Comparison of Beta--Hemolysis of Group A Streptococci, Effect of Different Blood Agar Media and Condition of Incubation

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INTRODUCTION:

Streptococcus pyogenes is an organism often encountered by clinicians. Also called beta hemolytic streptococcus of Lancefield Group A, it is of high virulence and can cause such diseases as pharyngitis ("strep throat"), sinusitis, otitis media, meningitis, pneumonia, erysipelas, scarlet fever, septicemia, and puerperal sepsis. It is common as a secondary invader and is also responsible for such "sequelae" diseases as rheumatic fever.

Obviously accurate laboratory detection and identification of this organism is very important to patient care. In most laboratories, detection of Strep. pyogenes in cultures is based on hemolytic properties, the parameter easiest to use in classifying the streptococci into large groups. Those streptococci producing alpha type hemolysis, that is partial hemolysis and usually some greening, are collectively called the viridans group. Those which produce no hemolysis of red blood cells are called gamma hemolytic and classified as the an-hemolyticus group. Those producing the beta type, or complete hemolysis, are called the hemolyticus group (1).

Strep. pyogenes belongs to the last group. Alpha and gamma streptococci are of low virulence and are often found as normal flora in various parts of the body. Though laboratory workers depend on the different hemolytic properties when looking for Strep. pyogenes on blood agar plates, many factors influence hemolysis, and sometimes Strep. pyogenes will fail to produce beta hemolysis on blood agar plates.

* The Term Paper for the Degree B.S. (M.T.) The School of Medical Technology Faculty of Medicine Chiang Mai University 1970
(Note: Due to limitation of space, this report has been reined and abridged. Data charts have been omitted)

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instead producing an alpha or gamma appearance. In this case the organism may be overlooked as "normal flora" or temporarily misdiagnosed.

Some of the factors influencing the growth of streptococci and their production of hemolytic zones are: species, age, and concentration of blood used in the blood agar, the blood agar base used, the thickness of the agar, the pH of the medium, and inoculation and incubation methods used. The purpose of this study was to examine each of these factors, using known strains of Strep. pyogenes as indicators, for their effect on hemolysis and to draw some conclusions perhaps useful in choosing a medium and a method for clinical laboratory detection of Strep. pyogenes.

MATERIALS AND METHODS:

Four strains of Strep. pyogenes stocked in cystine trypticase agar were used. One more strain was obtained from the throat swab of a patient. After the organisms had been checked for typical morphology and hemolysis on blood agar and for bacitracin susceptibility (2), they were used in the following procedures:

1. Comparison of Different Kinds of Bloods. Three kinds of blood were used, fresh sheep blood, fresh rabbit blood, and human blood, both fresh human blood and outdated blood bank blood. All fresh blood was defibrinated at the time of collection. In the human bank blood, sodium citrate was used as an anticoagulant. Fresh blood was collected from 3 separate human donors, and bank blood was also obtained from 3 separate donors.

Two types of blood agar base were used: Blood Agar Base (Difco) and Tryptose Blood Agar Base (Difco), prepared according to manufacturer's directions. The experiment was done as follows: After the base had been autoclaved and cooled to 48°C, 5% of the different kinds of blood were added to both Tryptose Blood Agar Base and Blood Agar Base. The media were gently mixed, and 18 ml. were poured into each sterile petri dish. Plates were then incubated 24 hours to check for contamination. The same day, each of the stock strains of Strep. pyogenes was streaked on blood agar to obtain isolated colonies. After 24 hours incubation, colonies were suspended in 1-2 ml. sterile distilled water and the inocula adjusted so each was the same turbidity. By means of a capillary pipette, one drop of each suspension was placed on media containing each of the different kinds of blood. The plates were then streaked with a platinum loop to ob-
tain isolated colonies and incubated at 37°C. The incubation time was set at 24 hours, since most clinical cultures are read after 18-24 hours incubation. After incubation, plates were examined visually for clarity of the hemolytic zone around colonies.

