A Miniature Anaerobic Jar for Tissue Transport or for Cultivation of Anaerobes

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ABSTRACT

Attebery, Howard R., and Finegold, Sydney M.: A miniature anaerobic jar for tissue transport or for cultivation of anaerobes. Am. J. Clin. Path. 53: 383-388, 1970. Miniature jars (35-mm. film containers), with acidified copper sulfate and steel wool for obtaining anaerobiosis, were found to be economical and convenient to use for the transport of tissue for anaerobic examination. These jars were compared with Brewer jars and gassed roll tubes. All were equally effective in keeping Bacteroides melaninogenicus and Fusobacterium fusiforme viable for six hours in inoculated tissue. The miniature-jar system can also be used for the cultivation of obligate anaerobes on agar slants or in roll-vials. Alka-Seltzer is an effective source of carbon dioxide.

ANAEROBES frequently infect man and animals. Examination of clinical material suspected of containing anaerobes should be made immediately after collection or, if this is not possible, the material should be kept in an anaerobic state until it is brought to the laboratory.

Tissue is usually transported anaerobically in liquid media such as fluid thioglycollate medium or Stuart's Transport Medium or in vials of pre-reduced medium into which bits of tissue have been injected. Tissue may also be transported in sterile containers placed in anaerobic jars or placed in gassed-out, oxygen-free tubes.

This report presents a modified copper-sulfate-steel-wool method for obtaining anaerobiosis that is used with metal 35-mm. film containers as miniature anaerobic jars (the "mini-jar" method).

Materials and Methods

Acidified 5% copper sulfate solution is prepared by dissolving 25 Gm. of cupric sulfate in 500 ml. of distilled water; then about 1.6 ml. of concentrated sulfuric acid is added to bring the pH to about 2.0. Following this, 1.0 ml. of polyoxyethylene sorbitan monooleate (Tween 80) is added and the solution is mixed thoroughly. Twodram vials are filled with this solution.

Pads of steel wool, approximately 1 Gm. in weight, are prepared from grade 0, fine, commercial steel wool. Alka-Seltzer* tablets are cut to form \( \frac{1}{8} \) inch square (approximately 0.15 Gm.) pellets.

* Composed of aspirin, mono-calcium phosphate, sodium bicarbonate, and citric acid; Miles Laboratories, Elkhart, Ind.
is placed in the jar with the vial lid loose. The Alka-Seltzer is attached to the top of the container with tape. Treated steel wool is packed around the vial and the jar lid is tightened. The jar is placed in a 37-C. incubator or a pocket to accelerate the oxygen-adsorbing reaction.

An aerobic Transport of Tissue in the Mini-jar. The kit for this purpose (Fig. 2A) consists of a two-dram vial of acidified copper sulfate solution attached to a mini-jar. Within the jar is a sterile one-dram vial and a steel wool pad. The tissue is placed in the vial, the lid of which is left loose. The subsequent procedure is identical to that for culture of anaerobes described above except that the carbon dioxide source (Alka-Seltzer) is not used.

Gassed-out Roll Tubes. These were prepared by flushing out 16 by 125-mm. roll tubes with oxygen-free nitrogen tank gas by the method of Moore.7 Tubes were stoppered with butyl rubber stoppers and autoclaved at 115 C. for 15 min. These were used for tissue transport.

Standard Anaerobic Jar. In the comparison studies, vials with tissue or vials inoculated with cultures were placed with lids loose in Brewer jars that had been flushed six times with nitrogen. Tank gas was used to produce a final concentration of 10% carbon dioxide and 90% nitrogen.

Growth of Anaerobes in Mini-jars. Broth cultures of ten species of obligate anaerobes (Table 1) were streaked in triplicate on blood-agar slants to provide comparable inocula on all slants. One series was incubated in a Brewer jar, one in mini-jars with the carbon-dioxide source, and the third in mini-jars with no carbon dioxide source. All were incubated at 37 C. for four days and examined for growth. The amount of growth was graded + to ++ + +.

Survival of Anaerobes in Mini-jars without CO₂. The species listed in Table 1 were

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†Eastman Kodak Co., Rochester, N. Y.
‡Kimble 600910, Owens-Illinois Co., Toledo, Ohio.
§Pfizer Diagnostic Laboratories, New York, N. Y.
¶The McBee Laboratory, Bozeman, Montana.
cultured on blood-agar slants in vials placed in Brewer jars. After incubation for three days the vials were transferred to individual mini-jars processed without carbon dioxide. Mini-jars were incubated for four hours, then stored at room temperature for six days. Vial cultures were subcultured to fluid thioglycollate medium with ascitic fluid.

