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# Anaerobic culture by Total Air Barrier: A preliminary study

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## RESEARCH

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## Abstract

**Background**
For study with obligate anaerobes, inoculated plates containing suitable reduced media need handling and incubation under strict anaerobic condition. Instead of ensuring a confined oxygen free chamber for placing seeded plates, same purpose may be achieved by creating total air barrier to the surface.

**Method**
Upper moist surface of freshly prepared anaerobic media in Petri plates were intimately covered with very thin transparent bacteriological inert sterile polyester sheets. Stock culture of *Bacteroides fragilis*, ATCC 23745 and *Clostridium sporogenes*, ATCC 11437 were grown in cooked meat broth and then sub-cultured on respective plates, after lifting the cover sheets. Sheets were again covered and incubated at 37°C ordinary incubator. To perform antimicrobial susceptibility test, similarly covered seeded plates with well inoculums were inverted en-block after stripping sides with the help of a spatula. Now antibiotic disks were placed on upper bare surfaces. After short pre-diffusion, plates were incubated keeping inoculated surface below. Same study was performed by conventional method using Gaspak.

**Results**
Good growths were noted in both sets of the study; however discrete colonies appeared more flat in nature in test set. Almost identical zones of inhibition were noted in both sets of sensitivity study. Seven days old growths in covered blood agar plates were found viable when sub-cultured in cooked meat broths.

**Conclusion**
Isolation, identification and susceptibility study for most clinically important obligate anaerobes may be performed by simple barrier method after appropriate standardization.

**Key Words**
Anaerobiosis, culture, sensitivity, polyester sheet

## Background

Surface culture of anaerobes is much more difficult and costlier than aerobes. That is why facilities for isolation, identification and drug susceptibility testing of anaerobes are not available in most laboratories. For successful culture of an obligate anaerobe, preconditions are low redox potential of medium, handling and testing of organism in an environment almost devoid of oxygen. [1] For that purpose clinical materials are to be inoculated promptly at collection point on appropriately reduced solid medium or into a transport medium. After inoculation, medium should be rapidly incubated in anaerobic environment which may be created either in the form of anaerobic cabinet or transiently created anaerobic container by displacing or exhausting oxygen from the confined chamber.[2] However an important limitation of the whole process is that the container chamber is to be re-conditioned after each handling and this will make whole procedure cumbersome and costlier.

According to Bulloch, about 300 different kinds of anaerobic apparatus were discovered in 30 years time from 1888 to 1918, based on permutation and combination of a few elementary principles. [3] These included removal or reduction of oxygen tension from contact air and its maintenance throughout incubation period either by using sealed chamber or by allowing growth at depth of medium or under a liquid or solid seal. Instead of attempt for evacuation or physical displacement of oxygen with inert gas or by combustion, chemical or biological consumption, if air space over inoculated plate itself is reduced to zero by intimate covering with very thin transparent inert polyester sheet, there will be no need for reducing oxygen tension or its maintenance by anaerobic incubation. Applying this technique a fresh arrangement after each handling may not be required. This new approach for anaerobic culture has been applied in the present study. In a similar attempt Knetemanin 1957 first used hard plastic films as oxygen barriers on the surface of agar pour plates. The technical drawbacks of his method are mostly eliminated in our present study. [4]

## Method

The project was approved by the Ethical Committee of IPGME&R, Kolkata.

*Barrier film*: The film used for creating air-interface barrier in the present study was prepared from commercial rolled transparent polyester sheets (De'Smat, Rational Business Corporation, New Delhi). The film was 40 micrometer thick nonporous bacteriologically inert (not influencing bacterial growth) as determined by control study. Circles of 86 mm diameter were cut-out which were convenient to lay over the surface of 90 mm size agar plates. Those were packed in Petri dishes, separated from each other by filter papers and sterilized by Plasma sterilizer (using plasma state of vaporized hydrogen peroxide).

*Media*: Reduced blood agar (BA) medium (Freshly prepared blood agar media is considered to be in reduced state .It is prevented from reoxidation by application of air barrier )was prepared by adding aseptically collected defibrinated sheep's blood (5%) to sterile nutrient agar (Hi-Media, India), that was melted and cooled to $50^{O}$C and $P^{H}$ adjusted to 7.2. Now in each 9 cm sterile Petri dish, 20 ml of medium was poured and allowed to set quickly in $4^{O}$C refrigerator. After solidification of medium, one sterile circular polyester sheet was placed on wet upper surface of agar gel. The sheet remained intimately adhered on surface of medium by bondage with microfilm of moisture. Any trapped air during overlaying the sheet was driven out by a sterile spatula on surface.

