Blood Agar Plates and Hemolysis Protocols

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Information

History

The history of blood agar, as we know it today, is uncertain. The inclusion of blood as a nutritive supplement in culture media may pre-date the use of agar. In their 1903 *Manual of Bacteriology*, Muir and Ritchie list its inclusion before they discuss “agar-agar” as a replacement for gelatin as a solidifying agent.

In the same discussion, however, they note that Robert Koch preferred plates poured by mixing bacterial inocula with melted gelatin rather than streaking material on the surface. Koch recommended media that were “firm, and where possible, …transparent…” It appears that pour plates were the standard procedure for many years due largely to problems with surface contamination upon incubation. (It should be noted that, initially, agar “plates” were, indeed, sterilized flat glass plates, not Petrie dishes as we know today.)

An interesting method of adding blood to agar media is described in Bulloch’s 1938 *The History of Bacteriology*:
Human blood or the blood of animals may be used. “Sloped tubes” of agar are employed. …Purify a finger first with 1-1000 corrosive sublimate, dry, and then wash with absolute alcohol to remove the sublimate. Allow the alcohol to evaporate. Prick with a needle sterilised by heat, and, catching a drop of blood in the loop of a sterile platinum wire, smear it on the surface of the agar. The excess of the blood runs down and leaves a film on the surface. Cover the tubes with India-rubber caps, and incubate them for one to two days at 37°C before use, to make certain that they are sterile. Agar poured out in a thin layer in a Petri dish may be smeared with blood in the same way and used for culture. In investigating the diseases of races other than the white, it appears advisable to use the blood of the race under investigation.
Any reader interested in the history of microbiology should explore Wolfgang Hesse’s biographical sketch of his grandparents, Walther and Angelina (“Lina”) (below). Walther was a protégé of Koch, and Angelina served as his assistant and illustrator. Sometime prior to the end of 1882, Walther was frustrated by the melting of his gelatin-coated culture tubes in the summer heat. He quizzed Lina about her jellies and puddings which maintained their solid consistency even at the warm temperatures. It seems that she had learned about the use of agar from a former neighbor (who had emigrated from Java where agar was a common food additive). Although there is no written record of such, it could easily be

imagined that Walther stirred blood into his cooled, melted agar in the same way that Lina did her fruit and meat juices!
(Rebecca Buxton with archival help from Jeff Karr)

ASM News article "Walther and Angelina Hesse - Early Contributors to Bacteriology" by Wolfgang Hesse and translated by Dieter H. M. Groschel (1992).

Purpose

Blood Agar is a general purpose enriched medium often used to grow fastidious organisms and to differentiate bacteria based on their hemolytic properties.

Theory

"Blood Agar" is not a consistently defined medium. The term "blood agar" generally refers to an enriched base medium to which defibrinated mammalian blood has been added.

In the US "blood agar" is usually prepared from Tryptic Soy Agar or Columbia Agar base with 5% Sheep blood. Rabbit or horse blood may be used for growth of NAD-requiring organisms, such as*Haemophilus* species, but the hemolytic patterns may be inconsistent with those on sheep blood. (Human blood is discouraged because of the increased possibility of exposure to human blood-borne pathogens such as HIV or hepatitis.)

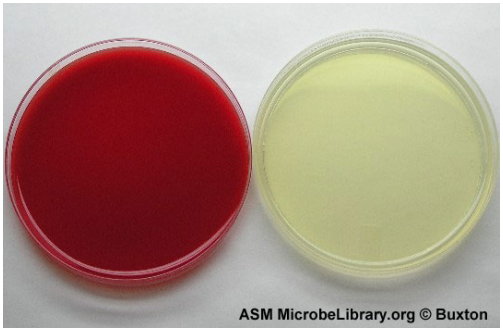


FIG. 1. Tryptic Soy Agar with and without sheep blood

RECIPE

One commonly used formula:

Soybean-Casein Digest Agar

(Also referred to as "Trypticase Soy Agar" or "Tryptic Soy Agar" or "TSA" or "Blood Agar Base")

Pancreatic digest of casein USP 15.0 g
Papaic digest of soy meal USP 5.0 g

NaCl 5.0 g
Agar 15.0 g
Distilled water 1,000 ml

Combine the ingredients and adjust the pH to 7.3. Boil to dissolve the agar, and sterilize by autoclaving.

Blood Agar

To sterile Blood Agar Base which has been melted and cooled to 45 to 50°C, add 5% (vol/vol) sterile defibrinated blood that has been warmed to room temperature. Swirl the flask to mix thoroughly, avoiding the formation of bubbles, and dispense into sterile plates, continuing to avoid bubbles and froth on the surface. (NOTE: Cooling the agar and warming the blood are essential steps in this procedure. Hot agar can damage red blood cells, and cold blood can cause the agar to gel before pouring.)

