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THE ISOLATION OF STRICT ANAEROBES: THE USE OF AN ANAEROBIC CABINET COMPARED WITH A CON- VENTIONAL PROCEDURE

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PLATES X AND XI

SPECIAL procedures developed for the culture of strictly anaerobic bacteria may involve the use of oxygen-free gas-flushing systems (Andresen, 1946; Hungate, 1950) or anaerobic cabinets such as those described by Socransky, MacDonald and Sawyer (1959), Rosebury and Reynolds (1964) and Drasar (1967). The techniques are widely used in studies of anaerobic bacteria of the rumen and intestine of animals (Bryant and Burkey, 1953; Moore, 1966; Barnes and Burton, 1970) and of the gastro-intestinal tract of man (Moore, 1966; Drasar, 1967; Drasar and Crowther, 1971). These special anaerobic techniques demand much time, equipment and materials, but it is implied that they are essential for maximum recovery of important groups of organisms. We have shown that it is possible to recover vegetative cells of exacting anaerobes by traditional procedures with an anaerobic jar at the bench (Collee, Rutter and Watt, 1971; Watt, 1972). The following studies were designed to determine whether experiments with some anaerobes of clinical importance might justify the more general use of an anaerobic cabinet in the service laboratory.

MATERIALS AND METHODS

Bacterial strains. The following test strains were maintained and subcultured in cooked-meat medium with regular purity check cultures on blood agar media (see below): *Clostridium tetani* type VI, no. NCTC9569; *C. oedematiens* (*C. novyi*) type D, no. NCTC8350; *C. welchii* (*C. perfringens*) type A, a β -haemolytic, non-heat-resistant strain, no. 1 BW.

Media. Cooked meat broth (CMB) was prepared as described by Cruickshank (1968), except that the infusion broth component was made from Nutrient Broth no. 2 (Oxoid CM67). Reinforced Clostridial Medium (RCM) was supplied by Oxoid Ltd (CM149). "Pre-steamed

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nutrient broth" (PNB) was used as a diluent in quantitative studies; it was steamed at 100°C for 30 min. to remove dissolved air, then cooled rapidly to 37°C.

Blood-agar plates were prepared with Oxoid Blood Agar Base no. 2 (CM271) enriched with equine blood (10%). In quantitative studies with *C. oedematiens* type D, cysteine-dithiothreitol equine blood agar (CDEBA; Watt, 1972) was used.

Blood. Defibrinated equine blood was supplied by Wellcome Laboratories.

Standard anaerobic procedure. Baird and Tatlock (BTL) jars were used. Each jar was equipped with three room-temperature catalyst sachets. The detailed procedure was that described by Collee *et al.* (1972); in addition, the jars were evacuated again after being filled with a mixture of 90% H₂ and 10% CO₂ and then refilled with this gas mixture before incubation. All cultures were incubated at 37°C overnight, except where indicated otherwise.

Gases. Cylinders of CO₂ gas were supplied by Distillers Company Ltd, and cylinders of compressed H₂ were supplied by the British Oxygen Company; cylinders of H₂ 90% with CO₂ 10%, and H₂ 3% with N₂ 97% were supplied by the British Oxygen Company's Special Gases Division, Deerpark Road, London SW19 3UF.

Catalytic purifier. A model D5/50 catalytic purifier was obtained from Englehard Industries Ltd, Valley Road, Cinderford, Gloucestershire.

Gas flow-meters. These were obtained from Rotameter Manufacturing Co. Ltd, Purley Way, Croydon.

Gas connexions. All gas connexions were made with special $\frac{1}{8}$ -in. (8-mm) gastight tubing ("Saffire" hose N.153478, British Oxygen Company); all connexions were sealed with "Araldite" (Ciba (A.R.L.) Ltd, Duxford, Cambridge) and secured with "Jubilee" clips.

