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Modified Human Blood Agar as Substitute for Sheep Blood Agar in Laboratories of Developing Countries

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Sheep blood (SB) has been well recognized as a standard blood supplement in blood agar (BA) preparation. Since it is expensive and inconvenient to obtain, expired human blood (HuB) from local blood banks is commonly used to prepare blood agar in the routine microbiology laboratory in many developing countries including Thailand. The major problem of human blood agar (HuBA) is misdiagnosis, especially in streptococci whose identification is based on true hemolysis. This may be due to the different size of sheep and human red blood cells, as well as to inhibitors in human serum that affect hemolysis. Developing a technique to prepare HuBA of equal quality to sheep blood agar (SBA) would be useful. HuBA for culture and isolation of streptococci and other medically important bacteria was prepared from washed and unwashed red blood cells at concentrations of 3–5% vol/vol. Bacterial growth characteristics, e.g. colony morphology, hemolytic pattern and colony count were evaluated in comparison to SBA. It was found that a concentration of 3% HuB in the preparation of HuBA showed results equivalent to SBA. Specialized identification tests, including CAMP test and satellite test were performed with 3% HuBA. HuBA prepared from washed cells produced correct results in the CAMP and satellite tests, possibly because serum inhibitors were removed in the wash step. Therefore, our study indicates that 3% washed-HuBA can replace SBA in the routine laboratory in developing countries and constitutes a ready available alternative at significantly reduced costs.

Keywords: Blood agar, hemolysis, culture, CAMP test, satellite test.

Accurate evaluation of microbial growth characteristics, colony morphologies, and hemolytic patterns on blood agar (BA) is a significant initial step in the identification of human pathogenic bacteria in routine clinical microbiology laboratory. Agar enriched with 5% defibrinated blood from wool sheep (SB) or horse (HB) has been well recognized as a gold standard medium in North America and Europe, respectively. Its properties supports the growth of various kinds of pathogens and allows true differential characterization of bacterial hemolytic patterns.[1] However, SB and HB are often not readily available to laboratories in resource-poor countries, either due to a lack of reliable sources of defibrinated blood or due to financial constraints.

Different animal blood, including from goat, pig, ram, and hair sheep, was used for preparation of BA and compared to standard SBA by microbial growth analyses and specialized identification tests.[2-5] Although the blood of these animals was shown to be suitable as a medium supplement with identical growth characteristics and specialized test results to SBA, it is difficult to adopt in a real-life situation which precludes its widespread application. Therefore, readily available expired human blood (HuB) from blood banks is still commonly used to prepare BA in developing countries as well as in Thailand. In addition, it does

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not require specific equipment for defibrination and is cost-free.[2, 5-6]

Major problems that have been reported in the use of expired HuB for BA are poor bacterial isolation rates due to the presence of serum inhibitors and the anticoagulant citric acid which both inhibit the growth of bacteria and alter hemolytic patterns due to morphological and functional changes of the stored red blood cells.[3-4] In a study to overcome the disadvantages of HuBA Magbojos *et al.* (2011) demonstrated that the quality of HuBA can be equal to that of SBA if the red blood cells are washed prior to BA preparation. The wash steps enhanced the morphology and hemolytic pattern of *Staphylococcus aureus* and *S. epidermidis*. Unfortunately, there is a lack of data on streptococci for which true hemolysis is very important for identification and other medically important human bacteria. We, therefore, aimed to evaluate BA prepared with either washed or unwashed HuB in respect to the growth of streptococci and other important pathogens and the results of specialized microbiology tests. Furthermore, we have analyzed the effect of different HuB concentrations on the test results because any discrepancies might be due to the different size of sheep and human red blood cells.[4] The most suitable HuBA preparation from our study will offer an accurate and affordable diagnosis in routine microbiology laboratory when SB is not easy to obtain.

