. cholerae* from various serogroups, providing an environmental reservoir of such genes. Therefore, under certain environmental conditions, non-toxigenic *V. cholerae* can transform into the toxigenic form with epidemic potential by acquisition of the VPIΦ and CTXΦ phages encoding the cholera toxin (Faruque and Nair, 2002; Faruque et al., 2006).

The objective of this study was to determine the presence of virulence genes and to establish a clonal relationship among environmental strains of *V. cholerae* isolated during the post-epidemic period of the disease in the State of Pernambuco, Brazil.

2. Materials and methods

2.1. Isolation and identification of *Vibrio cholerae*

Water samples from four aquatic basins (Figure 1) were collected weekly during the five-year period between 2001 and 2005, using the Moore technique (Barrett et al., 1980). Sterile swabs were immersed in the waters for 24 h and then transferred to bottles with 300 ml of alkaline-peptone-water, pH 8.6. The bottles were shipped at ambient temperature to the laboratory where the samples were processed within approximately 6 h according to standard bacteriological methods for the isolation and identification of *V. cholerae* (MS-Brasil, 1992). The strains isolated were confirmed serologically using *V. cholerae* antisera supplied by the National Reference Laboratory for Cholera from the Oswaldo Cruz Institute/Fiocruz, RJ, Brazil. Agglutination was double-checked after boiling the cell suspensions in a water bath at 100 °C for 20 min.

2.2. Extraction of genomic DNA

Total DNA of the cultures was extracted as described by Ausubel et al. (1987), without lysozyme to prevent precocious lysis of the bacterial cells and fragmentation of the DNA.

2.3. Detection of virulence genes

The presence of virulence genes was assessed by PCR using primers described by Keasler and Hall (1993) for amplification of the *ctx*A and *tcp*A genes, by Leal et al. (2004) for *zot* and *ace*, and by Coelho et al. (1995) for *tox*T under the conditions described by Leal et al. (2004).

2.4. Genomic profiling

The isolates were analyzed by the amplification pattern of the 16S-23S intergenic spacer region (ribotyping) as described by Chun et al. (1999). Only the bands from 500 bp to 900 bp that were shown to be clear and reproducible in all the experiments were considered for the construction of the ribotyping profiles. RAPD was carried out as described by Leal et al. (2004). Invitrogen synthesized all the primers.

3. Results and discussion

After 2 years without any isolation of *V. cholerae* O1, 10 presumptive *V. cholerae* strains were obtained during the first 6 months of 2001; identification was based on the results of oxidase tests and on the biochemical reactions on tryptic sugar indole and lysine iron agar media. Serological characterization showed, however, that two isolates (131 and 177) were *V. cholerae* O1 El Tor Ogawa and six were *V. cholerae* non-O1/non-O139. Furthermore, specific biochemical reactions revealed the other two strains to be *Aeromonas veronii* biovar *sobria* (Table 1).

It should be pointed out that the six *V. cholerae* non-O1/non-O139 and the two *A. veronii* strains exhibited an atypical pattern of agglutination with the O1 antiserum in which the clumps were thinner and lighter than usual. Of these six, two isolates (132 and 134) appeared rough, agglutinating with the R antiserum, and accordingly they displayed auto-agglutination after being boiled. The other four *V. cholerae* non-O1/non-O139 (123, 124, 125, 129) strains and the two *A. veronii* did not agglutinate with the R antisera, and did not auto-agglutinate nor re-agglutinate with the O1 antiserum after being boiled. The atypical agglutination of these cultures with the O1 antiserum could be due to an antigenic structure present in the thermo-labile envelope of the bacterial cells as described by Shimada et al. (1987).

In the period 2002—2005, eight *V. cholerae* O1 El Tor Ogawa isolates were obtained (Table 1). Most of the *V. cholerae* O1 isolates originated from the Capibaribe river basin; non-O1/non-O139 isolates were found in the four basins (Figure 1).