A COMPARISON OF METHODS FOR THE ISOLATION OF *CAMPYLOBACTER* SPECIES FROM STOOL SPECIMENS

KARNJANA FONGSIRI, ORASA SUTHIENKUL, DAVID N. TAYLOR,*
SOMSRI SAIBORISUTH**, SUMALEE SRICHARMORN,***
SUCHIN KONGVISAISOOK** and PETER ECHEVERRIA*

Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok 10400, *Department of Bacteriology, Armed Forces Research Insitute of Medical Sciences, Bangkok 10400, **Department of Pathology, and ***Department of Paediatrics, Bamrasnaradura Infectious Disease Hospital, Nonthaburi, Thailand.

INTRODUCTION

Campylobacter have been recognized as an important cause of diarrheal illness in man. Isolation of *Campylobacter* from stools was first performed by filtration to eliminate normal enteric flora (Dekeyser *et al.*, 1972). Subsequently, incorporation of antimicrobial agents into the media and incubation at 42 ° C became the standard method to inhibit the background flora (Skirrow, 1977). The three most commonly used selective media are Skirrow's (Skirrow, 1977), Butzler's (Butzler and Skirrow, 1979), and Campy-BAP (Blaser *et al.*, 1979). These media also contain a rich basal media supplemented with sheep or horse blood. Although these selective methods have made the routine isolation of *Campylobacter jejuni* possible, it has been demonstrated that some campylobacters are sensitive to cephalothin or colistin contained in the selective media or sensitive to the incubation temperature at 42 ° C (Fennell *et al.*, 1984; Ng *et al.*, 1985; Steele *et al.*, 1985; Tee *et al.*, 1987). To resolve this problem, a simple membrane filter method was developed that did not require an antibiotic selective media or incubation at 42 ° C (Steele and McDermott, 1984).

Differences in isolation techniques may be one reason why clinical and public health laboratories report varying rates of isolation of this organism in cases of acute enteritis (Janssen and Helstad, 1982; Ohashi, 1982; Wang *et al.*, 1982; Wang *et al.*, 1983; Steele and McDermott, 1984; Taylor and Blaser, 1987). There are few comparative studies of selective and non-selective media methods for isolation of campylobacters (Goossens *et al.*, 1986; Taylor *et al.*, 1987)

To elucidate optimal methods, both in terms of cost and time economy, we studied eight different *Campylobacter* isolation methods using the membrane filter methods and conventional selective antibiotic media methods under different atmospheric and temperature conditions. Examination of stools by direct microscopy was also studied as a rapid screening method for *Campylobacter*.

MATERIALS AND METHODS

From May to August 1987, stool specimens were collected from 270 children under 5 years old with acute diarrhea attending the outpatient department and pediatric ward at

Bamrasnaradura Infectious Disease Hospital, Nonthaburi, Thailand. Stool specimens were processed for direct microscopy and cultured within 3 hours of collection.

For direct microscopic examination a small amount of fecal material was smeared onto a glass slide and stained with 1% basic fuchsin for 45 seconds for microscopic screening of *Campylobacter* morphological characteristics (Park *et al.*, 1983).

The entire microbiological procedure is outlined in Table 1. The membrane filter method was carried out as described by Steele and McDermott (1984); plates were prepared using Brucella agar (BBL Microbiology Systems, Cockeysville, Md) with 5% sheep blood without antibiotics (BA−SB). Using the membrane filter method each fecal specimen was cultured under 5 different incubation conditions; gas generating envelope (GasPak without catalyst, BBL Microbiology Systems, Cockeysville, Md) in anaerobic jar at 37 ° C and 42 ° C, candle jar at 37 ° C and 42 ° C, and in an anaerobic jar with gas mixture at 37 ° C (Table 1). Stool specimens were also cultured directly onto Campy−BAP−HB (using 5% human blood) and Campy−BAP−SB (using 5% sheep blood). Specimens were also inoculated into Campy-thio and kept at 4 ° C overnight (Blaser *et al.*, 1979) and cultured only on a Campy−BAP−HB plate. Plates from this conventional method were incubated at 42 ° C in an anaerobic jar evacuating and refilling with a mixture of 5% O_2 10% CO_2 and 85% N_2. All plates were incubated for 48 hours. *Campylobacter* isolates were identified into species by using hippurate hydrolysis (Lior, 1984) and other biochemical tests (Morris and Patton, 1985; Taylor and Blaser, 1987; Tee *et al.*, 1987).

Statistical methods: The McNemar test or matched pair analysis was used.

RESULTS

Isolation rates

By one or more culture methods, *Cam-*

Table 1

Description of methods used to isolate *Campylobacter* from human fecal specimens.

