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Preparation of the Blood-Enriched Agar with the Use of Red Cell Suspension

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Abstract - A culture medium is said to support the growth and development of different microorganisms. Certain bacteria like *Staphylococcus aureus* and *Staphylococcus epidermidis* entail hemoglobin found in the red blood cells. Because of its cost effectiveness and availability, expired human blood is being utilized in some developing countries. Despite the widely accepted disadvantages of using human blood as enrichment agent, many laboratories still opt to use it due to the unavailability of sheep blood or due to budgetary reasons. This study determined if the washed expired human blood can be used as an alternative enrichment agent in the preparation of Blood Agar Plate (BAP) culture medium in the isolation of *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228. The cultural characteristics and hemolytic reactions of the selected microorganisms were recorded, assessed and compared with their growth in BAP. The stability of the washed expired human blood was evaluated in terms of

temperature and storage period. Results reveal that expired human blood with washing improved the morphologic and hemolytic pattern of *Staphylococcus aureus*. The washing of blood had no effect on *Staphylococcus epidermidis* because it is a gamma hemolytic bacterium. Both unwashed expired and fresh human blood produced gamma hemolysis due to the interferences still present in them. Both washed expired human blood and washed fresh human blood produced beta hemolysis. Washed expired human blood could be stored for seven days and still could be used for microbial culture.

Keywords - Blood, Blood Enriched Agar, Red Cell Suspension

INTRODUCTION

Like humans, microorganisms have their own preference when it comes to food. The cultivation and development of a particular organism will only be possible if their needs are fulfilled. Such needs are met in a culture medium.

Culture medium is a mixture of the nutrients needed by microorganisms. It is categorized based on its contents and composition. In diagnostic bacteriology, it is necessary to use several types of media for routine culture, particularly when the possible organisms are aerobic, facultatively anaerobic, and obligately. Unfortunately, these requirements pose logistic and economic problems, especially in resource-limited areas where bacteriological culture facilities are few and the allocation of fund is limited.

A variety of animal blood and banked human blood (BHB) is used to enrich microbiological culture media and to highlight growth characteristics such as hemolysis. In most clinical microbiology laboratories, the selection of colonies from primary cultures for further workup as putative beta-hemolytic streptococci (BHS) is made on the basis of the hemolytic reaction on blood agar (BA) as well as the colonial morphology (Anand et al. 2000).

Even with such advantages, sheep blood is still the most ideal for blood enriched agar. Many researchers reported disadvantages of using human blood such as variation in hemolysis production due to old red cells present resulting to misidentification, production of larger zones during antibiotic susceptibility testing, lack of characteristic morphology , and the difficulty of

discerning where the growth began (Russell et al., 2006; Changsri et al. 2001; Anand et al. 2000). The presence of citric acid as an anticoagulant, antibiotics, antibodies, or other anti-infective agents inhibits the growth of the desired bacterium (Russell et al. 2006).

Despite the widely accepted disadvantages of using human blood as enrichment agent, many public hospitals in the Philippines utilize outdated human blood as enrichment agent for its accessibility and cheaper price compared to sheep blood. There is a need, therefore, to develop an alternative technique to improve the use of human blood as enrichment agent. The possibility of removing by washing the interferences found in expired human blood that will be used as enrichment was never done by previous researches, thus became the main focus of this study.

Staphylococci are Gram-positive bacteria that tend to form grapelike clusters. These organisms are facultative anaerobic and grow on routine laboratory media such as blood (BAP), chocolate agar plate (CAP), colistinnalidixic acid (CAN), and phenyl ethyl alcohol (PEA) agars. Colonies are circular, opaque, smooth and have a butyrous (butter- like) consistency. *Staphylococcus aureus* colonies are often beta-hemolytic with a yellowish pigment (Bartelt 2000). The appearances of the colonies of *Staphylococcus aureus* are medium to large, smooth, slightly raised and translucent. Visible growth appears on 5% sheep blood and CAP incubated at 35 degree Celsius under carbon dioxide or ambient air usually within 24 hour. *S. aureus* is a major pathogen for humans. (Forbes et al., 2007).

