Method for Detecting Small Numbers of *Vibrio cholerae* in Very Polluted Substrates

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A method is presented for the indirect detection of *Vibrio cholerae* by the multiplication of two specific bacteriophages: φH74/64 for El-Tor vibrios, and phage group IV (Mukerjee) for classical vibrios. The product to be examined is seeded in alkaline tryptone water for enrichment, as in the classical method, and is then incubated for 6 h at 37 C. Thereafter, a loopful is transferred to each of two nutrient broth (pH 9) tubes. One of these receives a drop of phage φH74/64; the other receives a drop of phage group IV. The stock phages are diluted so as to contain about 3,800 plaque-forming units in one drop; this is the maximum amount which, when added to 10 ml of broth, will not be detected in a loopful of 1 mm diameter. The tubes containing phage φH74/64 are incubated at 42 C; those with phage group IV are incubated at 37 C. After 18 h the cultures are killed by agitation with chloroform, and a 1-mm loopful is deposited on a layer seeded with the detector strains: Makassar 757 for El-Tor phage and *V. cholerae* 154 for classical cholera phage. After 4 to 5 h at 37 C, lysis appears on the spots if there has been phage multiplication in the respective broth tubes. With experimentally contaminated sewage water, vegetables, or stools, 1 to 10 cholera vibrios were detected in every sample. In rare cases, false-positive results were obtained by multiplication of the phage on non-cholera vibrios.

One of the principles of preventing the spread of cholera into a non-endemic area is to discover the first case as early as possible. An indirect method to achieve this result is by regular examination of sewage water in exposed areas (5). Therefore, any improvements in the detection of cholera vibrios in sewage water may positively contribute to the control of cholera.

The search for *Vibrio cholerae* in sewage water or on sewage-contaminated vegetables is more difficult than the examination of the rice-water stools of cholera cases (1, 3, 10–12). The classical method, based on enrichment and selective media, is efficient during an epidemic but may fail at its very beginning, when only small numbers of cholera vibrios are present in sewage water.

Experiments carried out in our laboratory showed that the possibility of detecting small numbers of cholera vibrios in heavily polluted substrates depends not only on the number of cholera vibrios in the sample examined, but also on the general bacterial load as determined by the standard plate count. In sewage water with a low bacterial load (10⁴ to 10⁵/ml), positive results may be obtained, by the classical method, in the presence of as few as 1 to 10 *V. cholerae* organisms per ml (Fig. 1). To obtain a positive result in sewage water with an increased bacterial load, there must also be an increase in the number of cholera vibrios in the specimen. In a sample of sewage water with a standard plate count of about 10⁸, there must be at least 10² El-Tor vibrios or 2 x 10⁴ classical cholera vibrios per ml to detect their presence (Fig. 1). Such high numbers of *V. cholerae* organisms are not expected to be present in sewage water at the beginning of an epidemic. Increasing the volume of the sample examined does not substantially improve the result because it mainly depends on the ratio between the number of cholera vibrios and the number of other microorganisms in the sample.

The efficiency of the classical method may be improved by using a second enrichment, but even then less than 50 to 100 El-Tor vibrios per ml or 10² to 10⁴ classical cholera vibrios per ml cannot be regularly detected in sewage water with a high bacterial load.

To increase the sensitivity of the test to detect small numbers of cholera vibrios in the stools of carriers, two other methods have been used: the fluorescent-antibody method (20) and a method using the detection of precipitant antigens (8).
Both of these methods also need the presence of at least $10^9$ cholera organisms in 1 g of stool to obtain a positive result.

**Phage multiplication method.** In 1955, Schacter (17) published a method for the detection of *Salmonella typhi* in stool, using the multiplication of Vi bacteriophage. One year later, Timakov and Goldfarb (18) published a method for the detection of *S. typhi* that was based on a similar principle. This latter method has since been used for the detection of some other microorganisms (6, 7, 13, 14). In 1958, Domaradskii et al. (9) published an application of the same principle for the detection of *V. cholerae* in water. The authors indicated that the minimal number of cholera vibrios detected by this method was $10^9$/ml of water. In experiments carried out in our laboratory by the method as described by Domaradskii, positive results were obtained in the presence of more than $10^4$ El-Tor vibrios or $10^5$ classical vibrios per ml of sewage water (standard plate count: about $10^{10}$/ml) (Fig. 2).

