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ISOLATION, CULTIVATION AND IDENTIFICATION OF ANAEROBES IN AN ANAEROBIC BACTERIOLOGY LABORATORY

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Abstract

This review examines a simple, workable scheme which is within the capabilities of most small, laboratories to isolate, cultivate and identify anaerobic bacteria in an anaerobic bacteriology laboratory.

Success in the isolation, cultivation and identification of anaerobes depends more on the critical sense of the worker than the method he employs. Microscopic observation should be made of incoming material and of cultures after twenty-four and forty-eight hours' incubation, and the development of a critical eye for the morphology and staining reactions of anaerobes is imperative .

The subject of the isolation , cultivation and identification of anaerobes is one which the worker is inclined to approach with apologies.

Every month or so a paper appears in some journal in which a new and expeditious procedure for the separation of anaerobes is described.

There are many successful ways of isolating anaerobes and it is unwise to recommend any one method above all others.

This review is to analyze some of the principles governing the isolation , cultivation and identification of these organisms and to explain a few of the pitfalls which have caused many workers to believe that the securing of "absolutely pure" anaerobic cultures and cultivating and identifying it is a difficult matter. With a little practice and with the exercise of much discrimination, anaerobes may be isolated as quickly, or nearly as quickly, as aerobes.

INTRODUCTION

Anaerobic bacteriology is a very neglected area of routine diagnostic procedures. It has been our experience that once clinicians are aware that such procedures are available , the number of positive anaerobic cultures that are recovered from specimens greatly increases.Anaerobiosis is the study of anaerobic bacteria which includes their sample collection, transportation media, cultivation, incubation, subculturing and identification.Anaerobic bacteria in the environment are part of the indigenous microbial flora of the body. They cause diseases to virtually any body site, organ or tissue when condition favor penetration, survival and colonization of bacteria. Anaerobic bacteria don't require oxygen as terminal electron acceptor for growth or metabolic activities and their growth is inhibited by it.Obligatory anaerobic bacteria do not grow on nutritionally adequate blood agar, chocolate agar or other solid media in an ambient air incubator, or a candle jar or a 5-10% oxygen incubator. Their growth in culture or in nature is favored by the exclusion of oxygen from the system and by a low oxidation- reduction potential within the medium.Fundamental to good anaerobic microbiology is the requirement to ensure that samples are not exposed to oxygen during collection and transportation to the laboratory. Oxygen exposure reduces the culturability of the obligate anaerobes present in the sample.

Essentially, the use of special collection methods, transport media, and sensitive anaerobic techniques in anaerobic bacteriology is to ensure that the microorganisms are preserved, their viability is not lost and optimum growth is obtained from clinical specimens.

Anaerobic bacteriologic examination of such specimens in the absence of these special precautions may be negative or yield incidental aerobes, leading to mis-guided diagnosis.

In the isolation, cultivation and identification of anaerobic bacteria in a clinical microbiology laboratory, heating at 70 ºC of pipettes is to be recommended for routine work. A routine medium should be employed which will favour as many divers forms as possible. Chopped beef heart, preferably containing a little peptic digest both, the reaction at about pH 7.2, presents numerous advantages as a routine medium, for most of the anaerobes studied in a pathological laboratory.

If freshly boiled, it is usually quite unnecessary to incubate it anaerobically. Selective media may be employed for special purposes, and they offer many possibilities.

Isolation by means of guinea – pig inoculation, securing the organism from the heart's blood or from the affected tissues remote from the site the affected tissues remote from the site of inoculation, is preferable for invading pathogens, but may not be depended upon to give a true picture of the pathogenic flora of the material injected.

The making of dilution shakes in deep agar (method of Liborius and of Veillon) is to be preferred to the colony methods; care must be taken to isolate for a type of colony from an apparently pure culture.

A medium for dilution shakes should afford an opportunity for growth to just as many species as possible. Such a medium is peptone-liver agar which will be described in the text.

When once pure, a culture should be carefully kept pure. Re-incubation, prolonged incubation in closed jars, storing in close cans or in dusty places, are to be avoided.

Autoclaved media only should be employed for the preservation of type cultures; one cannot be too careful as to routine technique in the isolation, cultivation and identification of anaerobes.

This review presents a simple workable scheme for anaerobic bacteriology which is within the capabilities of most small laboratories.

CONTAMINATION OF ANAEROBIC CULTURES

Contamination occurs somewhat more frequently in anaerobic cultures than in those of aerobes. Contamination of originally pure cultures may be attributed to the following causes. (Achalme *et al* 1911). Insufficient sterilized media; anaerobe media are usually pasty and require more careful sterilization than others. (Ankershamiti *et al* 1905).

Inoculation transfer involving the exposure of the cotton plug and of the inoculums to the air. Researchers have noticed that the more dusty the air, the more frequent are contamination's, and the contamination flora may vary according to location. (Barbera *et al* 1914).

During incubation in closed jars the cotton plugs may become sufficiently moist for molds to grow through them; where a mold can grow a bacillus can follow. (Barber *et al* 1920). During prolonged incubation , water of condensation may even run into the tubes from the top of the jar.(Barber *et al* 1920) . If stored in closed cans, molds may grow through the plugs. Workers should take these points into consideration in planning their work. Anaerobic jars are exceedingly convenient and practical for periods of incubation under four or five days, and for much anaerobic study, twenty-four to forty-eight hours incubation is sufficient. Prolonged incubation should be made under Vaseline or in the case of sugar-free media in exhausted sealed tubes. Sealing of tubes is inadvisable where carbon-dioxide may be so confined that it produces an acid end-point. Re-incubation of cultures in exhaust jars should be cautiously undertaken so that the medium may not boil up to the cotton plugs. Anaerobic jars which do not require exhaustion are preferable for re-incubation of cultures.

The commonest contaminators of cultures have been cocci and molds, not anaerobes. The reason that anaerobic contamination of anaerobe cultures is so very common probably lies principally in the uncritical handling of such

cultures. If a coccus or mold contaminates a culture the worker immediately kills such an organism, but if an anaerobe enters the tube it proceeds to multiply unmolested. Daily watchful observation of the cultures studied is absolutely necessary for successful anaerobic work. There are no indications of any so-called symbiotic tendency that makes anaerobes more difficult to isolate than aerobes.

Anaerobes vary greatly in their behavior and requirements, and the method of isolation must be adapted to the problem in hand as it turns up. Each combination of two or more species of organisms presents different elements for consideration and for adaptation of technique. There is no one method that is always best, and it is only after a worker knows something about the nature of the particular organisms that he is dealing with, their cultural behavior, and their morphology in the medium in which he regularly grows them that he is able quickly and surely to isolate, cultivate and identify numbers of strains.

It is, of course, desirable to make use of methods that may be applied to the largest possible number of species, that are easy of manipulation, and moderate as to cost of time and material.