Enumeration procedures. Viable counts were performed by a spread-plate method (Watt, 1972); they were recorded as the mean obtained from six to ten plates seeded from a dilution that gave 100–700 discrete colonies per plate. To minimise errors in comparative studies, dilutions were prepared inside the cabinet with a pre-set constant volume pipette which was then passed out of the cabinet and used for preparation of dilutions at the bench. All plates were stored anaerobically for at least 24 hours until used. Total cell counts were performed as described by Collee, Rutter and Watt (1971).

Immunofluorescence microscopy. The identity of growths of the *C. oedematiens* strain was confirmed by direct staining of a heat-fixed smear with fluorescein-conjugated anti-*C. oedematiens* globulin (Wellcome Research Laboratories, Beckenham, Kent). The preparations were examined under ultra-violet light with a Zeiss RA-38 microscope.

Toxicogenicity testing. The haemolysin-neutralisation test described by Rutter and Collee (1969) was also used to check the identity of growths of the *C. oedematiens* type D strain. The toxicogenicity of growths of the *C. tetani* strain was confirmed by injecting 0.1-ml doses of a 5-day CMB culture supernate subcutaneously into an unprotected mouse and into a mouse protected with 0.5 ml equine antitoxin administered intraperitoneally.

Faecal anaerobes. Because stock cultures of anaerobes may not be representative of fresh isolates, comparative studies were made with wild strains of strict anaerobes derived from samples of human faeces. Freshly-passed samples were obtained from 12 volunteers; from each sample a 1-g portion was added to 100 ml PNB in a beaker which was then taken through the gas-lock (as described below) into the anaerobic cabinet. After homogenisation with a glass rod, 0.02-ml inocula from 10⁴ and 10⁵ dilutions of the suspension were seeded on to at least five replicate plates of RCM containing 1% Neutralised Liver Digest (Oxoid L 27) and 10% equine blood (Drasar and Crowther, 1971) that had been stored in the cabinet for not less than 24 hours before use. The plates were taken out of the cabinet in the modified BTL jar ready for incubation. At the same time, the dilutions were taken out and were used to seed a further series of plates of the same medium at the bench. These plates had been stored anaerobically at room temperature for at least 24 hours. The seeded plates were then placed in a separate BTL jar, and both jars were processed and incubated for 72 hours.

The design and construction of an anaerobic cabinet

The basic cabinet consisted of a steel glove-box (Model IIIc, Lintott Engineering, Horsham, Sussex) fitted with "Neoprene" gloves (fig. 1). A cylindrical compartment, bearing

a needle valve to allow evacuation and flushing, functioned as a gas-lock and "posting port". A mixture of 3% H_2 with 97% N_2 was regulated and mixed with CO_2 via separate gas flow-meters; the final mixture containing 10% CO_2 was rendered oxygen-free by passage through a catalytic purifier as suggested by Latham and Sharpe (1971). The H_2 - N_2 - CO_2 mixture was shown to be non-inflammable in air. Within the cabinet this anaerobic gas mixture was distributed through a length of copper tubing (4.5-mm bore), perforated at intervals and extending along two sides of the cabinet. A pressure gauge indicated the internal pressure. Exhaust gases passed through an adjustable oil-seal.

Preparation and operation of the cabinet

With the posting port open, a large polythene bag in the cabinet was inflated with the anaerobic gas mixture to displace most of the air. The outer seal on the posting port was then closed and the bag was collapsed by manipulation with the gloves; the cabinet was then flushed continuously with the anaerobic gas mixture for 72 hours at a rate of 0.4-0.5 litres per min. Catalyst sachets were then introduced through the gas-lock and distributed on the floor of the cabinet in glass Petri dishes. After a further 24 hours, the gas flow was stopped and the system was shown to be leak-proof. During use, eight to ten catalyst sachets were held in the cabinet and a further three were placed in the gas-lock. The sachets were replaced every 2-3 weeks.

During an experiment, the cabinet was flushed continuously with the anaerobic H_2 - N_2 mixture (0.3-0.4 litres per min.) and CO_2 30-40 ml per min., i.e., a CO_2 concentration of about 10%. At all other times, the flow rates were reduced to 50-100 ml and 5-10 ml per min. respectively. The pressure release system was arranged to maintain a constant positive pressure of 2.5 cm water irrespective of the gas-flow rates through the system.