*Test bacteria*: Reference strains lyophilized cultures of *Clostrdium sporogenes*, ATCC 11437 and *Bacteroides fragilis*, ATCC 23745 were obtained from Microbiologis Inc., USA. Five *Clostridium tetani* isolates were recovered from OT environment of IPGME &R, Kolkata. In our laboratory all these anaerobes were maintained in CMB (Cooked Meat Broth, Hi Media, India). Reference strain *Pseudomonas aeruginosa*, ATCC 27853 was used as control.

*Culture procedures*: Bacterial suspension from 48 hours growth in CMB was thoroughly mixed and by a sterile pipette 20 microlitre of each bacterial suspension was deposited on each test plate after lifting the barrier film. By a sterile loop, inoculum was smeared thoroughly on 1 cm x 3 cm area to form an area of well inoculum. The loop was re-sterilized and then drawn at $120^{O}$ angle from the well or immediate preceding strokes in three parallel lines onto fresh area of the media. The process was repeated and immediately after completion of inoculation the barrier film was well placed on the surface. Any trapped air was removed by dragging a sterile spatula on the surface of the film. For each organism five repetitions were done using common source of inoculum. Plates were incubated at $37^{O}$C at ordinary incubator. Another five plates were similarly streaked out and without barrier film overlaying, were incubated at $37^{O}$C incubator after placing plates in anaerobic jar containing Anero- Gaspak (Hi-Media, India). After 48 hrs of incubation discrete colonies from secondary strokes onwards on each plate were counted. The average number of colonies grown in barrier method was compared with that of conventional method.

Five clinical isolates of *C. tetani* and one *P. aeruginosa* were similarly seeded on reduced BA plates, one each for conventional anaerobic incubation and another for aerobic incubation with barrier film. All test anaerobic organisms and the control aerobe was cross-checked by inoculating on BA plates followed by aerobic incubation.

Colonies of anaerobic bacteria grown under barrier films were cross checked by sub culturing on reduced blood agar plates with and without barrier film overlay. Growths were also maintained for three months by repeated subculture following same barrier method at every seven days intervals.

*Antibiotic resistance test*: For the test organism of *B. fragilis* ten reduced blood agar plates were uniformly seeded by spreading 50 microlitre of 48 hrs growth in CMB. In control set of five plates, five test antibiotic disks

were placed at sufficient distance with each other and then promptly placed inside anaerobic jar with active Gaspak. The jar was incubated at 37 $^{O}$C for 48 hrs. In test set of 5 plates, barrier films were overlaid immediately after inoculation. Then the gel like media were inverted en-block (media was transiently placed inner side of upper lid and then promptly glided to lower lid of the petriplate) with the help of a sterile spatula, so that un-inoculated reverse surfaces appeared on top. Antibiotic disks were placed on uncovered surfaces. After 30 minutes pre-diffusion at 4$^{O}$C, plates were incubated at 37 $^{O}$C for 48 hrs. Zones of inhibition were measured. A zone of <15 mm was considered to indicate resistance.

The following antibiotic disks (Hi-Media, India) were used for testing *B. fragilis*; Neomycin 1000 µg / disk, Kanamycin 1000 µg/ disk, Metronidazole 5µg/ disk, Benzyl Penicillin 2 unit/ disk and Vancomycin 5 µg/ disk.

## Results

Test anaerobic organisms grew on all seeded plates placed in Gaspak containing anaerobic jars while *Pseudomonas* strain failed to grow there. Similar results were observed on plates incubated in non-anaerobic environment but with barrier film covers. Thus our preliminary study proved that optimal environment for growth of common Gram positive spore-bearing and Gram negative non-spore bearing obligate anaerobes can be achieved by easy alternative technique of applying oxygen barrier film on seeded reduced solid media. Clinical isolates of *C. tetani* grew equally on plates incubated anaerobically in Gaspak containing jar and those incubated aerobically with barrier film application. Colonies were more flat in nature when were grown under cover sheets. All anaerobes failed to grow on BA plates when incubated aerobically whereas control aerobic strain grew well.

A comparative result of bacterial growth by conventional and alternative method is shown in Table: 1, in terms of their numbers of isolated colonies on respective plates. Although these numbers are not indicating their total viable bacterial counts present in each 20 µl inoculum, but indicate number of colonies on secondary or tertiary stroke areas, such results may help to assess the status of the medium. Standard method for colony counting at various dilutions could be followed for the purpose provided a self-contained glove box anaerobic chamber could be used for control set to avoid varying period of air exposure during handling. The cover method was also found suitable for maintenance of the stock of test anaerobes at least for three months by repeated subcultures at every 7 days intervals.