The organisms present in material to be investigated may belong to any one of four large groups, which may be described:

Table 1: Grouping of organisms present in material

Organism	Undesirable	Desirable
Easily killed by heat	Non-sporulating aerobes, common, many species	Non-sporulating anaerobes: Welch bacillus is, in most media, the chief consideration
Not easily killed by heat	Sporulating aerobes, not common in pathological material; species are however numerous.	Sporulating anaerobes, species legion in number

Whatever be the material that is to be investigated, a microscopic examination of a Gram stain is first in order. Practice only will enable the worker to form judgments which will be of value to him. As hints to the beginner, one may suggest that there are an endless number of species of anaerobes and that specific diagnosis by microscopic examination is futile. There are frequently many species of anaerobes in the material that finds its way to a laboratory, and, unless a study of many strains is intended, the isolation or demonstration of a single species, whose nature is guessed at, must be attempted. If the microscope shows the probability of the presence of that species, matters are simplified. To seek a certain organism one should familiarize himself with a pure strain of that type of organism, or study photographs or drawings of it; verbal descriptions are not of much value. He should also learn the colony form of several strains of the type he desires to obtain. The employment of a medium in which the morphology of the organisms is varied and characteristic is imperative. Some laboratories uses chopped meat medium containing 5 per cent peptic digest broth (pH 7.2) for routine cultivation and this medium excels all other autoclaved media in the above respect. The use of oil over the medium to produce anaerobiosis should be avoided whenever possible for routine works, as it interests with the making of satisfactory smears; for long incubation and under certain circumstances demanded by technical considerations, Vaseline will be found very useful. Ghon and Sachs recommend the use of agar for stratification; liquid media should be frozen before the agar is poured.

HEATING OF INOCULUM

To free sporulating organisms from non-sporulating organisms heating is always restored to. Heating of inoculated media may be performed in one of two ways. Heavily inoculated media may be heated to 80^0 C in a water bath for fifteen to thirty minutes. This method is highly inaccurate, especially in case pasty media are used, but it serves on occasion. Or the material to be inoculated may be heated in a Pasteur pipette after the following fashion:
Sera, executes, and muscle extracts should be diluted with sterile saline. Cut the end of a Pasteur pipette off square with a file, flame it, then draw up the inoculum for about two inches by capillary attraction, and seal the pipette with less than a quarter of an inch of air space between the tip and the liquid. To kill non-sporulating organisms ,heat in a

waterbath for ten minutes at 70^0 C to 72^0 C. Then flame the pipette above the inoculum to kill organisms that may have been above the water-line, mark the tip in several places with the file or diamond, slowly flame the tip, insert it in the tube of fresh medium, flame a pair of light forceps and with them break the tip of the pipette against the inner wall of the tube and expel the material.

If a worker is certain that the type of sporulating anaerobe desired is always highly resistant to heat, he may use higher temperatures, in the neighborhood of 100^0C. for heating his culture. Northrup has repeatedly employed this method with success in the isolation of *Bacillus botulinus*. Von Hibler sowed mixtures containing such organisms, and even less resistant ones, directly into hot agar. Some strains of B. *botulinus* and of Novy's bacillus are highly resistant to heat.

SEPARATION OF NON-SPORULATING ANAEROBES FROM AEROBES

1. Heat to 56 ºC – 58 ºC for five or ten minutes. This occasionally serves the purpose.

2. Try to induce sporulation by growing the mixture on alkaline sugar-free medium, such as alkaline egg, or serum medium (von Hibler, 1908, p. 189). When the anaerobes form spores, heat. This procedure is a sure method of freeing *B. Welchii* from ordinary aerobes: incubate for four days. This organism is found in a sporulating condition in soil and in fecal material.

3. Try a pathogenicity test. If the organism sought is pathogenic, it may be recovered in pure culture from the animal tissues. Use this method for *B. Welchii*, *B. egens, B. Fallax*.

4. Use selective media. For the Welch bacillus use milk or 1 per cent glucose broth. Inoculate it with a pipette, a fresh tube of medium every twelve hours if possible.

5. Use good anaerobic methods. Cultivate the material on meat medium in strict anaerobiosis, inoculate in agar dilution tubes that have been thoroughly boiled, and fish the colonies. This technique is described in this text.

6. Northrup suggests the use of a 25 – cc. burette, in which the organisms of an inoculated mixture will, on short incubation, sort themselves out, the aerobes growing above, the anaerobes below, where they may be drawn off through a stopcock.

SEPARATION OF NON-SPORULATING ANAEROBES OR RELUCTANTLY SPORULATING ANAEROBES FROM OTHER SPORULATING ANAEROBES

1. Use selective media, milk, with short incubation periods, for *B. Welchii.*

2. Use animal inoculation.

3. Use shake cultures.

4. Use semi-anaerobiosis: The non-sporulating anaerobes are naturally more resistant to oxygen than the sporulating ones.

Aside from *B.Welchii* this sort of organism is rarely sought after or noticed. Few non-sporulating anaerobes are described, and the group has been generally neglected, but careful methods show that non-sporulating anaerobic rods and cocci are not uncommon.

SEPARATING EITHER VARIETY OF ANAEROBES FROM SPORULATING AEROBES

Sporulating aerobes are rather infrequently found in pathological material. One meets them frequently, however, in a medium that has been insufficiently sterilized. Encounters with sporulating aerobes have been so rare that it would

be wise to recommend that a worker always go back to the original material and test it for the presence of any sporulating aerobes that he finds in a culture with which he is working. Avoid sporulating aerobes, do not contaminate cultures with them, and isolate the anaerobes from the original material again.

1. Sporulating aerobes are of two classes: strict aerobes (any good anaerobic technique followed by a colony method will free a culture of these) and facultative anaerobes. There has never been a sporulating facultatively-anaerobic aerobe that grew better under strictly anaerobic anaerobic conditions than its accompanying anaerobes. Any strictly anaerobic colony method that will separate anaerobes from each other will separate them from aerobes. Experience has shown that trouble with abundantly growing aerobic organisms denote faulty anaerobiosis: the presence of a small amount of oxygen that permits the undue multiplication of the aerobes. Research could , however, been almost entirely with pathological material and may have failed to meet with the most troublesome aerobic organisms.

2. Kitasato and Weyi found that anaerobes were less sensitive to pyrocatechin, chinon, sodium formate, and sodium sulphindigotate than were the aerobes causative of cholera, typhoid and anthrax. Rivas continued this type of investigation.

3. Churchman has investigated the inhibitive effect of gentian violet on aerobic growth. Hall recommends the use of gentian violet in a dilution of 1 :100,000 to separate sporulating aerobes from anaerobes. This, I should think, would work very well for the heavy Gram positive organisms of the B. *subtilis* group, provided the desired anaerobe is not of the same nature.

4. The spores of aerobes may sometimes be satisfactorily germinated in broth in a Petri dish, the broth being then heated and inoculated into agar.

SEPARATION OF SPORULATING ANAEROBES FROM NON-SPORULATING ANAEROBES AND AEROBES

To separate sporulating anaerobes from non-sporutaling anaerobes and aerobes and aerobes Heat as described above.