Transfer of materials into the cabinet. With the inner seal shut, materials were placed in the gas-lock and the outer seal closed. The gas-lock was evacuated to -200 mm Hg, then filled with H_2 - N_2 mixture. This process was repeated four times, but a mixture of H_2 90% and CO_2 10% was used for the final filling so that an excess of H_2 was present. After a check at 10 min. that a secondary vacuum was being drawn (indicating catalytic activity), the gas-lock was left for at least 1 hour, and more H_2 + CO_2 was then admitted. The inner port of the gas-lock could thereafter be opened and the materials transferred into the cabinet.

A BTL anaerobic jar was modified to fit into the gas-lock (see fig. 2). Spreaders and pipettes were stored in the cabinet in foil-covered tins clipped to the copper gas-entry pipe. All media and diluents were held anaerobically in the cabinet for at least 24 hours before use. On occasion, this was preceded by anaerobic storage for 48 hours in BTL anaerobic jars on the bench.

RESULTS

Anaerobiosis in the cabinet

Electrometric determinations showed that the concentration of O_2 in the cabinet was less than 1%; as a more sensitive instrument was not available we made use of biological indicators of anaerobiosis. Plates seeded with a culture of *C. welchii* gave good growth when incubated at room temperature in the cabinet. Plates were seeded inside the cabinet from overnight CMB cultures of *C. tetani* type VI and *C. oedematiens* type D and, still within the cabinet, the plates were then placed in the modified BTL jar described above; on this occasion, however, the normal catalyst sachets had been removed and the jar contained the atmosphere of the cabinet, i.e., the jar was incubated at 37°C for 72 hours without further processing. Good growth of *C. tetani* was obtained on EBA and CDEBA plates, and growth of *C. oedematiens* type D was also obtained on both media. The atmosphere within the cabinet was therefore

sufficiently anaerobic to support the growth of very demanding anaerobes on blood-agar media.

Quantitative recovery studies

The recovery of demanding anaerobes such as *C. tetani* and *C. oedematiens* type D that we could obtain from CMB cultures by conventional methods at the bench was not apparently improved when dilutions were made and plates were seeded inside the cabinet. For more comprehensive assessment, we compared the quantitative recoveries obtained when cultures were manipulated in various ways. (A) Dilutions were prepared and plates seeded under anaerobic

TABLE I

The effect of different methods of processing inoculum and plates on the recovery of Clostridium tetani type VI from broth culture

Inoculum	Mean colony count, $\pm$ SE of counts, from samples processed according to methods*		
	A	B	C
0.02 ml of a 5×10^3 dilution	179.3 $\pm$ 7.9	190.6 $\pm$ 5.4	183.0 $\pm$ 13.1

- * A. Dilutions prepared, and plates processed, within the anaerobic cabinet. Count derived from nine replicate plates.
- B. Dilutions made within the cabinet were used to seed plates under aerobic conditions at the bench. Count derived from seven replicate plates.
- C. Dilutions prepared, and plates processed, under aerobic conditions at the bench. Count derived from seven replicate plates.

Inocula from an overnight cooked-meat-broth culture were plated on to blood-agar medium. All manipulations were performed at room temperature (19.0°C).

conditions in the cabinet; in this series the plates had been stored anaerobically within the cabinet for 24 hours. (B) The appropriate dilutions, having been made under anaerobic conditions in the cabinet, were used to seed plates under aerobic conditions at the bench; the plates in this series had been stored anaerobically in a BTL jar for at least 24 hours. (C) Dilution and inoculation were both performed under aerobic conditions at the bench, with plates that had been held anaerobically as in (B). The same pre-set constant-volume device was used for preparing dilutions in the cabinet and to prepare dilutions under aerobic conditions at the bench. All plates seeded at the bench were randomly distributed between four BTL anaerobic jars. These were then processed along with the jar from the cabinet and incubated for 18 hours (*C. oedematiens* type D) or 48 hours (*C. tetani*).

