

Comparison of Direct-Plating and Enrichment Methods for Isolation of *Vibrio cholerae* from Diarrhea Patients

MURAD LESMANA, EMILY RICHIE,* DECY SUBEKTI, CYRUS SIMANJUNTAK,
AND STEPHEN E. WALZ

U.S. Naval Medical Research Unit No. 2, Jakarta, and National Institution of Health Research and Development,
Indonesian Ministry of Health, Jakarta, Indonesia

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A direct-plating method on thiosulfate citrate bile salts sucrose agar (DIR-TCBS) in conjunction with enrichment in alkaline peptone water (APW) incubated for both 6 h and 24 h followed by subculture onto TCBS (APW6h-TCBS and APW24h-TCBS, respectively) was performed on 16,034 rectal swab samples for isolating *Vibrio cholerae*. A total of 2,932 (18.3%) rectal swab samples were positive for *V. cholerae* O1 biotype El Tor, with the Ogawa serotype constituting 99.2% of the isolates. There were no significant differences in *V. cholerae* O1 isolation rates between the three culture systems nor between the combinations of any two systems. However, direct plating plus enrichment demonstrated a significantly higher *V. cholerae* O1 isolation rate than DIR-TCBS alone ($P < 0.02$). Conversely, enrichment procedure, alone or in combination with DIR-TCBS, yielded significantly more ($P < 0.0001$) *V. cholerae* non-O1 isolates than DIR-TCBS alone. The length of incubation time of the enrichment broth, 6 h, offers no significant advantages over 24 h for the isolation of *V. cholerae* O1 and non-O1. A 24-h enrichment broth incubation period has the practical advantage of being easy to integrate into a normal laboratory workday, whereas 6-h broth enrichment, although more commonly recommended, requires that arrangements be made for after-hours subculture.

Laboratory protocols for the isolation of *Vibrio cholerae* from suspected cholera patients include both direct-plating and broth enrichment methods (7, 10). The most commonly used enrichment medium for the isolation of *V. cholerae* is alkaline peptone water (APW; 1% peptone, 1% NaCl, pH 8.4). Routine laboratory practice is to inoculate APW directly with either a stool swab or a rectal swab, incubate it for 6 to 8 h, and then subculture the broth onto a thiosulfate citrate bile salts sucrose (TCBS) agar plate. Incubation of the enrichment medium for more than 8 h has not been recommended because vibrios may be overgrown by other enteric bacteria present on the swab samples (3, 7, 8). However, reports comparing a 6- to 8-h period of incubation to a longer incubation time, namely, 20 to 24 h, for APW enrichment are scarce. Pazzaglia et al. (11) reported improved recovery of *V. cholerae* from Moore swabs in APW incubated at room temperature for 12 or 18 h compared to 6-h incubation, although the differences were not statistically significant. In an attempt to obtain maximal recovery of *V. cholerae* from diarrhea patients in the course of an oral cholera vaccine trial, we performed direct culture of the rectal swabs onto TCBS agar plates in conjunction with APW enrichment medium incubated for both 6 h and 24 h, followed by subculture to TCBS agar. The results of this study are reported herein.

MATERIALS AND METHODS

Specimen collection and bacteriology. From January 1994 through December 1995 rectal swabs were obtained from patients with watery diarrhea seen at any one of four participating hospitals in North Jakarta, Indonesia, a densely populated area where cholera is endemic. Regardless of the severity of illness (mild, moderate, or severe), participating hospital staff were instructed to obtain two rectal swabs from each patient. The two swabs were regarded as one sample. All swabs were immediately placed in Cary-Blair medium and held at room temperature until the end of the normal working day, at which time they were trans-

ported to the U.S. Naval Medical Research Unit No. 2, Jakarta. The specimens were held overnight at room temperature and processed the next morning. Thus, all rectal swabs were cultured within 24 h of collection. The two swabs from each patient were streaked directly onto the surface of a TCBS (DIR-TCBS) medium (Difco Laboratories, Detroit, Mich.) and thereafter placed in APW enrichment broth and incubated at $36 \pm 1^\circ\text{C}$. Enrichment broths were subcultured to TCBS after 6 h (APW6h-TCBS) and 24 h (APW24h-TCBS) of incubation. All TCBS plates were incubated at $36 \pm 1^\circ\text{C}$ for 18 to 20 h. Colonies resembling those of *V. cholerae* were picked and identified by conventional biochemical and serological methods (7, 10). *V. cholerae* isolates were motile; produced an alkaline slant over an acid butt on Kligler's iron agar; were oxidase, sucrose, indole, ornithine, and lysine positive; and were arginine negative. Isolates agglutinating in *V. cholerae* O1 polyvalent antiserum were further serologically characterized with Inaba and Ogawa specific antisera.

Data analysis. Significant differences in isolation rates for individual and combined culture procedures were determined by chi-square comparisons of the proportions of positive samples. The software package EpiInfo version 6 (Centers for Disease Control and Prevention) was used for statistical calculations.

