THE BLOOD-AGAR PLATE FOR SPORE-FORMING ANAEROBES

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The advantages of the blood-agar plate for the isolation and grouping of streptococci are well known. It now seems that the blood-agar plate may be equally useful in isolating and grouping another genus of bacteria, the spore-forming anaerobes. Under suitable conditions of anaerobiosis this medium has proved very satisfactory for securing growths, and the effect of these bacteria on hemoglobin resembles that obtained with streptococci, that is, some spore-forming anaerobes are hemolytic; some produce methemoglobin, and others have no effect on the red blood cells. It appears also that the plate method is more suitable for quickly securing pure cultures than the deep tube methods, so much used in the past, principally because the gas formed by many of these bacteria escapes from the thin layer of agar more readily than from the deep layer and thereby prevents mixing of the growths. Under similar physical conditions the anaerobic spore-formers should be as easy to secure in pure culture as, for example, the streptococci, although the literature gives the impression that pure cultures may be obtained only after long and tedious effort. More recently the Barber single cell method has been adopted by certain bacteriologists as the only reliable means of separating the anaerobes.

During the last year the blood-agar plate has been used as a routine for securing pure cultures of anaerobes at postmortem examinations with good results. I shall now present more particularly a tentative classification of pure cultures, obtained from
other sources, according to their effect on the blood-agar plate. Zeissler, used blood-agar-streak plates for growing Clostridium welchii and Clostridium oedematis-maligni and reports very characteristic surface colonies, stating that this method is most satisfactory for proving the purity of cultures. Richardson and Dozier speak of isolating delicate anaerobes on blood-agar plates, anaerobic conditions being obtained by the method of McIntosh and Fildes. They give no details as to cultures, but later Wagner, Dozier, and Meyer, using the same anaerobic method, describe the growth of Clostridium botulinum, Clostridium sporogenes and Clostridium tetani on poured blood-agar plates. Wheeler and Humphreys used blood-agar plates for growing anaerobes, securing anaerobic conditions in a jar exhausted with hydrogen. They were able to secure characteristic colonies of Clostridium welchii, Clostridium botulinum, Clostridium sporogenes, and Clostridium histolyticum, but found that Clostridium tetani and Clostridium putrificum grew poorly. Wagner reports that Pfenninger was unable to grow Clostridium botulinum type C, and the parabotulinus organism of Seddon on blood-agar plates. Hall has made extensive use of blood-agar slants for the cultivation of anaerobes, securing anaerobic conditions by Wright’s method.

EXPERIMENTAL DATA

The medium used was hormone agar made after Huntoon’s formula, with 1.5 per cent agar, and sterilized in flasks in amounts of 50 and 100 cc. The agar was melted and cooled to 45°C.

FIG. 1. CLOSTRIDIUM WELCHII (No. 2 HALL)
Double method, twenty-four hour culture. × 2

FIG. 2. CLOSTRIDIUM WELCHII (No. 2 HALL)
Double method, twenty-four hour culture. × 6

FIG. 3. CLOSTRIDIUM CENTROSPOROGENES (No. 76 HALL)
Double method, forty-eight hour culture. × 2

FIG. 4. CLOSTRIDIUM CENTROSPOROGENES (No. 76 HALL)
Double method, forty-eight hour culture. × 6

FIG. 5. CLOSTRIDIUM OEDEMATIS-MALIGNI (No. 18 HALL)
Double method, twenty-four hour culture. × 2

FIG. 6. CLOSTRIDIUM OEDEMATIS-MALIGNI (No. 18 HALL)
Double method, twenty-four hour culture. × 6
and 5 per cent human blood added; it was then dispensed into sterile tubes, 10 to 12 cc. to each tube. Serial inoculations were made from the material to be cultured, and plates poured. Anaerobic conditions were secured by means of a Novy jar exhausted with hydrogen, or by combining this with the alkaline pyrogallol method. In filling the jar with hydrogen the apparatus was so arranged that hydrogen entered the top of the jar while the air was drawn off from the bottom by means of a rubber tube attached to the glass tube in the stopper and long enough to reach to the bottom of the jar when the cover was in place. The ground-glass seals between the two halves of the jar and around the stopper were kept well coated with vaselin, and the jar, when in use, was held together with small clamps. A hydrogen cylinder fitted with a reducing valve was found to be the most convenient source of hydrogen. The hydrogen was allowed to run into the jar and the gas issuing from it was led into a pan of water by a tube with an inside diameter of between 4 and 5 mm. When the bubbles appeared at the rate of about 175 a minute it was assumed that the air could be largely removed from the jar in from twenty to thirty minutes, and if a tube of the gas was caught over water after this interval it would burn with very little explosion when ignited.