Staphylococcus epidermidis resemble *S. aureus* in morphology and in the Gram stain preparation. It appears to be Gram- positive cocci in clusters, white, creamy, raised growth on blood-enriched agar, coagulase negative, and DNase negative. *S. epidermidis* is normal flora of the skin and mucous membranes of humans and other animals. With the increasing cases involving this organism, it is now one of the Pathogenic bacteria (Delost 2004).

Bacteria have numerous nutritional needs that include different gases, water, various ions, nitrogen, sources of carbon and energy. Carbohydrates and proteins most commonly provide the latter two. In the laboratory, nutrients are incorporated into the culture media on or in which bacteria are grown. Bacterial growth after inoculation also requires that the medium be placed in optimal environmental conditions (Forbes et al. 2007). In addition to carbon, other elements are needed by microorganisms for the synthesis of cellular materials. The synthesis of DNA and RNA requires nitrogen and some

phosphorous, as does the synthesis of ATP; the molecule is important for the storage and transfer of chemical energy within the cell. Nitrogen makes up about 40 percent of the dry weight of bacterial cell, and sulfur and phosphorus together constitute about another 4 percent. They require very small amount of other mineral elements such as iron, copper, molybdenum, and zinc. Most are essential for the function of certain enzymes, usually cofactors, although these elements are sometimes added to a laboratory with tap water and other components of media. Even most distilled water contains adequate amounts; tap water is sometimes specified to ensure that these trace minerals will be present in culture media.

Media are categorized according to their function and use. Enrichment media contain specific nutrients required for the growth of particular bacterial pathogens that may be present alone or with other bacterial species in a specimen. Supportive media contain nutrients that support growth of most nonfastidious organisms without giving any particular organism a growth advantage. Selective media contain one or more agents that are inhibitory to all organisms except those being sought. Inhibitory agents can be in the form of dyes, bile salts, alcohols, acids and antibiotics. Differential media employ some factor that allows colonies of one bacterial species to exhibit certain culture characteristics that can be used to distinguish them from other bacteria growing on the same agar plate (Forbes et al. 2007).

Blood agar (nutrient agar plus 5% sheep red blood cells) and chocolate agar (nutrient agar plus powdered hemoglobin) are examples of solid-enriched media that are routinely used in the laboratories (Engelkirk and Burton 2007). Variety of animal bloods and banked human blood is used to enrich microbiological culture media and to highlight growth characteristics such as hemolysis. The use of pig and goat bloods was shown to be suitable alternative to sheep blood in the preparation of enriched culture media (Anand et al. 2000).

The isolation of some organisms requires blood as a culture medium supplement. Human blood agar is widely used in developing countries for the isolation of bacteria from clinical specimens. Defibrinated sheep, horse, pig or goat blood agar is recommended for the isolation of *S. pneumoniae* and *S. pyogenes*. Agar prepared with human blood is not suitable, partly because of the safety risk to the laboratory personnel, but mainly because it results in poor bacterial isolation rates, although there were few published data to support the method (Russell et al. 2006). The loss of RBC viability has been

correlated with the "lesions of storage," which is associated with various biochemical changes. These changes include a decrease in pH, a decrease in glucose consumption, a buildup of lactic acid, a decrease in ATP levels, and a reversible loss of RBC function (Harmening 2007). Despite this, human blood agar has been routinely used in bacteriology laboratories in seven developing countries in the Asia Pacific Region (Russell et al. 2006).

Packed red cells that underwent inadequate washing might possibly contain antigens, antibodies and complement proteins. The presence of antigen receptor in red blood cells can induct such actions, thus resulting in the lysis of the red blood cells.

Bacterial metabolism involves all the cellular processes required for the microorganism's survival and replication. Nicotinamide adenine dinucleotide serves as carrier molecule during the process of producing energy. 2-3, DPG is very important in glycolysis wherein the production of ATP is dynamic (Forbes et al. 2007). Washing the red blood cells removes free unbound serum globulins. Saline, used in washing, are stored for long periods in plastic containers has shown to decrease pH, which may increase the rate of antibody elution during the washing process. The expected hematocrit increase for washed red blood cell is the same as that for regular red blood cell unit (Harmening 2007).