RESULTS

A total of 16,034 rectal swab samples were obtained from patients during the course of the study. *V. cholerae* O1 biotype El Tor was recovered from 2,932 (18.3%) of the swab samples cultured. The overwhelming majority of these isolates (2,908 or 99.2%) were of the Ogawa serotype. The Inaba serotype was found in only 18 samples (0.6%), and the remaining 6 positive samples (0.2%) yielded mixed or multiple serotypes of Inaba and Ogawa. In only two of these six cases were Ogawa and Inaba serotypes identified on the same subculture TCBS plate. The isolation of multiple serotypes may be dependent on the use of multiple culture procedures, i.e., DIR-TCBS, APW6h-TCBS, and APW24h-TCBS. *V. cholerae* non-O1 was isolated from 850 (5.3%) samples, 179 (21.1%) of which were from patients who were also positive for *V. cholerae* O1 biotype El Tor.

The isolation frequencies of *V. cholerae* O1 and non-O1 for DIR-TCBS, APW6h-TCBS, and APW24h-TCBS and their combinations are shown in Table 1. The maximum number of rectal swab samples positive for *V. cholerae* O1 biotype El Tor by any method was 2,932 (18.3%). Although there was no

* Corresponding author. Mailing address: U.S. Naval Medical Research Unit No. 2, Jakarta, Box 3 APO AP 96520-8132. Phone: (62-21) 421-4458. Fax: (62-21) 424-4507.

TABLE 1. Results of three culture systems and their combinations in the isolation of *V. cholerae* O1 and non-O1

Culture system	No. of positive <i>V. cholerae</i> isolates (%) ^a	
	O1	Non-O1
DIR-TCBS	2,671 (16.7)	443 (2.8)
APW6h-TCBS	2,771 (17.3)	610 (3.8) ^c
APW24h-TCBS	2,706 (16.9)	641 (4.0) ^c
DIR-TCBS + APW6h-TCBS	2,845 (17.7) ^b	685 (4.3) ^c
DIR-TCBS + APW24h-TCBS	2,874 (17.9) ^b	741 (4.6) ^c
APW6h-TCBS + APW24h-TCBS	2,891 (18.0) ^b	801 (5.0) ^c
DIR-TCBS + APW6h-TCBS + APW24h-TCBS	2,932 (18.3) ^b	850 (5.3) ^c

^a From 16,034 rectal swab samples.

^b Significantly greater than DIR-TCBS alone ($P < 0.02$).

^c Significantly greater than DIR-TCBS alone ($P < 0.0001$).

significant difference in *V. cholerae* O1 isolation rate between the DIR-TCBS plus APW6h-TCBS and DIR-TCBS plus APW24h-TCBS methods, either combination was significantly better ($P < 0.02$) than DIR-TCBS alone. Also, the combination of APW6h-TCBS and APW24h-TCBS did not yield significantly more ($P > 0.48$) *V. cholerae* O1 isolates than either enrichment method in combination with DIR-TCBS. The combination of all three procedures (DIR-TCBS, APW6h-TCBS, and APW24h-TCBS) was not significantly better ($P > 0.16$) than any two procedures in combination.

The efficacy of APW enrichment was more pronounced for non-O1 *V. cholerae* than for the O1 strains, although again the length of enrichment incubation (6 h versus 24 h) was not significant ($P > 0.18$). Either enrichment procedure, alone or in combination with DIR-TCBS, yielded significantly more ($P < 0.0001$) *V. cholerae* non-O1 isolates than DIR-TCBS alone. In contrast to the isolation of the O1 strains, however, DIR-TCBS yielded a large proportion (5.8%) of non-O1 isolates that were not recovered from enrichment broth culture (Table 2). Also, although APW6h-TCBS and APW24h-TCBS yielded similar numbers of non-O1 *V. cholerae* isolates, 32.2% of these were uniquely detected by only one of the two enrichment procedures (12.8% by APW6h-TCBS and 19.4% by APW24h-TCBS). From Table 2 it can also be seen that 84.2% of the *V. cholerae* O1 isolates were detected by all three of the culture procedures employed compared to only 37.3% of the non-O1 isolates.

