The optimization of Human Blood Agar (HBA) for *Streptococcus pneumonia* growth

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**Abstract.** Sheep Blood Agar (SBA) is a standard media for microbiology examination. The difficulties of sheep blood resources on a tropical country and developing countries causing an obstacle in culturing *Streptococcus pneumoniae*. Beside usage of Human Blood Agar (HBA) was totally not recommended for culturation. The purpose of doing this observation is to modify HBA by washing human blood erythrocyte. Method experimental study with True experimental post-test only design. Colony of *Streptococcus pneumoniae* that had been checked purity were planted on HBA media and SBA media. Observation was done in 24 hours and 48 hours, comprising of colony diameters, hemodygest diameters, and the characteristics colony. After 24 hours, mostly result was found that *Streptococcus pneumoniae* colony diameters on SBA media have a significant difference (0.8 mm) with HBA media (0.6 mm). On the 48 hours observation, mostly colony diameters on HBA media (1.5 mm) were narrower than those on SBA media (1.8 mm). After 24 hours, the mean of *Streptococcus pneumoniae* haemolysis diameters, on SBA media (1.2 mm) were significantly different with HBA media (0.6 mm). After the 48 hours observation, haemolysis diameters were widest in SBA media (1.6 mm) and in HBA media (0.8 mm). The characteristics of colony in 24- and 48-hours observation were not having a significant difference between them. Conclusion HBA is an acceptable alternative media for culturing *Streptococcus pneumoniae* in poorly resourced laboratories.

1. **Introduction**

*Streptococcus pneumoniae* is found worldwide as bacteria that mostly do not causing the disease but sometimes can cause disease, especially pneumonia. These bacteria are a pathogenic microorganism in humans that frequently colonizes the airways and can cause meningitis, sepsis, otitis media, bacteraemia, peritonitis, sinusitis and community-acquired pneumonias. They spread through aerosol droplets from person to person. They mostly infect children, the elderly, and people who have an immune systems weakness. Until the year of 2000, these bacteria infections caused 100,000-135,000 hospitalizations for pneumonia, 6 million cases of otitis media, and 60,000 cases of invasive disease, which included 3300 cases of meningitis. Incidents in the United States show geographical variations of 21-33 cases per 100,000 people [1].

Efforts in the context of eradication of pneumonia disease required the proper management of diagnose and treatment of cases. For *Streptococcus pneumoniae*, the gold standard for diagnose is bacterial culture. However, there is still a lot of obstacles in the real activities because *Streptococcus pneumonia* is a fastidious bacterium that is only able to grow in certain environments and nutrients so that required standard media to culture these bacteria.
In Europe and North America, it has been used for the blood of sheep (ADD) and horse blood agar (ADK) as the standard medium for the culturation of *Streptococcus pneumoniae*. This is because both media are able to grow maximally the bacteria. But in developing countries including Indonesia, *Streptococcus pneumoniae* cultures still using human blood media (ADM) rather than ADD media for cost and tropical climate less suitable for sheep breeding [2].

Research by Magbojos, *et al.* [3] said that ADM less able to grow *Streptococcus pneumoniae* when compared with using ADD media. The differences in morphological structure and composition between sheep erythrocytes and human erythrocytes are the main cause of this problem. The content of complement and antibodies present in human erythrocytes can inhibit *Streptococcus pneumoniae* growth. Regular anticoagulants used to prevent blood clots of human donors also have antibacterial properties. Based on the above background, the researchers are interested to conduct research about “The Optimization of HBA for *Streptococcus pneumonia* Growth”.

2. **Method**

Preparation of culture media with defibrination of SBA and HBA. The process of SBA defibrinating used glass parell to separated serum and blood. For HBA that contain red blood cells have been separated with saline solution, rotated at 3300 rpm for 1.5-2 minutes as much as 3 times. The precipitate of washed red blood cells is 100% suspension. Preparation of media for bacteria growth that were Nutrient Agar and Blood Boullion. Sterilized in autoclave at 121°C for 15 minutes. After sterilization, they cooled in an incubator to a temperature of 40-50°C. For Nutrient Agar mix with sheep blood, human blood without washing, and washing human blood aseptically into Erlenmeyer, shaken until homogen. Poured into a sterile petri dish aseptically.