By this method the anaerobes which can tolerate small amounts of oxygen will grow well, but others grow poorly or only on very heavy inoculation. Repeating the process of exhaustion after half an hour to remove traces of oxygen which might diffuse out from the plates gave no better results. Therefore the remaining oxygen was absorbed in alkaline pyrogallate, which was done by placing an empty plate in the bottom of the jar to keep the others out of the liquid, and adding about 15 to 20 gm. of pyrogallic acid for a jar of approximately 3 liters capacity. It was necessary to have a jar whose diameter was somewhat larger than that of the plates. After exhausting with hydrogen about 50 cc. of 5 per cent sodium hydroxid was aspirated back into the bottom of the jar through the delivery tube, care being taken to turn the stopcock in the jar before air entered. It was found that cultures which grew poorly with the first method grew freely on the addi-
tion of the small amount of alkaline pyrogallate; it was therefore concluded that these cultures were inhibited by small amounts of oxygen. In some cases twenty-four hours is sufficient to secure well developed colonies, but for the majority forty-eight hours is preferable.

While the list of anaerobes studied by this method is not complete, enough of the more common ones have been examined to give a fairly good idea of the groups that may be expected. The difficulties attendant on securing authentic cultures for comparison make a complete study of all the described anaerobes a time-consuming project. Several platings were done on each culture, each being tried by both of the methods of anaerobiosis described. The following species have been studied, the numeral following each name indicating the number of strains: Clostridium welchii (10); Clostridium centrosporogenes (1); Clostridium oedematis-maligni (vibron septique) (2); Clostridium chauvei (1); Clostridium bifermentans (2); Clostridium histolyticum (1); Clostridium novyi (1); Clostridium tetani (2); Clostridium botulinum Type A (3); Clostridium botulinum Type B (3); Clostridium sporogenes (1); Clostridium tertium (3) and Clostridium putrificum (1). The deep colonies were preferable to surface colonies for differentiation, since surface colonies are so largely influenced by moisture, and since in certain cases surface colonies are very few as compared with deep colonies. The following grouping is proposed, to serve, first, as a rapid laboratory method of indicating the more important pathogens which belong to the hemolytic group, and, second, as a guide to their more detailed identification.

**BLOOD-AGAR GROUPING OF SPORULATING ANAEROBES**

Group I. Hemolytic

A. Grow well in anaerobic jar exhausted with hydrogen (single method)²

1. Zone of hemolysis large compared to deep colony
   a. Deep colony large, compact, often irregular, hemolysis clear

² For convenience the method of exhaustion with hydrogen is called the single method. When alkaline pyrogallate is also used it is called the double method.
(1) Zone of hemolysis surrounded by a deeper red zone
   Clostridium welchii
(2) Zone of hemolysis not surrounded by a deeper red zone
   Clostridium centrosporogenes
   b. Deep colony small, compact, often lens-shaped
      Clostridium oedematis-maligni (vibrion septique)
      Clostridium chauvei
2. Zone of hemolysis small compared to size of deep colony
   a. Deep colony filamentous on margin
      Clostridium bifermantans
   b. Deep colony compact and irregular
      Clostridium histolyticum
B. Grow well only under strict anaerobic conditions (double method)
   1. Zone of hemolysis large compared to size of deep colony
      a. Deep colony compact, irregular, hemolysis clear
         Clostridium novyi
      b. Deep colony, filamentous on margin
         Clostridium tetani
   2. Zone of hemolysis small compared to size of deep colony
      a. Deep colony compact, disc-shaped or irregular
         Clostridium botulinum
      b. Deep colony filamentous on margin
         Clostridium sporogenes
Group II. Not hemolytic, producing green zone around colonies
A. Grow well in anaerobic jar exhausted with hydrogen
   1. Deep colony small, compact, lens-shaped
      Clostridium tertium

