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Comparison of Preservation Media for Storage of Stool Samples†

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Transportation of clinical samples and long-term recoverability of pathogens are critical to epidemiological studies, particularly when conditions do not permit immediate processing. This study confirms that Cary-Blair medium (CB) is suitable for the preservation of *Salmonella* and *Shigella* isolates for more than 2 weeks at 25, 4, or −70 C. *Campylobacter jejuni* was not recovered after 2 days of storage in CB at 25 C when an inoculum of 12×10^8 cells per ml was used. Lower temperatures supported the recovery of this organism for 6 days. When individual pathogens were preserved with stools in CB and incubated at 25, 4, or −70 C, the *Salmonella* and *Shigella* concentrations dropped from 12×10^8 cells to 1×10^3 or 1×10^4 cells per ml within 2 days and then remained stable for the rest of the observation period (15 days). *C. jejuni* survived preservation with stools for 5 to 9 days. The addition of blood and glycerol to CB improved the recoverability of all enteropathogens, particularly *C. jejuni*, which was consistently detected for 7 to 9 days at the different preservation temperatures used. When trypticase soy broth-glycerol (freezing medium), with or without blood, was used, there was little or no decrease in the *Salmonella* and *Shigella* concentrations during 2 weeks of preservation with stools at −70 C. *C. jejuni* demonstrated a relatively sustained high concentration in Trypticase soy broth-glycerol with 5% blood. The use of defibrinated, laked sheep blood as a long-term freezing medium supported the recovery of low concentrations of *Salmonella* and *Shigella* spp. (10^2 to 10^3 cells per ml) for more than 14 weeks. Recovery of *C. jejuni* was consistent for 7 weeks when an initial concentration of 10^6 cells per ml was present in stools. Laked blood provided a simple, sterile, and inexpensive medium for the preservation of individual isolates and clinical samples.

Microbiology laboratories deal with a diversity of clinical samples collected from various parts of the body. Several transport media are available for short-term preservation of microbiological samples (8). Cary-Blair transport medium (CB) has been shown to maintain better viability of bacteria than Stuart medium (4, 5, 16). CB produced better recovery rates for *Neisseria gonorrhoeae*, *Neisseria intracellularis*, *Haemophilus influenzae*, *Bordetella pertussis*, streptococci, staphylococci, pneumococci, *Escherichia* spp., *Enterobacter* spp., and *Trichomonas vaginalis* (1, 13). Some workers recommended reducing the agar content of CB from 0.5 to 0.16% to prolong the viability of preserved *Campylobacter* strains for a few hours (2). The survival of pure cultures of *Vibrio* species in CB was reported to average 22 days at a temperature of 21 to 31°C (5, 10). *Salmonella* and *Shigella* species were isolated from CB after 49 days of storage at 28°C. Recently, Richardson et al. (14) reported on the recoverability of enteropathogens in stools by inoculation of CB, Amies medium, and buffered glycerol-saline. At 4°C, the viability of organisms was not consistently maintained beyond 1 month, with CB giving the best results. Wang et al. (17) found that freezing of stool specimens with or without preservation medium at −70°C effectively preserved the enteric pathogens (except *Campylobacter jejuni*) for 12 months. The recoverability of *C. jejuni* held at this temperature decreased gradually over a period of 3 months. Nair et al. (12) recommended preservation of *C. jejuni* cultures in an egg-based medium for up to 4 months at 4°C. Mahajan and Rodgers (10) maintained this organism at −70°C in 10% sorbitol with 1% calf serum. Wells and Morris (18) showed that buffered glycerol-saline was better than CB for the transportation of *Shigella* spp. Refrigeration of that medium supported the recoverability of *Shigella* spp. for several days to a few weeks.

Limited experience regarding the long-term storage of bacteriological specimens has been acquired (14), and information about the utility of CB, buffered glycerol-saline, or other media in the preservation of microorganisms is contradictory. In our laboratory, reduced sheep blood has been used effectively for the long-term preservation of anaerobic clinical samples and isolates (17a). This study was designed to evaluate the recoverabilities and viabilities of enteropathogens (*Salmonella* spp., *Shigella* spp., and *C. jejuni*) preserved with stools in different transport or storage media. The results of this study may improve our understanding of this field and offer an opportunity to better store samples.

