Practical Method for Isolation of Anaerobic Bacteria in the Clinical Laboratory

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An inexpensive but practical and simple method is described for providing an environment suitable for the maintenance and survival of clinically significant anaerobic bacteria, consistent with the needs of a large-volume laboratory. This modification of the GasPak procedure is based on two concepts. One involved the storage of "freshly prepared" plating medium under a constant stream of carbon dioxide. The other concept was to use an anaerobe jar, continuously flushed with oxygen-free carbon dioxide, as a "holding receptacle" for the inoculated media. Experience with this modification has shown that the number of isolations of anaerobic bacteria from clinical material can be increased significantly.

Recovery of clinically significant anaerobic bacteria in the laboratory has taken on new significance because of the increasing number of reports incriminating these organisms in diverse disease states. The use of a tightly sealed container from which the oxygen has been removed has been the main approach to the cultivation of these bacteria in most laboratories (3, 5, 13, 15). Indeed, several different types of jar and lid arrangements, including the more complex and expensive anaerobic incubators, have been in use for several years. Methods, such as the Hungate roll tube method or some modification of it, have been reported by several investigators (1, 5, 8-11, 13, 14) as being better suited for recovery of anaerobic bacteria from heterogeneous sources. Other workers (4, 7, 12) have found that, by using a glove box under conditions approaching continuous anaerobiosis, the ideal environment is provided for the detailed study of these organisms.

Although it is well recognized that both of these methods have contributed much to the isolation and subsequent characterization of these bacteria, it can be argued that it is not practical to apply either the Hungate roll tube method or the glove box procedure to routine use in most clinical bacteriology laboratories. Moreover, the expense incurred in processing specimens by either one of these methods could prohibit their use in many hospital laboratories.

This report describes an inexpensive but practical and simple method that can be used with the GasPak (BBL) procedure, which makes possible the isolation of most clinically significant anaerobic bacteria.

One of the most practical methods for achieving anaerobic conditions is the anaerobe jar. Among the more widely used is the self-contained, disposable, anaerobic system (GasPak anaerobic jar, BioQuest, Cockeysville, Md. 21030) of Brewer and Allgeier (2). This uses a safe, disposable hydrogen and carbon dioxide generator envelope, a disposable anaerobic indicator, and a platinum catalyst (the catalyst may be reactivated after each use by heating for 2 hr at 160°C; V. R. Dowell, Jr., personal communication).

To provide an environment which was more conducive to the maintenance and survival of anaerobes under the circumstances of a large-volume laboratory, two concepts were used. Although the need for freshly prepared plating media is well recognized for the isolation of anaerobes, the media which cannot be put into immediate use concerned us most. Even more important was the desire to relieve the burden of preparing large amounts of various kinds of plating media daily. In this regard, all of our plating media are stored until used, but no more than 72 hr, under a constant stream of CO₂ (0.5 liter/min) in a relatively air-tight cabinet; for example, we adapted an extra incubator for this purpose (Fig. 1). Periodic determinations on these media (5% sheep blood-agar, phenylethyl alcohol-blood-agar, and kanamycin-vancomycin-mendone-blood-agar) showed that at no time did the pH go below 6.8 during a 72-hr period of storage. Moreover, experiments designed to determine the ability of these stored media to support growth of representative strains, Peptococcus sp., Pepto-streptococcus sp., Eubacterium lentum, Bacteroides fragilis, Clostridium perfringens, C. tetani, C.
sporogenes, and C. sordelli, indicated no adverse effects. Indeed, growth characteristics of these bacteria with 10-fold dilutions (10⁻¹ to 10⁻³) were similar on both the stored media and the freshly prepared media after both 48 and 72 hr of incubation. In using these "stored" media, the technicians are instructed to anticipate their needs for it, thereby keeping exposure to oxygen at a minimum.

The other concept is shown in Fig. 2. When the medium has been inoculated, it is immediately placed in the anaerobe jar receiving O₂-free CO₂. The CO₂ is passed over copper heated to about 300 C in a thermostatically controlled oven (14). The volume of CO₂ delivered is not critical. However, we maintain a flow rate which produces a slow but steady formation of bubbles in water. The lid assembly is a Brewer lid which rests on the jar without any fastening. However, it is important that this lid assembly (or one similar to it) be heavy enough and the gas volume adequately regulated so that the lid rests comfortably on the lip of the anaerobe jar without bumping. Periodically, the gas flow should be checked by inserting one of the delivery tubes into water. At the flow rate we use, the heated copper will have to be reduced (14) every 3 to 4 weeks. Once this O₂-free CO₂ anaerobe jar is filled with inoculated media, a GasPak is quickly added and the jar is secured (as in the GasPak method); another anaerobe jar then serves as the O₂-free CO₂ container.