Culture method	Selective measure	Incubation		Cost per plate*
		temperature	atmosphere	
1	Membrane filter	37	gaspak	0.95
2	Membrane filter	42	gaspak	0.95
3	Membrane filter	37	candle jar	0.78
4	Membrane filter	42	candle jar	0.78
5	Membrane filter	37	gas mixture	0.79
6	Antibiotics + human blood	42	gas mixture	0.16
7**	Campy-thio -> Abc + human bld.	42	gas mixture	0.19
8	Antibiotics + sheep blood	42	gas mixture	0.26

* in $ U.S. not including cost of petri dish, jars, or gas tank

** placed into Campy-thio overnight before plating.

Abc = antibiotic; bld. = blood

pylobacter species were isolated from 30 (11%) of 270 children under 5 years old with diarrhea. Thirty-one *Campylobacter* species comprising 24 *C. jejuni*, three *C. coli*, and four atypical *Campylobacter* species were identified from 134 plates from 30 patients. Only 1 of 30 patients was infected with more than one *Campylobacter* species (*C. jejuni* and atypical *Campylobacter*). The biochemical reactions of the atypical *Campylobacter* strains are shown in Table 2. Strain K−19 was hippurate-negative, cephalothin-sensitive, and catalase-weakly positive. This strain resembled *C. upsaliensis* and was only isolated with the membrane filter method (Steele *et al.*, 1984; Tee *et al.*, 1987). Strain K−37 was also catalase-negative and was also only isolated with the membrane filter method. The other two strains were atypical *C. jejuni* strains. These two strains were also isolated on antibiotic containing media.

Campylobacter was isolated from 20 (11%) of 180 children less than 1 year old, 9 (17%) of 54 children 1−2 years old and from only one (3%) of 36 children over 2 years old. The youngest patient with *Campylobacter* enteritis was one month old. *Campylobacter* was isolated from 10% of 159 males and 13% of 111 females. The difference in isolation rates of *Campylobacter* between males and females was not significant ($P > 0.05$). The highest isolation rates were found in 12−23 months old females, which was higher than in males in the same age group

Table 2
Characteristics of 4 atypical *Campylobacter* strains from Thai children under 5 years old, 1987.

Characteristics	Atypical *Campylobacter* strains			
	K−19	K−37	K−79	K−138
Growth at 25 ° C	−	−	−	−
42 ° C	W	+	+	+
Hippurate hydrolysis	−	+	+	+
DNA hydrolysis	±	+	+	+
Susceptibility to:				
Nalidixic acid (30 ug)	S	S	S	S
Cephalothin (30 ug)	S	R	R	R
Oxidase	+	+	+	+
Catalase production	W	−	+	W
Nitrate reduction	+	+	−	+
H_2S in TSI[a]	−	−	−	−
H_2S on lead acetate paper	+	+	+	+
0.1% TMAO[b]	W	−	−	−
0.04% TTC	+	+	+	+

+ = positive, − = negative, W = weak, ± = narrow hazy zone
S = susceptible, R = resistant
[a] = Fresh (≤ 3-day-old) Triple Sugar Iron agar
[b] = Anaerobic growth in trimethylamine N-oxide hydrochloride
TTC = Triphenyltetrazolium chloride

(29% vs 9%).

Isolation methods

The isolation rates ranged from 4 – 8% for any single method (Table 3). The isolation rates of *Campylobacter* spp. using membrane filter method was 71% when incubated at 37 ° C (method 1) and 68% when incubated at 42 ° C (method 2). These isolation rates were significantly higher than was obtained using a candle jar at 37 ° C (method 3) (42%; $P < 0.05$) or by using the enrichment method (method 7) (36%; $P < 0.05$). There were no differences among positivity rates of *Campylobacter* spp. when methods 1, 2 were compared with methods 4, 5, 6 and 8, ($P > 0.05$). Eighteen specimens were positive for *Campylobacter* from only direct Campy–BAP using sheep blood (method 8), but this was not significantly different from method 6 using human blood.

C. jejuni and 2 of 4 atypical *Campylobacter* that resembled *C. jejuni* were isolated using either selective or non-selective media. Two other atypical strains could only be isolated on non-selective media at 37 ° C. None of 3 *C. coli* were isolated after incubation in candle jars. The best method only identified 71% of the total positive methods. The best combination was the membrane filter incubated at 37 ° C (method 1) and the standard antibiotic containing method incubated at 42 ° C (method 8). Method 8 was positive in 6 to 9 that were negative by method 1 so that 90% of the positive were identified when these two methods were combined.

