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Simple Procedure for Rapid Identification of *Vibrio cholerae* from the Aquatic Environment

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Biochemical tests commonly used to screen for *Vibrio cholerae* in environmental samples were evaluated, and we found that a combination of alkaline peptone enrichment followed by streaking on thiosulfate citrate bile salts sucrose agar and testing for arginine dihydrolase activity and esculin hydrolysis was an effective rapid technique to screen for aquatic environmental *V. cholerae*. This technique provided 100% sensitivity and ≥70% specificity.

Since *Vibrio cholera*e is autochthonous to the aquatic environment (3, 16, 18), monitoring this bacterium in water sources is important for control of cholera. A selective medium, such as thiosulfate citrate bile salts sucrose (TCBS) agar, eliminates most nontarget bacteria in clinical samples but is not satisfactory for environmental samples because many bacteria present in natural water sources can produce colonies on TCBS agar whose appearance is similar to the appearance of *V. cholerae* colonies. Furthermore, the series of biochemical tests commonly used to identify *V. cholerae* (1, 6, 9, 15) was originally designed for clinical samples in order to specifically detect pathogenic vibrios. Molecular methods, including PCR and DNA-DNA hybridization performed with probes specific for *V. cholerae*, provide more reliable identification (2, 13) but have limitations because of cost and the facilities required for analysis; these limitations are particularly significant for field studies involving large numbers of samples.

We report here the results of a study in which we used 13 generally accepted biochemical tests for identifying clinical strains of *Vibrio* spp. to determine the minimum number of such tests that is required to identify *V. cholerae* in environmental samples.

Isolation of presumptive *V. cholerae*. Water and plankton samples were collected from Chesapeake Bay in Maryland over a 7-month period (January through July 1998). Samples of water (250 ml) and plankton (25 ml) were collected and filtered by using 0.22-μm-pore-size polycarbonate membrane filters; this was followed by incubation of the membranes in an enrichment medium consisting of 100 ml of alkaline peptone water (1% peptone, 1% NaCl; pH 8.6) overnight at 30°C. The incubation temperature was lower than the temperature normally used for clinical samples in order to accommodate and reduce the stress for environmental isolates that inhabit lower-temperature environments. Two loopfuls of the culture broth, taken from the top layer of the alkaline peptone water, were streaked onto TCBS agar (Oxoid Ltd., Basingstoke, England) and incubated overnight at 37°C. Six to 12 yellow, flat, 1- to 3-mm-diameter colonies were picked from each sample and streaked on Luria-Bertani agar containing 2% NaCl. A total of 844 colonies were purified and stored for further testing.

Biochemical tests. Thirteen biochemical tests were performed for each isolate. Kligler iron agar slants (Difco, Detroit, Mich.) were prepared as recommended by the manufacturer, and 5-ml aliquots were dispensed into tubes (13 by 100 mm). The tubes were inoculated by stabbing. Results were read after incubation at 37°C for 24 h.

For the oxidase test bacterial colonies were picked with a platinum wire and streaked on filter paper saturated with 0.5% tetramethyl-*p*-phenylenediamine hydrochloride (BBL Co.). Rapid appearance of a dark purple color was considered a positive reaction.

The method used for the arginine dihydrolase test was based on Thornley's method (14). The medium used was Luria-Bertani broth containing 1% (wt/vol) L-arginine (pH 6.8). Phenol red powder (Difco) was added as an indicator (M. H. Matté, personal communication). After inoculation, the medium was covered with sterile mineral oil and incubated at 37°C for 24 h. Appearance of a red color was considered a positive reaction.

Lysine and ornithine decarboxylase assays were performed by using Moeller decarboxylase base medium (Difco) amended with an amino acid at a concentration of 1% (wt/vol) and adjusted to pH 6.8. The base medium, without an amino acid added, was inoculated as a control. After inoculation, the medium was covered with mineral oil and incubated at 37°C for 24 h. Positive reactions were indicated by a dark purple color compared to the color obtained with the base medium without an amino acid.

Cells grown in the presence of 0, 6, and 8% (wt/vol) NaCl in nutrient broth were used to determine the requirement for Na^+. The medium was lightly inoculated and incubated at 30 or 37°C for up to 7 days, and positive results were determined by examining the turbidity.

Acid production from 1% mannitol or arabinose was determined by using purple broth base (Difco) (pH 6.8), and the results were read after no more than 24 h of incubation at 37°C. A change in the color of the medium from purple to yellow indicated that there was a positive reaction.

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The methyl red reaction was tested by using MR-VP medium (Difco) incubated at 37°C for 48 h after inoculation, and the Voges-Proskauer test assay was performed by using a culture grown in MR-VP medium at 37°C for 48 h.

Esculin hydrolysis was tested by using heart infusion agar (Difco) containing 0.1% esculin and 0.05% ferric chloride. Blackening of the medium after incubation at 30 or 37°C for up to 3 days indicated that that there was a positive reaction.

