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A microbial culture system for use in remote field environments

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SUMMARY

Field studies of human flora carried out in remote environments are often compromised by problems associated with media, equipment or cargo limitations. For the International Biomedical Expedition to Antarctica an anaerobic culture system was developed based on sealed vials, pre-reduced anaerobically sterilized media, antibiotic selective media and compact processing equipment. The system proved simple to use in a harsh environment and gave results comparable with standard plate and roll-tube techniques. No problems with dehydration, contamination or oxidation were encountered. Furthermore, the system preserved viability of primary isolates for up to 6 months of storage.

INTRODUCTION

Microbiological investigations in remote regions are often restricted because methods, media and equipment cannot provide the quality, reliability or the quantitative accuracy of similar investigations in base-station laboratories. With particular reference to ice-bound regions, McLean's bacteriological studies of 1911–14 (1919) and Sladen's of 1947–59 (1965) were fraught with difficulties, including restriction of laboratory and cargo space, dehydration of media, fungal contamination, inability to prepare fresh media, fluctuating incubation temperatures, failure to maintain anaerobic conditions and isolates being non-viable when shipped home.

As part of the 1980/81 International Biomedical Expedition to the Antarctic (IBEA),* extensive microbiological investigations of human flora were required before, during and after the 12-man team's planned Antarctic isolation. To overcome the problems encountered by previous investigators, a culture system was developed for the isolation and enumeration of facultative and anaerobic bacteria. This was based on a modification of the sealed vial system (Miller & Wolin, 1974) and the utilization of pre-reduced anaerobically sterilized media (Holdeman, Cato & Moore, 1977). This paper examines the proficiency of the system as compared to standard plate-culture techniques, its ability to maintain viability of organisms over long periods of time, and its performance under the field conditions imposed by Antarctic isolation.

* The International Biomedical Expedition to the Antarctic (IBEA) was a scientific project organized by the Scientific Committee on Antarctic Research (SCAR) Working Group on Human Biology and Medicine.

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MATERIALS AND METHODS

The exingencies of IBEA dictated that the microbiology protocol be based on the use of growth media made and pre-poured in the 'home' laboratory (Adelaide, Australia). The Antarctic samplings required that the media be surface-transported to Antarctica and that all cultures be stored at 5 °C until arrival back in Australia. The system is based on the availability of a power source.

Preparation of growth media

Pre-reduced anaerobically sterilized (PRAS) growth media were prepared as outlined in the Virginia Polytechnic Institute manual of Holdeman, Cato & Moore (1977) (VPI Manual) but with the addition of selective antibiotics (Sutter, Vargo & Finegold, 1975). The media (and their respective purpose) were as follows: Brain heart infusion agar with hemin and vitamin K (BHIA – total anaerobes/facultative anaerobes); BHIA supplemented with 100 μg/ml neomycin (BHIAN-clostridia); BHIA with 100 μg/ml neomycin + 7·5 μg/ml vancomycin (BHIANV-Bacteroidaceae); and BHIA with 10 μg/ml naladixic acid + 400 μg/ml cycloserine (BHIANC-*Clostridium difficile*). Rogosa's agar was used for isolation of lactobacilli (Rogosa, Mitchell & Wiseman, 1951). The VPI Manual procedures were followed though the final stages of media preparation were carried out in an anaerobic chamber.

To prepare the above media 4 ml volumes of the brain heart infusion broth–antibiotic mixtures were dispensed into 10 ml glass antibiotic vials (Watts-Winter Co., Sydney) containing 0·1 g of agar. The vials were sealed aseptically in the anaerobic chamber using a Capsolut crimping tool (Schubert & Co, Denmark) and were then autoclaved. For BHIANC, naladixic acid and cycloserine were added aseptically after the BHIA vials had cooled to 50 °C

Field culture procedures

In the field, all manipulations of bottles, containers and pre-reduced fluid media were carried out under CO_2 cover. This was achieved by immediately inserting into the opened vessel a glass tube through which oxygen-free CO_2 was issuing Fig. 1). When the manipulation was completed, simultaneous closing of the vessel and withdrawing of the tube, enabled anaerobic conditions to be maintained.