A study conducted by Russell et al. in 2006 compared the efficacy of agars that used citrated sheep blood agar and outdated human blood agar with defibrinated horse blood agar and defibrinated sheep blood agar for the isolation and antibiotic susceptibility testing of reference and clinical strains of *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus*. Susceptibility testing for *S. pneumoniae* and *S. pyogenes* was performed on defibrinated sheep blood Mueller-Hinton agar, citrated sheep blood Mueller-Hinton agar, and human blood Mueller-Hinton agar plates. For all organisms, the colony numbers were similar on all agars. Substantially smaller colony sizes and absent or minimal hemolysis were noted on human blood agar for all organisms.

Antibiotic susceptibility results for *S. pneumoniae* were similar for the two sheep blood agars; however, larger zone sizes were displayed on human blood agar, and quality control for the reference strain failed on human blood agar. For *S. pyogenes*, larger zone sizes were demonstrated on human blood agar and citrated sheep blood agar than on defibrinated sheep blood agar. Poor hemolysis made interpretation of the zone sizes difficult on human blood

agar. Citrated sheep blood agar is an acceptable alternative for the isolation of these organisms.

The characteristic morphology is not evident, and hemolysis is poor on human blood agar; and so human blood agar is not recommended for use for the isolation or the susceptibility testing of any of these organisms. Citrated sheep blood agar-Mueller-Hinton agar may be suitable for use for the susceptibility testing of *S. pneumoniae*. The growth of a clinical isolate of *S. pneumoniae* was similar on all blood agars. The morphological appearances were similar for colonies on horse blood agar, citrated sheep blood, and defibrinated sheep blood. However, colonies were much smaller and alpha-hemolysis was not obvious for any strain on human blood agar. The numbers of colonies and growth of the *S. aureus* reference strains were similar on all blood agars at all dilutions. The appearances and the sizes of the colonies were similar on all agars, but hemolysis was not obvious for any strain on human blood agar and was only faint on horse blood agar.

The group of Changsri et al. (2001) examined effects of acid-citrate-dextrose (ACD) anticoagulant solution on quality of blood agar and determined shelf-life of sheep blood agar and human blood agar. The prepared blood agars were observed on sterility and their media appearance along the experiment in blood agar supplemented with different types of 5 % blood such as DF, ACD sheep blood and ACD human blood. The research showed that ACD does not affect the blood agar quality and is likely that the age of blood agar more of than 11 weeks old was not suitable for determining hemolysis patterns.

In 2000, Anand et al. showed the growth characteristics and colony morphologies of selected microorganisms and assessed them according to the medium type and incubation conditions for comparisons. The study examined the use of pig and goat blood as potential substitutes for sheep blood in blood-supplemented bacteriologic media. All eleven strains of *Enterococcus* species produced alpha-hemolysis on sheep blood agar, goat blood agar, and pig blood agar when incubated in carbon dioxide. Strains of BHS groups grew equally well and gave identical hemolytic reactions on the three blood agars although individual isolates displayed variation in the size of the hemolytic zone and/or sharpness of the zone edge on the different blood agars. Colonies of *S. pneumoniae* were dome-shaped and more mucoid on pig blood agar compared to colonies on sheep and goat agar, which were flat with a central depression.

Twenty-seven strains of VT-producing *E. coli* of 7 different O-serotypes, 74 strains of Verocytotoxin-nonproducing E. coli of 24 different O-serotypes and

one strain of O157 coded *Escherichia hermanii* were used for this basic study. In comparison of washing times of sheep blood with PBS, 5 times washing was better than 3 times, the original. In sheep blood concentration, supplement with 4% sheep blood was best for hemolysis observation. In experiment of addition of 2 divalent metal ions, Ca2+ and Mg2+, supplement with Ca2+ was more suitable than Mg2+ for hemolysis, and the supplement with 10 mM CaCl2, the original, was the best concentration. On the basal medium used in Beutin's sheep washed blood agar, 4 kinds of media were compared. In addition to Soybean-Casein Digest (SCD) agar, the original, Nutrient agar, Heart Infusion (HI) agar and Brain Heart Infusion (BHI) agar were examined, HI agar was the best blood agar among the four media.

