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Screening of Aquatic Samples for *Vibrio cholerae* Serotype O1 by a Dot-Blot Method and a Latex Agglutination Test

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A dot-blot, enzyme-linked immunosorbent method and a latex agglutination test were studied for their abilities to detect *Vibrio cholerae* serotype O1 in aquatic samples by testing artificially contaminated water as well as samples from natural potential sources. Water samples were preenriched with alkaline peptone and then enriched with Monsur peptone water. For the dot-blot test, enriched cultures of organisms in a small portion of the Monsur peptone water were transferred to a polyvinylidene difluoride membrane with a microfiltration apparatus. The enzyme-linked immunosorbent assay was performed by using biotin-labeled antibodies and avidin-biotin-peroxidase complex; brown dots developed in the wells that contained serotype O1 vibrios. Latex agglutination tests were performed by mixing 1 drop of the culture in Monsur with 1 drop of reagent coated with monoclonal antibody specific for antigen A. The sensitivities and specificities of the methods were compared with those of the colony-blot method, which identified individual colonies of *V. cholerae* O1 in mixed bacterial cultures on isolation media. Our results indicate that the dot-blot method is as sensitive as the colony-blot method and is useful for screening for *V. cholerae* serotype O1 even in specimens that are heavily contaminated with non-O1 vibrios.

Vibrio cholerae serotype O1 is the causative agent of cholera in humans. In recent years, *V. cholerae* El Tor serotype O1 has been isolated from aquatic environments in which cholera is not endemic, as reported in studies made in the United States (6), England (2, 11), Australia (15), and Japan (14). Although the significance of these isolates in terms of an environmental reservoir of cholera is uncertain, it is important to be able to ascertain whether the organism is present in samples from such aquatic environments, since the most likely vectors of the organism are seafood and water (4, 5, 9).

In fecal specimens, *V. cholerae* O1 can be readily identified and differentiated from coliforms by use of thiosulfate-citrate-bile salts medium. However, this culture medium is not useful for detecting the organisms in environmental samples because of the presence of non-O1 *V. cholerae*. Polymyxin-mannose-tellurite agar (PMT), which was developed for selection and identification of *V. cholerae* El Tor in environmental specimens that also contained non-O1 vibrios, is much more useful than thiosulfate-citrate-bile salts for this purpose (16). Plating on PMT can be very sensitive if background flora do not ferment mannose. However, in examinations of environmental samples, the plates are very often contaminated with mannose-positive organisms. Because *V. cholerae* O1 is detected by chance, microbiologists are required to pick large numbers of colonies and perform slide agglutination tests. To eliminate this time-consuming procedure, we developed a colony-blot, double-stain method that enables us to identify individual colonies of *V. cholerae* O1 in mixed bacterial cultures on solid media (13). However, it is laborious to perform the colony-blot assay with large numbers of specimens, and methods for more rapid screening are clearly needed.

A dot-blot, enzyme-linked immunosorbent method and a latex agglutination test were studied for efficiencies in screening for *V. cholerae* O1 in aquatic specimens, and the sensitivities and specificities of the methods were compared with those of the colony-blot method (13).

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MATERIALS AND METHODS

Organisms. Six strains (2 from patients, 4 from estuaries) of *V. cholerae* El Tor Inaba and 14 strains (9 from patients, 5 from estuaries) of El Tor Ogawa were used. A strain of serotype O45 from estuarine water was chosen and used as a representative of non-O1 *V. cholerae*.

Materials. Reagents and culture media used were as follows: alkaline peptone water (pH 8.8), Monsur peptone water (12), Dulbecco phosphate-buffered saline (PBS), triple sugar iron agar, PMT (16), and thiosulfate-citrate-bile salts from Nissui Pharmaceutical Corp., Ltd., Tokyo, Japan; diaminobenzidine tetrahydrochloride, enzyme-linked immunosorbent assay-grade bovine serum albumin (BSA), and Tween 20 from Sigma Chemical Co., St. Louis, Mo.; polyvinylidene difluoride filter paper (0.45-μm pore size; 9 by 12 cm) from Millipore, Ltd., Yonezawa, Japan; nitrocellulose filter paper (0.45-μm pore size; 82-mm diameter) from Toyo Roshi, Ltd., Tokyo; horseradish peroxidase-protein A (HRP-protein A; lot no. 70406), biotinylated protein A (biotin-protein A; lot no. 80100326), biotinylated HRP (biotin-HRP; lot no. 70805), and streptavidin (avidin; lot no. 70527) from Zymed Laboratories, San Francisco, Calif.; *V. cholerae* O1-specific antiserum (lot no. 52-712), monoclonal antibody against antigen A of *V. cholerae* O1 (18), and anti-A monoclonal antibody-sensitized latex reagent (lot no. 29-810) from Denka Seiken Co. Ltd., Tokyo.