Confirmation of the identities of presumptive *V. cholerae* isolates. For development of the method, the identities of presumptive isolates of *V. cholerae* were confirmed by PCR. The PCR was performed as described by Chun et al. (2). The PCR primer set used, prVC-F (5'-TTAAGCSTTTTCRCTGAGAATG-3') and prVCM-R (5'-AGTCACTTAACCATACAACCCG-3'), amplifies a portion of the intergenic spacer region between the 16S and 23S rRNA genes and is specific for *V. cholerae* (>100 strains tested) (2). In our evaluation of the simple biochemical test procedure, due to the vast number of isolates, a colony blot hybridization technique was used instead of PCR. The probe was based on a modification of PCR primer prVC-F. The oligonucleotide probe, pVCITS-1 (5'-GCSTTTTCRCTGAGAATG-3'), was end labeled with ^{32}P. This probe hybridized with all 201 *V. cholerae* strains tested and did not hybridize with 30 other *Vibrio* species and 10 non-*Vibrio* species (C. J. Grim, I. N. G. Rivera, N. Choopun, J. Chun, A. Huq, and R. R. Colwell, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. Q33, 2000). To evaluate the simple test, bacterial colonies were transferred to no. 541 filter paper (Whatman Inc.) and processed as previously described (17). The hybridization reaction was performed in sodium chloride-sodium citrate as described previously (5).

Selection of the biochemical tests. The effectiveness of each biochemical test was evaluated based on sensitivity and specificity, and 100% sensitivity was sought in order to eliminate false negatives. Sensitivity and specificity were calculated as follows: sensitivity = [(number of isolates positive as determined by biochemical tests and PCR)/(total number of isolates positive as determined by PCR)] × 100; and specificity = [(number of isolates negative as determined by biochemical tests and PCR)/(total number of isolates negative as determined by PCR)] × 100.

The tests that provided 100% sensitivity included the arginine dihydrolase and esculin hydrolysis tests, which had specificities of 72 and 26%, respectively (Table 1), and these tests were shown to be very effective in screening for *V. cholerae*. Of 844 bacterial colonies tested, 241 were identified as presumptive *V. cholerae* isolates by using only these two tests, and all were confirmed to be *V. cholerae* isolates by PCR. None of 13 isolates randomly selected from the isolates not identified as *V. cholerae* by this pair of tests was found to be a *V. cholerae* isolate when PCR was used.

Evaluation of the simple biochemical test procedure. After the test procedure was developed, additional water samples were collected from Chesapeake Bay from June through August 2000, and a total of 481 bacterial colonies were picked and subjected to both the simple test procedure and colony blot hybridization. Sixteen isolates were identified as *V. cholerae* by the simple test. The results were in complete agreement with the hybridization results.

In addition to the Chesapeake Bay samples, 67 presumptive

TABLE 1. Thirteen biochemical tests used to screen for *V. cholerae*[a]

Biochemical test	*V. cholerae* reaction[b]	% of isolates with same reaction as *V. cholerae*	Sensitivity (%)	Specificity (%)
Kligler iron agar[c]	K/A	98	100	2
Oxidase[c]	+	99	100	1
Arginine	−	49	100	72
Lysine	+	75	98	30
Ornithine	+	61	97	53
NaCl (0 %)	+	57	90	56
NaCl (6 %)	V	NA[d]	NA	NA
NaCl (8 %)	−	97	96	3
Mannitol	+	68	71	30
Arabinose	−	85	100	20
Methyl red	V	NA	NA	NA
Voges-Proskauer	+	56	80	53
Esculin	−	81	100	26

[a] A total of 844 bacterial isolates from Chesapeake Bay water and plankton samples were included in the study. See text for the methods used to calculate sensitivity and specificity.
[b] K/A, alkaline at the top and acid at the bottom; +, 90 to 100% of the isolates were positive; −, 0 to 10% of the isolates were positive; V, variable reaction.
[c] The Kligler iron agar and oxidase test results are the results obtained with 336 and 330 isolates, respectively.
[d] NA, not applicable.

V. cholerae isolates from Peru, isolated from environmental samples collected by using the procedures described above, were subjected to the arginine dihydrolase and esculin hydrolysis tests. Of these 67 isolates from Peru, 25 turned the esculin medium dark but were identified as *V. cholerae* by PCR. Normally, blackening of the medium results from reaction of esculetin, a product of esculin hydrolysis, with ferric ion in the medium, which results in a black phenolic compound-iron complex. Hydrolysis also results in a loss of the characteristic fluorescence of esculin that can be measured at 354 nm (11). These 25 isolates from Peru darkened the medium, but there was no loss of fluorescence, even 10 days after inoculation, demonstrating that the strains did not hydrolyze esculin. After stab inoculation, all of the isolates that hydrolyzed esculin completely blackened the medium by day 2, and there was a total loss of fluorescence by day 3.