**Materials and Methods.**

To compare the efficiency of different methods of detecting cholera vibrios in sewage water and to determine the influence of certain parameters on the method, an experimental model was used. Sewage water with different standard plate counts (from about $10^4$ to about $10^{10}$) was contaminated with decimal dilutions of a suspension of cholera vibrios to obtain final concentrations ranging from about 1 to about $10^7$ vibrios per ml. The detection methods were carried out in parallel on all the samples, and the positive results were correlated with the predetermined number of cholera vibrios added to the sample and with the standard plate count of the sewage water.

**Sewage Water.** Sewage water from four outputs from Jerusalem and from three outputs of Gaza was used; the standard plate counts varied between $10^4$ and $10^{10}$/ml.

**Cholera Strains.** The cholera strains were as follows: *V. cholerae* biotype El-Tor, serotype Inaba no. 1100/1970 (isolated during the outbreak in Jerusalem); *V. cholerae* biotype El-Tor, serotype Ogawa no. 1/1970 (from the same outbreak); *V. cholerae* biotype El-Tor, serotype Inaba Phl. 6973; *V. cholerae* (classical) serotype Ogawa no. 2313/1970 (isolated in Jerusalem); *V. cholerae* serotype Ogawa no. 41; and *V. cholerae* serotype Inaba no. 35A3.

**Cholera Phages.** The cholera phages were: phage 47H74/64 of Basu and Mukerjee (4) for multiplication on El-Tor vibrios, and phage group IV of Mukerjee (16) for multiplication on classical cholera vibrios.

**Indicator Strains.** The indicator strains were: *V. cholerae* biotype El-Tor, Makassar 757, sensitive to El-Tor phages; *V. cholerae* no. 134, sensitive to classical cholera phages. The cholera indicator strains and the phages were kindly supplied by A. K. Ghosh (Calcutta).

**Media.** The media were: alkaline tryptone water (pH 9), cholera medium (Oxoid), thiosulfate-citrate-ble-sucrose medium, and Monsur medium (15).

**Simplified Method of Detecting the Phage Multiplication.** The detection of phage multiplication by plating dilutions onto the indicator strain and counting the number of plaque-forming units before and after multiplication is time consuming and cumbersome. We used a more simple and direct method developed previously by one of us (17). To a tube containing 10 ml of broth enough phage is obtained to obtain a concentration of 3,800 to 4,000 plaque-forming units in the whole tube. This concentration is obtained by adding 1 drop (about 0.038 ml) from a
phage suspension containing $10^8$ plaque-forming units per ml to the 10 ml of broth in the tube. At this concentration, the phage is not detected at all when a small loop of 1 mm diameter (about 0.0025 ml) is deposited on a layer of the detecting organism. Rarely, one isolated plaque may appear, but it doesn’t signify multiplication of the phage.

Practically, the phage stock is diluted 10-fold to $10^{-4}$, and 1 drop of each dilution is added to sterile, 10-ml broth tubes. After mixing, a small loopful of broth from each tube is tested for phage activity on the detecting organism. The dilution to be used in this test is the lowest that shows no activity in a small loop.

If a sample contaminated with cholera vibrios is incubated in a broth tube with cholera phage at this concentration, the phage will multiply on the vibrio and, by testing the action of a loopful of the killed culture on the detecting organism, full confluent lysis or at least a significant number of plaques will appear.

**PARAMETERS OF THE PHAGE MULTIPLICATION METHOD**

The phage multiplication method was first studied by direct seeding of 1 ml of experimentally contaminated sewage water into 10 ml of nutrient broth and adding 1 drop of the respective phage in the established concentration. The broth was incubated overnight at 37°C and then treated with chloroform. After about 2 h of sedimentation, a small loopful (1 mm diameter) of the supernatant was deposited on a nutrient plate previously seeded with the respective indicator strain of *V. cholerae*. The plate was incubated for 4 to 5 h at 37°C and the results were read.