SEPARATION OF SPORULATING ANAEROBES FROM OTHER SPORULATING ANAEROBES BY CULTURAL METHODS

To separate sporulating anaerobes from other sporulating anaerobes by cultural methods heating may be employed as follows:

Table 2: Separation of aerobes by cultural methods

Anaerobic Bacteria	Proteolytic Group	Non-Proteolytic Group
Early sporulating species (18-24 hours)	Bifermentans group et alii. Do not occur very frequently	Nearly all sporulating organisms
Later sporulating species (24 – 48 hours)	Sporulating group et alii	Nearly all sporulating organisms.
Late sporulating species (48 hours only)	Tetanus group, botulinus group.	Nearly all sporulating organisms.

This diagram above shows that if proteolytic early-sporulating organism are absent, as is frequently the case, a saccharolytic form may be isolated or be rendered relatively far more abundant by heating eighteen to twenty-four-hour cultures successively. There are mixtures of *B. sporogenes* and organisms of the blackleg group that were not pathogenic for guinea-pigs because of the scarcity of *B. Chauvoei*. Two successive heatings and inoculations made black leg the predominant organism and the culture was highly pathogenic. This method is also excellent for organisms of the vibrion-septique group and for many non-pathogenic saccharolytic bacteria, as well the early-sporulating proteolytic ones.

SELECTIVE MEDIA

Isolation methods usually depend on securing a predominance of the organism sought. To increase the relative numbers of an organism with whose nature one is familiar, a medium should be selected on which the organism grows best. For saccharolytic species mixed with proteolytic ones, use sugar-containing media. Meat medium plus I per cent glucose is good, meat medium not neutralized in the making is also good. Ordinary meat medium, the culture being taken early in its development, is usually sufficiently selective. Sugar media selective for certain groups may be used, if the number of cultures to be isolated warrants the investigation of the sugars split by that group. I have found that culture in casein-digest liver-broth renders blackleg the most able guinea-pig invaderin a blackleg-vibrion-septique mixture. To increase the percentage of proteolytic organisms use meat medium or brain medium in a culture two to four days, old, or even older; or employ the medium of Achalme-Passini, salt solution or broth containing cubes of egg-white; or use serum medium or other sugar-free media; or a medium made up at pH 8.0 or above. For an organism whose morphology interests one and whose nature is not known, experiments should be tried with various media, and the behaviour of the mixture should be studied. Under laboratory conditions certain types always tend to disappear from mixed cultures. It must be kept in mind that conditions must exist in nature which favor the multiplication of such species or they would have died out long ago. For such organisms try media of vegetable origin.

In taking samples of pathological material enrichment with the tissue in which the organisms are found is advisable. Schottmuller isolated septicemic streptococci in blood-glucose agar shakes. The many tissue-containing media favor the growth of pathogens. (Media summarized by Pfuhl.) Tunnicliffe used serum and ascites agar for the anaerobic coccus found by her in measles cases. Plotz and his co-workers added ascites or hydrocele fluid to glucose agar for blood cultures from their typhus patients. Dick and Henry employed blood-glucoses agar for the various anaerobes found in the blood of scarlet fever patients. Leucowicz used serum-sugar agar for F*usiformis.*

Digest media are excellent for anaerobes. A number of such media are discussed by Stickel .

Serious problems sometimes arise. Thus, *B. tetani* is particularly difficult to isolate from gross mixtures, as it is not a tissue invader, and because it sporulates later than the organisms that usually accompany it. In case an organism like *B. tetani* grows excellent on a given medium but its accompanying organisms grow better than it does, try similar selective media of modified reaction, or make use of exhaust media of the type recommended by Tulloch. One may always grow the objectionable species or several species in a medium till growth ceases, filter the medium and then grow mixed culture in the filtrate. In case this fails one may add a minute quantity of some solid protein for a starter. Tulloch added a bit of rabbit kidney to an exhaust filtrate and found it highly selective for *B. tetani.* Von Hibler grew mixtures containing B. tetani on clotted rabbits' blood and stated it to be selective of the organism. But photographs of the organisms show his cultures to have been so badly contaminated that he may have been mistaken.

Modification of a medium of Rosenthal is excellent for the enrichment of soil anaerobes (sodium phosphate 0.05 per cent, ammonium sulphate 0.05 per cent , soluble starch 1 percent, calcium carbonate 0.5 per cent). The anaerobic flora obtained in such a medium after heating a soil emulsion is very different from that obtained in meat or other media of complex composition. By fishing large lenticular or modified lenticular colonies from 2 per cent shakes of this medium which have been incubated for four days, the large butyric acid bacteria of the genus (Clostridium may be isolated with comparative ease. Winogradsky (1902) recommends the use of media free of fixed nitrogen for the isolation of nitrogen fixing anaerobes *(Clostridium Pastorianum)*; this medium is described by Fred (1916) and Bredemann used it for the isolation of his Bacillus *amylobacter* which he considers to be the same organism as Winogradsky's. Milk may also be used as an enrichment medium for many organisms of this genus.

Omeliansky (1904) describes the following method for enriching cellulose fermenters: Place in a long-necked flask any cellulose substance, paper, cotton, flax; add chalk, and fill to the top with water which contain 0.1 per cent magnesium sulphate, and a little sodium chloride. Inoculate with slime or horse manure, cover, and set in the dark. In other publications (1899; 1902) he gives other formulae; several are given by Fred. Ankerschmitt used physiological salt solution containing cubes of potato to enrich splitters of hemicellulose. Choukevitch employed I per cent peptone broth with 5 per cent starch for starch splitting organisms. Silicate jelly as a substrate for such of these organisms as will not grow on agar is described by Omeliansky (1899) and formulae or similar jellies are given by Fred and by Kuster.

SYMBIONTS

Symbionts have been used to enrich certain types of anaerobes. Sturges found that *B. putrificus* flourished best in the presence of *Bact. Coli,* and used the latter as a symbiont for the former. Rhein used *Bact. Faecalis-alcaligenes* as a symbiont for anaerobes, cultivating them in the presence of air: this organism has several advantages. Wilson and Steer described a cocco-bacillus as an excellent anaerobes symbiont.

UNFAVOURABLE CIRCUMSTANCES

Another resource is to test the resistance of the desired species to unfavourable circumstances. Thus McCoy and Bengtson of United States Public Health Laboratory isolated many strains of tetanus with great ease by heating toxic strains at 70^0 C for a half hour and inoculating the spores in veal agar dilution shakes. This techniques is adverse for an anaerobe, but *B. tetani* appears to be hardy enough to withstand it. Modified highly acid or alkaline media, or media poor in protein may be used for such purposes. A pure strain of the desired organism is invaluable in testing out media of this sort.

ANILINES

Aniline dyes may be used to eliminate certain species of organisms and the possibilities which they offer are almost unlimited.

SELECTIVE TEMPERATIVES.

Selective temperatives may be employed for enrichment of various organisms. *B. botulinus* was long thought to produce toxin at low temperatures only, because the contaminating organisms in cultures outgrew it at 37^0 C. Thermophilic organisms are of various types, and are discussed by Bergy. Major W.J. Tulloch says that the flora obtained by incubating a mixture of anaerobes in meat medium at 42^0 C is quite different from that obtained at 37^0 C, slender, oval end-sporing organisms predominating. It is probable that anaerobic organisms will be found that grow at much higher temperatures than at 42^0 C.