2. Comparison of Blood Agar Bases: The following bases were used: Azide Blood Agar Base, Azide Dextrose Agar, Blood Agar Base (Infusion Agar), Brain Heart Infusion Agar, Nutrient Agar, Tryptose Blood Agar Base, Tryptose Phosphate Agar, Trypticase Soy Agar, and Crystal Violet Blood Agar Base. The first seven of these were from Difco and the Trypticase Soy Agar was from BBL. The Crystal Violet Blood Agar Base was prepared using the formula from Cruickshank (3). Tryptose Blood Agar Base with crystal violet added was also used, making a total of 10 different bases tested. In the case of the Nutrient Agar, it was necessary to add 5 grams/liter of NaCl to prevent hemolysis of the blood, as the commercial medium contained no NaCl and proved hypotonic to red blood cells. The plates were then inoculated as previously described and incubated at 37°C for 24 hours. Plates were then examined for clarity of hemolysis and diameters of colonies and hemolytic zones were then measured by using a widefield binocular microscope and eyepiece micrometer as Feller and Stevens had done in 1952 (4). About five isolated colonies and their zones were measured on each plate and an average diameter determined.

3. Comparison of Different Concentrations of Blood. Tryptose Blood Agar Base was used in this procedure. 17 flasks of the base were prepared and blood was added in different concentrations. These were 2%, 3%, 4%, 5%, 6%, 7%, and 8% defibrinated sheep blood and 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, and 10% human bank blood. The five strains of Strep. pyogenes were inoculated using the same method as in Procedures 1 and 2. After 24 hours incubation, plate were examined and diameters of colonies and hemolytic zones measured as before.

4. Comparison of Thickness of Blood Agar. R.E.O. Williams (5) prepared blood agar plates by pouring 8-10 ml. of blood agar base into a petri dish, allowing this to set, and then following it with 10-15 ml. of blood agar, thus obtaining “double layer” plates. Roberts and Sherris(6) prepared blood agar plates similar to those described by Williams, but the bottom layer was 6 ml. of blood agar base and the top layer was 12 ml. with 5% defibrinated sheep blood. In
our study, double layer plates were prepared using either Tryptose Blood Agar Base or saline agar (8.5 grams NaCl, 20 grams Bacto Agar in 1 liter distilled water) as the first layer and Tryptose Blood Agar Base with 5% sheep blood as the top layer. The thicknesses of the respective layers were varied from 3 ml./15 ml. to 15 ml./3 ml. Plate of varying respective thicknesses were also prepared using 8% sheep blood agar as the top layer. As previously, the stock strains were inoculated and the cultures incubated.

5. Comparison of pH of the Medium. Tryptose Blood Agar Base with 5% Sheep blood was used. The pH of various lots of media was adjusted using NaOH or HCl so that plates had the final pH range of 5.8, 6.4, 6.6, 6.8, 7.2, 7.4, 7.6, 7.8, 8.6. Inoculation and incubation were done as before.

6. Method of Inoculation. Culture plates were prepared as in Procedure 5. The 5 stock strains of Strep pyogenes were then inoculated using the technique given in Procedure 1. On one set of plates the organism were also stabbed into the agar with a wire needle. Four plates were prepared for each strain using the method of Bailey and Scott (7). Three sets of plates for each method were prepared and then used in the

following procedure.

7. Comparison of Atmospheric Conditions During Incubation. In addition to the plates prepared in procedure 6, double layer plates were poured and inoculated by the streak method. All plates were then incubated at 37°C, one set in ordinary air, another in 5-10% CO₂ in a candle jar, and the third in an anaerobic jar using illuminating gas as a reducing agent. After 24 hours incubation, plates were inspected and colony and hemolytic zone sizes were measured.

8. Comparison of Temperature of Incubation. Plates inoculated with the five strains were incubated at 30°C, 37°C, or 45°C for 24 hours and the results examined.

9. Comparison of Age of Blood. Media was prepared using sheep blood varying in age from fresh to 42 days old. Both single and double layer plates were prepared, inoculated, and incubated at 37°C for 24 hours.

RESULTS

1. Comparison of Different Kinds of Bloods. The clearest hemolytic zones were obtained with the media containing sheep blood. The next was rabbit blood, every organism giving the same result with these two bloods. Fresh human blood,
when results were averaged, seemed to give no better results than bank blood, and neither produced good clarity. The order of clarity was the same for the Tryptose Blood Agar Base plates and the Blood Agar Base plated, but Tryptose Blood Agar Base gave the clearest zones with all bloods.