By comparison with results obtained when manipulations were performed at the bench, there was no improvement in recovery when the anaerobic cabinet was used for the preparation of dilutions or when both preparation of dilutions and seeding of plates were performed within the cabinet (table I). The data for *C. oedematiens* type D, the most exacting strain, confirm that complete recovery

was obtained when all manipulations were performed under "aerobic conditions" at the bench (table II).

TABLE II

*The effect of different methods of processing inoculum and plates on the recovery of Clostridium oedematiens type D from broth culture**

Inoculum	Mean viable count $\times 10^6$ per ml, $\pm$ SE of counts, from samples processed according to method†		
	A	B	C
0.02 ml of a 10^2 dilution	6.6 ± 0.1	6.7 ± 0.1	6.7 ± 0.2

* Viable counts on cysteine-dithiothreitol equine blood agar from overnight cooked-meat-broth cultures. The total cell count was 6.0×10^6 per ml; the spore count was $< 6.0 \times 10^4$ per ml.

† A. Dilutions prepared, and plates processed, within the anaerobic cabinet. Count derived from nine replicate plates.

B. Dilutions prepared within the cabinet were used to seed plates under aerobic conditions at the bench. Count derived from ten replicate plates.

C. Dilutions prepared, and plates processed, under aerobic conditions at the bench. Count derived from six replicate plates.

All manipulations were performed at room temperature (18.5°C).

Anaerobes from faeces

Because colonies of aerobic organisms from faeces were fewer than 1% of the total, colony counts on anaerobic plates were taken to represent the "total

TABLE III

A comparison of the mean anaerobic viable counts derived from samples of human faeces from 12 subjects, and processed conventionally at the bench or in an anaerobic cabinet

Subject number	Mean viable count $\times 10^{10}$ per g faeces (with ranges) derived from replicate plates processed	
	on the bench	in the cabinet
1	2.7 (2.4-3.5)	2.0 (1.6-2.6)
2	1.3 (0.9-1.9)	1.4 (0.9-1.9)
3	2.4 (2.0-2.7)	2.6 (2.3-2.8)
4	4.4 (3.6-4.8)	3.4 (2.8-4.6)
5	2.1 (1.6-2.7)	1.9 (1.6-2.1)
6	1.8 (1.3-2.2)	1.8 (1.4-2.7)
7	4.1 (2.6-5.4)	5.2 (4.6-5.7)
8	2.6 (2.4-3.0)	2.7 (1.9-3.2)
9	1.2 (0.9-1.4)	1.5 (1.0-1.8)
10	3.3 (1.6-4.8)	3.2 (2.5-3.7)
11	3.7 (3.2-4.8)	3.8 (2.8-4.5)
12	0.9 (0.5-1.3)	0.8 (0.5-1.2)

anaerobes". The majority of these were morphologically typical of *Bacteroides* spp., although colonies that were apparently of lactobacilli were also seen. Almost identical counts were obtained, whether the specimens were processed

in the cabinet $\{(2.4 \pm 0.4) \times 10^{10}$ organisms per g $\}$ or by the standard procedure at the bench $\{(2.4 \pm 0.3) \times 10^{10}$ organisms per g $\}$. The numbers of organisms grown from each specimen are detailed in table III.

In a further series of experiments, counts from faecal samples were compared when the various manipulations were performed inside the cabinet or at the bench. The results (table IV) again demonstrate that no significant increase in growth was obtained when the anaerobic cabinet was used for the preparation of dilutions or for the seeding of plates or for both of these operations.

TABLE IV

The effect of different methods of processing inocula and plates on the yield of total anaerobes from faecal samples

Inoculum	Mean surface viable counts $\times 10^{10}$ of total anaerobes* derived from samples processed according to method†		
	A	B	C
0.02 ml of a 10^7 dilution	2.6 ± 0.7	2.4 ± 0.5	2.5 ± 0.7

* Viable counts made on at least five replicate plates of Reinforced Clostridial Medium containing 10% equine blood, expressed as the mean $\pm$ SE of the results of six experiments, with six different samples of human faeces.