MATERIALS AND METHODS

Samples. Thirty fresh clinical stool specimens (13 with *Salmonella typhi*, 4 with *Salmonella* serotypes C1 and B, 10 with *C. jejuni*, and 3 with *Shigella sonnei*) collected from patients with diarrhea and individual strains of *Salmonella typhimurium*, *Shigella flexneri*, and *C. jejuni* (five each) isolated from human stools were employed in this study.

Culture media. (i) CB (Difco Laboratories, Detroit, Mich.) was prepared according to the instructions of the manufacturer or with the addition of 5% sheep blood (CBB) or of 5% sheep blood and 15% glycerol (CBBG). (ii) Trypticase soy broth (TSB) (Difco Laboratories) was also utilized with 15% (wt/vol) glycerol (TSBG) for the freezing of samples at −70°C. In some experiments, 5% sheep blood was added to the medium (TSBGB). (iii) Sheep blood as a preservation medium was prepared by aliquoting 1 ml of sterile, defibrinated sheep blood and freeze-thawing it three or more times. The aliquots were kept in the freezer until they were thawed for sampling. (iv) Selenite F broth and MacConkey, Hektoen Enteric, xylose lysine desoxycholate, *Brucella*, and *Campylobacter* agars (Difco Laboratories) were used for the isolation and identification of enteric bacteria.

Preservation of enteric samples. To evaluate the recoverability of enteropathogens, 30 clinical stool samples known to be positive for enteric pathogens (see above) were preserved in TSBG at −70°C and then thawed at weekly intervals to assess the recoverabilities of the same organisms on suitable selective media.

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TABLE 1. Recoverabilities of pure enteropathogens preserved with pathogen-free stools at different temperatures in CB or TSB supplemented with blood or blood and glycerol

Days of storage	Organism[a]	Log_{10} recoverable concn (cells/ml) at:					
		25 and 4°C			−70°C		
		CB[b]	CBB	CBBG	TSBG	TSBGB	CBBG
2	*S. typhimurium*	3	5	8	ND[c]	ND	ND
	S. flexneri	4	7	8	ND	ND	ND
	C. jejuni	3	4	4	ND	ND	ND
5	*S. typhimurium*	3	4	8	8	8	8
	S. flexneri	4	6	8	8	8	5
	C. jejuni	3	4	4	4	8	8
7	*S. typhimurium*	3	5	8	9	9	9
	S. flexneri	4	5	8	9	9	9
	C. jejuni	1	4	2	5	8	3
9	*S. typhimurium*	3	5	8	ND	ND	ND
	S. flexneri	4	6	8	ND	ND	ND
	C. jejuni	1	6	1	2	7	1
15	*S. typhimurium*	3	5	8	7	8	7
	S. flexneri	4	6	8	7	8	6
	C. jejuni	0	0	0	1	5	1

[a] The original concentrations in the seed were 12×10^8 cells per ml.
[b] Samples preserved in CB at −70°C showed similar results.
[c] ND, not done.

To compare the viabilities of enteropathogens in different preservation media, groups of five TSBG tubes with and without blood (5%) were inoculated with 1 ml of individual enteric bacteria (at a McFarland turbidity of 4 [ca. 12×10^8 cells per ml]) and stored at −70°C. Another set of TSBG tubes was prepared with the addition of a swab containing a pathogen-free stool sample (ca. 0.5 g). Laked blood was inoculated in a similar way, preserved at −70°C, and cultured on a weekly basis. CB vials were also prepared as described above and incubated at room temperature (25°C) or at 4 or −70°C. Cultures were performed on the first day of storage and then every other day until day 15.

Cultivation and identification of bacteria. Swabs from preserved samples were emulsified in phosphate-buffered saline (pH 7.4), serially diluted (10-fold), and then cultured on appropriate selective media. In experiments utilizing blood as a freezing medium, colonies recovered at each dilution were enumerated and expressed as a CFU percentage of the original selective count before storage. Incubation of all media and identification of enteric pathogens were performed by standard methods (2, 6).

RESULTS

The recoverabilities of enteropathogens from 30 clinical stool specimens preserved in TSBG at −70°C for 1 week were assessed. Of 13 *S. typhi*-positive samples, 8 isolates (61.5%) were recovered (two of these [25%] required enrichment in Selenite F broth). Of the 17 other enteropathogen-positive samples, 1 isolate (5.9%) (1 of the 3 *Salmonella* serotype B-positive samples) was recovered; none of the 10 *Campylobacter* (*C. jejuni* and *C. coli*) or 3 *Shigella* (*S. sonnei*) strains or the 1 *Salmonella* serotype C1 strain was isolated after storage for 1 week in TSBG medium.