SELECTION OF ORGANISMS BEFORE SOWING

This was suggested by Stoddard, who shook his material with sea sand to separate encapsulated or autoagglutinated organisms. Northrup states that he has found such technique useful in isolating anaerobes from soil and from old meat cultures which had sporulated heavily. Such separation is not necessary when fresh cultures are used.

ISOLATION BY VARIOUS COLONY METHODS

Because of the confusion that exists as to the purity of cultures of anaerobes, it will be well to study the biological factors involved in the genesis of bacterial colonies. A colony is an aggregation of organisms that are prevented from mixing with other organisms by a physical obstruction. A colony may be defined as follows:

a. From one single organism – the ideal colony for isolation purpose.

b. From two or more organisms descended directly or indirectly from one organisms – a satisfactory colony for isolation purpose.

c. From two or more organisms of closely related strains – the most undesirable type of colony for isolation purpose.

d. Or from two or more organisms of unrelated strains – an undesirable type or colony for isolation purposes. This type or a contaminated pure colony is sometimes useful in procuring a new proportional mixture of strains.

Broadly speaking a colony may consist of any number of organisms from one to infinity. Technically speaking a colony consists of the organisms confined within a certain radius inside of or on the surface of the mass of colloid gel. For purpose of discussion, let us define a bacterial colony as the uncontaminated descendants of a very small number of organisms, irrespective of the medium in which they are found. It will be realized that this definition covers perfectly the biological factors involved in the derivation of any ordinary agar or gelatin colony.

Colony methods available for the isolation of anaerobes are the following:

1. Agar colonies – von Hilber and older workers used also gelatin.

   A. Surface colonies

   i. On plates

   ii. On tube colonies

   B. Deep colonies

   i. In Petri dishes

   ii. In deep agar tubes

2. Colonies in liquid media

   a. Isolated of a single bacillus by the India-ink method.

   b. Isolation of a single bacillus or of a small number of organisms by the technique of Barber.

   c. Isolation of a single bacillus by the technique of Schouten, of Hecker, of Holker, or that of Malone.

I*solation from surface colonies* has been employed by many workers with anaerobes. Veillon and Zuber list a large number of types of anaerobic apparatus, Von Hibler (1908) gives a bibliography of various plates and apparatus for purpose of anaerobic culture, Besson's textbook figures a number of arrangements, and Fildes describes various methods at the end of Mclntosh's report.

Henry uses plates of agar which he streaks with egg-albumen and incubates in hydrogen. Stoddard uses slants made of the modified egg medium of Smith made with tryptic broth and 1 per cent glucose. Zeissler, who at first used glucose agar plates containing human blood for the isolation of anaerobes, later employed horse blood and sheep blood agar plates. Many laboratories now make use of large slants of blood agar, kept under anaerobic conditions for the isolation of anaerobes. Isolation of nitrogen fixing organisms was accomplished by Wingradsky by inoculating cultures on pieces of carrot which he placed in vacuo, and Fribes isolated pectin fermenters on potato slants rubbed with chalk. Melntosh prefers agar slants to plates for isolation procedure. He reiterates: "it cannot here be impressed too strongly on the worker that the purity of a culture can only be tested and controlled by repeated surface

cultivation", and he speaks of the Veillon-tube method of continental workers as giving impure cultures. I have used plating occasionally and am familiar with technique necessary to make anaerobes grow on plates. In fact it was the first method for the isolation of anaerobes that I learned to use. It is a perfectly feasible method, but I find it to be less satisfactory than others for various reasons.

The difficulty of regulating the amount of moisture on the surface methods. Aerobic culture differ fundamentally from anaerobic ones in this respect. They are, so to speak, self-regulating in their moisture content. When a plate is poured, the surface of the agar is exceedingly moist, and the organisms planted in it grow rapidly till their growth is inhibited by the drying atmosphere of the incubator. Moisture conditions are fairly uniform in ordinary bacteriological technique; colonies, when few, are usually pure; the viable aerobes usually all form colonies, and the method as a whole is easy and practical. But with anaerobes the moisture content of the medium and the moisture on its surface become of great importance. I have known agar in deep tubes of medium, which had given perfect results with blackleg colonies, to refuse to give a growth of blackleg when it was somewhat old and dried out, though the agar had nowhere, as yet, separate from the side of the tube. The addition of sterile distilled water made the medium as fertile as soil as fresh agar. I have encountered aerobes which grow to the surface of the agar of would dryness affect the growth of the more delicate anaerobic organisms on the surface of a plate! Even the hardy tetanus organisms, which grow well in dry deep agar, often refuse to grow on its surface. In order to produce discrete anaerobic colonies plates must be dried after pouring. They must be dried just long enough and not too long. This period varies with the composition, age and thickness of the agar, with the humidity of the atmosphere, and with the moisture present in the anaerobic jar. It takes time and patience to learn to adjust the period for drying the plates. Then when the culture is sown and the plates are ready to incubate, what have we for anaerobic methods? A variety of available atmospheres for the growth of the organisms almost as great as is the number of workers in the anaerobic field: Hydrogen, carbon-dioxide, nitrogen, illuminating gas, nitrogen-hydrogen-dioxide and vacuum with varying degree of moisture, pressure and oxygen present. How can one hope to standardize type colonies under such conditions? and what, may we ask, is the proper moisture for the surface of a plate? There is no universal proper moisture. Agar moist enough to grow tetanus will allow the spread of *B. sporogenes* till the *B. sporogenes* has. Some mixtures of organisms allow isolation of their components by surface methods, and some do not. When discouraged with plates that have dried too long, the worker dries them less, and finds to his joy beautiful discrete colonies, some round and some lobed. He must fish them immediately onto plates or into a deep medium or they may die. But let him beware of a pitfall. Let him hold them to the light without a cover and look between the colonies. A slight film of moisture there may represent a spread of growth which contaminates all his colonies. But such a spread may be difficult or impossible of detection. A fragment of coverslip dropped between colonies may show bacilli. I venture to suggest that it is almost impossible to determine in an agar slant the non-existence of such a thin spread, an such a thin spreading film is far more likely to occur in the confines of a tube than on a plate.

Methods of spreading a culture on a surface do not separate the individual organisms from one another so well as does a shaking in liquid agar – in properly made shakes the colonies are beautifully distributed.

Other minor disadvantages of a surface method are that the plates must be incubated immediately after sowing and be fished immediately after opening; they are usually valueless when reincubated after opening for inspection because of too much drying, and they require the use of more glassware than do deep-tube methods, and also the use of an anaerobic jar or other anaerobic apparatus.

