The use of an anaerobic incubator for the isolation of anaerobes from clinical samples

PL BERRY, E TAYLOR, I PHILLIPS

From the Department of Microbiology, St Thomas's Hospital Medical School, London SE1 7EH

SUMMARY An anaerobic incubator was compared with a standard jar system for the isolation of anaerobes from clinical material. Seventy specimens were selected as likely to yield anaerobes: 342 different anaerobes were isolated in the incubator and 347 in anaerobic jars. These included *Bacteroides* spp (43%), *Peptococcus* spp (26%), *Peptostreptococcus* spp (13%), *Veillonella* spp (7%), *Fusobacterium* spp (7%), *Clostridium* spp (2%) and miscellaneous Gram-positive non-sporing bacilli (2%). Differences in isolation rates for each system were inconsistent and minor.

Sixteen anaerobes were chosen for quantitative tests at the beginning and end of the study period. Miles and Misra counts showed a slight advantage of the incubator for *F nucleatum*, but no difference for *B fragilis*, *B thetaiotaomicron*, *B uniformis*, *B bivius*, *B corrodens*, *F mortiferum*, *Ps anaerobius*, *P prevotii* or *Propionibacterium acnes*. In almost all cases, colonies in anaerobic jars were slightly larger than those in the incubator. Disc antibiotic sensitivity tests gave the same results in each system, at the beginning and end of the study period.

The anaerobic incubator provides an effective means of isolation of anaerobes in a clinical laboratory. However, several design features of the prototype would require change if the system were introduced.

Increased awareness in recent years of the role of anaerobic bacteria in common infections has led to demands for the isolation of these organisms in the diagnostic laboratory.¹⁻³ Most of the work can be carried out with anaerobic jars,⁴⁻⁶ but these require care and skill in use. Jars are often set up when there are sufficient plates to fill them, leading to delays in the critical early phase of incubation. Furthermore, diagnostic work in larger laboratories is usually divided into sections, with each having its own jars: this creates a problem when incubator space is limited. There is thus a possible need for an anaerobic incubator into which plates can be placed immediately they are inoculated and from which they can be removed without rendering the whole system aerobic. We have assessed the performance of such an incubator in comparison with that of anaerobic jars.

Material and methods

ANAEROBIC SYSTEMS

The anaerobic jars were converted six- or eight-litre pressure cookers.⁷ Plates were placed in the jar

along with two catalyst sachets each containing about 5 g catalyst previously heated to 160°C.⁸ The jars were evacuated with an Edwards type 2 vacuum pump to a reading of -760 mmHg, on a Gallenkamp pressure gauge and filled with 10% hydrogen, 10% CO₂ and 80% nitrogen.

The anaerobic incubator was a prototype produced by GV Planer Ltd. in co-operation with the Department of Health and Social Security. It consisted of a chamber of internal dimensions 61 cm × 63.5 cm × 35.5 cm, with a 62 cm × 32 cm window containing a 15.3 cm glove port fitted with a 70 cm size 9 neoprene glove (Hanfare Protective Clothing Ltd.). Inside the chamber were a 51 cm diameter turntable with space for 10 stacks of Petri dishes and a 23 cm × 28 cm shelf 25.5 cm above the base. Cultures were placed in the incubator via a 25.5 cm × 20.5 cm × 16.5 cm (internal dimensions) transfer port with a 51 cm transfer arm, an inner door (20.5 cm × 16.5 cm) and an outer door (17 cm × 13.8 cm) with a window. Two containers, containing approximately 200 g of Engelhard Deoxo "D" palladium catalyst pellets (Don Whitley Scientific) were used in the incubator, and the catalyst containers and silica gel were recycled in the same manner as jar catalyst. An Edwards type 2 Vacuum pump (British Oxygen

Company) was used for initial evacuation, and the incubator was rendered anaerobic by flushing with 10% hydrogen, 10% CO₂ and 80% nitrogen.

A Forma Scientific System model 1024 anaerobic work station was used for incubation of identification tests, including API-20A strips.

ISOLATION OF ANAEROBES FROM CLINICAL SPECIMENS

Specimens of pus were collected from patients in St Thomas' Hospital between July 1979 and May 1980 and transported immediately to the laboratory either in 25-ml bottles or on serum-coated swabs. They were examined for brick-red fluorescence in ultra-violet light^{9 10} and the odour noted. A Gram stain was performed and whenever possible unextracted, underivatised pus was examined for the presence of volatile fatty acids in a Pye Unicam GCD Chromatograph.¹⁰ Only specimens likely to contain anaerobes on the basis of these preliminary investigations were included in the study.

