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Evaluation of crayfish chaff charcoal agar as a transport medium for anaerobes

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A laboratory formulated crayfish chaff charcoal agar (CCCA) was evaluated both as transport and storage medium for anaerobic bacteria in parallel with Amies charcoal agar (ACA), cooked meat medium (CMM) and thioglycollate broth (TCB). The survival of anaerobes in swab obtained clinical specimens and viability of specific anaerobes in these media were assessed. Eight genera of anaerobes (*Bacteroides*, *Fusobacterium*, *Parvobacteroides*, *Porphyromonas*, *Prevotella*, *Clostridium*, *Peptoniphilus*, *Peptostreptococcus*) were isolated from ACA, CMM and CCCA, 7 (*Bacteroides*, *Fusobacterium*, *Parvobacteroides*, *Prevotella*, *Clostridium*, *Peptoniphilus*, *Peptostreptococcus*) from TCB transported specimens. Comparatively, the difference in isolation rate of anaerobes in aspirate (85%) and swab (75%) processed specimens was not significant ($p < 0.05$). Irrespective of storage temperature (-20°C or 30 ± 2°C), positive anaerobic cultures from 7-day stored swab specimens in transport media were TCB 10, CCCA 14, ACA and CMM 18 each. Anaerobes recovery from CCCA and ACA were comparable ($p < 0.05$). Quantitatively, *Bacteroides* was recovered after 6 weeks of storage in CCCA with counts of $10^{6.1}$ and $10^{5.6}$ CFU/ml at -20°C and 30 ± 2°C respectively. Similar pattern of recovery occurred with *Prevotella*, *Clostridium* and *Peptoniphilus* in CCCA, ACA and other transport media with no significant differences in viable counts ($p < 0.05$). The CCCA function is comparable with those of the other media and can be prepared and used in-house for transport of clinical specimens and short term storage of anaerobes.

Key words: Crayfish chaff charcoal, transport medium, anaerobes, survival

INTRODUCTION

The oxygen sensitive nature of anaerobes requires that specimens be cultured within the first hour of collection. In addition to this is that cotton swab which is the least acceptable specimen for anaerobic study ironically is the most frequently collected (Johnson et al., 1995; Jousimies-Somer et al., 2002). Cotton swab provides pockets of oxygen, contains toxic fatty acids and other compounds and dries out easily making recovery of anaerobes from swab collected specimens more difficult (Perry, 1997; Peterson, 1997). To overcome these set backs special swabs including albumin and charcoal coated swabs were introduced with varying success rates and clinical acceptability for use. The successful use of transport media in the transport of clinical specimens for

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Table 1. Sample distribution.

Clinical condition	Number of specimens collected			
	Swab/transport medium			Aspirate+
	In icebox*	at -20°C	30 ± 2°C	
Septic caesarian section	14	14	14	14
Suppurating dentoalveolar abscess	7	7	7	7
Orthopedic wound abscess	7	7	7	7
Total	28	28	28	28

*Icebox temperature ranged from 20-22°C.+Aspirate samples were inoculated at the patient beside or the consulting room.

Neisseria gonorrheae by Stuart, Cary and Blair, and Amies opened a new chapter in the cultivation of fragile bacteria (Sng et al., 1982; Stoner et al., 2004). The incorporation of charcoal by Amies to the mineral formulation of Amies charcoal medium help in neutralizing the toxic component in wooden and cotton swab and also mop-up residual oxygen in the swab transported specimen. With these development and others including the use of thioglycollate and cooked chopped meat as introduced by Robertson anaerobic bacteriology gradually picked up.

Though still regarded as the least acceptable specimen for anaerobic culture, it remains the easiest to collect and manipulate. Several studies reporting the use of swab specimens for anaerobic cultures have contained these swabs for brief periods in transport media before cultivation (Peterson, 1997; Stoner et al., 2008). Other studies have evaluated the viability of anaerobes in transport media maintained over a period of time and storage conditions (Dahlen et al., 1993; Ali et al., 1995; Piccolomini et al., 1998).

There exist the needs for evolving knowledge. In one of our reports we showed that crayfish broth was a good nutrient source for anaerobes (Egwari and Otegbeye, 2000). The medium was found to have good buffering property and rich in mineral salts. The chaff from the crayfish was used in formulating a new medium crayfish chaff charcoal agar which in this study was evaluated along side Amies charcoal agar, thioglycollate broth and cooked meat medium as both transport and storage media for anaerobes.

