



Alkaline peptone water plus 0.5% agar suitable for transport of *Vibrio cholerae*

Meiyanti*, Oktavianus Ch. Salim**, Julius E. Surjawidjaja***, and Murad Lesmana***

ABSTRACT

Microbiological procedures for *V. cholerae* isolation from clinical specimens are important factors in clinical and epidemiological management of cholera. The standard preservation medium for enteric pathogenic bacteria, including *V. cholerae*, is Cary-Blair medium (CB), a semisolid medium for preservation and transport of specimens containing intestinal bacteria. A special medium for *Vibrio* organisms is alkaline peptone water (APW), which is both a transport and an enrichment medium. The purpose of this study was to ascertain the suitability of APW supplemented with 0.5% agar (APW-0.5) as a sensitive preservation-transport medium for rectal swab specimens for isolation of *V. cholerae*. A total of 144 paired rectal swab specimens were collected from children and adults with acute diarrhea. Of each specimen pair, one was placed in CB and the other in APW-0.5, from which they were plated out to thiosulfate citrate bile sucrose (TCBS) agar. Altogether, from both CB and APW-0.5 transported specimens, *V. cholerae* non-O1 was present in 29 (20.1%) specimens, while only 2 (1.4%) specimens were positive in CB and 9 (6.3%) positive in APW-0.5 transported specimens. The number of *V. cholerae* non-O1 isolates from APW-0.5 transported specimens was significantly higher ($p=0.000$) as compared to that from Cary-Blair transported specimens. It may be concluded that for isolation of *V. cholerae*, specimen transport in APW-0.5 medium was more effective than transport in Cary-Blair medium.

*Department of Pharmacology,
**Department of Public Health,
***Department of
Microbiology, Medical Faculty,
Trisakti University
Jakarta

Correspondence
dr. Meiyanti, SpFK
Department of Pharmacology,
Medical Faculty,
Trisakti University
Jl. Kyai Tapa No.260
Grogol - Jakarta 11440
Phone. 021-5672731 ext.2805

Univ Med 2011;30:95-101.

Keywords: Alkaline peptone water, agar, *Vibrio cholerae*, rectal, swab

INTRODUCTION

Cholera is an acute diarrheal disease caused by serogroups O1 and O139 of *V. cholerae*. In its severest form the disease is characterized by profuse diarrhea with rice water stools, rapidly leading to dehydration.

Cholera has two special epidemiological features, namely its tendency to cause explosive epidemics, frequently at several foci simultaneously, and its capability for causing pandemics that progressively involve numerous parts of the world, as has been the case up to the present.⁽¹⁻³⁾

In several countries this disease has become endemic, from time to time causing epidemics.^(1,4-6) It is estimated that around 5.5 million cholera cases occur annually in Asia and Africa, 8% of these being of such severity as to need hospitalization, with 20% of these hospitalized cases terminating fatally, giving rise to a total annual mortality of 120,000.⁽⁷⁾ Consequently cholera is still considered to be a serious health problem in many developing countries with poor socio-economic conditions.^(8,9) Cholera surveys are still important instruments for determining behavioral trends of the disease in many areas around the globe and within a given country, in view of the fact that the majority of the reported numbers of cases are underestimates.^(8,9)

Among the enteric pathogenic bacteria *V. cholerae* is probably the easiest organism to identify, for which however a laboratory infrastructure is required. The accuracy of laboratory investigations, including the efficient use of material such as culture media,^(10,11) has a considerable impact on the exactness of bacterial identification and the ensuing accuracy of the report. Although treatment of cholera cases is not based on prior identification of the causal organism, the microbiological procedures for the isolation of *V. cholerae* organisms from clinical specimens are important factors determining the clinical and epidemiological management of cholera.⁽⁷⁾ The specimens, either from stools or rectal swabs, are collected at the earliest opportunity or at onset of the disease, prior to administration of antibiotics. If the specimens cannot be processed within two hours, they are placed in preservation media.^(7,12) The most common preservation medium used for enteric pathogenic bacteria, including *V. cholerae*, is Cary-Blair medium, which is a semisolid medium for preservation and transport of specimens containing intestinal bacteria. A special medium for *Vibrio* organisms is alkaline peptone water (APW), which is both a transport and an enrichment medium.^(7,12) However, APW is almost never used for transport of *Vibrio*