Antibiotic resistance test of anaerobes by Gaspak method and proposed alternative method showed very similar results in all plates. The *B. fragilis* strain used for the test showed almost no zone of inhibition against Kanamycin, Neomycin, and Penicillin, 6 mm zone of inhibition against Vancomycin and > 15 mm zone of inhibition against Metronidazole.

## Discussion

For exclusion of oxygen contact with the surface of inoculated solid medium, the easy solution is to incubate plates in anaerobic jar. To secure anaerobic environment within the jar many procedures are followed e.g. physical displacement of air by inert gases; evacuation and combustion; [5] chemical consumption and biological exhaustion. [6] In laboratories where facilities and experience in anaerobic bacteriology are lacking, commercially prepared hydrogen- carbondioxide generator “Gaspak” system may be simpler but costlier alternative. [7] Replacing all complicated procedures Kneteman in 1957 first introduced a simpler but cheaper method[4] of applying a barrier film over seeded surface. He used plastic film as oxygen barrier for the surface of agar pour-plates along with co-culture of micrococcus for better anaerobiosis. The technique was not so popular for several disadvantages. Shank in 1963 improvised the technique by using thyoglycollate instead of micrococcus co-culture. [8] This allowed use of suitable antibiotics for selective isolation of specific anaerobes. However by use of 1mm thick solid plastic film on pour plates, the desired results were not obtained and showed growth of anaerobes only under the central position of the film but little or no growth towards periphery. So there was possibility to grow facultative anaerobes or aerobic contaminants at that region. Although these earlier techniques were theoretically sound but failed in practice because of poor adherent force between thick hard film and medium as well as for the collective upward displacement thrust by bacterial growth.

These disadvantages can be overcome by using very thin flexible polyester films. The adherence force between moist agar surface and such film will be more firm than that of a hard plastic film. With the bacterial growth and liberation of gaseous metabolites a loosely adhered solid cover may be easily displaced while a flexible thin cover may be locally elevated remaining wrapped with the colony and gaseous products to a little extent may be absorbed into the medium by back-pressure. So the side-way air entry will remain negligible for sufficient period of time by application of thin flexible film.

The colonies of barrier film method are flatter in nature than identical growths in anaerobic jars. With the use of commercially prepared more thin elastic film or a nano-film, almost normal looking surface colonies may be obtained. Isolated colonies from such plates can be used for obtaining a pure growth and for further studied towards identification. The greatest advantage of the method is that for each time observation of growth and handling for further studies there will be no need for a fresh set up. The method will also minimize the time of exposure to air with clinical materials from collection to incubation, thereby ensuring better anaerobic growth. The proposed method of anaerobiosis is only a modification of culture technique, notwithstanding need for selective media. The only drawback of this method is that it may not be suitable for media where organisms grow with excessive gas production.

The presently practiced antibiotic resistance study by anaerobic jar method can be replaced by reverse inoculation method of antibiotic sensitivity study after appropriate standardization with a suitable reference strain. The recommended method for sensitivity study of anaerobic bacteria by Agar dilution technique is very cumbersome which may also be replaced by barrier method with reverse surface inoculation. [9] The ease and convenience of the barrier film method of anaerobiosis thus makes it suitable for laboratories where occasionally anaerobic cultures are required and also for field surveys.

## Conclusion

Covering of a freshly prepared solid medium with an air barrier sheet, not only helps to maintain reduced state of medium for a reasonable period of time but also to maintain its hydration state. Thus commercially prepared reduced medium with such barrier films can be used for growth of aerobes, facultative and obligate anaerobes either by post-inoculation overlaying of the sheet or not according to need.

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## PEER REVIEW

Not commissioned, externally peer reviewed.

## CONFLICTS OF INTEREST

The authors declare they have no competing interests

## Figures and Tables

**Table 1.** *Growth* of bacteria under barrier polyester film.*

| Organism | Reduced BA + Anaerobic jar | | Reduced BA + Film | |
|---|---|---|---|---|
| | Isolated colonies | Average | Isolated colonies | Average |
| ***C. sporogenes* ATCC-11437** | **453 in 5 Plates** | **90** | **422 in 5 plates** | **84** |
| ***B. fragilis* ATCC-23745** | **361 in 5 plates** | **72** | **334 in 5 plates** | **67** |
| ***C. tetani*** | | | | |
| **Isolate 1.** | **69** | **70** | **73** | **67** |
| **Isolate 2** | **76** | | **42** | |
| **Isolate 3** | **65** | | **77** | |
| **Isolate 4** | **57** | | **66** | |
| **Isolate 5** | **81** | | **75** | |
| ***P aeruginosa* ATCC-27853** | **No growth** | **0** | **No growth** | **0** |

*Number of isolated colonies following uniform inoculation,
BA = Blood agar