Culture of organisms. All strains were grown overnight at 37°C in Monsur peptone water. The organisms were fixed in Formalin (1% [vol/vol]) and refrigerated until the dot-blots were performed. The number of organisms in the culture was estimated by direct visual comparison with previously calibrated standards of Formalin-fixed *V. cholerae* Inaba.

Preparation of reagents. A portion (0.1 ml) of O1-specific antiserum was added to 5 μl of biotin-protein A (1 mg/ml). This mixture was kept at 4°C for 1 h and then diluted 1:100

(vol/vol) with PBS that contained 0.1% BSA and 0.05% Tween 20 (anti-O1–protein A–biotin complex). A portion (0.1 ml) of a 200-fold-diluted solution of monoclonal antibody specific for antigen A was added to 5 μl of biotin-protein A and treated as described above (anti-A–protein A–biotin complex). A sample (10 μl) of avidin (1 mg/ml) and 5 μl of biotin-HRP (1 mg/ml) were mixed and diluted with 15 ml of PBS (0.1% BSA, 0.05% Tween). The solution was used after incubation for 1 h at room temperature.

Dot-blot method. A polyvinylidene difluoride membrane filter was wetted with 1% BSA in PBS and set into the microfiltration apparatus (BIO DOT; Bio-Rad Laboratories, Richmond, Calif.). Samples were dispensed into individual wells of the apparatus in various amounts, such that 5×10^7 to 1×10^8 organisms were applied per well, and filtered under mildly reduced pressure applied with a water aspirator. Blocking solution (200 μl; PBS containing 1% BSA and 0.3% H_2O_2) was pipetted into each well and allowed to react for 15 min to eliminate nonspecific binding of antiserum to the organisms and to inhibit bacterial endogenous oxidase. After the blocking solution was drained from the wells by suction, each well was washed with 200 μl of 0.1% Tween in PBS; 100 μl of a solution of anti-O1–protein A–biotin was then added to each well and allowed to react with the filter for 60 min. Control wells into which a solution of the antiserum absorbed with O1 organisms was placed were included for each of the specimens. After a total of three washes with PBS-Tween, 100 μl of avidin-biotin-HRP complex was added to each well and allowed to stand for 60 min. After three washings, 100 μl of color development solution (PBS containing 0.05% diaminobenzidine tetrahydrochloride and 0.03% H_2O_2) was added to each well and allowed to stand for 15 min. The solution was then drained by suction, and all the sample wells were suction washed with 200 μl of distilled water to stop the reaction. The membrane filters were then removed from the apparatus.

Latex agglutination test. In accordance with the instructions of the manufacturer, latex agglutinations were performed by mixing 1 drop of culture in Monsur medium with 1 drop of antigen A-specific monoclonal antibody-sensitized reagent on the indicated circle of a black, plastic-coated card. Samples were recorded as negative if no agglutination was observed within 1 min. Latex beads coated with normal mouse immunoglobulin were always included as a control.

Colony-blot method. Colonies of *V. cholerae* O1 on PMT agar were detected and isolated by our colony-blot method (13). Briefly, a nitrocellulose membrane was wetted with PBS, carefully applied to the surface of the PMT plate, and left for 60 min. The disk was then removed, placed colony-side up in a petri dish that contained 20 ml of 1% BSA in PBS, and gently agitated for 30 min. After a brief rinse, the disk was immersed in color development solution (PBS containing 0.05% diaminobenzidine tetrahydrochloride, 0.03% H_2O_2, and 0.03% $CoCl_2$) for 15 min; gray dots developed at the site of replicated colonies as a result of the presence of endogenous oxidase. After three washings with PBS-Tween, the disk was immersed in 10 ml of anti-O1–protein A–HRP solution for 60 min with gentle shaking. After the washes with PBS-Tween, the disk was incubated for 15 min in color development solution without $CoCl_2$. Brown dots developed at the site of O1 colonies.