organisms, due to concern that longtime storage of specimens in APW at room temperature encourages overgrowth by organisms such as *Pseudomonas*, *Proteus* and *Vibrio* non-O1, which are capable of inhibiting growth of *V. cholerae*.^(7,11) In contrast, there are reports stating that specimens enriched in APW and incubated for more than 20 hours did not show suppression of *Vibrio* isolation rates by growth of commensals.⁽⁷⁾ As a consequence, APW may presumably be used for transport of *V. cholerae* within a period of 20-24 hours, yielding a substantially high sensitivity of isolation, due to the enrichment features of APW. Concern that APW in its liquid form is subject to spillage, resulting in contamination and infection by specimens containing *V. cholerae*, may be countered by the addition of 0.5% agar, thus transforming APW into a semi-solid medium. The purpose of this study was to ascertain the suitability of APW supplemented with 0.5% agar (APW-0.5) as a safe and sensitive preservation-transport medium for rectal swab specimens for isolation of *V. cholerae*.

METHODS

Study population

Subjects recruited for this study were patients with diarrhea attending the Mampang Prapatan primary health center in South Jakarta. The subjects were categorized as having diarrhea on the basis of a self-reported frequency of defecation of 3 or more times within 24 hours, with watery/liquid/soft stools.^(8,10) Before collection of the fecal specimens, the subjects were informed on the purpose of the study and were requested to sign a consent form for participation in the study. In the case of children, the consent form would be signed by their caregivers. Subsequently a clinical questionnaire was to be filled out by research personnel with data obtained by interviewing the subjects, comprising data on age, gender, duration of diarrhea, type of diarrhea, and clinical symptoms.

Preservation and transport medium

Standard Cary-Blair medium was prepared according to the manufacturer's instructions, while APW supplemented with 0.5% agar (APW-0.5) was prepared by adding 10 g peptone, 10 g sodium chloride, and 5 g agar to 1 L of distilled water (pH 8.4).

Specimen collection and transport

The stool or rectal swab specimens were collected prior to administration of antibiotics. Rectal swabs were obtained by means of cotton swabs mounted on sticks. For subject comfort, the cotton swabs were wetted in transport medium before rectal insertion. To obtain a rectal specimen, the cotton swab was inserted past the anal sphincter (around 2-3 cm), slowly rotated and withdrawn, and immediately placed in transport medium. From each patient one pair of rectal swabs was taken, one of the pair being placed in Cary-Blair medium and the other in APW-0.5 medium. The specimens were then stored at room temperature prior to being sent to the Microbiology Laboratory of the Medical Faculty, Trisakti University. In the case of patients with prior antibiotic therapy, this fact was recorded in the questionnaire for consideration in the subsequent data analysis.

Bacteriological procedure

On arrival at the laboratory the rectal swab specimens in Cary-Blair and APW-0.5 were immediately plated out on thiosulfate citrate bile salts sucrose (TCBS) agar, a selective medium for *Vibrio* organisms. The TCBS plates were then incubated aerobically at 37°C for 18-20 hours. Bacterial growth resembling *Vibrio* colonies were picked and processed according

to the following standard procedures for identification of *V. Cholerae*,^(8,12) viz. oxidase test, biochemical reactions (comprising Kligler's iron agar, sucrose, semisolid, motility, indole, ornithine, lysine, arginine), and serological tests with specific antiserum for determination of *V. cholerae* serotypes.

Statistical analysis

The significance of the difference in transport media was assessed by comparing the proportions of positive specimens. A p value of <0.05 was considered to indicate statistical significance. Computations were performed by means of Epi Info version 3.5.1.

RESULTS

Within a period of 10 months a total of 144 paired rectal swab specimens were collected from children and adults with acute diarrhea. From these paired specimens, *Vibrio* species were isolated from 44 (30.6%) rectal swab specimen pairs, comprising 40 isolates (90.9%) of *V. cholerae* non-O1 and 4 isolates (9.1%) of *V. parahaemolyticus*, without any isolates of *V. cholerae* O1 organisms being found in this study. There were therefore 100 (69.4%) paired rectal swab specimens with negative results, yielding no isolates of *Vibrio* species (Table 1).

