Identification and Strain Differentiation of *Vibrio cholerae* by Using Polyclonal Antibodies against Outer Membrane Proteins

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Cholera is caused only by O1 and O139 *Vibrio cholerae* strains. For diagnosis, 3 working days are needed for bacterial isolation from human feces and for biochemical characterization. Here we describe the purification of bacterial outer membrane proteins (OMP) from *V. cholerae* O1 Ogawa, O1 Inaba, and O139 strains, as well as the production of specific antiserum and their use for fecal *Vibrio* antigen detection. Anti-OMP antiserum showed very high reactivity and specificity by enzyme-linked immunosorbent immunodiagnostic assay (ELISA) and dot-ELISA. An immunodiagnostic assay for *V. cholerae* detection was developed; this assay avoids preenrichment and costly equipment and can be used for epidemiological surveillance and clinical diagnosis of cases, considering that prompt and specific identification of bacteria is mandatory in cholera.

**MATERIALS AND METHODS**

**Selection and evaluation of bacteria.** Four *Vibrio* strains were used for antibody production and as controls for the assays: *V. cholerae* O1 Inaba (CDC13), *V. cholerae* O1 Ogawa (CDC12), *V. cholerae* O139, and *Vibrio alginolyticus*. Bacteria were plated and grown in thiosulfate-citrate-bile salts-sucrose agar (TCBS; Dibico-Mexico) for 18 h at 37°C. Colonies were tested by biochemical methods (5a, 9, 13) and by agglutination using polyclonal antibodies against *V. cholerae* O1 prepared with Roshka antigens in accordance with Centers for Disease Control and Prevention protocols (20). Other enteric bacteria (*Aeromonas caviae*, *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Morganella morgani*, *Plesiomonas shigelloides*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enterica serovar Typhimurium*, *Serratia marcescens*, *Vibrio mimicus*, and *Vibrio para-haemolyticus*) were grown in Trypticase in soybean broth (TSB; Bionox-Mexico), pooled, and inactivated by boiling for 20 min before using them in immunological assays.

**Isolation of OMP.** Vibrios for antibody production were grown in TSB at 37°C for 6 h in a humid chamber; 1 ml was transferred to Erlenmeyer flasks in TSB and grown overnight to induce logarithmic-phase growth. Bacteria were processed as described by Puzzo et al. (18) and Tarsi and Puzzo (23). Briefly, the culture was washed three times by centrifugation at 10,000 × g (Beckman; JA-21 or JA-20) for 20 min at 4°C in 125 mM Tris-HCl, pH 6.8. The last pellet was dissolved in the same buffer, and, instead of using a French press, it was frozen immediately in liquid N2 and thawed. This procedure was repeated 10 times. The suspension was centrifuged at 10,000 × g, and the supernatant was processed to obtain OMP by centrifugation at 100,000 × g (Beckman; TL-100 or SN-402) for 40 min at 4°C. Proteins in the pellet were extracted in Tris-HCl with 0.5% Sarkosyl for 30 min at 20°C and centrifuged at 100,000 × g for clarification and Sarkosyl elimination. The final pellet containing OMP was resuspended in Tris-HCl, the concentration of the OMP was measured by the Coomassie micro-method (Bio-Rad protein assay), and they were separated in aliquots and kept at −20°C.

**Polyvalent antibody preparation.** New Zealand rabbits were injected subcutaneously with *V. cholerae* O1 Ogawa (52 μg), *V. cholerae* O1 Inaba (26 μg), *V. cholerae* O139 (22 μg), or *V. alginolyticus* (18 μg) OMP. For the first immunization OMP were mixed 1:1 with complete Freund’s adjuvant (Mircrob-L-Mexico); for the next two, performed with a 15-day interval, incomplete Freund’s adjuvant (Mircrob-L-Mexico) was used. Anti-Vibrio antibody production was determined by enzyme-linked immunosorbent assay (ELISA) using heat-inactivated bacteria as the antigen. After the third injection antibodies were detected at high dilutions; thus sera were obtained and kept frozen in aliquots.

**Standardization of a polyclonal antibody-based ELISA for bacterial antigen detection.** Bacteria were adjusted to 10⁸ CFU/ml with a McFarland nephelometer, and serial dilutions up to 10 CFU/ml were prepared. Maxisorb plates (Nunc) were activated using UV exposure for 10 min as suggested by Boudet et al. (4): 100 μl of one dilution per well was adsorbed at 4°C overnight in carbonate buffer, and wells were washed and blocked with phosphate-buffered saline (PBS), pH 7.2–1% Tween–1% bovine serum albumin for 60 min. A similar volume of anti-Vibrio serum at serial dilutions from 1:100 to 1:102,400 was incubated for 1 h at 20°C; this was followed, after washing, by a 1:1,000 dilution of anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (as recommended by the manufacturer’s protocol; Sigma), which was incubated in similar conditions. The enzymatic reaction was developed using H₂O₂ (0.012%) and orthophenylenediamine (400 μg/ml) in citrate buffer as the substrate.