In this laboratory, all specimens other than throat and vaginal swabs, urine, and stool are processed routinely for anaerobic bacteria; the other specimens are done only on special request by the attending physician.

When the standard GasPak system was being used in this laboratory, anaerobic bacteria were present in almost 40% of 1,223 specimens examined (16); of this total, 323 anaerobic bacteria were recovered, for an isolation rate of 26.4%.

In our experience with the new procedure during 1970, approximately 35% of the specimens received in the laboratory were positive for anaerobic bacteria (Table 1). From these positive specimens, however, approximately 5,000 isolations were made (49.3%) or a ratio of anaerobes of 1:4:1 isolated from each positive specimen. The percentages shown in Table 1 are calculated from the total specimens received, which included a sizeable number of specimens that were culturally negative for all bacteria. Preliminary data in this laboratory indicate that as high as 40% of the specimens on which anaerobic cultures are done are negative for both aerobes and anaerobes by Gram stain and culture methods. Table 2 shows the distribution of anaerobic bacteria isolated according to strain and source.

The method outlined in this communication appears to offer several advantages for the clinical laboratory. Excluding the GasPak system, the entire cost for this system to become operational was less than $200.

![FIG. 1. Constant-flow CO₂ cabinet for storage of "reduced" plating media.](image1)

![FIG. 2. Constant-flow CO₂ anaerobe jar assembly for holding inoculated media.](image2)

**Table 1. Summary of specimens received and anaerobic bacteria recovered during 1970**

| Month     | Total specimens (no.) | Specimens positive | Isolations |
|-----------|------------------------|--------------------|------------|
|           | No. | Per cent | No. | Per cent |
| January... | 628 | 227     | 36  | 54       |
| February.. | 733 | 291     | 40  | 53       |
| March..... | 785 | 313     | 40  | 55       |
| April..... | 825 | 358     | 43  | 56       |
| May....... | 801 | 404     | 50  | 64       |
| June...... | 866 | 317     | 37  | 51       |
| July....... | 964 | 324     | 34  | 46       |
| August.... | 927 | 269     | 29  | 40       |
| September. | 774 | 291     | 38  | 52       |
| October... | 1,075 | 279 | 26  | 36       |
| November.. | 934 | 294     | 31  | 48       |
| December.. | 879 | 217     | 25  | 38       |
| Total....  | 10,191 | 3,584 | 35.1 | 5,029 | 49.3 |

This laboratory, all specimens other than throat and vaginal swabs, urine, and stool are processed routinely for anaerobic bacteria; the other specimens are done only on special request by the attending physician.
TABLE 2. Summary of anaerobic bacteria isolated according to strain and source

| Strain                        | Isolates (no.) by source | Total isolations |
|-------------------------------|--------------------------|------------------|
|                               | Hand (arm) | Leg (foot) | Rectum | Cyst | Fluid | Abdominal wound | Abscess | Swab | Wound | Surgical specimen | Blood | Ear | Nose | Urine | Sputum | No. | Per cent |
| Bacteroides fragilis         | 17         | 70         | 92      | 1    | 25    | 233     | 91    | 35   | 212   | 122   | 121  | 0    | 1    | 14    | 17    | 1,051 | 21    |
| B. melaninogenicus           | 13         | 23         | 25      | 3    | 12    | 37      | 28    | 18   | 30    | 24    | 0    | 0    | 0    | 1     | 55    | 289  | 6     |
| Bacteroides sp.              | 7          | 44         | 58      | 2    | 12    | 97      | 57    | 21   | 87    | 59    | 16   | 5    | 2    | 7     | 36    | 510  | 10    |
| Fusobacterium fusiforme      | 13         | 10         | 23      | 1    | 6     | 35      | 17    | 13   | 37    | 21    | 7    | 1    | 2    | 0     | 39    | 225  | 4     |
| Fusobacterium sp.            | 2          | 11         | 8       | 0    | 8     | 30      | 15    | 4    | 34    | 16    | 2    | 0    | 1    | 0     | 14    | 145  | 3     |
| Veillonella sp.              | 14         | 18         | 6       | 0    | 4     | 24      | 5     | 6    | 30    | 9     | 2    | 1    | 1    | 1     | 18    | 139  | 3     |
| Peptococcus sp.              | 70         | 212        | 37      | 2    | 12    | 76      | 69    | 38   | 179   | 95    | 3    | 8    | 5    | 23    | 13    | 842  | 17    |
| Peptostreptococcus sp.       | 15         | 49         | 29      | 2    | 12    | 73      | 50    | 25   | 81    | 50    | 9    | 2    | 3    | 5     | 22    | 427  | 8     |
| Clostridium perfringens      | 17         | 20         | 24      | 0    | 10    | 50      | 10    | 7    | 34    | 57    | 10   | 2    | 0    | 2     | 6     | 249  | 5     |
| Clostridium sp.              | 13         | 8          | 11      | 1    | 8     | 23      | 3     | 5    | 20    | 17    | 22   | 2    | 0    | 2     | 1     | 136  | 3     |
| Propionibacterium acnes      | 42         | 117        | 4       | 2    | 89    | 29      | 40    | 40   | 129   | 155   | 146  | 6    | 9    | 6     | 14    | 828  | 16    |
| Propionibacterium sp.        | 3          | 5          | 3       | 0    | 8     | 11      | 3     | 0    | 6     | 10    | 2    | 0    | 0    | 0     | 1     | 52   | 1     |
| Eubacterium sp.              | 1          | 5          | 3       | 0    | 1     | 7       | 6     | 0    | 6     | 5     | 1    | 0    | 0    | 0     | 1     | 36   | <1    |
| Bifidobacterium sp.          | 0          | 0          | 10      | 0    | 2     | 10      | 5     | 0    | 5     | 10    | 1    | 0    | 0    | 3     | 6     | 52   | 1     |
| Anaerobic Lactobacillus sp.  | 1          | 0          | 2       | 0    | 2     | 9       | 1     | 1    | 2     | 3     | 0    | 0    | 0    | 1     | 3     | 25   | <1    |
| Catenabacterium sp.          | 0          | 1          | 5       | 0    | 3     | 3       | 2     | 2    | 3     | 1     | 0    | 0    | 0    | 0     | 3     | 23   | <1    |
| Total no.                    | 228        | 593        | 340     | 14   | 214   | 747     | 402   | 215  | 915   | 654   | 342  | 27   | 24   | 65    | 249  | 5,029|
| Per cent                     | 4          | 12         | 7       | <1   | 4     | 15      | 8     | 4    | 18    | 13    | 7    | <1   | <1   | 1     | 5     |      |

