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Combined Screw-Cap and Rubber-Stopper Closure for Hungate Tubes (Pre-reduced Anaerobically Sterilized Roll Tubes and Liquid Media)

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A double-closured method introduced for Hungate tubes eliminates clamps or special racks to hold the rubber stoppers in place while the tubes are being processed. The system prevents stoppers from being dislodged by gas-producing anaerobes and keeps the stoppers sterile so that the closed system of transfer can be conveniently utilized. Open and closed systems for handling a gas-producing anaerobe were compared. Ten tubes containing *Clostridium perfringens* were opened; the organism was detected in the air by use of an impingement collector. The gloved hand of the operator also revealed the test organism. A similar trial with culture material removed by the closed system (syringe transfer) resulted in no detectable contamination of the environment.

Roll tubes are test tubes with a nutrient medium solidified in a thin layer around the inner surface of the tubes. The roll tube, as originated by Esmarch (3), has not been a popular system for the isolation and enumeration of bacteria since the availability of petri dishes. However, a roll-tube method for counting bacteria in milk is still used (1).

Esmarch used roll tubes for anaerobic culturing by filling the hollow core with a solid medium, and Fränkel (5) and Ewell (4) used hydrogen gas methods to reduce the medium after sterilization. These methods were replaced principally by petri plates used with anaerobic jars.

Roll-tube use is now increasing, owing to Hungate's origination of a continuously maintained anaerobic roll-tube method (7). His conception of pre-reduced anaerobically sterilized (PRAS) habitat-simulating media and his development of culture techniques which avoid air contact with the media make this culture method very successful for anaerobes.

Hungate uses either a closed or an open system for inoculation (8). In the closed system, material is transferred from one closed tube to another by a gassed-out syringe, the needle passing through a rubber stopper, with a recess provided for easier penetration. Kistner et al. (10, 11, 13) used double-closured bottles for the cultivation of ovine rumen bacteria by the closed system.

The open system of transfer is accomplished by removing the rubber stopper and allowing a stream of oxygen-free gas (such as carbon dioxide or nitrogen) to enter the tube so as to preclude entry of air.

Roll-tube methods allow cultivation of fastidious anaerobes that have not been successfully cultured by petri plate and anaerobic jar methods (7, 8, 14). Roll-tube techniques have been used to study the rumen of herbivores and, as a result, the rumen is now the best ecologically understood anaerobic microbial habitat.

Roll-tube anaerobic counts have been compared to standard anaerobic jar plate counts by using normal flora of animals and man (14; M. Stutman and D. F. Gordon, Jr., Int. Ass. Dental Res. Abstr. 181, p. 86, 1969), and the roll-tube method has always given a significantly higher count.

The use of PRAS medium, rubber stoppered with the open system of anaerobic transfer, was recommended (2) for the isolation and characterization of anaerobes obtained from clinical specimens. Hungate recognized the problem of adapting his procedures for the cultivation of pathogens from clinical material (8); the medium normally covers the inside of the stopper, and contamination of the surroundings is a possibility when the stopper is withdrawn.

The combined use of a rubber-stopper and a

screw-capped closure is suggested for the safer handling of clinical material and concomitant ease of manipulation.

MATERIALS AND METHODS

Roll tubes used in our laboratory are usually 16 by 125 mm (Fig. 1A). PRAS liquid medium is usually tubed in 4.5-ml amounts in 13- by 100-mm tubes (Fig. 1B). A convenient substitute for both purposes is a 20- by 125-mm screw-capped tube (Fig. 1C). For roll tube use, 8 ml of medium is used with the 20- by 125-mm tubes. One may also use 20- by 150-mm screw-capped tubes for roll tubes (Fig. 1D). Butyl rubber stoppers (Fig. 1E), size 00 (no. 8820-B; A. H. Thomas Co., Philadelphia, Pa.), are used instead of red rubber stoppers or black rubber stoppers (A. H. Thomas Co., HR-108 Stock; reference 9), because they are relatively impermeable to oxygen and fermentation gases. These butyl stoppers have an 0.5-inch (1.27 cm) recess that allows easier needle penetration and placement flush with the rim of the 20-mm screw-capped tubes (Fig. 1C) as the rubber is displaced into the central air space. Butyl rubber is slippery and, especially when wet, can be easily pushed in flush so that a screw-cap may be applied over it (Fig. 1D).

Peptone-glucose-yeast extract broth (2) was dispensed in 9-ml amounts in double-closured tubes (Fig. 1C). Plastic caps (Fig. 1F) covered with aluminum foil were used; 4.5-ml amounts were used in single-closured tubes (Fig. 1B). Double-closured tubes were inoculated by the closed-tube method, and single-closured tubes were inoculated by the open-tube method. A 24-hr culture of *Clostridium perfringens* in fluid thioglycolate medium was used to inoculate 10 tubes each of the above two systems.