MATERIALS AND METHODS

Preparation of media

Four culture media were assessed for ability to maintain the viability of anaerobic bacteria in clinical specimens and also serve as storage medium for anaerobes under specified conditions; Amies charcoal agar (ACA; Oxoid), cooked meat medium (CMM; Oxoid), Thioglycollate broth (TCB; Oxoid) and crayfish chaff charcoal agar (CCCA; formulated in-house). The first three media are used for transport of clinical specimen for culture or as back-up media. These were prepared according to manufacturer's instructions. Crayfish chaff was obtained after the nutrient in finely powdered crayfish (*Nematopalaemon hastatus*) had been extracted as described by Egwari and Otegbeye (2000). Crayfish chaff charcoal agar (CCCA) was prepared by mixing charcoal, crayfish chaff and agar in 1:4:1 ratio for every 100 ml of water. Each medium was homogenized at 100°C and dispensed into screw cap long narrow bore tubes of 9 cm long and bore diameter of 1 cm to a depth of 5 cm and sterilized at 121°C.

Media used for recovery of anaerobes from the test-transport-storage media were anaerobic blood agar supplemented with vitamin K_1 (1 ug/ml) and L-cysteine hydrochloride (2ug/ml), neomycin blood agar (100 ug/ml neomycin), Wilkins-Chalgren agar and trypticase soy agar supplemented with 10% defribinated sheep blood. Other media used were brucella bile salt esculin agar (Oxoid) and laked blood kanamycin vancomycin agar (Oxoid). In all cases, pre-reduced thioglycollate broth (steaming for 10 min) was used as recovery medium for anaerobes in the transport media.

Specimens

Clinical conditions from which specimens were collected include septic caesarian section and suppurating dentoalveolar abscess. Specimens were also collected from orthopedic wound abscess around the hip joint. Two sets of specimen were collected; alginate swab and needle aspirate and in all cases only when there was sufficient exudation to allow for collection of adequate sample. Needle aspirates were inoculated onto anaerobic agar plates at the collection point and transported in GENbag pouches with 18% CO_2 generated with the GENbag anaer sachet (bioMerieux). The swab specimens were broken into the transport media immediately after collection, labeled and sent to the laboratory under ambient atmospheric conditions. The needle aspirates were collected as quality checks for the type of anaerobes present in the specimen and also evaluate the effectiveness of the transport media studied. Specimens were collected from three different hospitals in Lagos State: General Hospital Gbagada, Lagos State University Teaching Hospital, Ikeja, and Lagos University Teaching Hospital, Idi-Araba.

Qualitative study

Swab processed specimens were grouped according to clinical conditions (Table 1). Each clinical condition except caesarian section (with 29 swabs) contains 15 swabs per transport medium with a set of 7 stored at room temperature (30 $\pm$ 2°C) and the other set of 7 stored at -20°C. The extra swab specimen transported in

icebox (20 to 22°C) was cultured within the first 6 h of collection. Transport medium containing swab were labeled day 1 through 7 and on each designated day swabs were withdrawn and cultured on the anaerobic agar plates and incubated. Colony types were identified and compared with those on needle aspirate plates.

Quantitative study

Bacteria isolated and identified in the qualitative study were used. Quantitative assessment was done as previously described (Egwari and Rotimi, 1991). Briefly, pure culture of each isolate was harvested and suspended in pre-reduced phosphate buffered saline (PBS) and standardized with McFarland nephelometer to an approximate concentration of 1 x 10^9 cfu/ml. Alginate swabs were dipped into the bacterial suspension to soak for a minute and transferred into freshly prepared transport media. Two sets of transfer were done and one set stored at room temperature and the other at -20°C. At the end of each storage period, the swab was withdrawn and transferred into 4 ml pre-reduced thioglycollate broth incubated for 18 h in an anaerobic jar in the presence of 10% CO_2. After incubation, 2-fold doubling dilution of the broth culture was carried out in pre-reduced TCB. Then 2 ml were added to molten anaerobic agar, mixed thoroughly and poured into sterilized plates, incubated and colonies counted. The viable counts were performed in triplicate and the final concentration expressed in $\log_{10}$ colony forming unit (cfu)/ml.