Enteropathogenic isolates (*Salmonella* and *Shigella* spp.) were consistently recovered from CB during storage for 2 weeks at room temperature or at 4 or −70°C (data not shown). The organisms were detectable at concentrations ranging between 10^4 and 10^6 cells per ml. *C. jejuni* was not recovered after 2 days of preservation in this medium. Table 1 shows that the preservation of pathogens seeded with pathogen-free stools in CB led to a sharp drop in the concentration of bacteria within 48 h regardless of the storage temperature (25, 4, or −70°C). During this time, the viability of the mixture fell to 10^4 or 10^3 cells per ml and remained stable at this level for the rest of the preservation period. *C. jejuni* showed a similar drop during the first 5 days of storage, and then viability was gradually lost over 4 more days. When blood was added to CB, the concentrations of *Salmonella* and *Shigella* spp. were sustained at comparatively higher levels than those in CB only. *C. jejuni* was consistently recovered from CB supplemented with blood (levels of between 10^2 and 10^4 cells per ml) for 7 days regardless of the presence of glycerol.

TABLE 2. Recoverabilities of enteropathogens from seeded stools over 11 weeks of preservation in laked sheep blood (−70°C)

Week	Minimal recoverable counts (CFU)[a]		
	C. jejuni (12×10^5 cells/ml, 50 colonies)	*S. typhimurium* (12×10 cells/ml, 40 colonies)	*S. flexneri* (12×10^3 cells/ml, 252 colonies)
1	10 (20)	6 (15)	5 (2)
2	20 (40)	14 (35)	5 (2)
3	20 (40)	3 (7.5)	10 (4)
4	40 (80)	4 (10)	6 (2.4)
5	10 (20)	8 (20)	3 (1.2)
7	15 (30)	1 (2.5)	1 (0.4)
11	1 (2)	10 (25)	0
P[b]	0.35	0.96	0.66

[a] Concentrations and counts higher than those reported as minimal showed an uncountable number of colonies. Numbers in parentheses are the percentage of colonies recovered (relative to initial counts) after preservation.
[b] Differences between the percentages calculated for each column are not statistically significant.

Table 1 also shows the recoverabilities of enteropathogens preserved with stools in TSBG or CBBG at −70°C. Results with both media were similar, showing a decrease in the concentrations of *Salmonella* and *Shigella* spp. by one to two 10-fold dilutions over a 2-week interval. The viability of *C. jejuni* dropped to 10^4 cells per ml in 5 days and then to 10^2 cells per ml by day 9. The recoverability of this organism was inconsistent after 15 days of storage. The addition of 5% blood to TSBG caused no change in the original concentrations of *Salmonella* and *Shigella* spp. However, *C. jejuni* was detectable at relatively high levels (10^5 cells per ml) after 2 weeks.

Table 2 demonstrates the recoverabilities of enteric bacteria from laked sheep blood. *C. jejuni* at 12×10^5 cells per ml (50 CFU) survived storage for 11 weeks (ca. 3 months), and higher concentrations remained viable for 14 weeks. Lower concentrations showed an inconsistent recovery after storage for 1 week. *S. typhimurium* remained culturable for 14 weeks when an initial concentration of 12×10^3 cells per ml (800 CFU) was used. The inoculation of 120 cells of the organism per ml in stools resulted in a sustained recoverability for 11 weeks. A concentration of 12×10^3 cells (252 CFU) of *S. flexneri* per ml was culturable for 5 to 7 weeks of freezing at −70°C. Higher concentrations survived storage for 11 weeks.

DISCUSSION

To date, there is no ideal medium that preserves the viability of pathogens and flora without allowing the growth or inhibition of some species. TSBG has been employed in our laboratory for the preservation of clinical isolates at −70°C. When this medium was used to preserve whole stool samples that were positive for *Salmonella*, *Shigella*, or *Campylobacter* strains, only 61.5% of the *Salmonella* strains were recovered after 1 week of storage. None of the *Campylobacter* or *Shigella* strains was recovered in this experiment, which agrees with previous findings supporting the poor recoverability of these organisms after preservation. Nair et al. (12) reported that *Campylobacter* strains may lose viability during storage in TSBG. Other authors claimed that preservation of *Campylobacter* spp. leads to

the transformation of cells to nonmotile, unrecoverable coccoid forms (3, 17). *Shigella* spp. were shown to be vulnerable to the acidic changes produced by the cohabiting flora (14). However, our results disagree with those of Richardson et al. (14), who showed that storage of stool samples at −70°C or lower (without preservation medium) was effective in sustaining the viability of enteropathogens over a 12-month test period.