According to Gardam and Miller (1998), the combination of Trypticase soy agar and sheep blood agar is recommended for the presumptive identification of *Streptococcus pneumoniae*. Optochin or ethylhyrocupreine hydrochloride test is widely used as an inexpensive and reliable means to presumptively identify *S. pneumoniae*. The National Committee for Clinical Laboratory Standards guidelines regarding optochin inhibition recommended using a blood agar plate specifying neither the type of blood nor the type of agar. Three popular types of agar plates Trypticase soy agar (TSA), Columbia agar and Mueller- Hinton agar supplemented with sheep blood were tested for the optochin inhibition zone criteria. Seventy two clinical isolates of *S. pneumoniae* and twenty two isolates of *S. viridans* were used. The study showed that optochin sensitivity tests performed on different sheep blood agar media yield significantly disparate results. Using media other than TSA-sheep blood agar will yield in a substantial number of isolates with indeterminate zones, which will require further testing before the organisms can be identified as *S. pneumoniae.*

MATERIALS AND METHODS

The expired human packed blood cells used in this research was obtained from the Blood Bank of Batangas Regional Hospital. The freshly collected blood was obtained from one of the members of the research team. Sheep blood that served as the standard came from Research Institute for Tropical Medicine in Alabang, Muntinlupa City.

The researchers employed the red cell suspension technique in the preparation of blood agar having expired human blood as the main component.

Two different microorganisms, namely *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228, were inoculated in five different blood agars. These included sheep blood, unwashed expired human blood, unwashed fresh human blood, washed expired and fresh human blood. The morphologies and hemolytic patterns were compared. Each type of blood was added to Trypticase soy agar (Hi-Media, USA) in the preparation of BAP. BAP using washed expired human blood without test organisms and sheep blood with test organisms served as the negative control and positive control, respectively.

The growth of the microorganisms was evaluated in terms of the cultural characteristics and hemolytic reaction (Forbes et al., 2007). The washed blood agar was subjected to different storage duration to determine its stability. To determine the effect of the duration of storage, a batch of culture media was stored at a refrigerated temperature. The said culture media were then serially inoculated with *Staphylococcus aureus* everyday for seven (7) days.

RESULTS AND DISCUSSION

I. Washed Expired Human Blood

The assessment of the microbial growth characteristics of the test isolates in washed expired human blood was completed through the 24-hour incubation of the microorganisms at 37ºC. The obtained results are shown in the table.

Table 1. Assessment of microbial growth of *Staphylococcus aureus* in washed expired human blood

Plate Number	Cultural Characteristics	Hemolytic Reaction
w/ sheep blood	smooth, slightly raised and translucent	Beta
BAP without inoculum	no growth	No growth
1	smooth, slightly raised and translucent	Beta
2	smooth, slightly raised and translucent	Beta
3	smooth, slightly raised and translucent	Beta
4	smooth, slightly raised and translucent	Beta
5	smooth, slightly raised and translucent	Beta

Continuation of Table 1

6	smooth, slightly raised and translucent	Beta
7	smooth, slightly raised and translucent (but with contamination)	Beta
8	smooth, slightly raised and translucent	Beta
9	smooth, slightly raised and translucent	Beta
10	smooth, slightly raised and translucent	Beta

Table 1 presents the morphology and the hemolytic pattern produced by *S. aureus* in the washed expired human blood. After the 24- hour incubation of the ten plates with *S. aureus*, beta hemolysis and yellowish pigment were spotted in all the plates. The physical manifestation of the colonies was smooth, slightly raised and translucent. These observations correlate with the standard culture characteristics of the said microorganism (Forbes et al., 2007; Bartelt, 2000). The same cultural characteristics were observed in BAP with sheep blood, which served as the positive control. No growth of organisms was observed on BAP with washed expired human blood, which was not inoculated with the test organisms. This indicates that the prepared blood agar plates were not contaminated.

Results of the experiments indicate that expired human blood with washing is sufficient to show the morphologic and hemolytic pattern of *Staphylococcus aureus.* Washing the expired human blood can enhance its capability as enrichment for blood agar. It is possible that washing the red blood cells removes free unbound serum globulins (Harmening 2007). Additionally, the anticoagulant citrate, which interferes with bacterial growth (Russell et al., 2006), is likewise removed resulting to a greater microbial yield in all the plates using washed blood. Through washing, interferences hindering proper microbial growth are removed, enabling the microorganisms to flourish and display their optimum morphologic and hemolytic pattern. A sample plate of the said agar is shown in Figure 1.