Incubation

In all cases, anaerobic incubation was done in the BBL GasPak system comprising the GasPak jar (2.5 L) with a catalyst chamber containing new palladium catalyst and the GasPak anaerobe envelope. Catalysts were activated in a hot air oven at 140°C for 2 h before use. Anaerobic conditions in jar were obtained with the GasPak envelope and were monitored with disposable BBL dry Anaerobic Indicator Strips (Becton Dickinson). Duration of incubation was dependent on the bacterial species.

Identification of isolates

Isolates were Gram stained and further identified by growth pattern on special media, spore formation and swarming growth where applicable. A number of conventional biochemical test procedures and the API system were used for speciation.

Statistical analysis

Data on frequency of isolation of anaerobes by various collection and storage methods were analyzed with the Pearson Chi square test at P = 0.05. Analyzed were differences in recovery rate of anaerobes from aspirate samples and swab specimens in icebox, and recovery rate of anaerobes from CCCA and ACA stored samples.

RESULTS

Twenty four (85%) of the aspirate samples grew anaerobes either in pure or mixed cultures. The anaerobes were distributed in five genera of Gram negative bacilli (22 positive cultures), six positive cultures of *Clostridium,* a Gram positive spore forming bacilli and two genera of Gram positive cocci (14 positive cultures). Comparatively, icebox swabs cultured within 6 h of collection produced 21 (75%) positive anaerobic culture, with GNB isolated from 17, *Clostridium* 6 and GPC 12. The difference in recovery rate using aspirate samples and swab specimens transported in transport medium and cultured within 6 h is not significant ($p < 0.05$). On storage in transport media at two different temperatures (30 $\pm$ 2°C and -20°C) for 7 days, swab specimens sustained the viability of anaerobes as follows: CCCA 14 positive cultures, ACA and CMM 18 positive cultures each, and TCB 10 positive cultures. The distribution of the anaerobic genera in the positive cultures is given in Table 2. Evaluation of the preservation potential of CCCA as a transport medium for anaerobic specimens against ACA showed no significant difference between the two ($p < 0.05$).

Data on daily quantitative assessment of viability of anaerobes in the transport media at the storage temperatures are given in Table 3. Bacterial population of $\geq 1 \times 10^6$ cfu/ml was used as the critical threshold for determining sustainable viability within the medium for anaerobes. At this bacterial population, most genera of anaerobes were recovered on the sixth and seventh day at -20°C especially in CCCA, ACA and CMM. At the higher temperature of storage, where differences occurred it was just a day earlier than the lower temperature. *Porphyromonas* survived least in the transport media while TCB was less sustaining.

Table 4 shows the viable counts of selected anaerobes in the transport media over an extended period of 6 weeks. Analysis of the data showed that no difference exist in maintenances of anaerobes from one medium to another and between the storage temperatures ($p < 0.05$).

DISCUSSION

Swab remains the most frequently received specimen in clinical microbiology. It therefore becomes pertinent to device means of sustaining microorganisms in it before processing irrespective of its many deficiencies. Many studies describing the survival of bacteria (aerobic and anaerobic) in swab-transported specimen were done within the first 24 h of specimen collection (Perry, 1997; Piccolomini et al., 1998). These studies aimed at maintaining the viability of the organism in the specimen as much as possible without unduly altering its presentation at the site of infection. Though a barrage of transport systems have been evaluated with different degree of success recorded, no single system has provided the panacea. Therefore the need for continuous formulation and evaluation exist as we continue to understand the physiology and metabolic requirements of

Table 2. Recovery of anaerobes in aspirates and swab specimens.

Group/Genera	Aspirate	Icebox	Swab+			
			CCCA	ACA	CMM	TCB
Anaerobe positive culture*	24	21	14	18	18	10
GNB positive culture	22	17	11	15	16	9
Bacteroides	18[ac]	15	10 (10)	12 (14)	14 (15)	7 (9)
Parvobacteroides	14[ac]	13	8 (10)	9 (11)	10 (13)	8 (9)
Fusobacterium	10[b]	8	4 (5)	5 (5)	4 (5)	3 (4)
Porphyromonas	8[b]	6	0 (2)	2 (4)	1 (5)	0 (0)
Prevotella	12[b]	10	8 (9)	8 (9)	8 (10)	5 (5)
GPB positive culture	6	6	6	6	6	5
Clostridium	6[c]	6	5 (6)	6 (6)	6 (6)	5 (5)
GPC positive culture	14	12	8	11	11	7
Peptoniphilus	8[a]	8	7 (8)	8 (8)	7 (8)	5 (6)
Peptostreptococcus	11[ab]	9	6 (8)	8 (10)	8 (10)	4 (6)

*Values in table represent frequency of occurrence; Aspirate samples were cultured within 1 h of collection; Icebox samples were cultured within 6 h of collection; Swab+ were analyzed on the 7th day of storage in transport medium; Values in parenthesis represent storage at -20°C; Superscript alphabets indicate combinations of mixed cultures where they occurred.