Fresh faecal samples were obtained in a paper cup which had been pre-filled with CO_2. Under CO_2 cover, approximately 1 g of faeces (obtained via a pre-calibrated scoop) was suspended in 10 ml of PRAS brain heart infusion broth (BHIB) and vortexed (each BHIB contained several glass beads to facilitate disaggregation). Following this initial procedure, 1 in 10 dilutions were then carried out using sealed vials of pre-reduced BHIB as diluent. Dilutions and inoculations of the sealed vials were carried out via disposable 1 ml tuberculin syringes and 23 G needles which were flushed with oxygen-free CO_2 prior to use. Dilutions of 10^{-1}, 10^{-5}, 10^{-7} and 10^{-9} were prepared in this manner.

Prior to the faecal samplings being taken, the required number of agar-media vials were heated in a small saucepan of water over an aerosol can of butane gas and then kept molten at 52 °C in a small 'water bath'. The water bath was a modified blood-pack warmer (Tuta Laboratories, Sydney) in which the floor had

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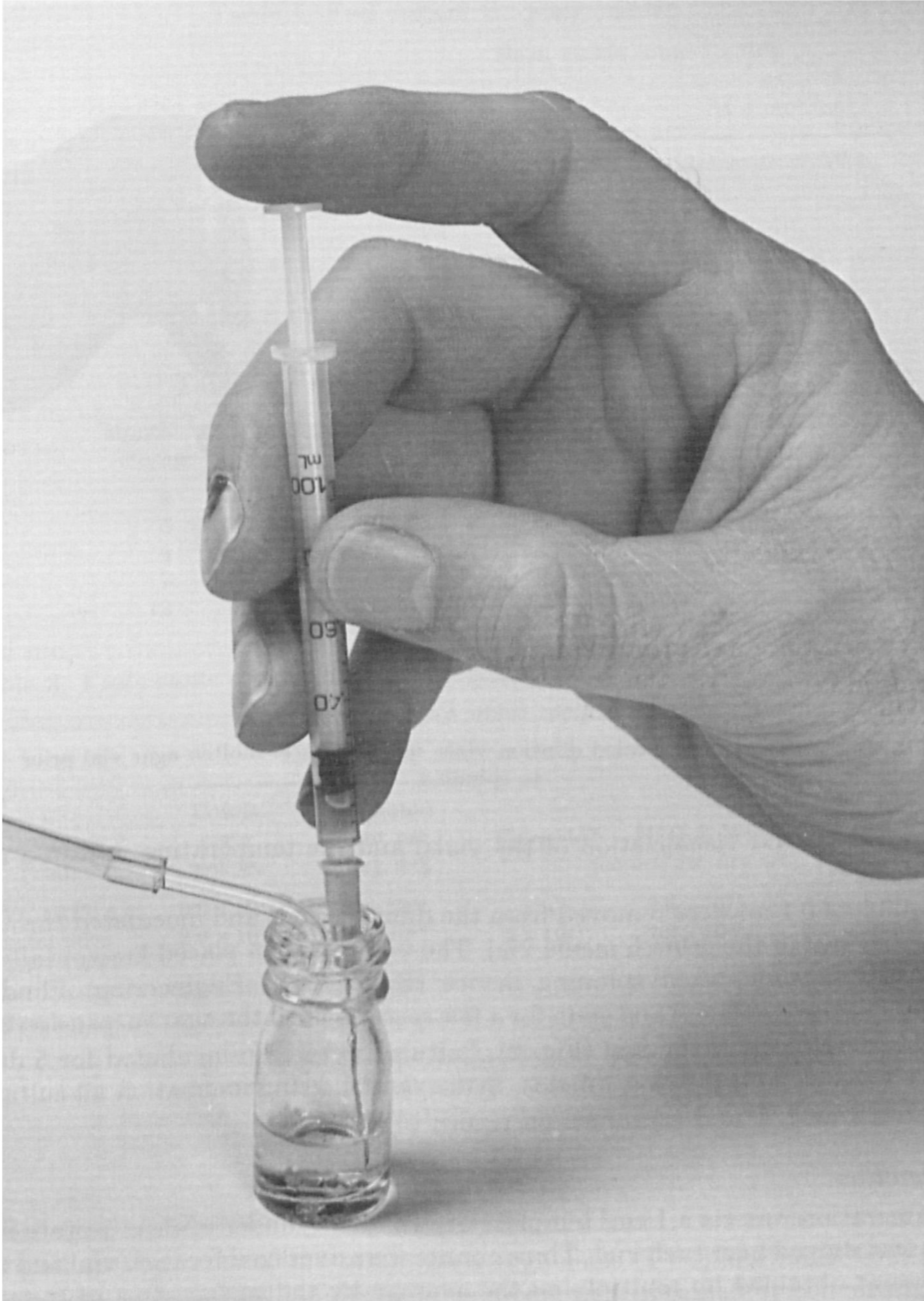

Fig. 1. Manipulations under constant CO_2 Cover.