The method of Marino should be recommended for organisms which form minute colonies, and for demonstration plates. Marino poured inoculated agar in the upper half of a Petri dish, and covered it directly with the inverted lower half, and covered the whole with a larger Petri dish. This method is convenient but not necessary for photographic work, as sections for that purpose may be cut from tubes of agar and may then be mounted between cover and slide. Fehrs and Sachs-Mucke used a similar method, covering the agar with a photographic plate. Krumwiede and Pratt used Marino's method satisfactorily for the isolation of fusiform bacilli, sealing the open crack with wax. Rhein used it with satisfaction for general anaerobic work, pouring as sterile agar layer on either side of the inoculated one. Dick use the method of Rhein, replacing the top dish by a layer of paraffin. Al these methods are probably preferable to surface plating for isolation purposes, but are somewhat cumbersome.

Foth complains that the invention of new anaerobic methods has become a sort of sport. Many procedures are too complicated to use, though most methods will save well for the cultivation of sticky black pyrogallic acid and alkali should be avoided, or at least only chosen in the medication of Lentz.

Certain workers with surface methods have charged that deep colony procedures do not give pure culture. Either type of procedure will give pure culture in the hands of the critical worker and impure ones in the hands of the uncritical one. Researchers have found that in making a large collection of anaerobes, that the cultures from laboratories whose isolation procedure was a deep colony method were more often pure than those from laboratories where surface methods were preferred, and they believe that, with the same amount of labor, the same expenditure of time and material, and the same degree of critique, the deep-colony methods are more successful than are surface ones.

Deep-colony methods have been described by the Hesses, by Liborius, and by Veillon and Zuber, and they have been used extensively by von Hibler, Burri, and by French workers. Von Hibler (1908) preferred deep colony isolation to plate methods because of the fact that water of condensation was likely to render plates worthless.

The selection of a suitable medium of deep-colony isolation is an essential to its success. For general work primary requirement is that the nutriment in the medium allow every anaerobe present to grow and form a colony. Otherwise colonies may be fished through agar that contains living invisible organisms of other species, and the most deceptive sort of contamination will take place. The medium should be clear and transparent. Our standard agar medium for routine work is made of beef liver. The usual proportion of one part of meat to two of water gave too active a growth and too much gas. The medium is made as follows:

One part of ground beef liver and four parts of distilled water are infused over night, boiled, and strained. To the broth add 1.5 per cent peptone, 0.5 per cent salt, and for ordinary purposes make up with 2 per cent agar pH 7.2 (faintly alkaline to litmus).

When unusually active gas-producers are present, high dilutions and short (twelve hours) incubation periods are resorted to. Such methods always suffice when rapidly growing species are the ones to be isolated. But when slowly growing species are sought in the presence of actively growing ones, other methods are available. To absorb hydrogen, I per cent potassium nitrate may be added to the agar (Veillon and Maze). To prevent the colonies of the rapidly growing types from outrunning the others, use 3 per cent agar or old agar that has partially dried out, or pay particular attention to enrichment of the desired species in the inoculum and employ abundant dilution tubes. Do not depend upon any colony method for the isolation of badly contaminated slowly growing tissues invaders, but resort directly to guinea-pig inoculation. For slowly growing non-pathogenic organisms mixed with rankly growing gas-producers, try a sugar-free agar (von Hilber, 1908).

There is an essential point in the employment of deep colony tubes which must be observed. Otherwise the method is of no more use than any other. Actively growing anaerobes frequently leave their colonies and grow in the agar as though it were a broth.

This happens more readily with some types of organisms than with others. B. *Welchii* is the chief offender and should be avoided by heating whenever possible. A tube in which this phenomenon has occurred is readily identified by holding it to the light with a control. Such tubes are to be regarded as “enrichment cultures.” Thus their colonies may be of great use when directly inoculated onto another agar series. They are of no use when inoculated into a liquid medium. The close observation of this phenomenon of “permeating growth” cannot be too earnestly insisted upon.

The deep colonies of anaerobes are highly characteristic. Surface colonies are quite characteristic but are obviously subject to many more outside influences than are deep one. Often colonies of different strains in the same species are different and sometimes colonies of one type of anaerobe resemble those of an entirely different type. But carefully made agar shakes often give a beautiful picture of the flora of a wound or of a culture. They are very easily observed with a hand lens and may be as closely approached as may surface colonies. Aerobic growth is easily distinguished from anaerobic growth. My routine method of testing for impurity of culture has been to make three dilution shakes on liver agar. The first and second tubes tell whether or not the culture is pure. The third usually

furnishes colonies suitable for fishing. I was able to isolate, in two series of three agar tubes each, a strain of *oedematiens* type that had been over-grown 1:500 by a vibron septique.

TECHNIQUES OF SOWING AND FISHING

Boil the tubes of agar for a minute or two, remove them from the water, shake them, boil them a little longer, shake them again to remove the air, them cool them to 45^0 C. Do not boil them for ten or fifteen minutes or the cotton will become saturated with moisture. For ordinary purposes use three tubes to each culture. For new and important material of doubtful nature or for shyly growing organisms among rankly growing ones, use more tubes. Inoculate tube 1 with one loopful of culture and roll it, tip it, and roll it four or five times. Take a Pasteur pipette[1] of large bore, flame it, draw up agar of tube.

It is to be noted that few laboratory workers today understand the making of strong and serviceable Pasteur pipettes, and I hope to be pardoned for describing so simple an operation. Meeker burners are best for this purpose. Heat the glass in the portion of the flame where the heat is nearly uniform for a considerable distance. In a blowpipe or Bunsen flame this is above the cone; in the flame of the Meeker burner it is half an inch above the base. Turn the glass 1, expel it, draw up fresh agar and expel it into tube2. For cultures containing abundant organisms, give tube 2 2 inches of agar measured in the capillary portion of the tube. For ordinary cultures give 5 inches, for *B. Novyi*, etc., give about two capillaries full. Place the inoculum throughout the length of the agar while withdrawing the pipette, but do not blow air into the agar of tube 2. Roll tube 2. Flame the Pasteur pipette. By means of it place agar from tube 2 in tube 3 to the amount of 0.5 to 1 inch on the upper or thick portion of the Pasteur pipette. Roll the tube. Incubate aerobically at 37^0. If actively growing species are present, incubate twelve hours. Otherwise incubate eighteen to twenty-four hours. For blackleg, clostridia, and unknown shy types, incubate four days. Examine the colonies with a hand lens. Look for permeating growth. It is better, in fishing from a tube containing more than one type of colony, to fish once more onto a series of agar tubes. Final isolation should be made from colonies of mixed cultures. Study the tubes carefully with a hand lens, noting minute colonies and aerobic growth. Select the tube to be fished, and, if possible, select the colonies desired. Take a well made, strong Pasteur pipette of fairly large bore, bend it at right angles where the capillary begins, break the tip, flame the whole capillary. Remove the plug from the tube and loose fibers of cotton from its opening, insert the Pasteur pipette along the side to the bottom, remove and empty it of agar; re-insert it, and blow the whole column of agar into a sterile Petri dish. The large Pasteur pipette may be used many times. One-half Petri dish serves for each tube. Take a short-stemmed Pasteur pipette, hold it in the flame, draw the capillary out to a hair-like tube, and break it off fairly short. Suck up the desired colony and expel it into a tube of meat medium of tube 1 of another agar series. Draw out the constantly but slowly in the same direction, not forwards and backwards. Continue till the hot portion softens and contracts to about four-fifths of its former diameter. Never pull the glass while it is in the flame. Remove the rod from too flame and wait a second, them pull slowly. If the glass is pulled too soon or too quickly the fine bore is formed from the hottest portion only, and not from all the heated glass, the bore is small, and its walls are thin and weak. An hour's continuous practice is necessary to begin with; the art, once learned, is extremely useful and is not forgotten pipette again, flaming it well, and use it to isolate two or three more colonies. Other workers employ other methods, which are probably ends with an autoclaved rubber stopper placed in the lower end. Some use the loop only for purposes of dilution. Some heat the end of the test tube and expel the agar column by force of the steam thus generated. It is necessary to break the tube at the bottom only when an aerobe is present. Burke used a dissecting lens with stand for fishing colonies; Northrup finds a binocular a great help in some cases. He sections the agar with a sterile blade when researching for minute colonies that are rare. Some workers prefer to attach a rubber tube or a teat to the pipette used in fishing. It is theoretically wrong to fish the colonies from the top of the column of agar without removing it from the tube, because the capillary may pass ungerminated organisms, but such a method might prove practical when used with discretion. Some workers fish the colonies with a platinum needle, but this would hardly prove as satisfactory as a pipette method.