Fig. 7. Clostridium chauvei (No. 6 Hall)
Double method, forty-eight hour culture. × 2
Fig. 8. Clostridium chauvei (No. 6 Hall)
Double method, forty-eight hour culture. × 6
Fig. 9. Clostridium bifermantans (No. 102 Hall)
Double method, forty-eight hour culture. × 2
Fig. 10. Clostridium bifermantans (No. 102 Hall)
Double method, forty-eight hour culture. × 6
Fig. 11. Clostridium histolyticum (No. 290 Hall)
Double method, forty-eight hour culture. × 2
Fig. 12. Clostridium histolyticum (No. 290 Hall)
Double method, forty-eight hour culture. × 6
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Group III. Without effect on red blood cells
A. Grow only under strict anaerobic conditions
   1. Deep colony punctiform
      Clostridium putrificum

DESCRIPTION OF CULTURES STUDIED

Clostridium welchii

Culture 2. (I. C. Hall) Twenty-four hours' incubation
   Good growth in twenty-four hours or less by both single and
double methods
   Surface colony: 2 to 3 mm., round, entire, convex
   Deep colony: 1 to 1.5 mm., lens-shaped or irregularly lobate
dense masses, with no filaments on margin (X 100)
   Hemolysis: Marked. A primary clear zone surrounded by a wide
diffuse zone, which is in turn bordered by a zone of darker red
than the surrounding medium (figs. 1 and 2)
   Morphology: Large rods of varying lengths, Gram-positive and
Gram-negative. Rods often appear granular

Clostridium centrosporogenes

Culture 76. (I. C. Hall) Forty-eight hours' incubation
   Grow well by both single and double methods, forty-eight hours
required for well developed colonies
   Surface colony: 1 to 2.5 mm., round, entire, or slightly irregular,
some with raised centers, some with depressed centers
   Deep colony: Dense irregular multilobulate masses, no filaments
on margin (X 100)
   Hemolysis: Clear zone whose diameter is 2 to 2.5 times that of
the deep colony (figs. 3 and 4)
   Morphology: Large rods, much like Clostridium welchii except
larger

Clostridium oedematis-maligni (Vibrio septique)

Culture 18. (I. C. Hall) Twenty-four hours' incubation
   Grow well by both single and double methods
   Surface colony: Variable in size and shape, some flat spreading
films covering a quarter of the plate; some small (1 mm.) with
root-like processes at margin, some small (0.5 mm.) round,
entire, convex
Deep colony: Punctiform to 1 mm., disc-shaped or irregular, with knobs or fan-like projections, no filaments on margin (× 100)

Hemolysis: Some colonies show a small clear zone slightly larger than the colony itself, some a large diffuse zone three to five times the diameter of the colony (figs. 5 and 6)

Morphology: Some long rods, others medium or short, Gram-positive but not strongly so. Bipolar staining, and lightly staining barrel-shaped forms with or without polar granules common

Culture 421. Isolated September 11, 1924, at postmortem from a case of peritonitis. Cultural characters, morphology and pathogenicity for rabbits on intramuscular inoculations indicated *Clostridium oedematis-maligni*

On blood-agar plates it behaved in all respects like the preceding culture

*Clostridium chauvei*

Culture 6. (I. C. Hall) Forty-eight hours’ incubation

Good growth by both single and double methods

Surface colony: 1 to 1.5 mm., round, entire, convex, slightly opaque

Deep colony: 0.2 mm., lens-shaped, compact, no filaments on margin (× 100)

Hemolysis: A wide zone compared to size of colony (2 to 3 mm.)

Many red cells still intact giving a diffuse hazy appearance to the zone (figs. 7 and 8)

Morphology: Medium sized Gram-positive rods, irregular staining, swollen, round or clubbed forms common

*Clostridium bifermentans*

Culture 102. (I. C. Hall) Forty-eight hours’ incubation

Good growth by both single and double methods

Surface colony: Some of small dew drop type, and others like a mat of filaments, rather dry looking

Deep colony: 0.25 to 1 mm., often showing a dense core set in a net work of filaments which contains coarse granules (× 100)

Hemolysis: The zone is not clear cut and extends but little beyond the line of growth (figs. 9 and 10)

Morphology: Large rods, Gram-positive and some partly decolorized. Granular forms common as in *Clostridium welchii*

Culture 507. Isolated at postmortem November 1, 1924, from the blood and spleen of a case of carcinoma of the ovary. The
results on coagulated egg, gelatin, brain broth, blood serum, litmus milk, and sugar broths indicate *Clostridium bifermentans*. This culture is nonpathogenic for rabbits when given intravenously or intramuscularly. On blood-agar plates it grows like culture 102, producing similar deep colonies and the same type of hemolysis.