The difficulty in interpreting the results of the esculin hydrolysis test was overcome by testing for fluorescence of the esculin instead of relying only on darkening of the medium. Blackening of the test medium by some *V. cholerae* strains, without esculin hydrolysis, can result from production of other compounds (e.g., melanin) (4). However, to produce melanin, the bacterium must be grown under highly aerobic conditions (4, 8, 10). Addition of 20 mM L-proline, which has been shown to inhibit melanin production in *V. cholerae* (4), did not prevent blackening of the test medium by the isolates from Peru. When stab inoculation is used, esculin hydrolysis is indicated by total blackening of the medium within 48 h and/or by a total loss of fluorescence by 72 h. When the latter parameter was used, 60 of the 67 *V. cholerae* isolates from Peru were identified as *V. cholerae* by using only the two biochemical tests, and 57 of the 60 strains were subsequently confirmed to be *V. cholerae* strains by PCR. The three false positives were then identified by 16S ribosomal DNA sequencing and by comparing the sequences obtained with sequences in the GenBank database.

TABLE 2. Results of the simple two-test procedure compared with the results of molecular analysis

Simple test result[a]	Molecular analysis (PCR-hybridization)[b,c]											
	1998 Samples			2000 Samples			Peru samples			All samples		
	No. positive	No. negative	Total no.	No. positive	No. negative	Total no.	No. positive	No. negative	Total no.	No. positive	No. negative	Total no.
+	241	0	241	16	0	16	57	3	60	314	3	317
−	0	13	13	0	465	465	0	7	7	0	485	485
Total	241	13	254	16	465	481	57	10	67	314	488	802

[a] +, positive for identification as *V. cholerae* as determined by the two-test method (i.e., both arginine negative and esculin negative); −, negative for identification as *V. cholerae*.
[b] PCR was used for 1998 and Peru samples; hybridization was used for 2000 samples.
[c] Both the sensitivity and the specificity for the 1998 samples and the 2000 samples were 100%. For the samples from Peru the sensitivity was 100% and the specificity was 70%. For all samples, the sensitivity was 100% and the specificity was 99.3%.

Two of these three strains were identified as *Vibrio metschnikovii*, and the third was identified as *Vibrio natriegens*. Although some strains of other *Vibrio* species might give false-positive results with this simple test, it is relatively easy to distinguish these strains from *V. cholerae* by a simple Na^+ requirement test. None of the other sucrose-fermenting *Vibrio* species can grow in nutrient broth without added Na^+. *V. metschnikovii*, which has a low Na^+ requirement, is oxidase negative (1); hence, it can be easily detected.

Table 2 shows a comparison of the results of the simple biochemical tests with the results obtained by using molecular methods. The simple pair of tests provided 100% sensitivity for both the Chesapeake Bay and Peru isolates and 100 and 70% specificities for the Chesapeake Bay isolates and the Peru isolates, respectively. The 100% sensitivity of this simple biochemical test procedure was meaningful because no false-negative results were obtained; therefore, all *V. cholerae* isolates were detected. Further field experiments, especially in endemic areas, will be used to assess the effectiveness of this simple procedure in specific areas. Two more tests, the Na^+ requirement and oxidase tests, may be included in the screening process in areas where there are high numbers of false-positive results.

Other selective media, such as cellobiose-polymyxin B-colistin agar and its modified formulas, modified cellobiose-polymyxin B-colistin agar and cellobiose-colistin agar, have been reported to be superior to TCBS agar for isolation of *Vibrio vulnificus* and *V. cholerae* (7, 12). These media may be useful for increasing the probability of isolation of *V. cholerae* from aquatic environments. However, whether these media can be used to isolate strains that are more susceptible to polymyxin B, such as the *V. cholerae* O1 classical biovar (1), has not been determined. In contrast to TCBS agar, which is routinely used in many laboratories, is commercially available, and requires only a boiling step, these media require more preparation steps, including pH adjustment, autoclaving, and filtration sterilization of the carbohydrates and antibiotics, and they must be freshly prepared before each use (because they become more inhibitory as they age) (12). These steps limit their use to larger laboratories with more resources.

In summary, aquatic samples can be screened rapidly for the presence of *V. cholerae* by enriching samples in alkaline peptone water, selecting yellow colonies on TCBS agar, and performing the arginine dihydrolase and esculin hydrolysis tests with the purified isolates. Isolates giving negative reactions in both of these tests (i.e., no color change in arginine medium and fluorescence in esculin medium) can presumptively be considered *V. cholerae* isolates.

The simple two-test procedure described here is useful because the results are easy to read and do not change with time, allowing more flexible operation. Furthermore, no additional steps are required to obtain the results. The simple two-test procedure is useful for field work, especially in developing countries where *V. cholerae* is endemic and facilities are limited.

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