Preparation of Prereduced Anaerobically Sterilized Media and Their Use in Cultivation of Anaerobic Bacteria

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Several modifications of the roll-tube method have made it simpler for routine use in the isolation and growth of anaerobic bacteria. These include use of a check valve for the production of prereduced anaerobically sterilized media; a Salvarsan tube under oxygen-free gas pressure for the dispensing of molten prereduced anaerobically sterilized agar medium; a Kelly infusion bottle with a graduated pipette side arm (also under gas pressure) for quantitative delivery of fluid prereduced anaerobically sterilized media; and screw-capped prescription bottles for the cultivation of anaerobes. Colonies of *Bacteroides melaninogenicus* were easily identified and counted by this method. Other anaerobic bacteria have also been cultivated successfully.

Recently there has been an increasing number of studies of anaerobic bacteria and a greater appreciation of their role in the disease process. For example, there is a current awareness that anaerobes are involved in infections in humans much more frequently than had generally been supposed (7). The introduction and development of new techniques have contributed to advances in this area. The established standard for cultivating anaerobic bacteria is the roll-tube method (4, 5), which features the use of prereduced anaerobically sterilized (PRAS) media. One of its difficulties is the total exclusion of air during the preparation and dispensing of PRAS media. Gosden and Ware (3) used a method which involved replacement of air in the autoclave with a hydrogen-nitrogen-carbon dioxide mixture. Fulghum (2) designed an all-glass dispenser for the PRAS media. This paper describes several modifications of the roll-tube method, making it amenable for routine use in the cultivation of anaerobic bacteria.

MATERIALS AND METHODS

Prereduced media were prepared as described in the *Anaerobe Laboratory Manual* (4). Dry ingredients, water, and resazurin were mixed in a vacuum flask. A cotton filter in glass tubing was attached to the side arm of the flask with a length of rubber tubing. A pinch clamp on the rubber tubing was removed to permit flushing through the side arm when desired.

Media were boiled, with refluxing into a chimney (4), until the resazurin was reduced. The flask was removed from heat, oxygen-free CO₂ was introduced through the side arm, and the chimney was replaced with a vented stopper to permit exit of CO₂. Media

were cooled to room temperature in an ice bath. Cysteine hydrochloride (0.05 g/100 ml) was then added, and the pH was adjusted to the desired value with HCl or NaOH. Agar, if required, was added. The vented stopper was then replaced with the chimney, and the medium was boiled, as before, until any oxidized resazurin was again reduced.

Before sterilization, the chimney was replaced with a stainless-steel check valve that has a nominal cracking pressure of 1 lb/in² (cat. no. SS-4CP2-1 Nupro check valve from Nupro Co., Willoughby, Ohio; in Canada, Laurentian Valve and Fittings Limited, Pointe Claire, Quebec). The valve was mounted on a rubber stopper (Fig. 1). The check valve permitted a blanket of nitrogen of 1 lb/in² above the medium. The gas was disconnected from the side arm and, after clamping, the flask assembly was autoclaved for the necessary time in the conventional manner.

A further modification involved the dispensing of sterile and reduced media after autoclaving by using a Salvarsan tube (Corning Glass Works, Corning, N.Y.). The check valve on the flask was replaced by a sterile single-glass tubing through a rubber stopper under continuous gas flow. (For ease of handling, the gas used from then on was a mixture of 90% argon, 5% H₂, and 5% CO₂.) At this point, sterile blood was added, if required. The glass tubing was then connected by sterilized rubber tubing to the Salvarsan dispenser (Fig. 2). In this manner, gas flushed freely through the Salvarsan tube.

With the delivery end clamped, the medium was transferred to the Salvarsan tube by tilting the flask. Gas continued to flush through the system. Gas was also directed to the sleeve assembly of the delivery tube (Fig. 3).

The culture bottles were 40-ml (2-ounce), rubber-lined, screw-capped glass prescription containers (cat. no. 16164-481; John's Scientific, Toronto, Ontario, Canada). A 10-ml amount was dispensed after flushing.

FIG. 1. Vacuum flask with a mounted check valve (arrow).

This amount provided a flat layer of agar medium which could be used for inoculation under a stream of oxygen-free gas.

When quantitative measurements of fluid were necessary, the Salvarsan tube was replaced by a Kelly infusion bottle (Corning) (Fig. 4). The exact volume of liquid medium was measured by means of the graduated pipette side arm and delivered as before (Fig. 3). Oxygen-free gas flushed freely through the apparatus at all times.