**Standardization of a bacterial antigen detection dot-ELISA with polyclonal antibodies.** The procedure used by Bosompem et al. (3) was followed with minor changes. Initially anti-OMP antiserum samples were evaluated; for this, serial bacterial dilutions, prepared in TSB as described above, were adsorbed to small disks (6 mm in diameter) of methanol-activated polystyrene (PVDF) membranes (Millipore), which were introduced into 24-microwell plates (Costar)
to perform the reactions. Membranes were blocked using PBS–1% Tween–1% bovine serum albumin and washed three times with PBS, and 1 ml of an anti-OMP antiserum was added. The membranes were incubated, and after they were washed and incubated with the second antibody, color was developed with H2O2 (0.012%) and 3',3-diaminobenzidine (400 µg/ml) in PBS. All incubations were performed for 30 min at 20°C.

For the detection of bacterial antigens in feces, defined concentrations of a dead bacterial strain were mixed with fecal samples from a healthy donor diluted in PBS (1:5) by soft stirring in an orbital shaker. The mixtures were immediately added to the PVDF disks, and the discs were processed as described above.

Evaluation of rectal swabs collected during surveillance activities. Feces were recovered using rectal swabs during field activities, enriched in alkaline peptone water, pH 8 (8 h, 37°C), and separated in three aliquots. One was cultured in TCBS (18 to 24 h, 37°C); the other two were kept frozen (−20°C) until use. After observation of cultures and biochemical species confirmation, bacteria were incubated for a further 18 to 24 h at 37°C and the characteristic metabolism of *V. cholerae* was confirmed and the serogroup was identified (5a, 8, 9). Finally 18 samples were subjected to ELISA and dot-ELISA.

RESULTS

Polyclonal antisera raised against OMP antigens showed very high absorbance values in ELISA at high serum dilutions (between 1:12,800 and 1:102,400) with homologous antisera compared to antibodies prepared with Roshka antigens (between 1:400 and 1:3,200), routinely used in our institute. Sera raised against *Vibrio* OMP did not react with other enteric bacteria (listed in Materials and Methods; Fig. 1). Specificity was further demonstrated in ELISA by reactions of bacteria at different concentrations and the highest OMP antiserum dilution (Fig. 2). Ten to 100 million Ogawa and Inaba bacteria were detected by their homologous antiserum diluted 1:50,000, while O139 at the same level was detected with a 1:150,000 dilution of its specific antiserum. No reaction was found with other enteric bacteria (listed in Materials and Methods), and a very low reaction (except with the Inaba antiserum) was found with the heterologous antiserum was found (Fig. 2).

The reactivity in dot-ELISA of anti-OMP antiserum was analyzed using several dilutions and 10⁶ CFU of *V. cholerae* or enteric bacteria (listed in Materials and Methods)/ml. OMP antiserum to Ogawa and O139 reacted against their specific adsorbed bacteria in all tested dilutions (1:500 to 1:16,000), but the anti-Inaba OMP reacted only in 1:500 and 1:1,000 dilutions (Fig. 3). Eighteen randomly selected blind peptonated enriched samples from those kept frozen after surveillance activities were analyzed by ELISA and by dot-ELISA; 8 were positive and 10 were negative in both assays. These results agreed with microbiological and biochemical results, suggesting 100% sensitivity and specificity for the assays.

DISCUSSION

The purpose of the study described here was to obtain a sensitive and specific immunodiagnostic assay for detection of
V. cholerae that would avoid preenrichment and costly equipment so that it can be used for epidemiological surveillance and clinical diagnosis of cholera cases. For this, OMP from V. cholerae Inaba and Ogawa serotypes (18, 23) were obtained by employing a technique similar to that used for V. alginolyticus (18). OMP antigens induced a higher humoral immune response in rabbits than antibodies produced with the Roskha extracts, similar to previous findings (18). OMP antibodies were specific, as could be seen by the reactivity in ELISA with the different antisera produced. Some degree of cross-reaction between anti-Inaba serum and the Ogawa strain was seen; this could be related to the transformation of Inaba to Ogawa (5, 22). Also the anti-Inaba antisera was less sensitive in the dot blot assay. Specificity of OMP has been shown previously (21), indicating that members of the Vibrioaceae family have different membrane antigens.

Whole bacteria were identified by ELISA and by dot-ELISA with OMP antisera using mixtures of known numbers of bacteria in human feces or frozen samples obtained from cases detected during surveillance activities. Concentrations of 10⁶ and 10⁸ CFU/ml were positive in dot-ELISA and ELISA, respectively. Probably the same sensitivity could be found with fresh human feces, but the feces obtained during epidemiological surveillance activities were, by routine, enriched in peptontated water, which increases the amount of bacteria. In natural infections bacterial concentrations of 10⁶ CFU/ml are usually found in feces; thus the assays developed in this study suggest that direct detection of bacteria in fecal samples is feasible. This sensitivity could not be confirmed in field or clinical studies due to the lack of recent cases. Anti-V. cholerae O139 antibodies as well as those of other enteric bacteria (listed in Materials and Methods) had very low cross-reactivity against the O1 V. cholerae serogroups used, supporting the specificity of the assay. Furthermore the amount of specific antisera produced in this study is enough for 15,000 dot-ELISAs.

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