Among the rectal swab specimens in Cary-Blair medium, *V. cholerae* non-O1 organisms were present in 21.5% (31/144) specimens and *V. parahaemolyticus* in 2.8% (4/144) specimens. In comparison, the rectal swab specimens transported in APW-0.5 yielded the following results: *V. cholerae* non-O1 was present in 26.4% (38/144) specimens, while the frequency

Table 1. Number of isolates of *V. cholera* non-O1 and *V. parahaemolyticus* recovered from 144 patients with diarrhea

Number of isolates (%)	N	Bacterial isolate	
		<i>V. cholerae</i> non-O1	<i>V. parahaemolyticus</i>
Positive	44	40 (90.9%)	4 (9.1%)
Negative	100	-	-

Table 2. Distribution of culture results of *V. cholerae* non-O1 and *V. parahaemolyticus* from 144 rectal swab specimens in Cary-Blair (CB) vs. alkaline peptone water (APW) + 0.5% agar

Culture results		<i>V. cholerae</i> non-O1		<i>V. parahaemolyticus</i>	
CB	APW+0.5%	Number	%	Number	%
Positive	Positive	29	20.1	4	2.8
Positive	Negative	2	1.4	0	0
Negative	Positive	9	6.3	0	0
Negative	Negative	104	72.2	140	97.2

of *V. parahaemolyticus* isolates was identical to the Cary-Blair isolates (Table 2). Table 2 also shows that 29 (20.1%) specimen pairs transported in Cary-Blair and APW-0.5 yielded positive results for *V. cholerae* non-O1, while only 2 (1.4%) rectal swab specimens were positive in Cary-Blair and 9 (6.3%) positive in APW-0.5 transported specimens. Results of McNemar's test indicated a significant difference between number of isolates of *V. cholerae* in Cary-Blair and that in alkaline peptone water ($p < 0.005$). Isolates of *V. cholerae* in APW ($9/40 = 22.5\%$) were substantially higher in number than isolates in CB ($2/40 = 5\%$) (Table 3).

DISCUSSION

The specimens used for isolation of *V. cholerae* are stools or rectal swabs, collected at the earliest opportunity or at onset of the disease, prior to administration of antibiotics, as the numbers of *V. cholerae* organisms in stools decline immediately upon antibiotic administration.⁽⁸⁾ The specimens should preferably not be collected from bedpans,

because they may have been contaminated by previous use or may contain traces of disinfectants after cleaning, leading to false positive or false negative results. For collecting stools, sterile stool cups may be used. Rectal swab specimens give as good results as stools in the isolation of *V. cholerae*, particularly in the acute phase of the disease.⁽⁵⁾ In addition, rectal swabs specimens are superior to stools for infants and field surveys, but are inadequate for convalescents or contacts.⁽⁷⁾

Incubation for 6-8 hours is the method recommended for APW,⁽⁵⁾ and is used in the majority of laboratories.^(8,13,14) In the initial 6-8 hours of incubation, *Vibrio* organisms show a rapid growth, but more than 8 hours of incubation is considered to result in growth of competing bacteria and inhibition of *Vibrio* organisms, impeding isolation of the latter. However, recent research points to the contrary, in that enrichment in APW for 24 hours, although promoting the growth of nonvibrio organisms such as *Proteus* to greater numbers than *Vibrio* organisms, yet did not decrease the growth of *V. Cholerae*.⁽⁴⁾ In the present study it was found that after 24 hours there was a large number of both non-vibrio and *Vibrio* colonies on the culture plates, the latter thus being easily picked and identified. Enrichment in APW for 6-8 hours does not yield significantly better results in comparison with enrichment in APW for 24 hours, with regard to capability for isolation of *V. cholerae*.⁽¹⁵⁾ It may be concluded that APW is superior as to frequency of isolation of *Vibrio* species, when compared to Cary-Blair medium.

Table 3. Number of isolates of *V. cholerae* non-O1 in Cary-Blair (CB) and alkaline peptone water (APW) + 0.5% agar