Comparison of direct smear and culture

Vibrio-like organisms were identified by direct basic fuchsin stained fecal smear in 24 of 30 children who has *Campylobacter* positive cultures. The direct smear was negative in 6 children who had positive cul-

Table 3

Comparison of results using 8 isolation methods for *Campylobacter* including their cost effectiveness.

Culture method	*C. jejuni* n = 24[a]	Atypical *Campylobacter* n = 4	*C. coli* n = 3	Total n = 31[b]	Cost per positive culture ($U.S.)
1	16	4	2	22 (71)[c]	11.66[d]
2	15	4	2	21 (68)	12.21
3	12	1	0	13 (42)	16.20
4	13	3	0	16 (52)	13.16
5	15	2	1	18 (58)	11.85
6	12	2	1	15 (48)	2.88
7	10	0	1	11 (36)	4.66
8	15	2	1	18 (58)	3.90

[a] isolates from all methods combined.
[b] 31 *Campylobacter* species isolated from 30 children.
[c] percentage of total *Campylobacter* isolates.
[d] cost per plate X 270 cultures ÷ no. of positive cultures.

tures and positive in 7 children who had negative cultures. Both the direct smear and the culture were negative in 233 children. Compared to culture, the direct smear was 80% sensitive and 97% specific. The predictive value of positive and negative results of the test were 77% and 87%, respectively.

Comparison of cost of isolation methods

The price of the various isolation methods is compared in Table 1. The membrane filters which cost $U.S. 0.58 and the GasPak which cost $0.17 per plate in Thailand (assuming 12 plates were incubated with 1 GasPak) increase the cost of isolation considerably. The cost per positive culture is reported in Table 3. The most cost effective method was method 6 which used antibiotic containing media and human blood. This method was approximately 4 times cheaper than the best membrane filter method.

DISCUSSION

In Thailand, the isolation rates of *Campylobacter* ranged from 4–18% in children with diarrhea (Lexomboon *et al.*, 1981; Supavej *et al.*, 1982; Wankijcharoen *et al.*, 1983; Simasathien *et al.*, 1984; Taylor and Blaser, 1987; Taylor *et al.*, 1988). In this study *Campylobacter* species were isolated from 11% of children with diarrhea when multiple isolation methods were used. This isolation rate was lower than that found in children of the same age in the study by Taylor *et al* (1988). This might be due to the use of an enrichment method in the previous study or due to seasonal differences in isolation rate. Since there is very little seasonal variation in isolation rates of *Campylobacter*, the use of enrichment media is the best explanation.

King (1957) previously reported that related vibrios (now *C. jejuni*) grew best in a microaerobic atomosphere and at 42 ° C. This required laboratories to use a 42 ° C incubator. This disadvantage led to many studies of normal incubation temperature (37 ° C) with various atmospheric conditions (Blaser *et al.*, 1979; Wang *et al.*, 1983; Steele and McDermott, 1984). It was shown that candle jars at 37 ° C resulted in a low isolation rate and were not suitable for isolation of *C. jejuni* from human stools. The present study confirms the suboptimal isolation rate with candle jars even when incubated at 42 ° C. The membrane filter method did not sufficiently increase the isolation rate from candle jar to make this method cost effective. Alternatively, *Campylobacter* could be detected in high rates at 37 ° C on media without antibiotics by using membrane filter method as described by Steele and McDermott (1984).

The price of gas generating envelopes is much higher than the price of a gas mixture. An atmosphere produced by gas mixture are normally employed in all laboratories with conventional methods using antibiotic containing media. The isolation rate from media without antibiotics incubated at 37 ° C under gas mixture conditions (method 5) was the same rate as direct plating on antibiotic containing media (method 8). Media without antibiotics and incubation temperature at 37 ° C could be used instead of media with antibiotics temperature at 42 ° C.

The use of antibiotic-containing media for the isolation of *C. jejuni* are effective (Skirrow, 1977; Blaser *et al.*, 1979; Butzler and Skirrow, 1979). Such media, however, are more difficult to prepare than ordinary blood agar, and errors in the quantity of inhibitory antibiotics being added could adversely affect the performance of the medium (Steele and McDermott, 1984). In this

study 4 (13%) of 31 *Campylobacter* isolates had atypical microbiologic characteristics. The conventional method using antibiotics incorporated in the media failed to detect 2 of 4 atypical *Campylobacter* strains. Enrichment might be beneficial with stool specimens under some condition, e.g. those delayed in transit or held at ambient temperature too long, those contained organism in low numbers from carriers, convalescent cases, patients who had been treated with antibiotics, or food and water samples (Morris and Patton, 1985). Doyle's enrichment have been reported to be useful to increase the isolation rate especially in carriers (Doyle and Roman, 1982; Taylor *et al.*, 1988).