DISCUSSION

The results of this investigation allow us to conclude with confidence that 6-h APW enrichment offers no significant ad-

TABLE 2. Test results of three culture systems in isolating *V. cholerae* O1 and non-O1 from rectal swabs

Culture result			No. of isolates/% of total	
DIR-TCBS	APW6h-TCBS	APW24h-TCBS	O1 ($n = 2,932$)	Non-O1 ($n = 850$)
+	+	+	2,470 (84.2)	317 (37.3)
+	+	-	127 (4.3)	51 (6.0)
+	-	+	33 (1.1)	26 (3.1)
+	-	-	41 (1.4)	49 (5.8)
-	+	+	116 (4.0)	133 (15.6)
-	+	-	58 (2.0)	109 (12.8)
-	-	+	87 (3.0)	165 (19.4)

vantage over 24-h APW enrichment for the isolation of *V. cholerae* O1 when the two methods are compared either as single procedures or as combined with direct plating of stool swabs on TCBS media. Also, in contrast to the opinion that the value of enrichment broth cultures may be limited to convalescing and/or antibiotic-treated patients who excrete *V. cholerae* in reduced numbers (5, 6, 12), we found that APW enrichment (for either 6 h or 24 h) significantly improves the recovery of *V. cholerae* from the acute cholera patient. Our results here were in agreement with those reported previously (9) which evaluated the use of APW enrichment for isolating *V. cholerae* and demonstrated that the combination of enrichment and direct TCBS plating was superior to direct plating alone. These findings are of more than just academic interest. In large-scale cholera vaccine efficacy trials, such as the one that provided the samples for this study, it is critical that all vaccinees who develop cholera be detected. At the same time, however, because of the large number of samples to be processed, it is important that laboratory protocols be efficient with regard both to technician time and to the supplies required. The APW6h-TCBS procedure is difficult to schedule into the normal laboratory workday since specimens arriving later in the day must be subcultured long after normal working hours. Conversely, the APW24h-TCBS procedure can be readily incorporated into most laboratory routines because specimens inoculated on one working day can be subcultured to TCBS during the following normal working day. Since the APW6h-TCBS procedure has little if any added value, research and clinical laboratories can confidently rely on a single 24-h APW enrichment in addition to direct plating on TCBS for detecting *V. cholerae* present in stool samples to save both technician time and laboratory supplies. Because the current study dealt solely with stool swabs transported in Cary-Blair media, the importance of 6-h APW enrichment of fresh stool with regard to the isolation of *V. cholerae* remains undetermined. Other investigators have expressed concern that APW enrichment in excess of 6 to 8 h may promote overgrowth by competing organisms (2, 4, 7, 8). We found that after 24 h of enrichment, although nonvibrio colonies, particularly *Proteus* sp., frequently outnumbered the vibrios, *V. cholerae* isolates were numerous and could easily be picked for biochemical and serological confirmation. This observation is supported by that of Barua (3), who reported no significant reduction in vibrio isolation when known positive samples were held in APW for 14 days at 22 to 27°C. A similar result was reported by Pazzaglia et al. (11), who described improved recovery of *V. cholerae* from Moore swabs APW-enriched for 12 to 18 h at ambient temperature, and by Spira and Ahmed (13), who incubated enrichment broths at 37°C and found that 18-h enrichments were superior to 6-h enrichments. Although the superiority of these 12-h and 18-h incubations over 6-h APW enrichment appears to conflict with the results of the present study, it should be noted that both of these investigations were limited entirely to the analyses of contaminated surface and sewer waters. It is likely that these samples contained fewer "healthy" *V. cholerae* organisms than the rectal swabs examined by us, and thus, longer enrichment incubation may have improved their chances of being recovered on TCBS agar.

As our results clearly show, the observation that approximately 6% of the non-O1 isolates were recovered from direct TCBS plating alone and that only 37% were detected by all three culture procedures emphasizes the diversity of this somewhat arbitrary grouping of potential pathogens. While for convenience we think of them as a related class of vibrios, on the basis of serotype alone they can be separated into 138 identifiable strains. The virulence and demonstrated epidemic po-

tential of serotype O139 dramatically highlight the extent to which one non-O1 serotype can differ from another. With this in mind, it is not surprising that the various conditions employed for their isolation selectively enhance or inhibit their recovery. The public health significance of this observation is difficult to assess. While in general non-O1 strains are not important causes of epidemic cholera, the emergence and spread of *V. cholerae* O139 since it was first reported in 1992 (1) have prompted a new respect for this class of low-level pathogens, historically and somewhat disparagingly referred to as "Nags" (non-O1 agglutinating). Since O139 strains have not been reported in Indonesia, the importance of 6-h versus 24-h APW enrichment for O139 isolation could not be evaluated. Nonetheless, based on our findings with regard to culture-technique-specific non-O1 isolation, it would be prudent for public health and research laboratories specifically interested in screening stool samples for potentially virulent non-O1 *V. cholerae* strains to employ a variety of culture media, enrichments, and incubation conditions.

Based on the results of this investigation we recommend the use of 24-h APW enrichment broth culture, in addition to direct plating on TCBS media, for the isolation of *V. cholerae* from acutely ill diarrhea patients when sensitivity of detection is critically important, namely, in the conduct of cholera vaccine efficacy trials. For investigations where non-O1 *V. cholerae* is of particular interest, we recommend 6-h and 24-h APW enrichment in addition to direct plating on TCBS. However, for routine isolation of *V. cholerae* from acutely ill patients, direct plating on TCBS provides adequate and cost-effective culture results within 24 to 48 h.

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