The efficiency of this method was compared with that of the method described by Domaradskii et al. (9) (Fig. 3). At different levels of bacterial loading of the sewage water, the method presented here detected lower numbers of El-Tor or classical cholera vibrios in sewage water than by the method of Domaradskii.

**Influence of incubation temperature.** In parallel experiments, the tubes seeded with contaminated sewage water and phage were incubated at 37, 40, and 42°C (Fig. 4). It was found that these temperatures differently influenced the detection of El-Tor and of classical cholera vibrios when the phage enrichment method was used. When the sewage water was contaminated with El-Tor vibrios, incubation at 42°C improved the results, and lower numbers of El-Tor vibrios were detected at this temperature. On the contrary, when the sewage water was contaminated with classical cholera vibrios, better results were obtained at 37°C than at higher temperatures.

**Influence of the pH of the broth.** The following pH values were examined: 7.6, 8.0, 8.5, 9.0, and 9.2. The phage multiplication in the presence of cholera vibrios in sewage water was faster at pH 9, and lower numbers of cholera vibrios were detected at this pH. Figure 5 presents the minimal numbers of *V. cholerae* necessary to obtain a positive result in sewage water with different bacterial load at pH 7.6 and at 9.0.

**Influence of sodium taurocholate or potassium tellurite.** By adding sodium taurocholate or potassium tellurite at the concentrations recommended for cholera media (0.5% and 0.5 x $10^{-4}$, respectively) to the phage enrichment broth, the multiplication of the phages was partially inhibited and greater numbers of cholera vibrios had to be present in the sample, to obtain a positive result (Fig. 6).

**Influence of pre-enrichment.** A significant improvement in the sensitivity of the method was obtained when the sample was not seeded
temperature of 42°C was optimal for the detection of El-Tor vibrios and 37°C was optimal for the detection of classical cholera vibrios.

**SPECIFICITY OF THE METHOD**

During the field application of the phage enrichment method, 660 samples of sewage water and 350 samples of vegetables were examined in a period when no cholera cases were present in the area. Only four false-positive results were obtained among the sewage water samples. From these samples we succeeded in isolating non-agglutinating vibrios that did not react with the OI serum of *V. cholerae* but that had the biochemical characteristics of El-Tor vibrios. Three of them permitted multiplication of phage φH74/64, and one multiplied the phage group IV (Mukerjee). Two of these strains were examined by the Ouchterlony method in the Cholera Laboratory of the Pasteur Institut (Paris). One of them was found to share three antigens and the other five antigens in common with cholera vibrios.

The phage multiplication characteristics of directly into the enrichment broth, but first was seeded into alkaline tryptone water as an enrichment medium for cholera vibrios. This improvement was more marked when a double-strength tryptone water was used. After 6 h of incubation of the contaminated sample in this medium, a loopful of the surface culture was transferred to each of two nutrient broth tubes (pH 9, 10 ml). To one of these tubes phage φH74/64 was added at about 3,800 plaque-forming units; to the other tube phage group IV was added in the same amount. The tubes were incubated for 18 h at 42°C for multiplication of El-Tor phage or at 37°C for multiplication of classical phage. By this method, less than 10 cholera vibrios were detected in 1 ml of sewage water, and the sensitivity of the method was almost independent of the bacterial load of the sample (Fig. 7).

The influence of the incubation temperature and the pH was again examined with the pre-enrichment method (Fig. 8 and 9). At pH 9 the sensitivity of the method was optimal for both El-Tor and classical cholera vibrios. The
these strains are in some respects different from those of the true cholera vibrios. (i) They are semiresistant (19) in that they do not form plaques on solid media. (ii) The titer reached by multiplication of cholera phage in broth culture on these strains is lower than by multiplication on true cholera vibrios. (iii) The multiplication of cholera phage is retarded on these non-cholera vibrios in comparison with the rate of multiplication on true cholera vibrios.