METHODS OF SINGLE- BACILLUS ISOLATION

Isolation of a single bacillus has been resorted to for the separation of anaerobes. Miss Robertson found that the India-ink method of Burri (Besson 1913) exposed the organisms too much and they failed to germinate. She used the Barber method for some time for blackleg and vibrion-septique organisms, and found that the exposure killed vegetative forms and that spores were necessary to give a growth. She fished from apparently pure cultures various numbers of organisms, from one to ten, into meat tubes and used for a type strain the tube that grew and had

received the fewest bacilli. She found the method wasteful of time. Material, eyesight, and nervous energy, and have abandoned it. Her employment of the apparatus was, however, far from being as skilled as that of Dr. Barber. She explained her difficulties to Dr. Barber and he (1920) has made a careful statistical study of the behaviour of various anaerobes when isolated by his technique. He was successful when inoculating various media with different anaerobes in securing 62 growths from 400 single bacilli, and 93 growths from 211 single spores. Vegetative rods represented, inoculate a series of guinea-pigs with antitoxic or antibacterial sera of the groups probably represented on the smears, in such a manner that for each type of organism there is a guinea-pig immunized against the other types only. Then inoculate the mixed material into all the guinea-pigs. This method was found successful by the Committee. In large war hospitals collections of guinea-pigs immunized by bacterial inoculation have been kept for diagnostic purpose.

It is best to inoculate guinea-pigs in the thigh muscles. Take cultures from various points in the body. The heart-blood culture is usually the most valuable. *Oedematiens*-group organisms and some other pathogens do not always become septicemic, however. Bifermentans-group organisms and other proteolytic types may become septicemic. Inoculate into another guinea-pig a culture from the heart-blood in smaller quantity than was used before. If this fails, isolate the proteolytic than was used before. If this fails, isolate the proteolytic organism, immunize a guinea-pig with it, then inoculate the mixture. For all animal work keep a careful record of the cultures inoculated, incubation periods, lesions in the animals, and, above all, make constant use of the microscope.

Anaerobic organisms should be sought in the following pathological condition:

- Infected wounds (rods or cocci)
- Gangrene
- Oedema
- Emphysema of muscles, connective-tissue, liver or other organs
- Haemorrhagic condition of muscles
- Pneumonic processes where anaerobic infection is suspected, pulmonary gangrene
- Necrosis of muscle or connective tissue (B. necrophorus et alii).
- Injection of serous surface, especially in ruminants
- Abortion in animals (search foetus for *Bact. abortion*)
- Endometritis, post abortum or post partum (*Streptococcus)*
- Appendicitis and various ulcerative and suppurative conditions
- Tetanus (in absence of wounds and uterine infection, search for peridental infection).
- Botulism, intestinal content and wall, liver, spleen, tools from patients.

Obscure fevers, measles, scarlet fever. Blood culture, look for various invaders, Rhinitis, Vincent's angins; mucous surface.

Make smears of affected tissue, make meat or brain cultures and make at the same time numerous shakes in deep liver-agar. Examine shakes twelve hours after incubation if possible, and examines meat cultures twenty to twenty-four hours after incubation. Blood cultures in broth, meat or agar should always be made, if possible, *ante mortem and post mortein.*

THE CULTIVATION AND IDENTIFICATION OF ANAEROBES

Smith and Holdeman have presented an admirable review of methodology, growth requirements, and identification of pathogenic anaerobic bacteria. With the exception of genus *Clostridium*, the role of anaerobic bacterial as etiological agents in diseases of veterinary importance has been poorly established. There are several reasons for this. Firstly, these organisms have been considered difficult to cultivate. Secondly, many pathogenic anaerobic bacteria are normal inhabitants of the bacterial flora at most sites of the animal body. Thirdly, it has been difficult to fulfil Koch's postulate and reproduce the disease condition in a susceptible host. A classical illustration of the importance of reproducing the disease condition from which an anaerobe was isolated, was establishing *F. nodosus* as the cause of foot rot in sleep .

Many anaerobic organisms can be isolated from clinical specimens. That such isolates may be contaminants from the normal flora often leads to their presence being ignored. A contaminated specimen implies poor collection technique and should, therefore, not be submitted for culture. The presence of anaerobes in a properly collected clinical specimen should not be ignored. They may not be the primary organism but they may have a marked influence on the severity of the lesion.

The approach and techniques described are listed below.

PROCEDURE FOR OBTAINING ANAEROBIOSIS

The procedure used is a modification of that developed by Rosenthal (.

(a) Reagents

1. Chromium powder
2. Sodium Carbonate
3. 15% Sulfuric acid

(b) Apparatus

1 Jar of container, 1.5 to 4 liter capacity with air-tight lid

2 Small beaker approximately 100ml capacity

(c) Quantities of Reagents Required

1. Jar capacity, 1.5 liters 22.5mls, 15% sulfuric acid, 2.25gms, chromium powder, 0.75gms, sodium carbonate
2. Jar capacity, 4 liters, 58.5mls, 15% sulfuric acid, 5.85gms, chromium powder, 1.95gms, sodium carbonate

(d) Procedure

1. Pour the sulfuric acid into a small beaker.
2. Add the chromium powder.
3. Place the sodium carbonate in a test tube or bottle cap and suspend within the beaker in such a way as to be easily tipped into the acid mixture.
4. Place the beaker and its contents into jar, lay the inoculated plate or plates on or alongside the beaker, and screw the lid firmly onto the jar.
5. After 30 – 45 minutes, if the lid is sufficiently air-tight, moisture will have formed on the inside of the beaker and often on the jar; if moisture does not condense on the walls, repeat the procedure.

When moisture is evident, tip or shake the suspended cap of sodium carbonate into the acid-chromium mixture.

