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Comparison Of Different Bacteriological Media and Blood Types For The Easy And Accurate Identification Of *Streptococcus pneumoniae* In Diagnostic Microbiology Laboratory Tests Of Developing Countries

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Abstract:
Background*: For preparing blood agar various types of media are used in different microbiology laboratories. Type of media and the blood supplement used for preparing the blood agar affect the growth characteristics of microorganisms. So, in this experiment we have prepared blood agar plates from three different most commonly used media along with sheep blood and human blood.*
Materials and methods*: Blood agar plates were prepared from unwashed and washed human and sheep blood to see the effect of washing on microbial growth characteristics. One set of blood agar plates were incubated at 37°C temperature in candle jar to provide CO_2 enriched atmosphere. Second set of plates were incubated at 37°C temperature at ambient air as CO_2 incubator is not normally available in most of the microbiology laboratories. Bacterial growth characteristics mainly the colony morphology, pattern of hemolysis and Optochin test results were evaluated.*
Results: *We found the blood agar plates prepared from blood agar base and sheep blood gives the most promising results irrespective of presence of CO_2 during incubation period. We have also observed that presence of CO_2 during incubation time enhances the process of hemolysis.*
Conclusion: *Blood agar plates prepared from blood agar base and unwashed human blood incubated in CO_2 enriched environment showed results equivalent to blood agar plates prepared from blood agar base and sheep blood which makes the identification of hemolysin producing bacteria like Streptococcus pneumoniae easier and more accurate.*
Key words: *Blood Agar, Colony Characteristics, Hemolysis, Human and Sheep Blood, Streptococcus pneumoniae*

I. Introduction

Proper and accurate evaluation of microbial growth characteristics, colony characteristics and pattern of hemolysis on blood agar (BA) is a first and most crucial step in the identification of many significant human pathogenic bacteria in routine clinical microbiology laboratory [1-3]. For preparation of BA, sheep blood (ShB) has been used as a well-recognized standard blood supplement. But ShB is expensive and inconvenient to procure [4-7]. So, expired human blood (HuB) from local blood banks is commonly used for preparing the BA plates in routine microbiology laboratory in many developing countries. Expired HuB is readily available from blood banks and it does not require any specific equipment for defibrination and is cost-free [4,6,8]. But the main disadvantage of BA plates prepared from expired HuB is that it may lead to misdiagnosis mainly in case of *Streptococcus* spp. whose identification is based on type of hemolysis obtained on BA [9,10]. Such BA plates leads to major issues in accurate microbial diagnosis like poor bacterial isolation rate due to presence of serum inhibitors and the anticoagulant citric acid which both inhibit the bacterial growth and also the hemolysis is hardly visible or no hemolysis at all due to morphological and functional changes of the stored RBCs [1,6,11-14]. On BA, variation in hemolysis pattern produced by microorganism depends on the blood type used to prepare media. *e.g.,* organisms such as *Haemophilus haemolyticus* (a commensal of the human respiratory tract, rather than a pathogen) also produce hemolytic colonies, which on the basis of morphology, can be confused with those of pathogenic β-hemolytic streptococci [15]. Thus, the selection of inappropriate colonies for further testing could increases the total turnaround time and also rises technical cost for processing the clinical specimens [15]. So, care must be taken while preparing blood agar as it plays a crucial role in the diagnosis of hemolysin producing human pathogens. So, each and every component for preparing the BA must be selected very

carefully. Many researchers have observed that use of washed blood for preparation of BA enhances the morphology and hemolytic pattern of many hemolysin producing bacteria [1].

In addition to type of blood, the base medium used to prepare blood agar also plays a significant role in microbial growth and morphological characteristics. Along with medium and blood type used for preparation of BA, one another factor plays an important role in the accurate identification of hemolysin producing bacteria mainly α-hemolytic *Streptococcus pneumoniae i.e.,* CO_2 enriched atmosphere during the incubation period [16,17].