CONCLUSIONS

The phage multiplication method is not intended to be used alone, but together with the classical bacteriological methods. The transfer from the enrichment medium to the phage multiplication broth is performed at the same time as the transfer onto solid selective media. The results of the phage multiplication method are striking, and a positive result is read in a glance. Such results oblige the worker to reexamine the selective plates of the same sample to detect any missed colony of cholera vibrios or non-agglutinating vibrios.

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, New York.
2. Barua, D., and W. Burrows. 1974. Cholera. W. B. Souders Co., Philadelphia.
3. Barui, R. K., W. H. Mosley, and W. M. McCormack. 1972. A comparison of purging and multiple rectal swabs in the detection of cholera infection. Bull W. H. O. 46:257–262.
4. Basu, S., and S. Mukerjee. 1970. A specific phage for pathogenic Vibrio cholerae, biotype El-Tor (H 74/64). Bull W. H. O. 43:509–512.
5. Benenson, A. S. 1971. The control of cholera. Bull. N. Y. Acad. Med. 47:1204–1210.
6. Birzu, Al., V. Beslea, and V. Dobre. 1967. The increase of the phage titer, a useful method for experimental research on the viability of some Salmonella in foods. Probl. Patol. Comp. 12:51–56.
7. Bogomolov, B. P. The use of the phage-titer-increase test for the diagnosis of bacillary dysentery in children. Zh. Mikrobiol. Epidemiol. Immunobiol. 32:1142–1144.
8. Dodin, A., J. Wurt, and M. Dugut. 1971. Les antigènes précipitants de Vibrio cholerae. Leur rapport avec les antigènes agglutinants et immunofluorescents. Bull. Soc. Pathol. Exot. 64:596–603.
9. Domaradzki, J. V., C. V. Anokhina, V. L. Kulikova, L. K. Denisenko, and O. N. Mosolova. 1958. The use of the phage titer increase reaction for the rapid detection of Vibrio comma. Zh. Mikrobiol. Epidemiol. Immunobiol. 29:1124–1128.
10. Finkelstein, R. A. 1973. Cholera. C.R.C. Crit. Rev. Microbiol. 2:513–623.
11. Gerichter, Ch. B., I. Sechter, and D. Cahan. 1972. Laboratory diagnosis of cholera, during the Jerusalem outbreak, August-September, 1970. Israel J. Med. Sci. 8:531–539.
12. Gerichter, Ch. B., I. Sechter, D. Cahan, and A. Gavish. 1971. Laboratory investigations during the cholera outbreak in Jerusalem and Gaza. 1970. Brith Hatzibur (Public Health) 14:26–38.
13. Goldfarb, D. M., and Z. S. Ostrovskaias. 1957. The detection of typhoid bacilli in water, using the increase of phage titer reaction. Zh. Mikrobiol. Epidemiol. Immunobiol. 26:636.
14. Leonescu, M., and T. Horodniceanu. 1961. Valoarea fagodiagnosticului in dizenterie. Microbiol. Parazitol. Epidemiol. 6:271–277.
15. Monaur, K. A. 1967. Bacteriological diagnosis of cholera under field conditions. Bull W. H. O. 28:287.
16. Mukerjee, S., D. D. Guha, and R. U. K. Guha. 1957. Studies on typing of cholera by bacteriophage. I. Phage typing of V. cholerea from Calcutta epidemics. Ann. Biochem. 17:161–177.
17. Sechter, I. 1955. Une méthode pour la recherche de la présence du bacille typhique dans les fèces à l’aide du bactériophage Vi. Stud. Cercet. St. Acad. RPR. fil. Iasii, 3:499–510.
18. Timakov, V. D., and D. M. Goldfarb. 1956. Bacteriophage and the problem of demonstrating pathogenic bacteria. Vestn. Akad. Med. Nauk SSSR 2:28–34.
19. Wahl, R. 1963. La semi-résistance aux bactériophages. Ann. Inst. Pasteur Paris 84:51–59.
20. Zinnaka, Y., S. Shimodori, and K. Takeya. 1965. Application of fluorescent antibody technique to the detection of cholera vibrio. Jpn. J. Infect. Dis. 20:51.