2. Comparison of Blood Agar Bases. The results varied among the strains tested, but the best hemolysis was invariably obtained with Tryptose Blood Agar Base (with or without cystal violet), followed by Tryptic Soy Agar and Tryptose Phosphate Agar in that order. However colonies on medium prepared with Blood Agar Base or Brain Heart Infusion Agar were larger than those growing on media prepared with Tryptose Blood Agar Base.

3. Comparison of Different Concentration of Blood. Hemolytic zones were clear on media containing 1-5% sheep blood. At higher concentrations, clarity decreased and at 10%, zones could not be measured due to lack of clarity. Hemolytic zones were invariably clearer with sheep blood than with human blood of the same concentration.

4. Comparison of Thicknesses of Blood Agar. The best results were obtained with the double layer plates using Tryptose Blood Agar Base blood agar over Tryptose Blood Agar Base. Diameters of zones and colonies were smaller, and hemolysis less clear, on the plates using a saline agar base for the first layer. Best ratio for the double layer plates was 10 ml. Tryptose Blood Agar Base for the bottom layer and 8 ml. 8% sheep blood agar for the top.

5. Comparison of pH of the Medium. pH variation in the range 6.4-7.7 had no effect on hemolytic zones. At pH 5.8 red blood cells began to hemolyse and at pH 8.6 the organisms grew poorly and produced no hemolysis at all.

6 and 7. Method of Inoculation and Incubation: The streak plate method gave better results on double layer plates than on single layer although on the latter, colonies stabbed into the agar gave clearer hemolysis than surface colonies. Pour plates resulted in clear hemolytic zones, colonies near the surface producing less clear zones than those deep in the agar. Anaerobic incubation produced the largest hemolytic zones on streak plates, though colonies size was the same under all condition tested.

8. Comparison of Temperatures of Incubation: The organisms did not grow on the plates incubated
at 45°C. At 37°C hemolytic zones were clearer and both colonies and zones were larger than at 30°C.

9. Comparison of Ages of Blood. The diameter of hemolytic zones was small on media containing old bloods. Fresh blood gave the larger hemolytic zones. Although clarity of hemolytic zones was the same with all ages of blood, old blood looked brownish after 24 hours incubation, and the contrast between medium and hemolytic zone was difficult to see.

DISCUSSION AND CONCLUSION

Sheep blood was the most suitable for preparing media, with the next best being rabbit blood. Human blood did not give clear hemolytic zones. Some have suggested that there may be a factor in human blood which inhibits growth and hemolysis of Streptococcus pyogenes. Antistreptolysin-O could partially inhibit hemolysis by deep colonies and for those growing under anaerobic conditions and has been reported to be present in high titers in the blood of persons from endemic streptococcus disease areas. Cultures from 20 patients were made on both sheep and human blood agar by Nussle, Wright, and Jones. Strept pyogenes was detected on human blood agar plates in only ten, but on all 20 with sheep blood plates (8).

Sheep blood agar was used by Krumwiede and Kuttner (9) because of its inhibitory properties for the Hemophilus group. Hemophilus hemolyticus colonies, which often resemble those of hemolytic streptococci, are not a problem on sheep blood agar. Sheep blood is also superior to heman blood in distinguishing "green" or alpha hemolysis from beta hemolysis.

The temptation to use discarded human bank blood to prepare blood agar plates is great in some laboratories because it is readily available and economical. However, in addition to the disadvantages shown in this study, such blood is usually citrated, and citrate ions are sometimes inhibitory to the growth of Strept pyogenes and other streptococci (10).

Of the bases tested, Tryptose Blood Agar Base with 5% sheep blood was the most suitable medium for detection of beta hemolysis of Strept pyogenes. Hemolytic zones were the clearest, although the diameters of the colonies were not the largest. Colonies on blood agar prepared with Tryptose Blood Agar Base tended to be smaller, while the zones of hemolysis were larger, than on similar plates prepared with Blood Agar Base or Brain Heart Infusion Agar. Blood Agar containing dextrose is probably
unsuitable for hemolysis studies. When 1% glucose was added to blood agar, hemolytic zones of Strep pyogenes were green and therefore easily confused with Streptococcus viridans or pneumococci (11). Azide blood agar and crystal violet blood agar are selective media for streptococci, since growth of other bacteria is inhibited. Crystal violet in a 1 : 500,000 concentration inhibits some bacteria, notably staphylococci, while allowing the growth of streptococci. When added to Tryptose Blood Agar Base blood agar in this concentration, it did not interfere with the clarity of hemolytic zones.