MATERIALS AND METHODS

Blood collection and preparation

Freshly expired human whole blood (HuWB) used in this study was obtained from the blood bank of Thammasat University Hospital and used to prepare BA at concentrations of 3–5% HuB. In detail, aliquoted HuWB was centrifuged at 3,000 rpm for 5 min and the supernatant was discarded. In the next step, the cells were resuspended in sterile 1× PBS and pelleted using identical centrifugation conditions. The supernatant was discarded and the wash step was repeated two times. Finally, the cells were resuspended in 1× PBS and the cell suspensions were immediately used for BA preparation at the indicated blood cell concentrations.

Medium preparation

HuBA was prepared from washed and unwashed HuB at the indicated 3–5% final HuB concentrations using blood agar base No. 2 (Oxoid, Australia) according to the manufacturer's instructions. Freshly poured plates were incubated overnight at 37°C and plates that showed contaminations were discarded. Defibrinated SBA was purchased from Clinical Diagnostic Ltd., Thailand (Lot. No. 150605) and used as positive control. All BA plates were stored at 2–8°C until used.

Bacterial strains

Bacterial strains used in this study were either obtained from ATCC, USA or were clinical samples collected at Thammasat University Hospital (Table 1). All organisms were cultured on SBA and incubated overnight at 37°C, 5% CO_2. A fresh subculture of each strain was diluted to 0.5 McFarland turbidity in sterile normal saline and used for medium inoculation.

Microbial growth analysis

Bacteria were plated in each of six different HuBA plates (5% washed-, 4% washed-, 3% washed-, 5% unwashed-, 4% unwashed-, 3% unwashed-HuBA) as well as SBA. A full loop of bacterial inoculum was streaked on plates for single colony isolation. In addition, each inoculum was serially ten-fold diluted to 10^3 cfu/ml and 100 µl of the final dilution was plated in triplicate on each agar. The inoculated plates were incubated at 37°C in 5% CO_2 for 18 to 20 h. Colony morphology, size, hemolysis, and number of colonies counted (cfu) on each BA type were recorded.

CAMP and satellite test

For the CAMP test, a single streak of *S. aureus* was made across the center of a blood agar plate. Group B streptococci (positive control) and other streptococcal isolates were tested by making a single streak perpendicular to the line of *S. aureus*, but without touching it. The plates were incubated at 37°C for 18–24 h. A positive CAMP reaction was indicated by an arrowhead or triangular shaped area of synergistic hemolysis in the intersecting area. A negative reaction appeared as a bullet-shaped zone of slightly or not increased hemolysis.

For the satellite test, *H. influenzae* was streaked all over the plate and *S. aureus* was single-streaked across the middle of the inoculated plate.

The plates were incubated at 37°C, 5% CO_2 for 18–20 h.

Biosafety approved

This study was approved by the Biosafety Committee, Thammasat University (approval no. 032/2015).

RESULTS

Comparison of the six different HuBA plates with 5% SBA by physical observation and microbial growth analysis

In the physical observation the degree of color intensity and opacity of the six different HuBA plates correlated with the percentage of added blood. HuBA plates at 5% HuB content showed the darkest red and highest opacity while plates at 4% and 3% blood concentration showed less red darkness and opacity, respectively. Differences in the appearance of washed- and unwashed-HuBA could not be observed at all blood concentrations. However, 3% HuBA showed a similar appearance to 5% SBA (data not shown).

In the microbial growth analysis eight medically important pathogens were evaluated on the six different HuBA types in comparison to 5% SBA for their growth characteristics, including colony morphology, hemolytic pattern, and colony count as detailed in Table 2. Significant differences in the morphology and size of colonies as well as colony count were not observed between the plates. However, for streptococci, some differences were noted in the hemolytic patterns between washed- and unwashed-HuBA at 5% and 4% but not at 3% of HuB content. Hemolytic patterns could be easier read on washed-HuBA than unwashed-HuBA. In addition, clearer and more accurate hemolytic patterns and wider hemolysis zones were observed at less than 5% HuB. HuBA at 3 % HuB showed the most obvious and accurate hemolytic patterns when compared to standard SBA as shown in Figure 1.