After 16 hr of incubation, during which time there was abundant gas production in all tubes as evidenced by froth and numerous gas bubbles, the tubes were processed as follows. The foil cover was removed from a double-closured tube, and a plastic disposable 10-ml syringe fitted with a 19-gauge, 1.5-inch (3.8 cm) Yale disposable needle was used to remove 2 ml of the culture. This procedure was accomplished by first inserting the needle through the stopper, holding the syringe with the plunger in the palm of the hand so that the gas pressure could not force the plunger out of the syringe. The plunger was allowed to be pushed out so that the pressure within the tube came to atmospheric pressure, and then the needle was withdrawn. The gas was slowly ejected from the syringe into a bunsen flame. The needle was then reinserted into the stopper, and 2 ml of culture was withdrawn.

A single-closured tube was opened by removing the stopper and placing it on a germicide-saturated towel, top down. The tube lip was momentarily flamed in the bunsen burner, and then the cap was replaced on the tube. The contents were not sampled and the tube was not gassed, as the purpose of this procedure was to determine whether contamination of the surroundings resulted from opening pressurized tubes.

Two systems were used for contamination detection—an impingement method to sample the air above the work area and swabbing of the gloved hand holding the tubes.

Impingement samplers were constructed by using 12- by 150-mm test tubes with a side arm and fitted with one-holed stoppers with autoclavable plastic tubing reaching near the bottom of the tube. A cotton filter was placed in the side arm, and a short piece of rubber tubing was attached. A piece of rubber tubing was also attached to the impinger tube, and the end of this tube was plugged with cotton. A 5-ml amount of fluid thioglycolate medium was dispensed in each impinger tube, and these were autoclaved at 12 lb/in^2 for 15 min. Immediately after autoclaving, the rubber tubes were clamped and the bottoms of the tubes were placed in cold water until cool.

Impingers were placed 14 inches (35 cm) above the work surface and directly over the area in which the culture tubes were processed. The impingers were connected to a vacuum pump and the flow meter was adjusted to 3 liters per min. The end of the rubber tube containing the cotton filter was then cut with sterile scissors.

Prior to the start of each evaluation, the air was sampled for 10 min, with a new impinger used each time. After each air sampling was completed, the contents of the impinger tube were removed with a sterile pipette, and a 2-ml amount was placed in each of two tubes containing 9 ml of fluid thioglycolate medium. These tubes were incubated for 5 days at 37 C.

Sterile gloves were used during each evaluation. After each procedure, a sterile cotton swab moistened with fluid thioglycolate medium was rubbed over the upper surfaces of the thumb and index finger of the gloved left hand which held the tubes. The swab was placed into a tube of fluid thioglycolate medium and rotated against the side of the tube. These tubes were also incubated for 5 days.

All tubes of fluid thioglycolate medium showing growth were subcultured, and definitive identification was carried out on isolates.

RESULTS

The results are summarized in Table 1. Air sampling for 10 min before each evaluation resulted in no recovery of *C. perfringens*. The test organism was not recovered from the air or glove sampling when the closed system was used. *C. perfringens* was recovered from both the air and glove sampling during the open-tube procedure. Small droplets were evident on the gloved hand after the open-tube series was completed, but were absent following the closed method.

DISCUSSION

Disadvantages of using only the rubber stopper as a closure, as given below, are eliminated by the use of a screw-capped closure with a rubber stopper.

(i) Clamps, presses, or special racks (6, 12) need to be used during autoclaving to hold the rubber stoppers in place as changes in pressure can blow out the stoppers.

(ii) During melting of the agar for roll tube

TABLE 1. *Comparison of closed and open methods of entering roll tubes with regard to contamination of environment with the test organism (C. perfringens)*

Method	Duration of air sampling	*C. perfringens* in air sample	*C. perfringens* on left-hand glove
	min		
Control	10	Absent	Absent
Closed method	7	Absent	Absent
Control	10	Absent	Absent -
Open method	4.5	Present[a]	Present

[a] Present in both tubes of fluid thioglycolate medium.

preparation, the stoppers should be clamped, as they can be dislodged.

(iii) The exposed glass lip of the roll tube is subject to contamination, as is the exposed rubber stopper. A screw cap over the stopper keeps the tube lip and stopper sterile, so that it is not necessary to flame the stopper or tube before inoculating or subculturing.