**Survival of Anaerobes in Tissue**

*Fusobacterium fusiforme* strain B153 and *Bacteroides melaninogenicus* strain B931 were grown in fluid thioglycollate medium (BBL01-140) with 25% ascitic fluid (final concentration). Forty-eight-hour cultures were diluted with 0.05% yeast extract that had previously been steamed for 10 min. and cooled. Dilutions were prepared so that 0.05 ml. contained approximately 150,000 viable *Fusobacterium* cells or 10,000 viable *Bacteroides* cells. Breed counts were used as a guide in dilution and viable counts were confirmed by blood-agar plate counts.

The liver from an adult rat was removed aseptically and sectioned into square pieces weighing approximately 0.15 Gm. Dilutions of the organisms (0.05 ml.) were injected into each piece. Inoculated tissue and uninoculated tissue controls were placed in separate one-dram vials and processed, one vial to a mini-jar. Tissue blocks were also placed in gassed-out tubes (one per tube) with tubes continually flushed with oxygen-free gas during the procedure. Three vials were placed in each of eight Brewer jars (two inoculated and one control tissue). All materials were incubated at 37 C. Pieces of liver were also placed in vials with aerobic atmospheres; the lids were tightened on these vials.

A three-hour period elapsed from the time of removal of the liver until all procedures were completed. Gassed-out tubes and vials with only lids tightened (no anaerobic treatment) were completed within 15 min. after inoculation of tissue; mini-jars were completed one hour later and the Brewer jars 30 min. after the mini-jars.

Material from all of the above set-ups was obtained for subculture at intervals of 2, 4, 6, 14, and 24 hr., and at 5 and 7 days. The entire piece of tissue was emulsified in fluid thioglycollate medium with ascitic fluid using a glass rod. This was subsequently incubated (aerobically) and checked for growth.

**Results**

**Growth of Anaerobes in Mini-jars**

Table 1 summarizes the data regarding growth of anaerobes in the mini-jar system, with and without carbon dioxide, and in Brewer jars with carbon dioxide. The growth of strains in mini-jars with carbon dioxide was equivalent to growth in the Brewer jar. *B. oralis* did not grow in the mini-jar system without carbon dioxide and *B. fragilis*, *B. melaninogenicus*, and *F. fusiforme* showed poorer growth without carbon dioxide. All of the strains remained viable during the storage period in the mini-jars. This suggests a method for convenient shipment of anaerobic cultures.

**Survival of Anaerobes in Tissue**

The survival time of *B. melaninogenicus* in tissue in the non-liquid transport systems tested is noted in Table 2. *B. melani-
TABLE 1. Comparison of Growth of Anaerobes in Mini-jars, with and without CO₂, and in Brewer Jars

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Mini-jar Without CO₂</th>
<th>Brewer Jar (90% N₂, 10% CO₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veillonella alcalescens</td>
<td>A 128</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Bacteroides oralis</td>
<td>B 311</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Bacteroides fragiis</td>
<td>B 3</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Bacteroides melaninogenicus</td>
<td>B 515</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sphaerophorus maccrophorus</td>
<td>B 540</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Fusobacterium fusiforme</td>
<td>B 153</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peptostreptococcus anaerobius</td>
<td>A 130</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>BB-12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Corynebacterium acnes</td>
<td>GPR-31</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>A-61</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*nogenicus* survived 24 hr. in both the Brewer jar and the mini-jar systems. It did not survive five days in the mini-jar; it was not cultured after five days in the Brewer jar because of dehydration. Gassed-out tubes held these bacteria viable for only six hours. No viable organisms were recovered from the tissue placed in vials that were not subjected to reducing methods.

The results of survival of *Fusobacterium fusiforme* in tissue stored in the various test systems are listed in Table 3. *F. fusiforme* survived five days, but not seven, in the mini-jar system, the closed vial, and the gassed-tube method. This strain survived one day in the Brewer jar and was not studied at five and seven days because of tissue dehydration.

**Discussion**

The use of iron wool treated with copper sulfate solution was introduced by Parker to remove oxygen from the atmosphere of sealed containers. Specific carbonate solutions were used to provide desired final concentrations of carbon dioxide to the jar atmosphere.

The steel-wool method has been used successfully for the cultivation of obligate anaerobes by many investigators. Ueno presented a steel-wool method for culturing a single test tube anaerobically. He steamed out and cooled the medium, then inoculated the cotton-plugged tube and placed it within a larger tube. A steel-wool treated pad was added to the outer tube, which was sealed with a rubber stopper.