Specimens were inoculated on blood-agar (Columbia agar, Oxoid CM331, plus 10% defibrinated horse blood) and on our own selective medium consisting of brain heart infusion agar (Oxoid CM375) plus 0.05% cysteine hydrochloride, 1% vitamin K-haemin solution, 0.5% yeast extract, 10 µg/ml nalidixic acid (Winthrop), 7.5% whole defibrinated horse blood and 2.5% lysed (by freezing and thawing) horse blood.

The plates were incubated in the two systems and aerobically. A stock culture of *Pseudomonas aeruginosa* inoculated on Simmon's citrate agar (Oxoid CM155) was used as a biological indicator of anaerobiosis in each of the systems.⁷ After 48 h, cultures were removed for observations on colony morphology, Gram staining, and subculture aerobically and anaerobically of probable anaerobes. All these manipulations were performed on the bench under normal atmospheric conditions. Identification of anaerobes was based on the methods set out in the VPI Manual¹¹ and included in addition to Gram staining and observation of colonial morphology, determination of antibiotic susceptibility, gas-liquid chromatography and the range of biochemical tests included in API-20A strips, the latter used only for bacilli.

COLONY COUNTS AND SIZES OF PURE CULTURES

A suspension of a 24–48 h blood agar culture of the isolates listed in Table 3 was made in saline to give a turbidity of 5 International Opacity Standard Units (Wellcome opacity tubes). Serial five- and tenfold dilutions of this suspension were prepared, and 0.2 ml of each inoculated on quadrants of blood agar plates, incubated within 20 min in parallel in the

incubator and jar systems for 48–72 h. Colonies were then counted in cultures from appropriate dilutions and sizes of 24 discrete colonies measured with a microscope with graduated eyepieces.

pH

The surface pH of inoculated and uninoculated, incubated and uninoculated 10% horse blood agar plates was measured with an Ingold e07 403-30-M3 surface electrode (3 mm separation) and a Pye model 78 pH meter, with an accuracy 0.1 pH units. Readings were made after 2 min contact and recorded as averages for three readings.

ANTIBIOTIC SENSITIVITY

For disc sensitivity tests, the inoculum was prepared as for counts with a density of about 10⁶ CFU/ml and inoculated on 10% horse blood agar plates. Discs (Mast Laboratories) of clindamycin (2 µg), metronidazole (2.5 µg), erythromycin (60 µg), tetracycline (25 µg), ampicillin (10 µg) and chloramphenicol (10 µg) were added and plates incubated in parallel in the two systems. After 48 h zone radii were measured. Two sets of investigations were carried out, the second six months after the first, on a total of 28 isolates of 18 different species of Gram-positive and Gram-negative anaerobes.

Results

Of the pus samples examined, 70 were studied in detail: the overall results are shown in Table 1. A single anaerobe in pure culture was isolated from only one specimen, mixed anaerobes from a quarter of the specimens, and mixed aerobes and anaerobes from the rest.

Control cultures of *Ps aeruginosa* in the entry port grew after 24 hours indicating a degree of failure of anaerobiosis. Cultures within the chamber remained negative, indicating satisfactory anaerobiosis, provided the chamber was used regularly, but growth was obtained when the incubator was left over a weekend, indicating that oxygen was gaining entry. Regular recharging by regular use was thus essential with this prototype incubator.

Cultures in the incubator yielded 342 anaerobes and those in jars yielded 347. Detailed results are shown in Table 2. There are few differences between the two systems, usually explicable in terms of failure to detect organisms in complex mixtures, failure to detect pairs of organisms that eventually were found to vary in antimicrobial susceptibility, or of failure to proceed with the identification of one of a pair of organisms initially thought to be the same.