CB	APW	
	+	-
+	29	2
-	9	104

This is presumably because APW is both an enrichment and a transport medium. In its original form, APW is a liquid medium. Supplementation with 0.5% agar minimizes spillage or leakage, thus preventing environmental contamination by enteric pathogens. However, the results of transport of *V. cholerae* in agar-supplemented APW for a period of more than 24 hours cannot as yet be predicted. There is a possibility that after 48 hours the commensal organisms start to reproduce rapidly, thus inhibiting *V. cholerae*, ultimately leading to negative isolation results for *V. cholerae*.⁽¹²⁾ In that case, specimen transit times in APW for days or even for more than one week, as is common with clinical specimens in Cary-Blair medium, will cause APW to be ineffective as transport medium for *V. cholerae*. In Cary-Blair medium *Vibrio* organisms remain viable for 4 weeks, without any possibility of inhibition by excessive growth of commensal organisms.⁽¹²⁾ According to this line of reasoning, Cary-Blair medium is to be preferred for transport of stool or rectal swab specimens for isolation of enteric pathogenic bacteria, including *V. cholerae*, whereas enrichment-transport media are to be used only when the field-collected specimens can be cultured within 12-24 hours after collection.^(7,12) If it can be demonstrated that specimen transit times in enrichment-transport media of more than 48 hours or even more than one week does not impair the viability of enteric pathogenic bacteria such as *Vibrio* organisms, use of such media for preservation and transport of *Vibrio* organisms may be taken into consideration.

The present study succeeded only in isolating *V. cholerae* non-O1 at a frequency of 27.8%, whereas no *V. cholerae* O1 were found. This may be due to the drastic decline in the *V. cholerae* O1 population in Indonesia since 2000, from 10.5% - 18.3% in the period of 1993-1998⁽¹⁶⁾ to < 2% after the year 2000,⁽¹⁷⁾ for unknown reasons. *V. cholerae* O1 could probably be isolated with a larger sample.

However, because *V. cholerae* O1 and *V. cholerae* non-O1 have identical culture characteristics, the results on the viability of *V. cholerae* non-O1 in Cary-Blair and APW may be assumed to refer also to *V. cholerae* O1. Both strains may be said to differ only in serology, clinical picture, and epidemiology. Serologically, *V. cholerae* non-O1 does not show agglutination with anti-O1 antiserum.⁽¹²⁾ With respect to clinical manifestations, *Vibrio* O1 causes profuse diarrhea, while *V. cholerae* non-O1 causes a milder diarrhea, but tends to result in extra-intestinal infections and bacteriemia.^(7,19) Epidemiologically, *V. cholerae* O1 frequently causes epidemics, whereas *V. cholerae* non-O1 is sporadic and non-epidemic in nature.⁽¹⁸⁾

Reports on evaluations of APW as an enrichment and transport medium for *V. cholerae* have never been encountered after the year 1997,⁽¹⁵⁾ making it difficult to obtain descriptions of more recent studies on APW. In spite of this, there have presumably been few changes since the last published reports on this topic. However, the discovery of a new variant of *V. cholerae* O1 that is a hybrid of the classic and El Tor biotypes⁽²⁰⁻²³⁾ poses a new threat for the occurrence of epidemics of pandemic proportions. Therefore, there should be increased efforts at maximal isolation of *V. cholerae*, particularly *V. cholerae* O1, using various culture and transport systems.


The efficacy of enrichment in APW is reportedly higher for *V. cholerae* non-O1 as compared with O1 strains. Enrichment in APW results in significantly higher yields of *V. cholerae* non-O1 isolates, in comparison with direct plating.⁽¹⁵⁾ Our study results are similar to those of previous studies on the evaluation of enrichment in APW for isolation of *V. cholerae*, demonstrating the superiority of enrichment to direct plating. Consequently, enrichment media with features of transport media, such as APW, have a higher effectivity and sensitivity for isolation of *Vibrio* organisms.

One limitation of the present study is the relatively short transport time of the specimens in APW (less than 48 hours), which cannot describe the ultimate fate of commensal organisms in the APW. Further long-term studies are necessary to resolve this question.

CONCLUSION

For isolation of *V. cholerae*, specimen transport in APW-0.5 medium is significantly more effective than transport in Cary-Blair medium.

ACKNOWLEDGEMENTS

The investigators thank the Dean and Vice-Deans of the Medical Faculty, Trisakti University, for the funding of this study. Thanks are also due to the doctors and staff of the Mampang Prapatan primary health center, and to all assisting parties for the successful completion of this study. 