Accuracy of the most suitable HuBA type in CAMP and satellite colony formation tests

The 3% washed- and unwashed-HuBA plates were used to evaluate the results of specialized tests in comparison to 5% SBA. In the CAMP test, *S. agalactiae* (group B streptococci) presented a synergistic hemolysis as seen by an arrowheaded area when tested on SBA and 3% washed-HuBA. Notably, a positive CAMP reaction was not observed in the CAMP test on 3% unwashed-HuBA (Figure 2). In the satellite test, *H. influenzae* grew on 3% washed- and unwashed-HuBA and 5% SBA as small satellite colonies adjacent to *S. aureus* (Figure 3).

DISCUSSION

This research investigated the application of HuBA for routine microbiology laboratory. Specifically, an easy washing protocol of HuB was established and used in a comparative analysis of 3–5% washed/unwashed-HuBA to 5% SBA. Physical appearance of the prepared BA and microbial growth analysis of different hemolytic

Table 1. Sources of bacterial strains

Organism	Source[a]	Test
Streptococcus pyogenes (group A)	ATCC 19615	Growth study and CAMP test
Streptococcus agalactiae (group B)	Clinical strain	CAMP test
*Streptococcus pneumonia*e	Clinical strain	Growth study
Enterococcus spp.	Clinical strain	Growth study and CAMP test
Group D streptococcus	Clinical strain	CAMP test
Staphylococcus aureus	ATCC 25923	Growth study, CAMP and satellite test
Escherichia coli	ATCC 25922	Growth study
Neisseria meningitidis	ATCC 13077	Growth study
Bacillus cereus	ATCC 11778	Growth study
Pseudomonas aeruginosa	ATCC 27853	Growth study
Haemophilus influenzae	Clinical strain	Satellite test

[a] The clinical strains were isolated from patient samples at Thammasat University Hospital, Thailand.

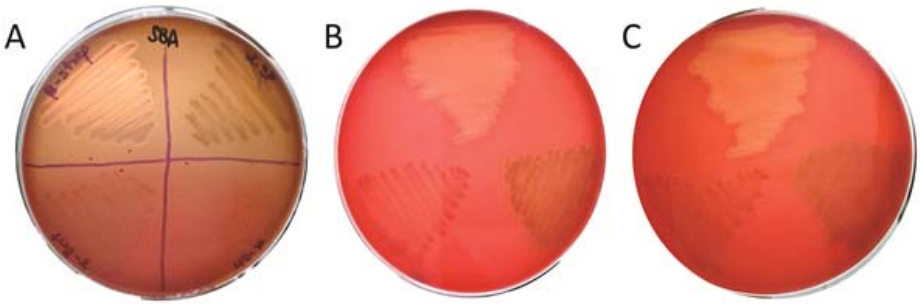
Table 2. Appearances of colonies, hemolysis, and colony counts for tested organisms on six different HuBA plates

Organism and characteristics[a,b]	Blood agar plates						
	SBA	Washed-HuBA 5%	Unwashed-HuBA 4%	3%	5%	4%	3%
S. pyogenes							
Hemolysis pattern	Beta with sharp and clear zone (1 mm)	Beta with narrow zone (0.5 mm)	Beta with narrow zone (<1 mm)	Beta with sharp and clear zone (1 mm)	Minimal beta-hemolysis	Minimal beta-hemolysis but clearer than 5%	Beta with sharp and clear zone (1 mm)
Colony appearance	Gray-white and translucent, size 0.5 mm						
Colony count	~100 cfu						
S. pneumoniae							
Hemolysis pattern	Alpha with clear green zone (>1.5 mm)	Minimal alpha-hemolysis with narrow zone	Clearer alpha-hemolysis than 5%	Alpha with clear green zone (>1.5 mm)	Absent or faint alpha-hemolysis	Minimal alpha-hemolysis with narrow zone	Alpha with clear green zone (>1.5 mm)
Colony appearance	Mucoid and gray, size 1-1.5 mm						
Colony count	~100 cfu						
Enterococcus spp.							
Hemolysis pattern	No hemolysis was observed (Gamma hemolysis)						
Colony appearance	Dry and gray-white, size 0.5 mm						
Colony count	~100 cfu						
S. aureus							
Colony appearance	Smooth, opaque white and slightly raised, size 1 mm						
Colony count	~100 cfu						
E. coli							
Colony appearance	Smooth and gray-white, size 1.5-2 mm						
Colony count	~100 cfu						
N. meningitidis							
Colony appearance	Smooth and gray-white, size 1 mm						
Colony count	~100 cfu						
B. cereus							
Colony appearance	flat or slightly convex with irregular edges, size ~3-5 mm						
Colony count	ND						
P. aeruginosa							
Colony appearance	slightly convex, irregular edges with a fluorescent greenish color, size ~3-5 mm						
Colony count	ND						