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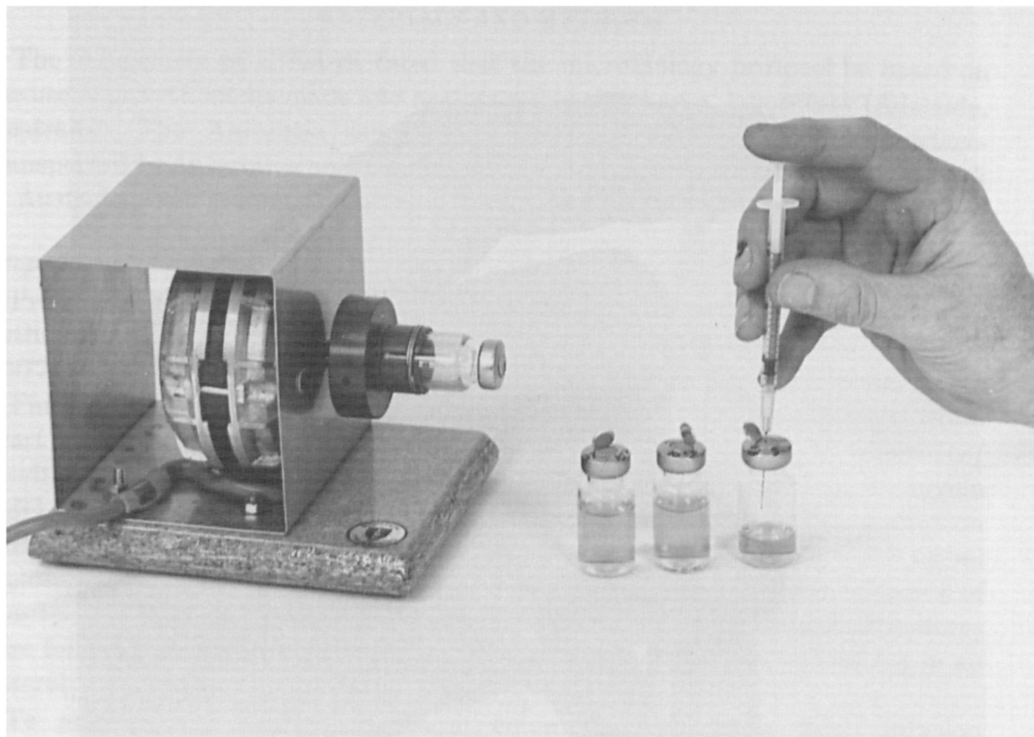

Fig. 2. Spinning device, sealed dilution vials, inoculation of molten agar vial prior to spinning.

been raised (to aid visualization of the vials) and the temperature adjusted and fixed at 52 °C.

Aliquots of 0·1 ml were removed from the dilution vials and inoculated through the rubber seal of the growth media vial. The vial was then placed horizontally in a specially manufactured spinning device (Biochemical Engineering, Flinders Medical Centre, Adelaide) and spun for a few seconds until the agar suspension had set against the wall of the vial (Fig. 2). Cultures were then incubated for 5 days in a 35 °C portable incubator, (Medos, Sydney). Following incubation all cultures were stored at 5 °C and examined on return to Australia.

Enumeration

Enumeration was via a 1 cm^2 template drawn on a cylinder of clear acetate film which was slipped over each vial. Three counts were averaged for each vial and the total count obtained by multiplying the average by the surface area of the vial (26·3 cm^2) and the dilution factor. Identification of isolates was according to the VPI Manual using biochemical tests and gas liquid chromatographic identification of volatile fatty acids where applicable. *Bacteroides* spp., *Lactobacilli* spp. and *Clostridium*, spp. were individually estimated by randomly sub-culturing and identifying 10 colonies from each vial of BHIANV, Rogosa and BHIAN respectively. Detection of *C. difficile* was via maceration of the vial contents, heating to 80 °C for 10 min and replating onto BHIANC.