In many anaerobic laboratories, the 3 1b peanut butter jar, 1.5 liter capacity, available from any supermarket, and without modification, has been found ideal. Because evolution of carbon dioxide and hydrogen increases the gaseous pressure within the jar, a heavy oil or mercury seal may be attached to the lid to provide pressure control. A seal is preferable to a balloon as a method for accommodating excess gas.

For good growth most anaerobic bacteria require an enriched media. All anaerobes likely to be encountered in the routine clinical laboratory will grow on blood agar. Most suitable general media is that developed by Schaedler *et a*l . Commercial Schaedler's media may have 5% blood added to it so that hemolytic reactions may be observed. The advantage of Schaedler's agar as compared to blood and slightly larger colonies are obtained. A number of satisfactory media are available commercially.

PROCEDURES FOR PROCESSING SPECIMENS FOR ANAEROBIC CULTURE

Whenever possible minimize time for exposure to oxygen and avoid dehydration of specimen. Therefore, it is best to inoculate the media immediately after collecting the specimen. At this hospital, the anaerobe jar and culture media are taken to the animal, the specimen collected and transferred to agar and broth culture media and immediately placed in the anaerobic environment. A sealed mass removed during surgery is brought to the laboratory for culturing. It is ideal but not essential for clinical work, to keep plated media stored in an oxygen free environment. With each anaerobic culture, two blood agar plates are also prepared and incubated at 37%. Slides are also prepared for staining by Gram's and Giemsa's procedures. A wet smear for bright and dark field examination is made. If the interest is specifically in the anaerobic bacteria, staining and wet smear examination is not performed. When microaerophilic organisms such as Vibrio or Brucella spp. are not suspected, culture in this atmosphere is unnecessary.

As many aerobes are facultative anaerobes, comparison between growth of each culture in the different environments is necessary. Absence of growth theologlycollate broth establishes that no anaerobes were present in the original specimen. Alternatively, when only a few strictly anaerobic colonies are found on solid media they may be recovered from the thiologycollate broth.

TENTATIVE IDENTIFICATION OF ANAEROBIC BACTERIA

The key used for recognition of common pathogenic anaerobic bacteria is based on morphologic characteristics, Gram stain, motility, and catalase production. The catalase test is performed after the culture has been removed from anaerobic environment for 30 minutes by adding 3% hydrogen peroxide (H^2O^2) to the bacterial growth on the agar surface and observing closely for continuous bubbling. Most bacteriology texts advise not to perform the catalase reaction on blood agar because hemolysis liberates erythrocytic catalase. In this paper where catalase production is mentioned as a characteristic of non-hemolytic organisms, all give very strong catalase positive reactions and are therefore not likely to be confused with the very slow reaction that is seen due to the red blood cell. It has been our experience that condition determined under these conditions from the blood agar plate is entirely satisfactory.

Reactions of anaerobic cocci as well as precise identification of member species of genera *Peptostreptococcus* and *Peptococcus* is likely to be necessary in the routine laboratory. Two species of genus *Veillonella* are recognized. Differentiation is based on their catalase reaction.

Members of only two genera, Corynebacterium and Propionibacterium are Gram positive, catalase positive, nonspore-forming rods. Organisms in these genera are slow growing, requiring up to one week before colony growth is observed. Member species are pleomorphic. Smith and Holdeman have shown that in general the ability to hydrolyze esculin, the failure to liquify gelatin and the inability to reduce nitrates, distinguish the *Propionbacterium* from the *Corynebactrium. Propionibacterium spp.* grow poorly in a microaerophilic atmosphere whereas anaerobic *Corynebacterium spp.* are strictly anaerobic. The anaerobic *Corynebacterium spp.* are normal inhabitants of many parts of the body. *C. acnes*, a skin inhabitant of most animals, is the only member of these two genera which we have isolated. Propionibacterium spp. have been isolated from dairy products but have yet to be demonstrated as pathogenic to animals.

The genus *Clostridium* contains all anaerobic Gram positive, catalase negative, spore-forming bacteria. Table II presents cultural and biochemical characteristics of members of this genus which my have veterinary importance. *Cl. Novyii* and *Cl. Hemolyticum* are frequently considered the most fastidious of the anaerobic bacteria. It has been our experience that to isolate the latter organism, freshly prepared media (and ideally held under anaerobic conditions immediately after preparation) must be used.

Five genera, Actinomyces, Ramibacterium, Catenbacterium, Cillobacterium, and Eubacterium which are Gram positive, catalase negative, nonspore-forming rods can be subdivided on the basis of branch or chain formation.

Members of genus Actinomyces are non-motile, produce branching mycelium in early stages of growth, produce acid but not gas in theologycollate dextrose broth, and attack sugars fermentatively. Some are facultative microaerophils. A bovis is found in mouth and jaw-bone infection of ruminants and may be responsible for pulmonary actinomycosis in pigs. *A. Israeli* is actinomycosis in pigs animals; A. *baudetti* is mentioned as pathogenic for dogs and cats in Bergey's manual. But this organism is not mentioned in other recognized texts because most authors accept these organisms as identical. *A. suis* has been isolated from udder infection of pig .

Cells of genus *Ramibacterium* show false branching being arranged in Y or V shapes referred to as flying bird formations. All members are strictly anaerobic, nonmotile, produce gas in culture media, and attack glucose but not lactose or maltose to produce acid. *R. ramosoides* has been found to be pathogenic for laboratory animals. Table IV shows the differentiating reactions of *R. ramosoides* from *R. ramosum,* a non-pathogenic member of this genus.

The genus *Catenabacterium* contains nonmotile, chain-forming, plemorphic rods. C. contortum, C. lottii, and C. nigrum are pathogenic for guinea pigs or mice, whereas other species of this genus are nonpathogenic . Genus *Eubacterium* has characteristics very similar to those of genus *Catenabacterium.* Veterinary isolates of genus *Eubacterium* are found in single cells, masses or short chains, whereas members of genus Catenabacterium have long chain formations. Table V shows differentiating features of possible pathogenic members of these two genera. All members of genus Cillobacterium are motile, nonspore-forming, Gram positive rods. Smith and Holdeman stress that an extensive search for spores should be made before identifying an organism as belonging to genus Cillobacterium. As they are rare isolate they will not be discussed further. Skerman has pointed out that characters providing for uniform differentiation of genus Bacteroides from other genera of the anaerobic, Gram negative rods are lacking. In Tables 6 and 7 the terminology give by Skerman has been followed. The morphological features shown in Table 6 should be considered as a general guide only. All organisms in Table 6 and 7 are catalase and nitrate negative.

Table 3 Key for the differentiation of A. bovis from A. Isreali.