The modifications described above were made in the course of studies with specimens of dental plaque collected from patients with periodontal disease. These were diluted in a reduced fluid medium, and portions were spread on the surface of blood agar medium enriched with hemin and vitamin K. The medium was contained in either petri dishes or prescription bottles (as described above). Incubation of the petri dishes was by the GasPak method (Becton, Dickinson & Co., Rutherford, N.J.), whereas the prescription bottles were placed in a conventional incubator. Black-pigmented colonies of *Bacteroides melaninogenicus* were counted in this study after 10 days of incubation at 37°C, but pigmentation of *B. melaninogenicus* was visible after 48 h of incubation.

RESULTS

The data in Table 1 show the efficiency of isolation of *B. melaninogenicus* by the GasPak anaerobic jar technique and by the modified

FIG. 2. Salvarsan tube with rubber stopper containing two glass tubings. The bent one extends below the stopper and is connected to the vacuum flask. The other open tubing serves as a vent. (Flask may also be put into a small water bath if necessary.)

method as described above. It may be seen that, in relation to the total cultivable flora, the modified method gave consistently higher isolations of *B. melaninogenicus* than did the GasPak method. In addition, colonies on the medium in the anaerobic bottle were larger and better pigmented than those incubated in the GasPak jar. The culture bottle (Fig. 5) shows some of the varieties of discrete colonies of anaerobic bacteria from a dilution of plaque sample—such colonies are easily visualized and enumerated. Furthermore, pure cultures of anaerobic bacteria have been successfully cultivated. These include *B. fragilis*, *Clostridium novyi*, *C. septicum*, *C. sporogenes*, and *C. tetani*.

DISCUSSION

There are several improvements in the preparation of PRAS media by the technique de-

FIG. 3. Delivery tip from Salvarsan tube (or Kelly infusion bottle) with a sleeve or jacket for gas influx from side arm.

scribed above. The most important of these is in the use of a check valve. It retains a constant atmosphere of N_2 (or any other O_2 -free gas) over the medium before and during autoclaving. When the steam is turned off, the difference in vapor pressure within the flask and the autoclave chamber is relieved by the check valve, eliminating any danger of explosion. In addition, media may be made in bulk because all the ingredients, such as agar, heme, and menadione, may be added at one time. The dispensing of PRAS media is facilitated by the use of the Salvarsan tube or Kelly infusion bottle under a gas stream which completely excludes oxidation by air. The gas mixture (90% Ar , 5% H_2 , and 5% CO_2) used is a nonexplosive mixture. The H_2 helps in maintaining a reduced atmosphere as well as in keeping the copper column reduced.

FIG. 4. Kelly infusion bottle with a graduated pipette side arm. Its rubber stopper has three short glass tubings: one bent one is connected to the vacuum flask; the other bent one is connected to the graduated pipette; the straight tubing is open to the air and serves as a vent for O_2 -free gas. The pipette tip is connected to the delivery mouth of the infusion bottle by means of a Y-joint and butyl rubber tubing. (Compare this apparatus with that of Fig. 2.)

The presence of CO_2 meets the growth requirement of some anaerobes. Pure CO_2 was not used because it would lower the pH of the medium.

The use of ordinary flat prescription glass bottles was efficient and practical, as has been reported by others for the isolation of treponemes (8) and clinically significant anaerobes (1). The 4-ounce medicine bottles described by Davis et al. (1) were closed with rubber stoppers. We found that the rubber liner of the screw cap maintained the media in the reduced state, even during storage. The method described above for

FIG. 5. Surface colonies of anaerobic bacteria from dental plaque growing in prescription bottle.

dispensing PRAS media into sterile bottles also circumvents the problem of breakage encountered when prereduced media are autoclaved in prescription bottles. We found that, in the bot-

TABLE 1. Comparative isolation of *B. melaninogenicus* on brain heart infusion blood agar medium by using the GasPak anaerobic jar and the "anaerobic bottle" method from specimens of subgingival plaque

Sample	<i>B. melaninogenicus</i> ($\times 10^2$ /total cultivable flora)	
	Anaerobic bottle method	GasPak method
1	42	3
2	9	4
3	39	21
4	17	5
5	22	0
6	43	4
7	64	17
8	28	6
9	34	11
10	3	2

tles, colonies were easily recognized and counted, and subsequent isolation of colonies for pure cultures was easily done.

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