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Table 1. *Vial culture* vs. *plate culture*

Vial	Challenge organism	Mean viable count (× 10⁹)			
		At preparation		At 4 months	
		Plates	Vials	Plates	Vials
BHIA	*P. indolicus*	5·0	4·4	2·8	2·6
BHIANV	*B. fragilis*	3·0	3·4	4·1	3·6
BHIAN	*C. perfringens*	1·2	1·0	2·4	3·0
BHIANC	*C. difficile*	1·5	2·0	1·8	2·3
Rogosa	*L. rogosae*	3·4	2·9	3·2	3·6

Table 2. *Viability of colonies on vial storage*

Months of storage	No. colonies subcultured	No. colonies viable (%)
2	144	138 (95·8)
5	230	219 (95·2)
6	168	160 (95·2)
7	120	102 (85·0)
12	117	33 (28·2)

Table 3. *Vial-counts of total organisms, bacteroides and Lactobacilli (including comparison with other methods)*

Organism (vial)	IBEA		Finegold*	H. G & M†	M & H‡
	Count per g wet wt	Estimated count per g dry wt§	(count/gm dry wt)		
TVC (BHIA)	$9{\cdot}6 \times 10^{10}$	$4{\cdot}0 \times 10^{11}$	N.A.	$2{\cdot}6 \times 10^{11}$	$4{\cdot}8 \times 10^{11}$
Bacteroides (BHIANV)	$2{\cdot}0 \times 10^{10}$	$8{\cdot}0 \times 10^{10}$	5×10^{11}	$7{\cdot}9 \times 10^{10}$	$9{\cdot}0 \times 10^{10}$
Lactobacilli (Rogosa)	$6{\cdot}9 \times 10^{8}$	$2{\cdot}8 \times 10^{9}$	$1{\cdot}3 \times 10^{10}$	$1{\cdot}2 \times 10^{9}$	$11{\cdot}0 \times 10^{9}$

§ Assuming faecal solids approximate 25% of wet weight (see text).
* Finegold *et al.* 1977 (Plated media).
† Holdeman, Good & Moore, 1976 (roll-tube).
‡ Moore & Holdeman, 1974 (roll-tube).

Proficiency of vial system

Overall mean counts of the total viable organisms (TVC) and mean counts of bacteroides and lactobacilli of the team members were obtained by converting the mean value obtained after a log transformation on each of the sample values. The proficiency of the culture system was gauged by comparing the means obtained prior to embarkation for Antarctica (when subjects were 'normal') with mean flora counts in normal individuals as found by others using standard culture systems.

Four uninoculated vials of each medium were stored for 2 years as a check on sterility, dehydration, and anaerobic condition. At preparation and again after 4

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months of storage, a further two vials of each medium were challenged with a 0·1 ml inoculum (approx. 10^5/ml suspension) of an organism for which the medium was designed. The same inoculum was also cultured on anaerobic media in conventional plates and incubated in an anaerobic chamber at 35 °C.

The ability of the vial system to maintain the viability of organisms during prolonged storage and transport was assessed by comparing the subculture yield after the Antarctica-inoculated vials were stored at room temperature (22 °C) for 2, 5, 6, 7 and 12 months.

RESULTS

Vial integrity. On examination at 1 and 2 years after sealing, none of the four sterile vials of each medium exhibited signs of dehydration or reduction in anaerobic conditions. These uninoculated vials, when incubated for 5 days at 35 °C, remained free of growth. During IBEA, 969 sealed vials were used. None exhibited any evidence of dehydration, reduced anaerobic condition or contamination and none was broken.

Media proficiency. The vials, when challenged both at preparation and again 4 months later gave counts within 0·2–0·6 $\log_{10}$ of the values obtained using the same inoculum on conventionally plated fresh anaerobic media incubated in an anaerobic chamber for the same time.

Organism viability. With particular reference to BHIANV and *Bacteroides* spp., Table 2 depicts the viability of colonies after prolonged storage of the BHIANV vials sealed during IBEA. Twelve species of bacteroides were identified.

Culture counts

Clostridium *sp. count*: not quantifiable (see text).
Clostridium difficile *count*: nil detected.