Field studies. Water samples were collected in sterile glass bottles from nine sites on rivers in Osaka City from April to August 1989. One liter of each sample was added to double-strength alkaline peptone water and preenriched by incubation at 37°C for 6 h. A sample (5 ml) of the enriched alkaline peptone water was added to 50 ml of Monsur peptone water and incubated at 37°C overnight (16). A loopful of the Monsur peptone water was serially streaked on two plates of PMT. Next, 2 ml of the top layer of the broth was transferred to a test tube, and then clumps of the organisms were disrupted by pipetting them several times with a volumetric pipette. After fixation in Formalin (1% [vol/vol]), the dot-blot screening was performed as described above. PMT plates incubated at 37°C overnight were scrutinized, and presumptive colonies were picked and inoculated into triple sugar iron for further identification. The colony-blot assay was then performed on PMT plates for direct identification of *V. cholerae* O1.

Detection of *V. cholerae* O1 in the presence of aquatic flora. Two systems were used to estimate the abilities of the above-mentioned methods to detect *V. cholerae* O1 in the presence of bacterial flora.

(i) System A. Samples (1.0 ml) of various dilutions of *V. cholerae* El Tor Inaba were added to 1 liter of collected specimens to give 100 to 300, 10 to 30, or 1 to 3 CFU/liters. Controls received 1.0 ml of a sterile solution of physiological saline. These samples wee then cultured and examined as described above.

(ii) System B. A loopful of culture in Monsur peptone water, originally obtained in the field studies, was transferred to fresh Monsur peptone water and incubated at 37°C. Cultures were then diluted 1:100,000 and used as vibrio flora. Sample (1 ml) of various dilutions of *V. cholerae* El Tor Inaba were added to 9 ml of the bacterial flora; the proportions in the different mixtures ranged from 1:10 to 1:10,000 Inaba vibrios to flora. Samples (1 ml) of the mixtures were inoculated into 10 ml of Monsur peptone water, which was then incubated for 15 h at 37°C. These samples were then examined as described above.

RESULTS

Dot-blot, enzyme-linked immunosorbent method. For the development of the dot-blot method, optimal conditions were established for the *V. cholerae* El Tor and *V. cholerae* serotype O45 strains. A solution of antibodies composed of 0.1 ml of O1-specific antiserum and 5 μl of biotin-protein A in 10 ml of buffer was sufficient. The addition of less than 1.25 μl of biotin-protein A often resulted in a weaker reaction. The avidin-biotin-HRP complex (10 μl of avidin and 5 μl of biotin-HRP in 15 ml of PBS) was adequate: the higher the dose, the higher the background. Representative results are shown in Fig. 1. Spots containing 2×10^5 or more *V. cholerae* O1 cells were consistently positive by macroscopic examination (Fig. 1), and no instrumentation was necessary to detect a positive reaction. The O1-specific polyclonal antiserum gave a stronger reaction than the monoclonal antibody specific for antigen A (data not shown).

Detection of *V. cholerae* O1 in artificially contaminated specimens. *V. cholerae* O1 was not detected in river water during the current field study. In our examination of 231 artificially contaminated specimens, the dot-blots, latex agglutination test, and colony-blots gave positive results for 167, 141, and 166 specimens, respectively. The sensitivities of the dot-blot and colony-blot methods were the same. Both methods gave positive results for most of the samples when 10 CFU or more of *V. cholerae* O1 was present in 1 liter of the specimens of water (Table 1) or at a ratio of *V. cholerae* O1 to background vibrios of 1:100 or more (Table 2). By contrast, screening by the latex agglutination test was somewhat less sensitive than either the dot-blot method or the colony-blot method.