Table 3 lists the results of comparisons of yields of organisms in pure culture. There was a significant

Table 1 Specimens investigated and overall isolation rates of aerobes and anaerobes

Specimen	No of specimens	No yielding:	
		Aerobes and anaerobes	Anaerobes only
Perianal	20	15	5
Buttock			
Ischiorectal			
Perineal			
Pilonidal			
Abdominal	17	16	1
Abdominal wall			
Appendix			
Liver			
Peritoneum			
Subphrenic	25	14	11
Skin			
Axilla			
Breast			
Groin			
Limb			
Neck			
Paronychia			
Pinna			
Sebaceous cyst			
Respiratory	4	3	1
Tonsil			
Quinsy			
Pleura			
Miscellaneous	4	3	1
Hydrocoele			
Labia			
Placenta			
Urethra			
Total	70	51	19

Table 2 Isolation of anaerobes from clinical specimens

Isolate	No of isolates in:	
	Incubator	Jar
<i>Bacteroides fragilis</i>	21	19
<i>thetaiotaomicron</i>	4	4
<i>ovatus</i>	4	4
<i>distasonis</i>	3	2
<i>uniformis</i>	4	5
<i>vulgatus</i>	1	1
<i>bivius</i>	9	8
<i>rumincola</i> ss <i>brevis</i>	3	4
<i>oralis</i>	2	2
<i>disiens</i>	1	1
<i>corrodens</i>	36	34
<i>asaccharolyticus</i>	33	33
<i>melaninogenicus</i> ss <i>intermedius</i>	4	4
sp	21	20
<i>Fusobacterium nucleatum</i>	10	9
<i>necrophorum</i>	4	4
<i>mortiferum</i>	1	1
<i>varium</i>	2	2
sp	5	7
Anaerobic Gram-negative bacillus	2	2
<i>Veillonella parvula</i>	25	24
<i>Clostridium perfringens</i>	3	3
<i>clostridiumforme</i>	4	4
<i>Eubacterium lentum</i>	1	1
<i>alactolyticum</i>	2	2
<i>Lactobacillus catenaforme</i>	1	1
<i>Propionibacterium</i> sp	3	2
<i>Peptococcus prevotii</i>	51	52
<i>asaccharolyticus</i>	15	18
sp	23	23
<i>Peptostreptococcus anaerobius</i>	21	22
sp	22	27
Anaerobic Gram-positive coccus	1	1

Table 3 Colony counts and sizes in incubator (I) and jars (J)

Organism	Date	System	No of organisms × 10 ⁹ ± SD*	p	Colony size mm ± SD†	p
<i>B fragilis</i>	July	I	3.01 ± 0.4	NS	2.44 ± 0.2	<0.001
		J	2.77 ± 0.5		2.84 ± 0.3	
	Aug	I	1.88 ± 0.1	NS	2.68 ± 0.1	<0.001
		J	1.77 ± 0.25		2.83 ± 0.2	
	Nov	I	2.23 ± 0.3	NS	2.22 ± 0.3	<0.001
<i>B thetaiotaomicron</i>	July	J	2.27 ± 0.3	NS	2.58 ± 0.2	NS
		I	1.59 ± 0.3		1.88 ± 0.1	
	Nov	J	1.83 ± 0.3	NS	1.9 ± 0.2	NS
		I	1.99 ± 0.4		1.86 ± 0.1	
	July	J	1.83 ± 0.3	NS	1.94 ± 0.2	
<i>B uniformis</i>	July	I	2.57 ± 0.1	NS	0.9 ± 0.1	<0.001
		J	2.57 ± 0.2		1.1 ± 0.1	
<i>B bivius</i>	Sep	I	5.98 ± 1	NS	1.51 ± 0.1	NS
		J	6.45 ± 1		1.46 ± 0.1	
<i>B corrodens</i>	Aug	I	4.13 ± 0.4	NS	ND	
		J	4.06 ± 0.4			
	Nov	I	1.37 ± 0.2	NS	1.31 ± 0.1	NS
<i>F nucleatum</i>	July	J	1.41 ± 0.2	<0.001	1.28 ± 0.1	<0.001
		I	0.05 ± 0.01		1.48 ± 0.1	
	Aug	J	0.04 ± 0.01	<0.04	2.15 ± 0.1	
		I	0.17 ± 0.04		ND	
	July	J	0.13 ± 0.01			
<i>F mortiferum</i>	July	I	1.39 ± 0.3	NS	ND	
		J	1.32 ± 0.1			
<i>Ps anaerobius</i>	July	I	0.6 ± 0.02	NS	1.1 ± 0.1	<0.001
		J	0.76 ± 0.01		1.5 ± 0.1	
<i>P prevotii</i>	Aug	I	0.04 ± 0.01	NS	0.48 ± 0.04	<0.001
		J	0.04 ± 0.01		0.66 ± 0.03	
<i>Pr acnes</i>	July	I	1.37 ± 0.1	<0.1	ND	
		J	1.28 ± 0.1			

*Means of 8 observations.