*Clostridium histolyticum*

Culture 290. (I. C. Hall) Forty-eight hours' incubation
Good growth by both single and double methods
Surface colony: Some 1 mm., round, entire, convex, white (resembling staphylococcus colony), others 3 mm. to 1 cm., flat, spreading, with finger-like projections
Deep colony: 0.5 mm., compact, lens-shaped, heart-shaped, or irregular, with clear-cut margins having no projecting filaments (× 100)
Hemolysis: Zone clear and sharply defined, its diameter being about twice that of the colony (figs. 11 and 12)
Morphology: Medium sized Gram-positive rods, some appear granular

*Clostridium novyi*

Culture 140. (I. C. Hall) Forty-eight hours' incubation
Growth poor by single method, but rapid by double method
Surface colony: 3 to 4 mm., round, flat, with raised center, margin torn and irregular, color yellowish
Deep colony: Dense irregular masses 0.5 to 1.0 mm. with few short projecting hair-like filaments (× 100)

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**Fig. 13. Clostridium novyi (No. 140 Hall)**
Double method, forty-eight hour culture. × 2
**Fig. 14. Clostridium novyi (No. 140 Hall)**
Double method, forty-eight hour culture. × 6
**Fig. 15. Clostridium tetani (No. 1 Hall)**
Double method, forty-eight hour culture. × 2
**Fig. 16. Clostridium tetani (No. 1 Hall)**
Double method, forty-eight hour culture. × 6
**Fig. 17. Clostridium botulinum type A (No. 9 Park)**
Double method, forty-eight hour culture. × 2
**Fig. 18. Clostridium botulinum type A (No. 9 Park)**
Double method, forty-eight hour culture. × 6
**Fig. 19. Clostridium botulinum type B (No. 178 Hall)**
Double method, forty-eight hour culture. × 2
Hemolysis: Zones very large and clear, eight to ten times greater in diameter than the deep colony. There is often a darker red ring just surrounding the hemolytic zone (figs. 13 and 14)
Morphology: Large rods, some curved. Occasional shadow forms, or swollen forms with bipolar staining. Gram-positive

**Clostridium tetani**

Culture 1. (I. C. Hall) Forty-eight hours' incubation
Growth poor by single method, sometimes a few colonies develop after very heavy inoculation. Growth very rapid by double method
Surface colony: Flat, dry, irregular growths composed of matted filaments
Deep colony: 0.25 to 1 mm. Larger colonies very filamentous ($\times$ 100) with large granules among the filaments
Hemolysis: Zone large (eight to ten times greater in diameter than the colony), and hazy, without a clear-cut margin (figs. 15 and 16)
Morphology: Large rods and filaments, Gram-positive

**Clostridium botulinum Type A**

Culture 9 Orr. (W. H. Park) Forty-eight hours' incubation
Growth occurs by double method only
Surface colony: Some are spreading, leaflike or rhizoid, others circular, entire, convex
Deep colony: Some heavy disc-shaped with entire margins 0.5 to 1 mm. Some small to punctiform, irregular, dense masses with or without a few filaments from margin ($\times$ 100). The colony shapes of all cultures of *Clostridium botulinum* are more variable than those of any other species studied (figs. 17 and 18)
Hemolysis: The larger disc-shaped colonies have a relatively narrow zone of hemolysis compared to the size of the colony, while the small colonies have a comparatively wide zone. The hemolysis is clear and the zone has a definite margin

Culture Memphis (W. G. McCoy) Forty-eight hours' incubation
Like the preceding culture it produces both spreading and convex
surface colonies, and also disc-shaped and punctiform deep colonies

Culture 372. (I. C. Hall) Forty-eight hours’ incubation

The same varieties of deep colony as previously described, also certain ones resembling Clostridium bifermentans. The hemolysis is similar in all, that is, with relatively narrow clear zones

Clostridium botulinum Type B

Culture Dixon (W. H. Park) Forty-eight hours’ incubation
Culture Nevin (W. G. McCoy) Forty-eight hours’ incubation
Culture 178 (I. C. Hall) Forty-eight hours’ incubation (See figures 19 and 20 of Culture 178)

What has been said of the type A cultures applies equally well to the type B cultures. The principal types of colony found in all botulinus cultures may be summarized as follows:

Surface: (1) spreading and (2) round, convex colonies

Deep: (1) disc, or lens-shaped colonies with or without coarse projections (fig. 20), and (2) punctiform or small irregular colonies

It was thought at first that these different varieties might indicate a mixed culture; consequently the different types were carefully picked to start new cultures. When these cultures were plated, the same variations occurred as in the original culture. As a further check these cultures obtained by picking different well isolated colonies were tested for toxicity. In all, nine subcultures representing the different colony types to be found in two type A and two type B strains were proved to be toxic for guinea pigs on feeding, and no nontoxic strains were found. This seems to indicate that Clostridium botulinum is quite variable as to colony form. The hemolysis, however, is a constant factor.