(iv) Gas production by aerogenic anaerobes can easily blow the stoppers from unclamped tubes and create aerosols which can contaminate incubators and other laboratory areas. A screw-capped closure used at all times prevents this occurrence. When testing suspected or known gas producers such as *Clostridium* or *Sphaerophorus*, it is suggested that the solid screw cap be replaced by a sterile screw cap (Fig. 1F) that has had a 5/16-inch hole drilled through the top and which is, in addition, covered with sterile aluminun foil. The hole allows convenient needle access, yet sufficient cap material remains to hold the stopper in position.

(v) The screw-capped closure also offers a convenient and safer system for transport and shipment of cultures. Tape should not be relied upon to hold slippery, butyl rubber-stoppered tubes in position during shipment, especially when gas-producing strains have to be forwarded in liquid medium (e.g., some spirochetes).

If it is desired to use the open system of inoculation with the two-closure system, a disadvantage is that more time is needed to remove the rubber stopper which is flush with the tube. These stoppers must be removed with flamed needle-nosed pliers. Although this is not difficult, the time required for inoculating a large number of tubes would make the practice impractical. A screw-capped closure should be made available in an extra-tall form with threads extending the entire length of the cap. The rubber stopper could then protrude 0.25 inch (0.6 cm) from the tube, so that either the open or the closed method of inoculation could be used, retaining the advantages of the double-closure system.

Before inoculation or subculture by the closed method, the tube contents have to be equilibrated so that the contents are at ambient pressure. It is difficult to accurately inject a measured amount of material into a tube not at ambient pressure. It is also not safe to remove inocula of pathogenic organisms from a pressurized tube because of the problem of gas being forced out of the needle as the syringe contents expand, owing to pressure decrease.

Needle penetration in simplified by thrusting obliquely through the recessed area as a thinner area of rubber is entered.

Commercially available media in double-closured tubes would allow small clinical laboratories to use the closed system for isolation and identification of anaerobes without necessitating the purchase of tank gas and a reducing furnace, which are needed for the open inoculation method. We believe a closed system allows a safer means of

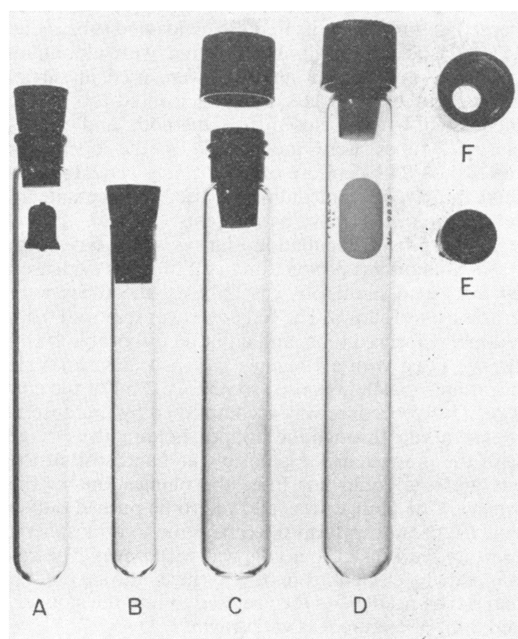

FIG. 1. *Tubes for closed and open system of inoculation showing rubber stopper closure and combined closure with screw caps. (A) Standard roll tube, 16 by 125 mm; (B) 13- by 100-mm tube for PRAS liquid medium; (C) large screw-capped tube—short form, 20 by 125 mm; (D) large screw-capped tube—long form, 20 by 150 mm; (E) butyl rubber stopper with recessed area; (F) screw cap, modified to hold stopper in place under pressure yet allow contents to be withdrawn safely.*

culturing pathogenic anaerobes than the open system and offers certain other conveniences. The closed system is not convenient for inoculating a large number of tubes.

To recover colonies from roll tubes, the tube would have to be opened and exposed to air briefly until the colonies could be transferred to other PRAS tubes for biochemical tests, etc. To accomplish this, one would remove the colony from the roll tube, emulsify it in a small volume of PRAS medium, aspirate this into a syringe, expel all air bubbles, and then inoculate a PRAS tube. Subsequent growth in this tube can be handled by the closed technique. It is preferable, however, if nitrogen or carbon dioxide tank gas is available, to immediately gas the tube upon opening it. Colonies are picked while the tube is being gassed. The tank gas should be run through a column of heated, reduced copper turnings to remove traces of oxygen. The gas should also be run through a sterile cotton filter and sterile apparatus down-stream from the filter (2).

We have kept double-closured media on hand for more than 6 months without evidence of oxidation (resazurin remaining in the colorless dihydroresorufin form). The double-closured method has been effective in our hands for the isolation and classification of obligately anaerobic spirochetes.

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