Blood agar containing 1-5% sheep blood was found to give clear beta hemolytic zones but with media containing only 1%, 2%, 3% blood, the color of the media was pale, making it difficult to see the difference between midium and hemolytic zones. The question arose as to whether alpha streptococci might produce clear hemolytic zones on this medium. So media containing these percentages of blood were prepared and inoculated with Strep pyogenes and Streptococcus viridans. Both the beta streptococci and alpha streptococci appeared to give clear hemolytic zones when 1%, 2% or 3% blood was used. The only apparent difference was in diameter of the hemolytic zones, the average diameter of Strep pyogenes zones being 3 mm. while the diameter of the Strep. viridans zones averaged 2 mm. 4% blood media could separate the alpha from beta streptococci, but when 5% was used, the medium was red and the hemolytic zones of the Strep pyogenes were clear. The alpha hemolytic zones were very green (this "greening" was less pronounced when human blood was used). Thus the most suitable concentration of blood appeared to be 5%, except in cases of low hematocrit.

Culture plates poured in double layer best utilized first 10 ml. of Tryptose Blood Agar Base and then 8 ml. of tryptose blood agar containing 8% sheep blood. As in the previous procedure, the question arose as to whether alpha streptococci might give clear hemolytic zones on this medium. They did not, instead producing typical greenish partial hemolysis.

Double layer plates were found to give clearer zones than single layer plates. A probable explanation for this is illustrated in Fig. I and Fig. II.
Since microorganisms require enough nutrients and moisture for growth, the total amount of medium should not be less than 15-20 ml. (about 2-3 mm thick). In a single layer plate, if the diameter of the hemolytic zone is smaller than 4 mm, some red corpuscles may remain unhemolyzed at the bottom of the plate, thus making the zone less clear than that of the double layer plate, with its thinner layer of medium containing red blood cell.

Comparisons of media differing only in pH showed that a large range was acceptable. Ginsberg, Bentwich and Harris (12) incubated Streptolysin S with red blood cells at different pH in a range from 3 to 11. Plotting a curve between pH of the medium and hemolytic units, they obtained a bell-shaped curve, with the highest activity at pH 7. The present study showed a wide range around pH 7 was satisfactory.

Incubating plates anaerobically yielded a larger hemolytic zone, but the diameters of colonies were not larger than those incubated in air or in 5-10% CO₂. The explanation for this phenomenon is probably that ordinarily only Streptolysin S, an oxygen stable hemolysin, is active in producing the hemolytic zone of Strep. pyogenes on blood agar. Streptolysin O has no effect because it is oxygen labile. Thus anaerobic incubation preserves streptolysin O activity and results in the larger hemolytic zones. Pour plate cultures accomplish much the same purpose as anaerobic incubation, but this method is less convenient than streak-plate in the clinical laboratory, both for inoculation and for subsequent subculture of colonies. When colonies on streak plates were stabbed into the agar, the purpose was the same as in incubating in an anaerobe jar, with the added advantage that hemolysin could diffuse in all directions from colonies inside the agar and hemolysis was thus more likely to extend through the depth of the medium.

The optimum temperature for incubation was 37°C. Alouf and Raynaud (13) showed that erythrocyte lysis by Streptolysin O is markedly
dependent on temperature. Results of the present study imply that Streptolysin S activity is also dependent upon temperature.

Fresh blood can be recommended from the results of the ninth procedure. Blood which had been stored in the refrigerator 2-3 weeks could be used, but blood stored more than 3 weeks was unsatisfactory because Streptococcus grew poorly on media prepared with blood of this age. Sheep blood six weeks old was almost completely hemolysed. Gentle shaking during defibrination, as well as storage at a constant temperature near 0°C, helps keep hemolysis to a minimum before media is prepared.