CB supported the survival of *Salmonella* and *Shigella* isolates for a 2-week observation period, which is in agreement with the findings of previous studies (5, 10). When a stool specimen (negative for *Salmonella*, *Shigella*, and *Campylobacter* spp.) was mixed with the cultures and stored at 25 or 4°C, the viabilities of pathogens decreased sharply within 2 days. The pH and biochemical activities of stool microbiota (aerotolerant anaerobes) have been reported to be detrimental to the viabilities of pathogenic organisms in fecal samples (8). However, *Salmonella* and *Shigella* spp. were recoverable throughout the entire observation period, supporting the results of Neumann et al. (13), who claimed detectability of these species for 49 days by preservation in CB at 28°C. The observed decrease in the concentrations of enteric pathogens may limit the role of unsupplemented CB in the preservation of clinical samples. This suggestion may disagree with the view that the low nutrient content of CB and utilization of phosphate as a buffering agent may provide suitable sample viability and control of contaminants that may mask the recovery of true pathogens (4, 7). In our experiments, *Campylobacter* strains showed a pattern of low recovery after 5 days of preservation. For an experiment using a different stool composition, Luechtefeld et al. (9) reported the recovery of *C. jejuni* from the cecal contents of turkeys when preserved for 15 days at 4°C. Those authors noticed improved results when transport media were used, with the most efficacious being CB. Low recoverability (20%) of *C. jejuni* was reported when specimens were frozen for 24 h at −20 or −70°C without a transport medium. However, the results from our study suggest that a high concentration of *C. jejuni* ($>10^9$ cells per ml) would be required for the organism to survive the storage conditions used by Luechtefeld and coworkers (9).

The addition of blood and glycerol to CB (CBBG) caused no loss in the viabilities of *Salmonella* and *Shigella* strains. The survival of *Campylobacter* strains was also improved. Blood may have enhanced the buffering and enzymatic mechanisms of the medium with minimal enrichment. The presence of glycerol in CBB appears to be important for maintaining the original inoculated concentrations of *Salmonella* and *Shigella* spp. without loss of viability. The compound may exert a limiting bacteriostatic action on the cohabiting flora or stimulate certain metabolic reactions or pathways to support the viability of pathogens at different temperatures.

In the case of TSBG, the addition of 5% blood to the formula (TSBGB) improved the recoverabilities of all pathogens used. Concentrations of *Salmonella* and *Shigella* species were not changed during storage for 2 weeks. Results with this medium were similar to those with CBBG. *Campylobacter* spp. maintained the original viability levels for the first 9 days of storage in TSBG and then decreased to 10^2 cells per ml at the end of the second week. This finding argues for the use of this medium for the storage of stool samples for 2 weeks or more.

When laked blood was used as a preservation medium, a minimal concentration of 12×10^5 *Campylobacter* cells (50 CFU) per ml was required for the organism to be recovered for more than 3 months of storage. The medium appears to be suitable for the preservation of stool samples without the need for other transport media. *S. typhimurium* was viable after 11 weeks of storage even when relatively low cell concentrations were preserved. *S. flexneri* required at least 10 times more bacterial cells than *Salmonella* spp. to be recovered for 7 weeks. While blood as a supplement or as a preservation medium seemed advantageous for enteric pathogens, the specific reason for this observation is unknown. One possibility could be the presence of iron, which is necessary for the recoverability of certain bacteria, especially *C. jejuni* (15). Another possibility could be the presence of an immense number of electrolytes, metabolites, nutrients, proteins, enzymes, hormones, and electron carrier systems in the plasma. These factors may contribute to the buffering, osmotic, homeostatic, and cryoprotective properties of this medium (11).

The present investigation shows that unsupplemented CB was not a very useful medium for the preservation of *C. jejuni* at the different concentrations employed. TSBGB and whole sheep blood supported the recovery of enteric pathogens, particularly *C. jejuni*. The use of blood in this study provided a simple, easy, and inexpensive method for better preservation of samples containing facultative or microaerophilic species.

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