Clostridium sporogenes

Culture 90. (I. C. Hall) Forty-eight hours’ incubation

Growth poor by the single method, but abundant by the double method

Surface colony: 0.25 to 1 cm., fern-like or arborescent, some flat, lobate or ameboid
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Deep colony: A dense core 0.2 to 0.5 mm., round or slightly irregular, surrounded by a radiating network of tangled filaments (× 100)

Hemolysis: A clear sharply defined zone 1.5 to 2 mm. in diameter (figs. 21 and 22)

Morphology: Medium-sized rods, Gram-positive

Clostridium tertium

Culture 518. (I. C. Hall) Twenty-four hours' incubation

Growth occurs by either single or double method, but seems to be better if anaerobiosis is not too complete. Incubation for a longer period than twenty-four hours is unnecessary in the cultures studied

Surface colony: Most are round, 0.5 to 1 mm. convex, entire, and slightly milky in color. Some are flat, spreading, 0.5 to 2 cm. with irregular lobate margin

Deep colony: Oval, disc-like, compact bodies, 0.5 mm. without filaments on the margin (× 100)

Hemolysis: Negative. If plates are incubated by the single method there is a narrow faint green zone surrounding the colonies, more marked after the plates have been removed from the jar for an hour or two and the blood has regained its bright red color. Clostridium tertium is a representative of the group of anaerobes producing green zones on blood-agar plates (figs. 23 and 24)

Morphology: Long slender rods, usually partly decolorized by gram stain

Culture 441. Isolated September 19, 1924, at postmortem, from a case of gangrene of the retroperitoneum. This culture corresponds

Fig. 20. Clostridium botulinum type b (No. 178 Hall)
Double method, forty-eight hour culture. × 6

Fig. 21. Clostridium sporogenes (No. 90 Hall)
Double method, forty-eight culture. × 2

Fig. 22. Clostridium sporogenes (No. 90 Hall)
Double method, forty-eight hour culture. × 6

Fig. 23. Clostridium tertium (No. 518 Hall)
Single method, twenty-four hour culture. × 2

Fig. 24. Clostridium tertium (No. 518 Hall)
Single method, twenty-four hour culture. × 6

Fig. 25. Clostridium putrificum (No. 38 Hall)
Double method, six-day culture. × 20
morphologically and culturally with *Clostridium tertium*, and gives the same effect on blood-agar plates as the preceding culture

Culture 542-2. Isolated November 23, 1924, at postmortem from the lung in a case of terminal bronchopneumonia. All cultural characters are like those given above. In addition to these strains of *Clostridium tertium* other anaerobes producing green zones on blood agar have been isolated. As yet they have not been identified with any species described at present

*Clostridium putrificum*

Culture 38. (I. C. Hall) Six days’ incubation

Growth was slow and was obtained only by the double method

Surface colony: None

Deep colony: Very tiny lens-shaped or oval compact masses

Hemolysis: None (fig. 25)

This culture grows very slowly in any medium. It is a representative of the nonhemolytic group. Other members of this group have been encountered in routine anaerobic culturing, but thus far they have not been identified with any described species. It is hoped that in time enough of them may be collected to make possible a comparative study.

**SUMMARY AND CONCLUSIONS**

The anaerobic blood-agar plate, when used as described, offers a very good general means of isolating and cultivating spore-forming anaerobes. It is also an aid in judging the purity of cultures.

Three groups of the anaerobes can be made, classified according to their effect on red blood cells: (1) the hemolytic group, comprising all of the well known pathogenic forms investigated in this work; (2) the group producing methemoglobin, or green zone colonies, and (3) that without effect on hemoglobin.

The blood-agar plate offers a rapid method of judging the significance of spore-forming anaerobes which may be encountered in clinical bacteriology, since the common pathogenic forms are hemolytic.
As an additional cultural method for identification of anaerobes, the blood-agar plate should be as valuable, if not more valuable, than any one of the test media commonly employed, such as brain broth, coagulated egg broth, and litmus milk.

REFERENCES