	A. bovis	A. Israeli
Groth in thiogylycollate		granular
Medium		broth clear
Colony morphology	Flat, granular	spider forms
(24 – 48 hrs. growth)	non-adherent	adherent
Microscopic appearance	diphtheroid, branching	branching,
(from theology broth)	rare; few filaments	filamentous
Nitrate reduced	-	+
Mannitol fermented	-	+ -

Table 4: Key for differentiation of *R. ramosoides* R. *ramosum*

	R. ramosoides	*R. ramosum*
Indole production	+	-
Litmus milk	slowly +	+
Coagulation	+	-
Hemolysis	+	-

Table 5: Key to The Differentiation of Members of Genera Catenabacterium and Eubacterium

Organisim	Morphology	Colony	Gas Production Black Media	Gelatin in Culture Faction	Lique- Odor	Fetid
C. contorium	chains	-	+	-	-	
C. lottii	chains	-	-	+	-	
C. nigrum	chains	+	-	-	+	
E. disciformans	small masses	-	-	no growth	-	
E. obitii	single rods	-	+	-	+	

Table 6: Morphologic and Cultural Feature of Some Anaerobic Gram Negative Bacilli

Organism	Morphology	Black Colonies	Haemolysis	Foul Odor	Motility
Sphaerophorus	pleomorophic rods;	-	+	+	-
Necrophorus	blunt ends; sphaerules or round bodies				
Dialister	minute rods;	-	-	-	-
Pneumosinies	rather pointed ends				
Fusobacterium	long rods; chains;	-	+	-	-

Fusiforme	pointed ends	_	-	+	-		
Fragilis							
Bacteroides	Cocobacilli		-	-	+	-	
Melaninogenicus							
Bacteroides	short rods		-	-	+	-	
Terebrans	short rods		-	-	+	-	
Variobilis bacteroids	thich rods		-	-	+	+	
Girans	chains						

Table 7: Biochemical Characteristic of Some Anaerobic Gram Negative Bacilli

Organism	Glucose	Lactose	Gelatin liguefaction	Mannitol	Indol	Gas in thioglycolate dextrose
S. necrophorus	A -		-	-	+	+
D. pneumosintes	A =		-	-	+	-
F. fusiforme	A -		-	-	-	-
B. fragilis	A -		-	-	-	+
B. Melanogenicus	A A		A	-	+	-
B. terebrans	A A		A	-	-	+
B. variabilis	A A		-	-	+	+
B. girans	A A		-	+	-	+

Table 8 gives the sensitivity results of organisms found in the cases.

Table 8: Anaerobic bacteria isolated from clinical cases: Sensitivity to chemotherapeutic impregnated discs measured as width of zone of inhibition (mm) of confluent growth of organism.

	B. giran s	*C. acne s*	*C. acne s*	*Corynebacteri um*	*Peptostreptococc us*	*Cl. chauvo ei*	*Cl. chauvo ei*	*Cl. septicu m*
Auremycin 30 mcg	10	11	11	10	11	8	11	-
Chloromyce tin 30 mcg	10	11	11	10	10	10	11	10
Furadantin 100 mcg	10	10	10	10	11	10	11	11
Kanamycin 30 mcg	-	3	-	-	5	-	-	-
Neomycin 30 mcg	-	5	2	3	-	-	-	-
Penicillin 10 units	-	9	10	10	10	11	10	10
Polymyxin B 300 units	-	-	-	-	-	-	-	-
Furaltadone 30 mcg	9	2	3	11	-	5	10	11
Sulphathiaz ole 10 mcg	-	-	-	-	-	-	3	-
Terramycin 30 mcg	10	9	10	10	9	5	6	10
Tetracycline 30 mcg	-	10	11	10	-	8	8	11
Tylosin 30 mcg	-	10	10	10	-	6	-	-
Case	2	3	4	5	7	8	9	9

CLINICAL CASES

The following nine cases were taken from clinical records. They illustrate some of the conditions in which an anaerobic organism may be involved.

Case 1

Specimen: fluid obtained by abdominal paracentesis.

Species: bovine

Clinical diagnosis:peritonitis

Laboratory Results:

(a) Direct smear – two organisms were seen: Gram positive cocci in chain formation; pleomorphic Gram negative bacilli.

(b) Culture – after seven days incubation, the cocci showed good zones of beta hemolysis and the bacilli formed raised, white, soft colonies without hemolysis; cocci grew in the presence of air; bacilli were strict anaerobes.

Organisms: *Streptococcus; Sphaerophorus necrophorus*

Case 2

Specimen: fluid obtained by thoracentesis

Species: feline

Pathologic diagnosis: exudative pleurisy

Laboratory results:

(a) Direct smear – two organisms were see: Gram positive cocci in pairs; Gram negative rods, rounded ends, uniform in appearance with only a rare filamentary form and a few swollen forms.

(b) Darkfield examination – motile rods.

(c) Culture – liquid media did not support growth; both cocci and bacilli were strict anaerobes; cocci showed hemolysis and demonstrated long chain formations; bacilli were non-hemolytic and had distinctively foul odor.

Organisms: Peptostreptococcus spp.; Bacteriodes girans

Case 3

Specimen: urine obtained by cystocentesis

Species: feline

Clinical diagnosis: urethral blockage due to struvite calculi; within a few hours of making urethra patent and flushing out of bladder, urethra repeatedly became impacted.

Laboratory results:

(a) Direct smear of urinary sediment – negative.

(b) Culture – initial isolation from thioglycollate broth; delayed, non-hemolytic growth in earliest subcultures with increasing hemolysis and more rapid growth in later cultures; Gram positive bacilli.

Organism: Corynebacterium acnes

Case 4

Specimen: urine obtained by cystocentesis

Species: feline

Clinical diagnosis: urethral blockage due to struvite calculi; animal had three crises within one month.

Laboratory results:

(a) Direct smear of urine sediment – negative

(b) Culture – strict anaerobic; Gram positive bacilli, strong catalase reaction; flying bird formations observed.

Organism*: Corynebacterium acnes*

Case 5

Specimen: direct culture with loop

Species: canine

Clinical diagnosis: chronic foot infection with fistula

Laboratory results: Gram positive diptheroid; became more aero-tolerant with sub-culture.

Organism: *Corynebacterium spp*.; several reactions were consistently inconclusive

Case 6

Specimen: swab from socket of premolar took on right jaw.

Species: equine

Clinical diagnosis: strict anaerobic; non-hemolytic organism; Gram positive cocci in long chains.

Organism: *Preptostreptococcus anaerobius*

Case 7

Specimen: blood

Species: equine

Clinical diagnosis: slow-healing abscess following castration

Laboratory results: two organisms were in chains; Gram negative bacilli which grew only in thioglycollate broth.

Organisms: *Peptostreptococcus anaerobiuss*; morphology suggested bacillus was *Bacteroides spp.*

Case 8

Specimen: blood

Species: canine

Clinical diagnosis: acute nephritis with secondary endocarditis

Laboratory results: uniformly shaped, medium size, Gram positive rod showing wide beta hemolysis was isolated.

Organism: *Clostridium chauvoei*

Case 9

Specimen: swab from peritoneum of aborted fetus

Species: canine

Clinical diagnosis: abortion

Laboratory results: two strict anaerobes isolated:

(a) Gram positive bacilli showing as spreading, beta hemolytic colonies which appeared dark by transmitted light;

(b) Gram positive bacilli, slender and longer than (a), showing as beta hemolytic, non-spreading colonies which appeared colorless by transmitted light.

Organism*:* *Clostridium chauveoi: Clostridium septicum*

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