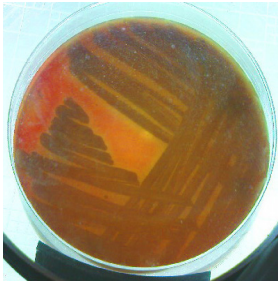

Figure 1. *Staphylococcus aureus* in washed expired human blood agar

Table 2. Assessment of microbial growth of *Staphylococcus epidermidis* in washed expired human blood

Plate Number	**Cultural Characteristics**	**Hemolytic Reaction**
w/ sheep blood	white raised colonies	Gamma
BAP without inoculum	no growth	No growth
1	white raised colonies	Gamma
2	white raised colonies	Gamma
3	white raised colonies	Gamma
4	white raised colonies	Gamma
5	white raised colonies	Gamma
6	white raised colonies	Gamma
7	white raised colonies	Gamma
8	white raised colonies	Gamma
9	white raised colonies	Gamma
10	white raised colonies	Gamma

In Table 2, *Staphylococcus epidermidis* created gamma hemolysis. White raised colonies were observed and recorded, which correlated with the expected culture characteristics for the said test isolate (Delost 2004). This shows that there are no distinct changes in the morphologic and hemolytic reaction of *Staphylococcus epidermidis* when grown in washed expired human blood agar. Thus, washing of blood has no effect on *Staphylococcus epidermidis*

because it is a gamma hemolytic bacterium. No growth of organisms was observed on BAP with washed expired human blood, which was not inoculated with the test organisms.

Figure 2. *Staphylococcus epidermidis* in washed expired human blood

II. Points of Differences

To determine the disparity in the use of different types of blood as enrichment to blood agar, the researchers decided to use unwashed expired human blood, washed fresh human blood, unwashed fresh human blood, and sheep's blood as its standard. The results of the experimentation are presented below.

Table 3. Growth characteristics of *Staphylococcus aureus*

Blood Agars	Cultural Characteristics	Hemolysis
BAP without inoculum	no growth	No growth
Sheep's blood	medium to large, smooth, slightly raised and translucent	Beta
Washed expired blood	medium to large, smooth, slightly raised and translucent	Beta
Unwashed expired blood	large, smooth and raised	Gamma
Unwashed fresh blood	medium and flattened colonies	Gamma
Washed fresh blood	large, smooth and green colonies	Partial beta

Table 3 shows the differences in growth of *Staphylococcus aureus* in four different blood agars. Each blood agar was tested in multiple trials. In

sheep's blood agar, the appearances of the colonies of *Staphylococcus aureus* were medium to large, smooth, slightly raised, and translucent as expected for the said bacterial specie as shown in figure 4.3 (Forbes et al. 2007). In the unwashed expired and fresh human blood shown in figures 4 and 5, different patterns were observed, which could be attributed to the interferences still present in the agars.

In washed fresh blood shown in figure 6, the result was unique. The morphologic characteristics of the isolate were slightly expressed but the hemolysis was not complete. No growth of organisms was observed on BAP with washed expired human blood, which was not inoculated with the test organisms. This indicates that the prepared blood agar plates were not contaminated. This study suggests that maybe adenosine triphosphate level in the blood plays a role in hemolysis.

When red blood cells are ATP-depleted, calcium and sodium are allowed to accumulate intracellularly, and potassium and water are lost, resulting in dehydrated rigid cell that decreases red blood cell survival (Harmening 2007). This low survival is increased hemoglobin presence in the agar extracellularly. The accumulation of hemoglobin during the growth of bacteria results in the formation of different hemolysis patterns.

In unwashed fresh blood agar, gamma hemolysis was observed probably because the ATP level was still high, which means most of the cells were still intact. In unwashed expired blood agar, gamma hemolysis was observed that could be due to the presence of interferences, thus hindering the flourishing of the isolates. The washed fresh blood agar displayed partial beta hemolysis due to the combination of ATP levels and washing. Its ATP levels were high, resulting in cells that were intact with hemoglobin kept inside. The interferences were removed through washing, thus allowing the bacteria to grow more easily but not abundantly. In washed expired blood agars, the ATP level was low plus washing allowed the microorganisms to abundantly grow.