The purity of stock bacteria was prepared. When it is pure, the bacteria are multiplied in Blood Boullion and incubated at 35°C and 5% CO₂ for 24 hours. After multiplied, the bacteria was suspended with 1 Mc Farland the concentration. About 100-micron suspension of 1 McFarland bacteria was grown on SBA plate, HBA without washing (HBA standard) plate, and washing HBA plate by four streak zone method. All plates were incubated at 35°C and CO₂ 5%. Observed colonic growth of the bacteria after incubation for 24 hours and 48 hours.

3. **Result and Discussion**

![Figure 1. (a) The Gram staining of *Streptococcus pneumonia* indicated diplococcus; (b) The Optochin test of *Streptococcus pneumonia*.](image)

*Streptococcus pneumonia* (pneumococcus) is Gram-positive, lance-shaped coccus (elongated cocci with slightly pointed outer curvature). Usually, they are seen at Figure 1 as pairs of cocci (diplococci), but they can also occur singly and in short chains. When cultivated on blood agar, they are alpha hemolytic, mucoid, and silvery. Individual cells are between 0.5 and 1.25-micrometers in diameter. They don't form spores and they are nonmotile [4]. *Streptococcus pneumoniae* is a fastidious bacterium, growing best in 5% carbon dioxide. Nearly 20% of fresh clinical isolates require full anaerobic conditions. In all cases, growth requires a source of catalase (eg blood) to neutralize the
large amounts of hydrogen peroxide produced by the bacteria. These bacteria are an aerotolerant anaerobic fermentation. It is usually bred in a medium containing blood. In agar, pneumococcus grow as a sparkling colony or a draughtsman colony, about 1 mm in diameter. Typical colonies produce alpha (green) hemolysis zones [5].

Figure 2. (a) The growth of *Streptococcus pneumonia* in SBA plate; (b) The growth of *Streptococcus pneumonia* in HBA with washing erythrocyte plate.

Figure 2 shows the differences between the bacteria colony after incubation 24 hours and 48 hours in either SBA, and washing HBA. Figure 3 indicate the result showed that SBA media with washing HBA is not different from Sig = (0.154)> 0.05. While the HBA standard media is different from SBA and washing HBA media with Sig = <0.05. The colony diameter of 24-hour incubation in washing HBA was greater (0.9 ± 0.06 mm) than growth in SBA (0.8 ± 0.10 mm) and HBA standard (0.6 ± 0.10 mm). After incubation for 48 hours, the bacteria colony diameter appears on each species to become wider. However, the largest was found in SBA (1.8 ± 0.12 mm) followed by washing HBA (1.6 ± 0.08 mm), and HBA standard (1.5 ± 0.12 mm). These results show significant differences with p = 0.000 (analysis by Two Way ANOVA).

Figure 3. The colony diameter of *Streptococcus pneumonia* after incubation for 24 hours and 48 hours in every medium.
The ability of SBA media to grow germs is supported by morphology and the composition of sheep erythrocytes. The sheep's erythrocyte diameter is smaller and the cell membrane is thinner than the human blood erythrocytes so, the hemolysis process will progress more easily in sheep blood [6].

The use of expired blood from whole human blood can change the shape and content of substances present in erythrocytes. Along with long-term human erythrocyte storage, erythrocytes will change in shape initially spherically into eclocyte form and result in decreased pH, increased lactic acid, increased glucose consumption, decreased 2-3 DPG (2-3 diphosphoglycerate), elevated extracellular potassium levels resulting in damage to NaK pumps, and decreased ATP which can decrease the ability of erythrocytes so that erythrocytes will be easy to lysise. Human red blood cells are also known to contain antigen receptors, antibodies, anti-infective agents, and serum globulins that can inhibit bacteria to grow maximally in HBA media [1].

Figure 4. The haemodigestion diameter of *Streptococcus pneumonia* after incubation for 24 hours and 48 hours in every medium.