† A. Dilutions prepared, and plates processed, within the anaerobic cabinet.

B. Dilutions prepared within the cabinet were used to seed plates under aerobic conditions at the bench.

C. Dilutions prepared, and plates processed, under aerobic conditions at the bench. All manipulations were performed at room temperature (18.5°C).

DISCUSSION

Oxygen-free gassing devices or anaerobic cabinets have been recommended in recent years for the growth of exacting anaerobes of clinical interest. We can now recover the vegetative cells of an organism as exacting as *C. oedematiens* type D quantitatively at the bench with a simple anaerobic jar (Collee, Rutter and Watt, 1971), so we are reluctant to accept the implication that ultra-specialised equipment is obligatory. The present study is an attempt to evaluate the need for such special equipment in the medical service laboratory for the isolation of exacting anaerobic organisms of man.

The design of the anaerobic cabinet that we developed took account of the potential hazards of heated reducing columns, the toxic effects of carbon dioxide for man and the fire hazards of hydrogen. Our system was based on a commercially-available glove-box and used gases that were rendered oxygen-free by a catalytic purifier; the system was more compact and less expensive than many others that have been described, and our final gas mixture was not inflammable in air. The oxygen content of the cabinet atmosphere was demonstrably less than 1%; and the fact that we could grow *C. oedematiens* type D and *C. tetani* on blood agar incubated in the atmosphere of the cabinet, without a catalyst in the sampling jar, was accepted as signifying an exceedingly low oxygen tension.

Although this system appears to be acceptable in terms of its safety and function, its routine use would require much time and skill and the running costs for a service laboratory would be considerable. We chose to use this model rather than a Hungate (1950) gassing procedure, because it seemed that air would be more reliably excluded at all stages of manipulation in a cabinet.

Our results encourage us to believe that growth of an exacting anaerobe will be satisfactory if viable cells are brought promptly to a suitable medium that has been freshly poured and rendered adequately anaerobic. The anaerobic cabinet could not improve upon the recoveries of *C. oedematiens* type D and *C. tetani* achieved by conventional procedures. Moreover, we could often grow as many colonies as the estimated numbers of cells present in inocula of the exacting *C. oedematiens* strain. This indicates either that very exacting anaerobes are potentially recoverable by our procedures if the correct media are found, or that *C. oedematiens* type D is not as sensitive to oxygen as are other anaerobes handled by various workers with whom we appear to disagree.

Moore, Cato and Holdeman (1969) claimed that they isolated strictly anaerobic bacteria in large numbers from abscesses that would have been regarded as sterile if pre-reduced media and an oxygen-free gassing system had not been used. These workers agree with us that many anaerobes are able to remain viable, at least transiently, in the presence of relatively high concentrations of oxygen. They believe that in some natural environments anaerobes do not seem to be as sensitive to oxygen as the conditions necessary for their laboratory isolation would imply. They conclude that exacting anaerobes removed from their direct association with other bacteria will not grow in pure culture unless the environment is already reduced.

Many other workers have reported that recoveries of exacting anaerobes are markedly enhanced when their special procedures are used (e.g., Rosebury and Reynolds, 1964; Spears and Freter, 1967; Aranki *et al.*, 1968; Barnes and Burton, 1970). Barnes and Impey (1971) found that the Hungate technique enhanced the growth of anaerobic flora from chickens, but noted that the technique did not seem to be essential for the growth of any of the strains in pure culture. McMinn and Crawford (1970) reported that use of pre-reduced media and oxygen-free gassing procedures was at least twice as effective in the isolation of anaerobic species from clinical specimens as were conventional procedures employing aerobically stored agar plates and an anaerobic jar.