[a] Colony count was done in triplicate plates.
[b] Hemolysis patterns of *S. aureus*, *E. coli*, *N. meningitidis*, *B. cereus*, and *P. aeruginosa* were not recorded.
Abbreviation: ND; not done.

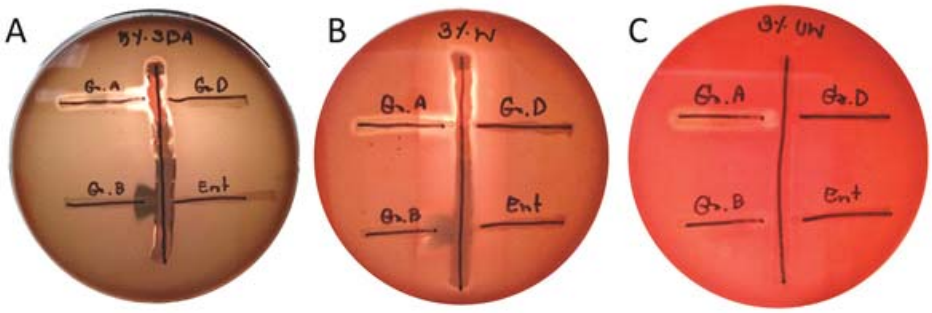
patterns caused by streptococci and other medically important bacteria were recorded. It was found that the physical characters, including intensity of color and opacity, of washed- and unwashed-HuBA at the same blood concentration were not different. However, at 3% blood content both HuBA types exhibited the same color and opacity as 5% SBA. Normally, HuBA is supplemented with 5% HuB following the standard protocol for SBA preparation but different to 5% SBA the agar has a dark red and turbid appearance. The mean corpuscular volume (MCV) of human red blood cells (80-100 fl) is higher than the MCV of sheep red blood cells (19-35 fl) and this causes the darker red turbidity.[7] Thus, HuBA of lower blood content will show less turbidity and our study showed that 3% HuB was the optimal concentration and resulted in an identical appearance of HuBA to 5% SBA.

In the microbial growth study, all HuBA types did not show significant differences in the morphology and size of colonies as well as in colony count in comparison to SBA. These findings are consistent with the results reported by Egwuata *et al.* (2014) but inconsistent with the work of Russell *et al.* (2006) who reported smaller colony sizes of the tested organisms on HuBA. However, a minimal or unclear hemolysis of streptococci was mostly observed on 5% HuBA particularly the unwashed-type. Hemolysis was more obvious on washed-HuBA at less than 5% HuB content due to the decreased opacity of medium. Furthermore, interfering compounds hindering microbial growth, including free unbound serum globulin, antibiotics, complement proteins, or citric acid anticoagulant, were either removed in the washing steps or diluted by decreasing the amount of HuB used per plate. This caused an improved bacterial growth with optimum colony morphology and hemolytic patterns.[6] From our data, we recommend that 3% washed/unwashed HuBA types are appropriate and support bacterial growth that results in true and clear hemolysis which is very important for accurate diagnosis in



(A) SBA, (B) 3% washed-HuBA and (C) 3% unwashed-HuBA were inoculated with *S. pyogenes* (beta-hemolysis), *S. pneumoniae* (alpha-hemolysis), and *Enterococcus* spp. (gamma-hemolysis).