Figure 4 shows the different of haemodigestion diameter between 24 hours and 48 hours incubation in either SBA, HBA standard, and washing HBA. We found that there is no significant differences haemodigestion diameter between SBA media with washing HBA media at 24 hours and 48 hours incubation with Sig = (0.084)> 0.05. While HBA standard media is significant with SBA and washing HBA media at Sig = <0.05. The haemodigestion diameter of 24 hours incubation on washing HBA media (1.2 ± 0.14 mm) was statistically significant with growth in SBA media (1.2 ± 0.08 mm) and greater than HBA standard (0.6 ± 0.10 mm). After incubation for 48 hours, the haemodigestion diameter of *Streptococcus pneumoniae* appears in each type to be wider. The bacteria growth in washing HBA media (1.7 ± 0.12 mm) was greater than in SBA media (1.6 ± 0.09 mm). The haemodigestion diameter of HBA standard media (0.8 ± 0.06 mm) is much smaller than other media. The mean rate of haemodigestion at 24 hours and 48 hours incubation showed significant difference with p = 0.000 (Two-way ANOVA).
The characteristics of Streptococcus pneumoniae colonies were assessed to see if the morphology and growth patterns typical in HBA standard and washing HBA were as good as standard SBA media in terms of size, pigmentation, shape, edges, and elevation of colonies. This bacteria colonies with good characteristics of small round shape, greenish grey, at the first 24 hours of dome shaped colonies or flat, looks wet, but in the next 24-48 hours the colony will progressively develop to form the centerplate or bend in the middle with an elevated edge. Figure 5 shows that at 24 hours and 48 hours incubation of overall colony growth on SBA, HBA standard and washing HBA have similarly good colony characteristics. These results showed no statistically significant differences between colonies characteristics on 24 hours and 48 hours incubation with Sig => 0.05 values on all media.

Streptococcus pneumoniae forms small round colonies, first dome-shaped, and then be develops a plateau center with an elevated edge. Colonies of this bacteria in blood agar are known as draughtsman colony. Based on the protein content of M, Streptococcus is divided into colonies of Matt and Glossy. The colony of Matt is formed if there is a protein content of M and usually this type is virulent bacteria. It does not contain M protein, so the colony is Glossy. It produces hemolytic α in the blood agar that visible shadow of greenery around the colony that indicates the cell is not fully lysis [7].

HBA standard is not optimal to growing Streptococcus pneumoniae compared with SBA and washing HBA. The diameter of colony and haemolysis in HBA standard were smaller than SBA and washing HBA. The washing HBA media can grow these bacteria better than HBA standard and equally well with SBA media at 24 hours incubation. The colony diameter of washing HBA in the first 24 hours was greater than SBA, although at 48 hours the diameter of HBA standard colonies and washing HBA became almost identical. The haemolysis diameter at 48 hours incubation showed washing HBA was superior to SBA and HBA standard. Associated with the characteristics of colonies, the observation of 24 hours colonies grown on SBA and washing HBA has a visible characteristic of the colony. This is in contrast to the existing colonies on the HBA standard plate where observations should be made up to 48 hours. Therefore, the use of washing HBA as an alternative medium of SBA to grow these bacteria has the advantage, that colonies can be observed in 24 hours incubation with large colony diameter and haemolytic diameter patterns and characteristic of colonies that are clearly visible [8].

The washing of erythrocytes removes heat stable compounds in erythrocytes, such as antigens and antibodies that can only be lost through erythrocyte leaching, insufficient by heating. The washing of human blood erythrocytes was done by using a saline solution that had been stored in a plastic
container for a long period of time. Citrate Phosphate Dextrose (CPD) content in human blood erythrocytes will decrease or disappear during erythrocyte leaching so as not to interfere with bacterial growth in ADM washing. The bacteria will thrive, morphology and haemolysis process will optimum [9,10].

4. Conclusion
There are differences in growth of colony diameter, haemodigestion zone, and colonic characteristics in 24 hours and 48 hours incubation in the three mediums of SBA, HBA standard, and washing HBA. Washing HBA media was suitable for use as an alternative substitute for SBA to Streptococcus pneumoniae culture because the colony grow optimally, the haemodigestion diameter is wide and still display typical characteristics as well as SBA and better than HBA standard. So, it was easier to identify Streptococcus pneumoniae although only observed after 24 hours incubation.

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