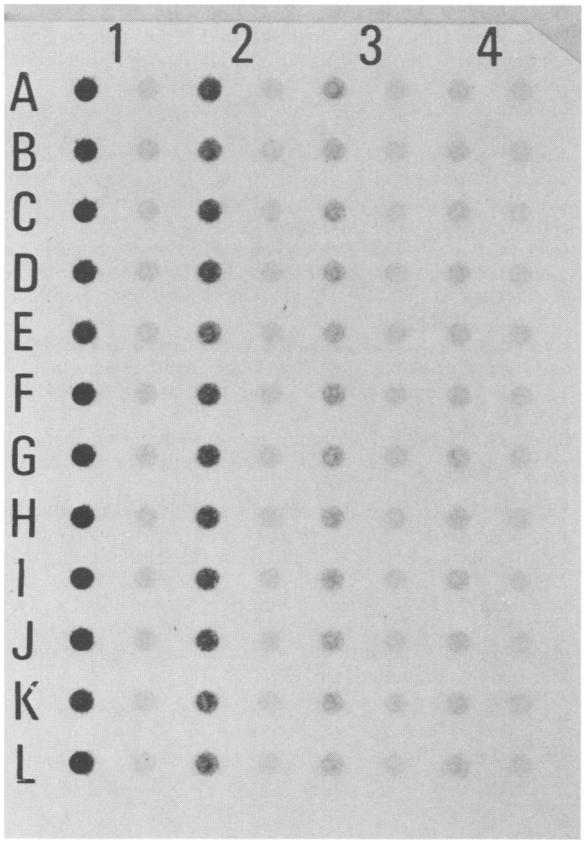

FIG. 1. Membrane filter showing representative dot-blot, enzyme-linked immunosorbent assay results of 12 strains (A through L) of *V. cholerae* O1. Columns 1 through 4 contained 2×10^7, 2×10^6, 2×10^5, and 2×10^4 *V. cholerae* O1 cells, respectively, each mixed with 2×10^7 *V. cholerae* O45 cells. Spots in right row of each column were used as controls. Compared with the control of each spot, spots containing 2×10^5 or more *V. cholerae* O1 cells were consistently positive by macroscopic examination.

DISCUSSION

The dot-blot method is useful for screening for *V. cholerae* serotype O1, even in specimens that are heavily contaminated with non-O1 organisms. The procedure requires little technical expertise, and commercially available reagents may be used without purification. The dot-blot method can be employed for the detection of *V. cholerae* O1, as can methods such as microscopic examination (1, 3, 7) and coagglutination with antibody-coated staphylococcal cells (10, 17).

The dot-blot required 2×10^5 or more *V. cholerae* O1 cells per well for production of a macroscopically visible deposit of insoluble product on membranes (Fig. 1). Since 5×10^7 to 1×10^8 organisms can be applied per well, at least 0.2 to 0.4% of a bacterial population in Monsur cultures must be made up of *V. cholerae* O1 if a positive result is to be obtained. In the screening of artificially contaminated water, however, the dot-blots gave positive results when as few as 10 CFU of *V. cholerae* O1 were present in 1 liter of the specimen (Table 1). The initial ratio of *V. cholerae* O1 to background flora in these specimens was approximately 1:100,000,000, because standard plate counts of the specimens gave values of about 10^6/ml. It is suggested that the two enrichment steps effectively increased the number of *V. cholerae* O1 cells and contributed to the increase in sensitivity of the dot-blots. Accordingly, if specimens contain significant numbers of bacteria that can proliferate in Monsur peptone water, the sensitivity of our system may be reduced. When vibrio flora were used as background flora, an initial ratio of 1:100 or more *V. cholerae* O1 to flora was required to ensure the detection of the organisms (Table 2). The present results indicate that the most important factor for the detection of *V. cholerae* O1 by our screening method was not the density of organisms in the water but the ratio of *V. cholerae* O1 to other vibrios.

TABLE 1. Detection of *V. cholerae* O1 in artificially contaminated water

No. of *V. cholerae* O1[a] cells (CFU)	No. of positive samples		
	Dot-blot	Latex agglutination	Colony-blot
100–300	29	28	29
10–30	26	22	25
1–3	14	14	10

[a] The number of *V. cholerae* El Tor Inaba cells inoculated into 1 liter of river water. At each level, 29 samples were tested.

TABLE 2. Detection of *V. cholerae* O1 in artificially contaminated water

Initial ratio of *V. cholerae* O1 to flora[a]	No. of positive samples		
	Dot-blot	Latex agglutination	Colony-blot
1:10	36	35	35
1:100	31	26	32
1:1,000	21	10	22
1:10,000	10	6	13

[a] A preparation of *V. cholerae* E1 Tor Inaba was added to the bacterial flora in various amounts such that the ratios in the different mixtures ranged from 1:10 to 1:10,000. The mixtures were inoculated into Monsur peptone water, which was then incubated for 16 h at 37°C. At each ratio level, 36 samples were tested.

Gustafsson (8) developed an enzyme-linked immunosorbent assay with monoclonal antibodies to screen for *V. cholerae* O1. However, the method is rather complicated and the monoclonal antibody is not commercially available. We aimed to develop a simple method with commercial reagents for use in the clinical laboratory. Although the latex agglutination test is rapid and easy to perform, it was somewhat less sensitive than the dot-blot method and is almost three times as expensive.

In dot-blot screening, polyclonal antiserum gave a stronger reaction and was preferable to the monoclonal antibody raised against the A antigen. Since the monoclonal antibody reacts with only one kind of epitope on the O1 antigen, the reaction cannot help but be weaker.

In conclusion, we recommend that specimens be screened by the dot-blot method, with subsequent application of the colony-blot method to the specimens that give positive results in the screening. These methods permit the effective analysis of aqueous specimens and aid in the detection of *V. cholerae* O1 organisms whose presence has heretofore been obscured by non-O1 vibrios.

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