DISCUSSION

IBEA was designed to study human responses to the stress of Antarctic cold and isolation. The Antarctic phase comprised a 90 day, 400 km journey inland on motorized two man toboggans with supplies towed behind. Experimentation was carried out in a small mobile van. Cargo and laboratory space were precious. With ambient temperatures reaching −29 °C and van temperatures around 8 °C, it was essential to keep culture manipulations simple and to a minimum. Given these conditions and the isolation of the IBEA camp, culture systems dependent on plated media and anaerobic chambers (or jars) or standard roll-tubes and equipment were unsuitable. The most promising alternative was to adopt the miniaturized toll-tube system of Miller & Wolin (1974), couple it with the PRAS techniques of the Virginia Polytechnic group (Holdeman, Cato & Moore, 1977) and use the selective media of the Wadsworth group (Sutter, Vargo & Finegold, 1975).

The preparation of reliably anaerobic vials was not without problems. Despite many attempts at (and modifications of) the VPI Manual's procedures for preparing PRAS media in the small vials, in a small proportion of them the anaerobic atmosphere lasted only a few days. This failure rate was unacceptable

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for Antarctic research. The problem was solved competely by locating the equipment in an anaerobic chamber and carrying out the final dispensing and sealing of the vials in the chamber.

The proficiency of the anaerobic vial system when challenged with known inocula was not significantly different from that when standard plate techniques were used (Table 1). Furthermore, the system's ability to preserve viability of organisms with long-term storage (apart from Rogosa's medium) was excellent. Everyday laboratory experience indicates that, with plated media, close to 100% recovery would be expected if subculture were carried out within 48 h of the appearance of primary culture colonies. The vial-system's viability rate of 95·8% after 2 months storage may indicate a small loss of viability or could be due to the subculture technique of inserting a bent wire through the vial neck and dissecting out colonies many of which were subsurface. With this technique it was occasionally difficult to determine whether an inoculum was present on the wire. This viability rate remained unchanged for up to 6 months of storage (Table 2). Viability may have been prolonged further had the vials been stored at 5 °C rather than room temperature.

Under field conditions the system's ability to isolate clostridia was poor not through failure of the vials but in the inability of BHIAN to effectively select clostridia. Although several clostridial species were identified, the majority of randomly selected colonies were *Bacteroides* sp. With poorly selective media this was not unexpected, as in the gut, bacteroides counts are several orders of magnitude higher than clostridia (Finegold *et al.* 1977). Clostridia counts and species could not therefore be compared with other studies. With regard to *C. difficile* however, the lack of isolates is most likely a real finding as the selective media has proved reliable in our laboratory.

Table 3 depicts the total viable count of anaerobes/facultative anaerobes, and the counts of bacteroides and lactobacilli as compared to published studies using conventional techniques. The conversion of counts per wet faeces to counts per gram dry faeces was based on the average faecal solids figure of 25% in the comparative studies. The count of the BHIANV vial was corrected to allow for the 13% of isolates identified as non-bacteroides (98 of 752 isolates). The comparison indicates that even in remote field conditions the sealed-vial system is able to match the performance of standard anaerobic culture methods.

The BHIANV medium proved particularly valuable for determining the *Bacteroides* sp. profile. Twelve species were identified together with two biochemical profiles of unidentified species. Ten of these species are listed in the 12 most common *Bacteroides* sp. in human faecal flora (Finegold *et al.* 1977, Holdeman, Good & Moore, 1976).

All colonies sampled in the Rogosa vials were Gram-positive rods resembling lactobacilli but the great majority were non-viable. All of the identified viable isolates were *Lactobacillus* spp. In the author's experience when Rogosa cultures are stored more than 4 weeks, the medium is virtually self-sterilizing through acid-production by the lactobacilli. As the medium is excellent for selection of lactobacilli the counts were presumed to be of this genus. Although five different species of lactobacilli were identified, the paucity of viable colonies precluded a species profile comparison with other studies.

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In another phase of the IBEA microbiology project, BHIA vials were utilized in skin and throat-flora studies, and viable colonies of streptococci, staphylococci, corynebacteria and facultative gram-negative rods were recoverable in high numbers. The vial system is eminently suitable for studies of anaerobic human flora in remote field environments and appears to be equally suitable for studies of facultative organisms.

One further advantage of the vial system is the compactness. There were 135 vials accommodated in a box 0·005 m^2. The same number of plated media would have occupied more than three times this space. On completion of IBEA, it was evident that the reliability of the vial system against dehydration, oxidation or contamination was excellent. Furthermore no vial suffered breakage or cracking. The vial system, given sufficient attention to preparatory detail, and coupled with the spinning and water-bath equipment as developed, may be effective for a wide variety of flora studies in remote areas particularly where the field excursion or culture storage is prolonged, where cargo space or weight is limited and where reliability is essential.

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