Comparison of various methods to isolate *Campylobacter* in this study showed that membrane filter method at 37 ° C and at 42 ° C could detect *Campylobacter* in the highest frequency. However, some *Campylobacter* species have been reported to be inhibited by incubation at 42 ° C (Fennell *et al.*, 1984; Ng *et al.*, 1985; Steele *et al.*, 1985; Tee *et al.*, 1987). The advantages of membrane filter method are: (1) nearly total elimination other enteric bacteria, (2) high rates of detection, (3) a relatively large amount of specimen could be inoculated onto the plates, (4) detection of *Campylobacter* species sensitive to antibiotics or 42 ° C incubation temperature, and (5) simple to prepare and require less quality control than a medium containing several antibiotics. The disadvantage of the filter method is that its cost is several times higher than the conventional method even when the higher isolation rate is taken into account. If a less expensive filter could be obtained the filter method would be the method of choice for *Campylobacter*. In this study a 0.45 μ pore size filter was used. Bolton *et al.* (1987) have found substantial increases in isolation rates using a 0.65 μ pore size filter. Thus use of the larger pore size filter may also increase the cost effectiveness of this method. While the filter method was the single best method for *Campylobacter* isolation from human stool, its combination with the standard method led to a substantial increase in isolation rate.

SUMMARY

Eight methods used for isolation of *Campylobacter* species were compared. Using a combination of methods *Campylobacter* species were isolated from 30 (11%) of 270 children with diarrhea seen at Bamrasnaradura Infectious Disease Hospital, Nonthaburi, Thailand. The membrane filter method using a gas generating envelope at 37 ° C identified 73% of the total positive specimens and was found to be the best isolation method for *Campylobacter* species from stool specimens. This method identified two strains that failed to grow on antibiotic containing media, and also gave a higher isolation rate of *C. jejuni* than could be isolated with conventional methods. The combination of the membrane filter method and a selective antibiotic method identified 90% of all isolates. At present the cost of the membrane filter method is higher than other methods. Therefore, the selective antibiotic method (Campy-BAP) with sheep blood under gas mixture at 42 ° C is recommended for laboratories with limited supplies.

Diagnosis by direct smear with 1% basic fuchsin revealed high degree of sensitivity and specificity. This rapid, inexpensive, and simple method could be used to make a presumptive diagnosis of *Campylobacter* enteritis when isolation methods are unavailable.

ACKNOWLEDGEMENTS

The authors acknowledge with thanks the staff of the Department of Bacteriology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand, Dr. Santisuk Vibulbandhitkij and the staff of the Department of Pathology, Bamrasnaradura Infectious Disease Hospital for their support and assistance.

REFERENCES

BLASER, M.J., BERKOWITZ, I.D., LAFORCE, F.M., CRAVENS, J., RELLER, L.B. and W.L.L. WANG. (1979). *Campylobacter* enteritis: clinical and epidemiologic features. *Ann. Intern. Med., 91* : 179.

BOLTON, F.J., HUTCHINSON, D.N., PARKER, G. (1987). A reassessment of methods for the isolation of campylobacters from feces. Abstract 26 IVth International Workshop on *Campylobacter* infections, Goteborg, Sweden.

BUTZLER, J.P. and SKIRROW, M.B. (1979). *Campylobacter* enteritis, *Clin. Gastroenterol., 8* : 737.

DEKEYSER, P., GOSSUIN-DETRAIN, M., Butzler, J.P. and STERNON, J. (1972). Acute enteritis due to related vibrio: first positive stool cultures. *J. Infect. Dis., 125* : 390.

DOYLE, M.P. and ROMAN, D.J. (1982). Recovery of *Campylobacter jejuni* and *Campylobacter coli* from inoculated foods by selective enrichment. *Appl. Environ. Microbiol., 43* : 1343.

FENNELL, C.L., TOTTEN, P.A., Quinn, T.C., PATTON, D.L., HOLMES, K.K. and STAMM, W.E. (1984). Characterization of *Campylobacter*-like organisms isolated from homosexual men. *J. Infect. Dis., 149* : 58.

GOOSSENS, H., de BOECK, M., COIGNAU, H., VLAES, L., VAN DEN BORRE, C., BUTZLER, J-P. (1986) Modified selective medium for isolation of *Campylobacter* spp. from feces: comparison with Preston medium, a blood-free medium, and a filtration system. *J. Clin. Microbiol., 24* : 840.