PROTOCOL

Interpretation* of Hemolysis on Blood Agar Plates

(*) To read the hemolytic reaction on a blood agar plate, the plate must be held up to a light source and observed with the light coming from behind (transmitted light).

Beta hemolysis (β) is defined as complete or true lysis of red blood cells. A clear zone, approaching the color and transparency of the base medium, surrounds the colony. Many species of bacteria produce toxic by-products that are capable of destroying red blood cells.

FIG. 2

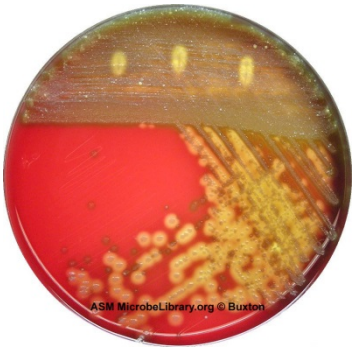


FIG. 3

FIG. 4

FIG. 2. Beta hemolytic Streptococcus species, *Streptococcus pyogenes* (transmitted light) (Lancefield group A).

FIG. 3.Normal Upper respiratory flora mixed with beta-hemolytic *Streptococcus* species. (The presence of beta-hemolytic colonies indicates the possibility of *Streptococcus pyogenes*infection.)

FIG. 4. Same blood agar plate as Figure 2 demonstrating that the beta hemolysis of *Streptococcus pyogenes* is so complete that print my be read through the resulting transparent medium.

Some species produce multiple toxins or display varying degrees of beta hemolysis.

One example is the occasional strain of *Streptococcus pyogenes* which produces only a hemolysin that is oxygen labile (“Streptolysin O”). In other words, the hemolysin is only active in conditions of low oxygen. Hemolysis can be demonstrated by a pour plate or agar overlay technique, or incubating in an anaerobic environment. A very simple way of producing an anaerobic “pocket” on an agar plate is to “stab” the inoculating loop vertically into the agar after streaking the plate. (Most strains of*Streptococcus pyogenes* also produce the oxygen stable hemolysin “Streptolysin S” which produces lysis in ambient air, as in Figure 3 above.)

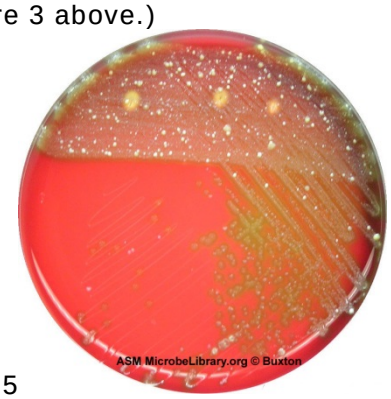


FIG. 5

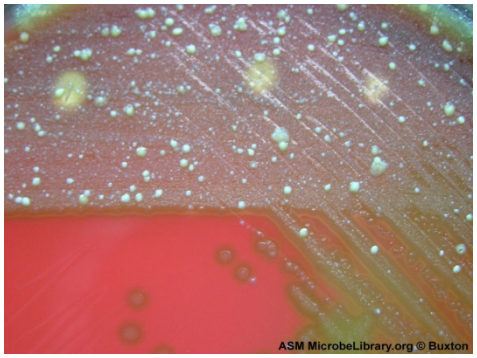
FIG. 6 

FIG. 5 and FIG. 6. Normal Upper respiratory flora mixed with Streptococcus pyogenes demonstrating production of Streptolysin O. Beta hemolysis is only evident where the agar was “stabbed”.

Another example is found in *Streptococcus agalactiae* (Lancefield group B) and *Listeria monocytogenes*. For these species, the hemolysin may be very slowly produced or weakly reactive. The visible hemolysis may be so subtle that it is only apparent directly beneath the colony (rather than broadly diffused as in *S. pyogenes,* above). In order to visualize this very weak reaction, the colony may be removed with an inoculating loop, allowing one to view the lysed cells directly below where the colony had been growing. (For further information on *Streptococcus agalactiae* and multiple hemolysins, see CAMP procedure.)

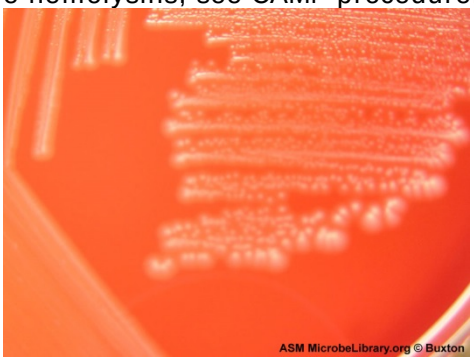
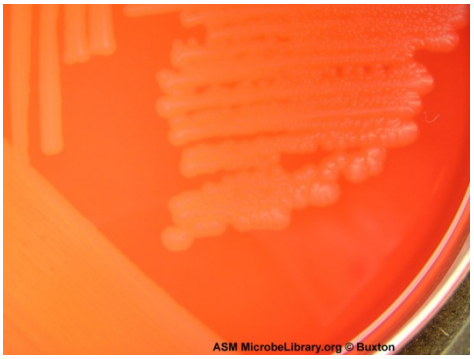
FIG. 7 