Drasar (1967) noted that, of several stock anaerobes tested by him, only *Bacteroides melaninogenicus* showed enhanced growth when media prepared under oxygen-free conditions were used. Wild *Fusobacterium* strains from saliva grew better on such oxygen-free media than on a conventional anaerobic medium. Only two of 40 strains of anaerobic lactobacilli, and one of 40 strains of *Bacteroides* species isolated in the cabinet from faeces could survive manipulations on the open bench. He claimed that his special techniques achieved a 100-fold improvement in comparison with conventional procedures. Drasar (1967) concluded that strains isolated by conventional techniques from faeces are necessarily the less oxygen-sensitive variants, and he considered that these methods are unsuitable for the enumeration of the total bacterial population of

any sample. It may be argued that the use of media prepared entirely anaerobically has not been investigated by our methods, but some workers do not feel that such preparation is an essential step in their routine work (Dr B. S. Drasar, personal communication). However, Drasar *et al.* (1969) remained convinced that "the use of an anaerobic cabinet enhances the yield of anaerobic bacteria many-fold" when human intestinal contents are cultured.

The counts that Drasar and his colleagues (1969) have reported in studies with faecal samples are of the order of 10^{10} – 10^{11} per g, and in the present study the mean of the counts that we achieved with our conventional procedure was 2.4×10^{10} , with some counts as high as 4×10^{10} per g. We were unable to achieve significantly higher counts with the cabinet, and the mean counts were the same. On occasion, in other studies, we have obtained counts of 10^{11} per g by conventional procedures (unpublished results). Our results in the present study compare favourably with those obtained in a previous survey by Dr Drasar's group in which several of our subjects participated (Hill *et al.*, 1971).

We have been careful to derive our viable counts from results with replicate plates bearing adequate numbers of colonies and we have borne in mind the limitations of total cell counts, even when these are performed in duplicate with a suitable counting chamber and a research-grade microscope (Norris and Powell, 1961; Cook and Lund, 1962; Meynell and Meynell, 1970). Nevertheless, we are well aware that our ability to recover stock strains of exacting anaerobes quantitatively from our cultures and our successful isolation of very large numbers of the "total anaerobes" from faeces are not guarantees that we can grow all of the potentially viable anaerobes from a faecal suspension. We suggest that colleagues with more extreme views must demonstrate and characterise the organisms of clinical interest that we may be failing to recover. It may be that organisms damaged by exposure in transit have an increased requirement for anaerobic handling and this merits further study (Collee and Watt, 1973).

Several precautions are now recognised as important for the growth of anaerobes. It is clear that if we were to use an unstandardised anaerobic procedure with poorly serviced anaerobic jars and to try to grow anaerobes in an atmosphere without added CO_2 on plates that had been stored aerobically for some time, we would have many failures. In comparative studies, all of the steps in anaerobic procedure must be defined. Our present results with conventional methods that take account of these factors encourage us to persevere with this approach until the need for more specialised procedures in clinical anaerobic bacteriology is clearly justified.

SUMMARY

An account is given of the design and development of an anaerobic cabinet for the manipulation of anaerobic cultures. Results of comparative studies on the recovery of anaerobic bacteria show that the quantitative recoveries obtained from cultures of strict anaerobes such as *Clostridium oedematiens* (*C. novyi* type D) and *C. tetani* were not improved by the use of the cabinet. Total counts

STUDIES WITH AN ANAEROBIC CABINET

FIG. 1.—The anaerobic cabinet (60.5 cm × 45 cm × 45 cm at maximum height, volume 110 l); a cylindrical transfer port (volume 5.7 l) is attached at the left side and gas supply and exhaust tubes at the right.

STUDIES WITH AN ANAEROBIC CABINET

FIG. 2.—Side view of the cabinet showing cylinders of compressed gas and BTL anaerobic jar modified to fit in transfer port.

of anaerobes from faecal samples, whether manipulated in the cabinet or at the bench by a careful conventional procedure, were almost identical. It is submitted that the quantitative growth of many anaerobic human pathogens does not require the use of ultra-specialised equipment.

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