*a Does not include abscess, surgical specimen, or wound isolates. These are included under appropriate columns.
Whatever method one considers for recovery of anaerobic bacteria, the major factors limiting the efficacy are the proper collection and transporting of specimens to the laboratory. If neither of these is adequate, there is little chance of isolating these bacteria. Efforts to improve on this aspect of the problem have been the subject of reports by others (1, 5, 6).

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LITERATURE CITED

1. Attebery, H. R., and S. M. Finegold. 1969. Combined screw-cap and rubber-stopper closure for Hungate tubes (pre-reduced anaerobically sterilized roll tubes and liquid media). Appl. Microbiol. 18:558–561.
2. Brewer, J. H., and D. L. Allgeier. 1966. Safe self-contained carbon dioxide-hydrogen anaerobic system. Appl. Microbiol. 14:985–988.
3. Dowell, V. R., Jr., and T. M. Hawkins. 1968. Laboratory methods in anaerobic bacteriology. Government Printing Office, Washington, D.C.
4. Draar, B. S. 1967. Cultivation of anaerobic intestinal bacteria. J. Pathol. Bacteriol. 94:417–427.
5. Finegold, S. M. 1970. Isolation of anaerobic bacteria, p. 265–279. In J. E. Blair, E. H. Lennette, and J. P. Truant (ed.), Manual of clinical microbiology. American Society for Microbiology, Bethesda, Md.
6. Fulghum, R. S. 1971. Mobile anaerobe laboratory. Appl. Microbiol. 21:769–770.
7. Gordon, J. H., and R. Dubos. 1970. The anaerobic bacterial flora of the mouse cecum. J. Exp. Med. 132:251–260.
8. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14:1–49.
9. McMinn, M. T., and J. J. Crawford. 1970. Recovery of anaerobic microorganisms from clinical specimens in prerduced media versus recovery by routine clinical laboratory methods. Appl. Microbiol. 19:207–213.
10. Moore, W. E. C. 1966. Techniques for routine culture of fastidious anaerobes. Int. J. Syst. Bacteriol. 16:173–190.
11. Moore, W. E. C., E. P. Cato, and L. V. Holdeman. 1969. Anaerobic bacteria of the gastrointestinal flora and their occurrence in clinical infections. J. Infec. Dis. 119:641–649.
12. Rosebury, T., and J. B. Reynolds. 1964. Continuous anaerobiosis for cultivation of spirochetes. Proc. Soc. Exp. Biol. Med. 117:813–815.
13. Smith, L. D., and L. V. Holdeman. 1968. The pathogenic anaerobic bacteria. Charles C Thomas, Publisher, Springfield, Ill.
14. Virginia Polytechnic Institute and State University Anaerobe Laboratory. 1970. Outline of clinical methods in anaerobic bacteriology, 2nd review. Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg, Va.
15. Willis, A. T. 1964. Anaerobic bacteriology in clinical medicine, 2nd ed. Butterworth & Co., Ltd., London, England.
16. Zabransky, R. J. 1970. Isolation of anaerobic bacteria from clinical specimens. Mayo Clin. Proc. 45:256–267.