Fig. 1. Comparison of hemolysis patterns on SBA and 3% washed/unwashed HuBA types

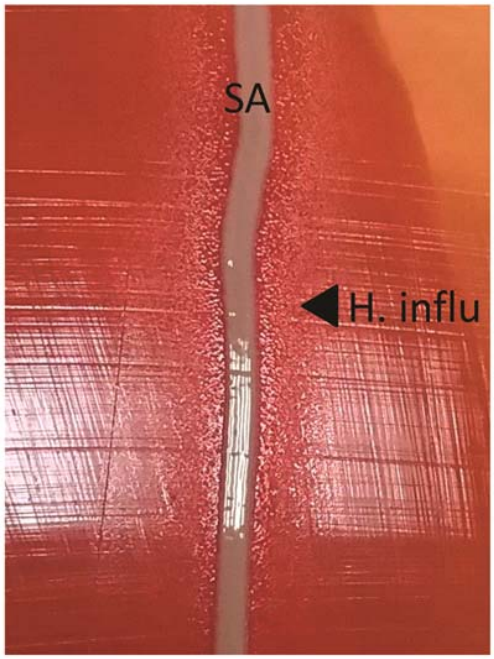


Group B streptococci showed a positive result in the CAMP test with synergistic hemolysis as seen by an arrowheaded area when tested on SBA and 3% washed-HuBA.

Fig. 2. Comparison of CAMP test on (A) SBA, (B) 3% washed-HuBA, and (C) 3% unwashed-HuBA

microbiological laboratory.

In the specialized identification CAMP and satellite colony formation tests only the 3% washed- and unwashed-HuBA were used for evaluation in comparison to standard SBA. These tests are simple and inexpensive to perform but can be significant in identification of some microorganisms. The CAMP test is used for identification of group B streptococci that secrete CAMP factor contributing to increased hemolysis in the arrowheaded area when grown near β-toxin producing *S. aureus* on BA. This test is useful especially in developing countries, where antiserum for streptococci serogrouping is often unavailable.[2] Although there are some previous reports that indicated that HuB cannot be used in the CAMP test[8-9], our study showed accuracy of positive and negative results when using 3% washed-HuBA. This may be due to the removal of β-antitoxin in the washing steps while this antitoxin was still present in 3% unwashed-HuBA.[10] However, such difference between 3% washed/unwashed HuBA types was not observed in the satellite test. *H. influenzae* was able to grow on both agars as small satellite colonies adjacent to nicotinamide adenine dinucleotide (NAD) essential factor for *H. influenzae* secreting *S. aureus*.



Small satellite colonies (arrowhead, H. influ) of *H. influenzae* grew adjacent to *S. aureus* (vertical streak, SA) when tested on SBA and both 3% HuBA types

Fig. 3. The satellite test for identification of *H. influenzae* on SBA and 3% washed/unwashed HuBA types

Despite the equal quality of 3% unwashed-HuBA and 3% washed-HuBA in respect to microbial growth characteristics and hemolytic results as well as allowing *H. influenzae* to grow in the satellite test, there is an important drawback, namely 3% unwashed-HuBA cannot be applied for the CAMP test. Thus, we recommend 3% washed-HuBA as the most suitable alternative medium to 5% SBA for use in clinical microbiology laboratory. The described addition of a simple repeated washing step allows laboratory staff to produce high quality enriched medium with standard equipment. When considering the costs, media prepared from expired HuB averages five Baht per plate which is six to ten times less than media prepared from commercial defibrinated SB or SBA. Although there are concerns about the exposure risk to blood-borne pathogens when using HuB in the laboratory it should be considered that 'Nucleic acid Amplification Technology' (NAT), a molecular technique with high sensitivity and specificity, has been used as standard method since the year 2010 for pathogen detection in Thailand.

In conclusion, we are confident that 3% washed-HuBA can replace SBA especially in the developing world where SB or HB is not readily available. The use of HuBA offers a safe, cost-effective, and accurate diagnosis in clinical microbiology laboratory. In addition, further work is required to assess whether 3% washed-HuB can be applied for antibiotic susceptibility testing or is suitable for chocolate agar preparation.

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