†Means of 24 observations.

ND not done.

NS no significant difference.

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difference between colony counts only with *F nucleatum* which on two occasions gave higher counts in the incubator.

Table 3 also shows the results of comparisons of colony size in the two systems. There was a significant difference in size in seven out of eleven tests, in each case the organisms being larger in the jar. There was no significant size difference for *B thetaiotaomicron* (twice), for *B bivius* and for *B corrodens*.

Measurement of surface pH showed no difference between uninoculated plates from the incubator or from jars. In each case for plates having a pH 7.5 before incubation, the pH rose slightly from pH 6.9–7 to pH 7.3 over a period of one hour after removal. Nor were there significant differences between inoculated plates incubated in the two systems, although there was a considerable difference in pH for different organisms, ranging from pH 5.7–5.9 for *B fragilis* to pH 7.4–7.6 for *V parvula*.

Disc sensitivity tests almost invariably gave zone radii within 4 mm or less for both methods of incubation in 27 sets of tests on the six antibiotics, 17 of which were carried out after the incubator had been in use for six months. On only one occasion, ampicillin radii differed by 5 mm for an isolate of *F nucleatum*. The application of Student's *t* test to the two batches of sets of sensitivity tests showed no significant differences between them. On the first occasion the mean zone radii were 16.5 mm for the jar and 16.47 mm for the incubator (SD = 7.8 and 8, *t* = 0.14 *p* < 0.99) and on the second radii were 19.4 mm and 18.8 mm (SD = 7.3 and 7, *t* = 0.46 *p* < 0.65).

Discussion

The overall results of this investigation suggest that the Planer anaerobic incubator is a suitable alternative to the jar system in terms of bacteriological performance. There were no significant differences in isolation rates of different species from clinical specimens which were chosen for their likelihood of yielding relatively fastidious anaerobes. Thus *B corrodens* and *B asaccharolyticus* were isolated more frequently than *B fragilis*. The relative paucity of Gram-positive bacilli in comparison with other reported investigations^{10 12 13} may also be a result of this specimen selection. Nor were there, with few exceptions, differences in the counts of organisms from pure cultures (though *F nucleatum* was more numerous in the incubator) nor in disc sensitivity tests for a variety of anaerobes with six antimicrobial agents. The fact that surface pH changes were similar in the two systems may well explain the similarity of performance in sensitivity tests.^{14–17}

The only consistent difference was between colony sizes for a number of organisms, which were significantly but not much smaller in the incubator. *B bivius*, *B corrodens* and *B thetaiotaomicron* were not affected in this way. We are unable to explain these findings but would comment that we saw no pH differences, that oxygen was probably more rapidly removed from cultures in the incubator than in jars,¹⁸ and that we saw no evidence from other investigations carried out after six months, of a build-up of toxic metabolites—and in any case, the differences were observed at all stages of the use of the incubator. It is possible that there were humidity differences, but we did not investigate this. Nor did we vary amounts of catalyst, which might have affected growth. Another possibility is that colonies were affected by the entry of oxygen via inadequate door seals, but again we did not investigate this specifically.

If the cabinet functions from a bacteriological point of view, its practicability in a diagnostic laboratory should be assessed. We worked with a prototype and clearly changes are intended as a result of our experience. Our cabinet was too deep, and we found that the entry port was too small—for example, for API-20A strips. Furthermore there was evidence that the door seals were not sufficiently gas tight, resulting in the entry of oxygen fairly rapidly into the entry port, and more slowly into the chamber when its atmosphere was not recharged by use. We also found the turntable and transfer arm system wasteful of space and would prefer shelves and two glove ports instead. If these changes were made, we feel that the cabinet would be useful in a diagnostic laboratory. The advent of anaerobic work stations does not change this decision as we feel that if plates can be examined without removing them from the incubator, all other manoeuvres for identification and sensitivity testing can be carried out on the open bench, provided cultures are returned promptly to an anaerobic chamber. This is satisfactory for the jar system and should be equally satisfactory for the incubator, which would be an attractive option if it were less expensive than a work station.

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Requests for reprints to: Professor Ian Phillips, Department of Microbiology, St Thomas's Hospital Medical School, London SE1 7EH, England.