REFERENCES

1. Simanjuntak CH, Larasati W, Arjoso S. Cholera in Indonesia in 1993-1999. *Am J Trop Med Hyg* 2001;65:788-97.
2. Lesmana M. Recent developments of cholera infections. *J Kedokter Trisakti* 2004;23:101-9.
3. Salim A, Lan R, Reeves PR. *Vibrio cholerae* pathogenic clones. *Emerg Infect Dis* 2005;11:1758-60.
4. Nguyen BM, Lee JH, Cuong NT, Choi SY, Hien NT, Anh DD, et al. Cholera outbreaks caused by an altered *Vibrio cholerae* O1 El Tor biotype strain producing classical cholera toxin B in Vietnam in 2007 to 2008. *J Clin Microbiol* 2009;47:1568-71.
5. Krishna BV, Patil AB, Chandrasekhar MR. Fluoroquinolone-resistant *Vibrio cholerae* isolated during a cholera outbreak in India. *Trans R Soc Trop Med Hyg* 2006;100:224-6.
6. Theophilo GN, Rodrigues DP, Leal NC, Hofer E. Distribution of virulence markers in clinical and environmental *Vibrio cholerae* non-O1, non-O139 strains isolated in Brazil from 1991-2000. *Rev Inst Med Trop Sao Paulo* 2006;48:65-70.
7. Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. *Lancet* 2004;363:223-33.
8. Agtini MD, Soeharno R, Lesmana M, Punjabi NH, Simanjuntak C, Wangsasaputra F, et al. The burden of diarrhea, shigellosis, and cholera in North Jakarta, Indonesia: findings from 24 months surveillance. *BMC Infect Dis* 2005;17:341-50.
9. Kosek M, Bern C, Guerrant RL. The global burden of diarrhoeal disease, as estimated from studies published between 1992-2000. *Bull World Health Organ* 2003;81:197-204.
10. Thielman NM, Guerrant RL. Acute infectious diarrhea. *N Engl J Med* 2004;350:38-47.
11. World Health Organization. Cholera 2001. *Wkly Epidemiol Rec* 2002;77:257-68.
12. Farmer JJ III, Janda JM, Birkhead K. *Vibrio*. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Tenover FC, Tenover FC, editors. *Manual of Clinical Microbiology*. 8th ed. Washington DC: American Society for Microbiology;2003.p.706-18.
13. Bhuiyan NA, Qadri F, Faruque ASG, Malek MA, Salam MA, Nato F, et al. Use of dipstick for rapid diagnosis of cholera caused by *Vibrio cholerae* O1 and O139 from rectal swabs. *J Clin Microbiol* 2003;41:3939-41.
14. Tamrakar AK, Goel AK, Kamboj DV, Singh L. Surveillance methodology for *Vibrio cholerae* in environmental samples. *Int Environ Health Res* 2006;16:305-12.
15. Lesmana M, Richie E, Subekti D, Simanjuntak C, Walz SE. Comparison of direct plating and enrichment methods for isolation of *Vibrio cholerae* from diarrhea patients. *J Clin Microbiol* 1997;35:1856-8.
16. Lesmana M, Subekti DS, Tjaniadi P, Simanjuntak CH, Punjabi NH, Campbell JR, et al. Spectrum of *Vibrio* species associated with acute diarrhea in North Jakarta, Indonesia. *Diagn Microbiol Infect Dis* 2002;43:91-7.
17. Oyofe BA, Lesmana M, Subekti D, Tjaniadi P, Larasati W, Putri M, et al. Surveillance of bacterial pathogens of diarrhea disease in Indonesia. *Diagn Microbiol Infect Dis* 2002;44:227-34.
18. Cariri FAMO, Costa APR, Melo CC, Theophilo GND, Hofer E, de Melo Neto OP, et al. Characterization of potentially virulent non-O1/non-O139 *Vibrio cholerae* strains isolated from human patients. *Clin Microbiol Infect* 2010;16:62-7.
19. Dalsgaard A, Forslund A, Hesselbjerg A, Bruun B. Clinical manifestations and characterization of extra-intestinal *Vibrio cholerae* non-O1, non-O139 infections in Denmark. *Clin Microbiol Infect* 2000;6:626-8.

20. Pourshafie M, Grimont F, Kohestani F, Grimont PAD. A molecular and genotypic study of *Vibrio cholerae* in Iran. J Med Microbiol 2002;51:392-8.
21. Nair GB, Faruque SM, Bhuiyan NA, Kamruzzaman M, Siddique AK, Sack DA. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. J Clin Microbiol 2002;40:3296-9.
22. Lizarraga-Partida ML, Quilici ML. Molecular analyses of *Vibrio cholerae* O1 clinical strains, including new nontoxigenic variants isolated in Mexico during the cholera epidemic years between 1991-2000. J Clin Microbiol 2009;47:1362-71.
23. Nair GB, Qadri F, Holmgren J, Svennerholm AM, Safa A, Bhuiyan NA, et al. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. J Clin Microbiol 2006;44:4211-3.