Table 3. First week data of anaerobes in transport media at storage temperatures.

Genera	Day bacterial count in media was $\geq$ 1 x 10^6 cfu/ml			
	CCCA	ACA	CMM	TCB
Bacteroides	7 (7)	7 (7)	7 (7)	6 (7)
Parvobacteroides	6 (6)	7 (7)	7 (7)	6 (6)
Fusobacterium	5 (6)	6 (7)	7 (7)	4 (5)
Porphyromonas	3 (4)	4 (5)	4 (5)	0 (0)
Prevotella	5 (6)	6 (7)	6 (7)	4 (5)
Clostridium	7 (7)	7 (7)	7 (7)	6 (7)
Peptoniphilus	6 (7)	6 (7)	7 (7)	6 (6)
Peptostreptococcus	6 (7)	7 (7)	7 (7)	6 (6)

Values in parenthesis are for storage at -20°C.

Table 4. Viable counts of anaerobes in transport media at storage temperature and over 6 weeks.

Genera	CCCA			ACA			CMM			TCB		
	2*	4	6	2	4	6	2	4	6	2	4	6
Bacteroides	6.5[+]	6.4	5.9	6.8	6.5	6.1	6.9	6.6	6.4	6.3	6.1	5.7
	5.5	5.1	4.8	6.4	6.1	5.6	6.6	6.3	6.0	5.2	4.7	3.8
Prevotella	5.8	5.5	4.7	6.0	5.7	5.2	6.0	5.3	4.8	5.6	3.9	3.1
	4.2	3.7	2.9	5.1	4.2	3.9	5.5	5.1	4.7	3.5	3.0	2.8
Clostridium	6.8	6.5	6.2	6.9	6.6	6.4	7.0	6.8	6.5	6.6	6.4	6.2
	6.4	6.3	6.1	6.7	6.3	6.2	6.8	6.4	6.3	6.0	5.7	5.2
Peptoniphilus	5.7	5.5	5.2	5.9	5.8	5.5	5.9	5.7	5.5	5.7	5.4	5.2
	5.0	4.4	3.9	5.3	4.9	4.2	5.4	4.8	4.1	5.1	4.6	3.7

*value represents week of recovery experiment; +values as expressed in Log_{10} cfu/ml; Lower values in table represent count at 30$\pm$2°C.

organisms especially anaerobes as evaluated in the present study.

Amies charcoal agar, cooked meat medium and thioglycollate broth are established transport media for specimens for anaerobic culture (Hindiych et al., 2001; Jousimies-Somer, 2002; Stoner et al., 2004). Thus this study was not to evaluate their effectiveness, but rather to use those in evaluating the effectiveness of the newly formulated crayfish chaff charcoal medium for same purpose. This study by extension also considered the survival of anaerobes in the media studied over an extended period of 6 months making them suitable as short term preservation media. The results showed that CCCA is comparable with Amies charcoal agar and cooked meat medium both as transport and short term storage medium. The difference observed in both the qualitative and quantitative studies were not statistically significant to undermine the usefulness of any. However, further improvement may be necessary for upgrade and better coverage for a broader spectrum of anaerobic bacteria.

In cooked meat medium, the chopped devitalized meat particles provide the anaerobic niche, thioglycollate the reducing atmosphere in TCB, charcoal and thioglycollic acid in Amies. In the formulated CCCA the crayfish chaff and charcoal combine to provide the anaerobic conditions for the anaerobes. Charcoal is also a good toxin neutralizer and oxygen scavenger; these dual properties will accentuate the usefulness of CCCA if properly standardized. The new medium contains no mineral salts and its buffering capacity is inherent in the minimal concentrations of Ca^{2+} and K^{+} in crayfish chaff after infusion (Egwari and Otegbeye, 2000). The addition of agar help in reducing convention current and the containment of the medium in a narrow tube is to maintain anaerobiosis for a prolonged period after medium preparation.

The promising preliminary results obtained with CCCA and its simplicity both in formulation and cost makes it a useful addition to the compendium of transport media for anaerobic bacteria.

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