This suggests that washing removes interferences for microbial growth. Both unwashed expired and fresh human blood show gamma hemolysis due to the interferences still present in them. Washed expired human blood produces beta hemolysis due to the removal of interferences plus the low ATP level that indicates low red blood cell survival, which means easy hemolysis for the red cells. Washed fresh human blood produces a partial beta hemolysis due to the removal of interferences, but can hardly makea full beta hemolysis due to high ATP levels that leave the red blood cell survival higher.

Figure 3
S. aureus in sheep's blood

Figure 4
S. aureus in unwashed expired blood

Figure 5
S. aureus in unwashed fresh human blood

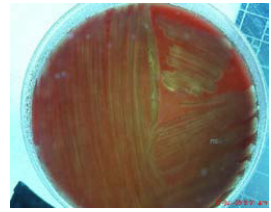

Figure 6
S. aureus in washed fresh human blood

Table 4. Growth characteristics of *Staphylococcus epidermidis* in different culture media

Blood agar	Cultural Characteristics	Hemolysis
BAP without inoculum	no growth	No growth
Sheep's blood	white raised colonies	Gamma
Washed expired blood	white raised colonies	Gamma
Unwashed expired blood	white raised colonies	Gamma
Unwashed fresh blood	white raised colonies	Gamma
Washed fresh blood	white raised colonies	Gamma

Table 4 presents the morphologic and hemolytic pattern of *Staphylococcus epidermidis* in different blood agars. The results were all the same because of the fact that this microorganism is naturally a gamma hemolytic organism (Delost, 2004). No growth of organisms was observed on BAP with washed expired human blood, which was not inoculated with the test organisms. This indicates that the prepared blood agar plates were not contaminated. Sample pictures are shown in Figure 7 to 10.

Figure 7
S. epidermidis in sheep's blood

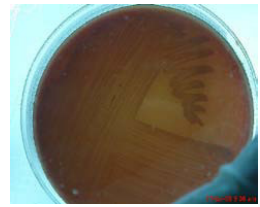

Figure 8
S. epidermidis in unwashed expired human blood

Figure 9
S. epidermidis in unwashed fresh human blood

Figure 10
S. epidermidis in washed fresh human blood

III. Stability of Washed Expired Human Blood Agar

The researchers decided to test the stability of washed expired human blood when stored in a refrigerated temperature for one week.

Table 5. Stability test for washed expired human blood with *Staphylococcus aureus*

Length of Storage (Days)	Result
With sheep blood	With growth
BAP without inoculum	No growth
1	With growth
2	With growth
3	With growth
4	With growth

Continuation of Table 1

5	With growth
6	With growth
7	With growth

Table 5 presents the length of time the blood agar prepared with washed expired human blood and be stored and still retains its capacity to grow microorganisms. From Day 1 to Day 7, colonies of *S. aureus* were observed. As revealed, washed expired human blood could be stored for seven days and still could be used for microbial culture. No growth of organisms was observed on BAP with washed expired human blood, which were not inoculated with the test organisms. This indicates that the prepared blood agar plates are not contaminated. Figure 11 to 13 are samples of growth on Day 5 to Day 7.

Figure 11
S. aureus in Day 5

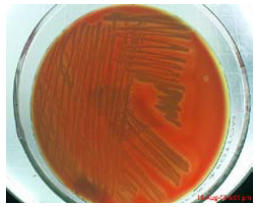

Figure 12
S. aureus in Day 6

Figure 13
S. aureus in Day 7

CONCLUSIONS

Results reveal that expired human blood with washing improved the morphologic and hemolytic pattern of *Staphylococcus aureus.* The washing of blood had no effect on *Staphylococcus epidermidis* because it is a gamma hemolytic bacterium. Both unwashed expired and fresh human blood produced gamma hemolysis due to the interferences still present in them. Both washed expired human blood and washed fresh human blood produced beta hemolysis. Washed expired human blood could be stored for seven days and still could be used for microbial culture.

NOTE:

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