By keeping in mind, the importance of all the above mentioned factors, we have compared and evaluated following parameters in this study: **1.** Bacteriological media used to prepare BA (Nutrient Agar- NA, Tryptic Soya Agar- TSA, and Blood Agar Base-BAB), **2.** Blood used to prepare BA (ShB and HuB), **3.** Washed and unwashed blood, and **4.** Incubation condition (incubation in Candle jar -to provide CO_2 enriched atmosphere and at an ambient atmosphere).

BA plates prepared from above mentioned parameters were tested for its performance characteristics when cultivating α-hemolytic *S. pneumoniae* ATCC® 49619™, β-hemolytic *S. aureus*, and β-hemolytic *Streptococcus dysgalactiae subsp. equisimilis.* We also tested its suitability for antibiotic susceptibility testing (AST) and Optochin test for *S. pneumoniae*; however, our main focus was the growth and colony characteristics and hemolysis pattern of *S. pneumoniae.*

II. Material And Methods

This study was carried out at Department of Microbiology & MLT, Arts, Science and Commerce Collage, Kholwad, Surat, Gujarat, India from February 2021 to March 2021. The study was approved by the Institute Ethics Committee.

A. Blood agar plate preparation

Bacteriological Media:

NA, BAB and TSA are commonly used media for the preparation of blood agar plates in routine microbiology laboratory and were evaluated in our study. All bacteriological media were procured from HiMedia *Laboratories Pvt. Ltd*., Mumbai. NA (M001-HiMedia): Ingredients (Gms/Lit): Peptone (5.0), Sodium chloride (5.0), HM peptone B (1.5), Yeast extract (1.5), Agar (15). Final pH (at 25°C) 7.4±0.2., BAB (M1989-HiMedia): Ingredients (Gms/Lit): Tryptone (7.5), HM peptone (2.5), Sodium chloride (8.0), L-Lysine (0.040), Potassium dihydrogen phosphate (0.250), Disodium hydrogen phosphate (1.750), Sodium bisulphite (0.10), Agar 13.50, Final pH (at 25°C) 7.0±0 and TSA (M1968-HiMedia): Ingredients (Gms/Lit): Tryptone (17), Soya peptone (3.0), Sodium chloride (5.0), Dextrose (Glucose) (2.5), Dipotassium hydrogen phosphate (2.5), Agar (15), Final pH (at 25°C) 7.3±0.2.

Blood source:

ShB and HuB were collected aseptically in a blood bag containing anticoagulant Citrate Phosphate Dextrose Adenine solution (CPDA). ShB was collected by jugular vein puncture from antibiotic free sheep housed in animal farm. To mimic the circumstances in developing countries, citrated HuB was taken instead of defibrinating. Defibrination is unmanageable in many developing countries, and the most conveniently available blood from human blood bag is also citrated. [11,12] In this experiment, HuB was procured from regional blood bank near expired date. The blood was stored at 4 °C.

Blood processing:

In this experiment ShB and HuB were used directly (without washing) and with washing. Washing of blood was proceeded as, aliquoted whole blood was centrifuged at 3,000 rpm for 5 min and the supernatant was discarded. In the next step, the cells were resuspended in sterile 1X 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 sterile 1X PBS to prepare blood agar plates. Whole washing steps were performed by strictly following the aseptic conditions to avoid microbial contamination of blood and subsequently the blood agar plates [1, 7].

Preparation of blood agar plates:

Different types of media were prepared by following identical microbiological techniques with identical ingredients according to the manufacturers' recommendations [18]. Blood agar plates were prepared using washed and unwashed HuB and ShB at 5% final blood concentrations. NA, TSA and BAB were prepared

according to the manufacturer's instructions. After sterilization and cooling down to 45°C-50°C, sterile blood 5% v/v were added in strict aseptic conditions to the media and plated. To check the contamination, freshly prepared plates were incubated at 37°C temperature for 18-20 hours. Next day, plates were examined. Plates that showed contaminations were discarded and checked sterile blood agar plates were stored at 2-8°C temperature until used.

B. Inoculation of media:

Bacterial strains:

Bacterial strains used in this study were *S. pneumonaie* ATCC® 49619™ obtained from American Type Culture Collection (ATCC), *Streptococcus dysgalactiae subsp. equisimilis* and *S. aureus* were isolated from clinical samples and identified by Vitek 2 Compact machine.