FIG. 8 

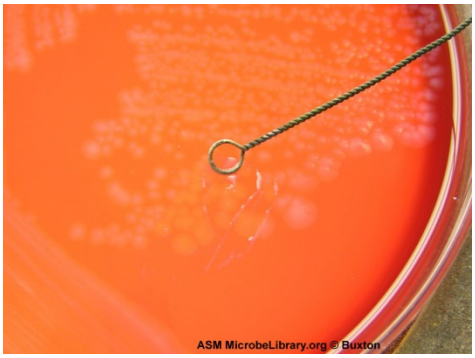


FIG. 9

FIG. 7. *Streptococcus agalactiae* (Lancefield group B) viewed with incident light: No obvious hemolysis.
FIG. 8. *Streptococcus agalactiae* (Lancefield group B) viewed with transmitted light: Subtle hemolysis.
FIG. 9. *Listeria monocytogenes*, removing colonies to see the subtle pink hemolysis directly beneath the colonies

Alpha hemolysis (α) is the reduction of the red blood cell hemoglobin to methemoglobin in the medium surrounding the colony. This causes a green or brown discoloration in the medium. The color can be equated with "bruising" the cells. Microscopic inspection of alpha-hemolyzed red blood cells shows that the cell membrane is intact, so it is not, in fact, true lysis. Some text book authors refer to alpha as “partial hemolysis,” which may be confusing to the student. It is most important to not confuse this “partial” or “incomplete” hemolysis with the “weak” or “subtle” lysis of *Streptococcus agalactiae* or *Listeria monocytogenes*, as seen above. Beta hemolysis will never include the brown or green discoloration of the cells in the surrounding medium. On prolonged incubation, many alpha hemolytic organisms will begin to appear more clear, but if the surrounding medium contains any shades of brown or green the “hemolysis” is still considered “alpha.”

FIG. 10

FIG. 11

FIG. 10. Alpha-hemolytic *Streptococcus* species “Viridans group” streptococci, including species such as the *Streptococcus mutans, mitis,* and *salivarius* groups display alpha hemolysis.

FIG. 11. Alpha hemolysis of *Streptococcus pneumoniae* (Encapsulated strain).

Gamma hemolysis (γ) is somewhat self-contradictory. Gamma indicates the lack of hemolysis. There should be no reaction in the surrounding medium.

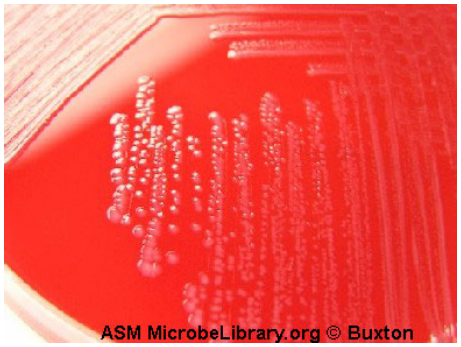


FIG. 12

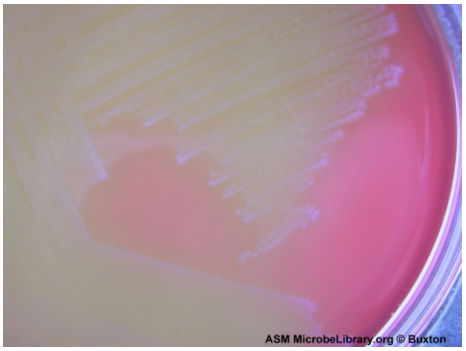


FIG. 13

FIG. 12. "Gamma *Streptococcus*" or *Enterococcus faecalis* (24 hours, non-hemolytic). "Gamma*streptococcus*" are usually non-hemolytic after 24 hours of incubation, but many eventually display weak alpha hemolysis. (The genus *Enterococcus* was once a part of the *Streptococcus* genus, and was considered a "gamma *Streptococcus* species". Enterococci usually reacts as Lancefield group D.)

FIG. 13. The same *Enterococcus* strain as Figure (12), shown with transmitted light at 48 hours incubation demonstrates the alpha

hemolysis of some “gamma streptococci.”

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

COMMENTS AND TIPS

This section is to evolve as feedback on the protocol is discussed at ASMCUE. Please contact the project manager for further information.

REFERENCES

For Recipes and Protocol:

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1. **Muir, Robert** and **James Ritchie**. 1903. *Manual of Bacteriology*. The MacMillan Company, London. (p. 226-229)
2. **Bulloch, William**. 1938. *The History of Bacteriology.* Oxford University Press, London. (p. 42).
3. **Hesse, Wolfgang**. 1992. Walther and Angelina Hesse – Early Contributors to Bacteriology. *ASM News.* **58:** 425-428.

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