In clinical pathology, there is a need for a simple, efficient, nonliquid system for the anaerobic transport of tissue specimens. The mini-jar system was developed to meet this need. Present methods of anaerobic transport of tissue usually consist of placing tissue in fluid thioglycollate medium or a fluid version of Stuart’s Transport Medium. These liquid systems have two possible disadvantages that are avoided in the mini-jar system. The liquid permeates the tissue specimen and changes the weight of the specimen so that quantitation of anaerobes would not be possible. Also, constituents of the holding medium entering the tissue may produce undesirable changes.

The miniature-jar system accomplishes anaerobiosis by chemical reactions which remove oxygen; water vapor and a small
amount of hydrogen gas are produced (to be published).

The amount of water vapor produced is sufficient to keep the contents of the jar humid, but does not allow washing of the tissue or dilution of organisms present in the tissue.

We have modified Parker's solution concentrations for economy of space with no sacrifice in effectiveness. The amount of steel wool and copper sulfate solution used provides a large reserve capacity for oxygen adsorption (this amount can effectively remove the oxygen from a 300-ml. container). The use of a large amount of oxygen-gathering material ensures rapid adsorption of oxygen with a reserve to reduce oxidized material.

The small-jar system using copper-sulfate-steel-wool has proven to be an efficient method for culturing the anaerobes so far tested. Blood-agar slants used with the mini-jar system are convenient for maintaining working stock cultures because the cultures are in individual containers and can be used independently. After use, the slant culture is replaced in the mini-jar and a new copper sulfate-treated steel wool pad is added.

Roll vials have been used with the mini-jar. They are more useful than slants in that more surface area is available for streaking, so that colony isolation from a mixed flora is possible. A vial roller and streaker can be made by modifying the apparatus used by Snyder. After streaking, the vial is placed in the mini-jar with the vial lid loose and the jar activated. Roll vials can also be used for quantitation of anaerobic flora, but are limited in usefulness by their small size. Prereduced media and the roll-tube methods of Hungate and Moore are more satisfactory for the isolation of anaerobes. However, the simplicity, ease of use, and effectiveness of the mini-jar method offer convenience for the cultivation of many anaerobes. The mini-jar method should be particularly useful for field work and for small laboratories.

It is important to include carbon dioxide in any system used for the primary isolation and cultivation of anaerobes, as well as for many aerobes. Brewer and Allgeier used a tablet (citric acid and sodium bicarbonate) to supply carbon dioxide to their jar. The incorporation of approximately 0.15 Gm. of an Alka-Seltzer tablet taped to the top of the jar supplies an adequate amount for growth of carbon dioxide-requiring anaerobes. As oxygen is removed chemically from the jar atmosphere, water is produced. Water vapor then acts on the dry Alka-Seltzer to liberate the carbon dioxide.

The mini-jar method compares favorably with the other anaerobic methods tested to keep the organisms inoculated into rat tissue viable. Tissue for anaerobic culture should be processed as soon as possible. We do not mean to imply from the duration of the tests utilized in this study that tissue may be kept that long before examination without loss of any organisms which are present. Immediate processing would be ideal; the delay should be no longer than six hours after collection.

From the data, it appears that Bacteroides melaninogenicus is more fragile in storage than Fusobacterium fusiforme. The latter survives well in closed vials for five days without benefit of anaerobic treatment. In comparison, B. melaninogenicus did not survive for two hours in the closed

<table>
<thead>
<tr>
<th>Time</th>
<th>Mini-jar</th>
<th>Brewer Jar</th>
<th>No Jar, Vial Cap Closed</th>
<th>Uninoculated Tissue Control in Mini-jar</th>
</tr>
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<tbody>
<tr>
<td>2 hours</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>6 hours</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>14 hours</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24 hours</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 days</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7 days</td>
<td>-</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Specimen dehydrated in closed Brewer jar; not cultured.
vial. *B. melaninogenicus* survival increases with anaerobic storage so that cultivation is possible after six hours.

It was not possible to process the liver tissue stored in the various containers simultaneously, but it happened that the tissue processed last (the evacuation-replacement jar, and the mini-jar) yielded the best results.

We have found the miniature-jar system to be safe, economical, and convenient to use in the transport of tissue specimens for anaerobic examination. The anaerobic kit is compact, and the chemicals have a shelf-life exceeding a month.

The procedure for producing anaerobiosis is simple and, due to the large oxygen-adsorbing capacity per volume of jar space, anaerobiosis is obtained quickly.

This nonliquid system offers a means of keeping tissue in a more natural state than liquid transport media, and is suitable for quantitative studies.

Miniature jars, Brewer jars, and gassed roll-tube methods were all equally effective, during a six-hour period, in keeping *Bacteroides melaninogenicus* and *Fusiformis fusiforme* viable in tissue. The ease of use of the miniature system, plus the fact that only simple, compact equipment is needed, makes the miniature-jar system the logical choice for transport of tissue specimens.

**References**