SUMMARY

This study investigated the role of multiple factors influencing hemolytic zones of Streptococcus pyogenes on blood agar. It was found that optimum results were obtained when the culture plates were poured in double layers, utilizing first 10 ml. of Tryptose Blood Agar Base, and then 8 ml. of Tryptose Blood Agar Base containing 8% fresh defibrinated sheep blood. This medium gave the clearest hemolytic zones. Single layer plates using 5% sheep blood agar, 18 ml. per 9 cm. plate, were also suitable but hemolytic zones on such plates were not as clear as those on the double layer plates. Anaerobic incubation produced slightly larger hemolytic zones than aerobic incubation. Pour plate cultures also resulted in clear hemolytic zones, but this method is less convenient and thus less suitable for routine laboratories. The optimum temperature of incubation was 37°C.

Acknowledgements

The authors wish to express appreciation to Dr. C. Evans Roberts Jr., and Miss Patricia Forsyth for their supports, advices and viewing the Manuscript.
REFERENCES


ข้ออภัคคณ์ถาวรข้ามกลุ่ม

เนื่องจาก beta-streptococci group A มีลักษณะในการทำให้เกิดโรคได้มากที่สุด และอาการรุนแรงกว่า alpha streptococci คดินนี้ จึงจำเป็นที่จะต้องเลี้ยงเชื้อนี้ในอาหารเลี้ยงเชื้อที่มีคุณภาพเหมาะสมต่อการเจริญเติบโตของเชื้อ และในสาระที่สำคัญ ซึ่งเลี้ยงเชื้อขันเลี้ยงสามารถแยกได้ว่า เป็น streptococci ชนิดใดอย่างชัดเจน มี factor หลายอย่างที่สำคัญจะช่วยกัน beta hemolysis ของ Streptococci group A ดังนั้น จึงได้ทำการทดลองต่อไปใน

1. เปรียบเทียบเกี่ยวกับชนิดของเลือด ที่จะนำมาเลี้ยงอาหารเลี้ยงเชื้อโดยใช้เลือดคน เลือดแกะและเลือดกระดูก อาหารเลี้ยงเชื้อ

2. เปรียบเทียบ blood agar base ชนิดต่างๆ

3. เปรียบเทียบของสารส่วนของเลือด ที่จะผสมลงในอาหารเลี้ยงเชื้อ

4. ผลลัพธ์ของความหนาของอาหารเลี้ยงเชื้อ

5. เปรียบเทียบ pH ของอาหารเลี้ยงเชื้อ

6. เปรียบเทียบวิธีวิธีการใช้ inoculate เซลล์บนอาหารเลี้ยงเชื้อ

7. เปรียบเทียบปริมาณอาหารเลี้ยงเชื้อใช้ใน การเพาะเลี้ยงเชื้อ

8. เปรียบเทียบแบบที่ใช้ในการเพาะเลี้ยงเชื้อ

9. เปรียบเทียบผลลัพธ์ของเลือด

ผลที่ได้ปรากฏว่า อาหารเลี้ยงเชื้อที่ผสมนมทุกสัตว์ คืออาหารเลี้ยงเชื้อที่เตรียมแบบผสมนม ซึ่งคือขันเลี้ยงใช้เฉพาะ Tryptose Blood Agar Base 10 ม.ค. ขันเลี้ยง Tryptose blood agar ซึ่งมี fresh defibrinated sheep blood 8% ผสมกับ Tryptose Blood Agar Base ทับลงไป 8 ม.ค. อาหารเลี้ยงเชื้อทำเตรียมแบบซึ่งดียิ่งขึ้นเลือด 5% ก็เพียงพอแล้ว แต่ beta hemolytic zone ไม่ชัดเจน เห็นเตรียมแบบซึ่งขัน การเตรียมอาหารเลี้ยงเชื้อแบบ pour plate ก็ได้ hemolytic zone ฟักส่าล่าและโดยผลลัพธ์ไปแต่ไม่เหมาะสมใน routine lab. อุณหภูมิที่เหมาะสมที่สุดในการอนุญาต คือ 37 องศา ขันเลี้ยงใน anaerobic condition จะได้ hemolytic zone กว้างขึ้นเล็กน้อย