JANSSEN, D. and HELSTAD, A.G. (1982). Isolation of *Campylobacter fetus* subsp. *jejuni* from human fecal specimens by incubation at 35 ° and 42 ° C. *J. Clin. Microbiol., 16* : 398.

KING, E.O. (1957). Human infections with *Vibrio fetus* and a closely related vibrio. *J. Infect. Dis., 101* : 119.

LEXOMBOON, U., ECHEVERRIA, P., SUVONGSE, C. and DUANGMANI, C. (1981). Viruses and bacteria in pediatric diarrhea in Thailand: a study of multiple antibiotic-resistant enteric pathogens. *Am. J. Trop. Med. Hyg., 30* : 1281.

LIOR, H. (1984). New, extended biotyping scheme for *Campylobacter jejuni, Campylobacter coli,* and *"Campylobacter laridis" J. Clin. Microbiol., 20* : 636.

MORRIS, G.K. and PATTON, C.M. (1985). *Campylobacter. In:* Lennette, E.H. (ed.) Manual of Clinical Microbiology, 4th ed. American Society for Microbiology: Washington, D.C., p. 302.

NG, L.K., STILES, M.E. and TAYLOR, D.E. (1985). Comparison of basal media for culturing *Campylobacter jejuni* and *Campylobacter coli. J. Clin. Microbiol., 21* : 226.

OHASHI, M. (1982). *Campylobacter* enteritis. *In:* Chew, A. (ed.) Gastrointestinal infections in Southeast Asia (IV). SEAMIC

publication: Japan, p. 114.

PARK, C.H., HIXON, D.L., POLHEMUS, A.S., FERGUSON, C.B., HALL, S.L., RISHEIM, C.C. and BARRIE, A.C. (1983). A rapid diagnosis of *Campylobacter* enteritis by direct smear examination. *Am. J. Clin. Pathol., 80* : 388.

SIMASATHIEN, S., SUPAVEJ S., SAMAKOSES, R. and VIRIYATAWEEKUL, W. (1984). *Campylobacter jejuni* enteritis in children in Pramongkutklao Hospital. *R. Thai Army Med. J., 37* : 77.

SKIRROW, M.B. (1977). *Campylobacter* enteritis: a "new" disease. *Br. Med. J., 2* : 9.

STEELE, T.W. and MCDERMOTT, S.N. (1984). Technical note: the use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter jejuni* from feces., *Pathology. 16* : 263.

STEELE, T.W., SANGSTER, N. and LANSER, J.A. (1985). DNA relatedness and biochemical features of *Campylobacter* spp. isolated in Central and South Australia. *J. Clin. Microbiol., 22* : 71.

SUPAVEJ, S., LEXOMBOON, U. and SIMASATHINE, S. (1982). Occurrence of *Campylobacter* enteritis in Thai children. *Southeast Asian J. Trop. Med. Pub. Hlth., 13* : 489.

TAYLOR, D.N. and BLASER, M.J. (1987). *Campylobacter* infections. *In:* Evans, and Feldman, R.A. (eds.) Revision for 2nd edition of bacterial infections of Humans. Plenum Publishing: New York.

TAYLOR, D.N., BLASER, M.J., ECHEVERRIA, P., PITARANGSI, C., BODHIDATTA, L. and WANG, W.L.L. (1987). Erythromycin resistant *Campylobacter* infection in Thailand. *Antimicrob. Agents Chemother., 31* : 438.

TAYLOR, D.N., ECHEVERRIA, P., PITARANGSI, C., SERIWATANA, J., BODHIDATA, L. and BLASER, M.J. (1988). Influence of strain characteristics and immunity on the epidemiology of *Campylobacter* infections in Thailand. *J. Clin. Microbiol., 26* : 863.

TEE, W., ANDERSON, B.N., ROSS, B.C. and DWYER, B. (1987). Atypical campylobacters associated with gastroenteritis. *J. Clin. Microbiol., 25* : 1248.

WANG, W.L.L., LUECHTEFELD, N.W., BLASER, M.J. and RELLER, L.B. (1982). Comparison of CampyPak II with standard 5% oxygen and candle jars for growth of *Campylobacter jejuni* from human feces. *J. Clin. Microbiol., 16* : 291.

WANG, W.L.L., LUECHTEFELD, N.W., BLASER, M.J. and RELLER, L.B. (1983). Effect of incubation atmosphere and temperature on isolation of *Campylobacter jejuni* from human stools. *Can. J. Microbiol., 29* : 468.

WANKIJCHAROEN, S. (1983). Serotyping of *Campylobacter jejuni* causing gastroenteritis in children. Master's thesis, Tropical Medicine, Faculty of Tropical Medicine, Mahidol University.