Inoculum preparation: Each strain was inoculated in Brain Heart Infusion (BHI) broth (M210-HiMedia) to make a suspension equal to 0.5 McFarland standard (R092-HiMedia).

Media Inoculation:

To see the morphological characteristics: A full loop of each bacterial inoculum as prepared above was streaked on different media by using a streak plate method for single colony isolation and to study growth and morphological characteristics of above mentioned different bacterial strains.

To perform Optochin test: With the help of sterile disposable swab (PW005-HiMedia), different blood agar plates were inoculated with culture of *S. pneumoniae* ATCC® 49619™. Optochin (P) disks (6 mm, 5 µg) (DD009-HiMedia.) was placed within the streaked area of the plate.

To perform antimicrobial susceptibility test (AST):

Blood agar media is mainly used to determine AST for the microorganisms belonging to *Streptococcus* and *Enterococcus* group of organisms. In this experiment, we have performed AST only for α-hemolytic *S. pneumoniae* ATCC® 49619™, with the help of sterile disposable swab (PW005-HiMedia), different blood agar plates were inoculated for lawn growth.

Antibiotic discs:

The Hexa Pneumococci 2-HX020 (HiMedia) ring containing different antibiotics *i.e.*, Vancomycin (VA): 30µg, Cefuroxime (CXM): 30µg, Levofloxacin (LE): 5µg, Gentamicin (GEN): 10µg, Ampicillin/Sulbactam (A/S): 10/10µg, Co-Trimoxazole (COT): 25µg. These discs are coated with antibiotics that aid in AST of pneumococci were applied each on different types of BA plates.

C. Incubation:

To see the effect of CO_2 enriched atmosphere and ambient air on the growth characteristics, 1st set of inoculated plates were incubated at 37°C temperature in candle jar to provide CO_2 enriched environment. 2nd set of inoculated plates were incubated at 37°C temperature under ambient air (outside the candle jar) for 18 to 20 hours. Ambient air was chosen along with CO_2 containing atmosphere because this is the common way in developing countries.

D. Microbial growth analysis:

After overnight incubation at 37°C temperature in a candle-jar and at ambient atmosphere different test results were analyzed.

To see the morphological characteristics:

Growth and colony characteristics of different microorganisms on different types of media were analyzed. The appearance of bacterial colonies was observed under impinging light, and hemolysis was observed under transmitted light. The pattern of hemolysis was evaluated visually.

Optochin test:

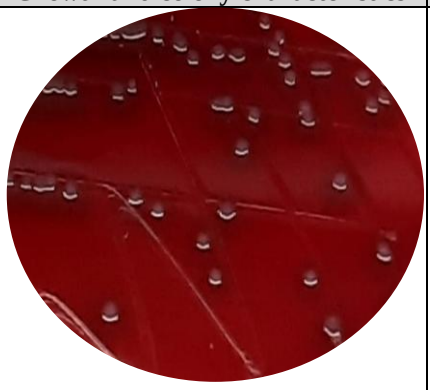
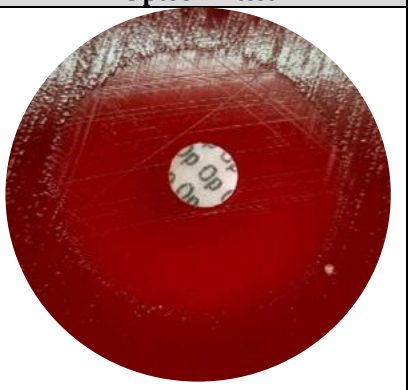
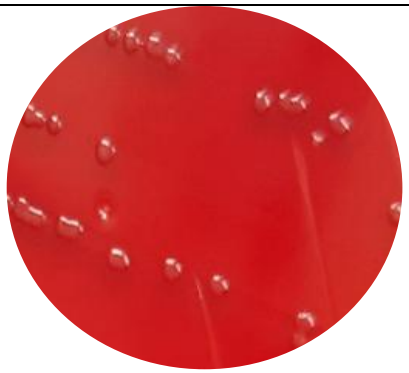
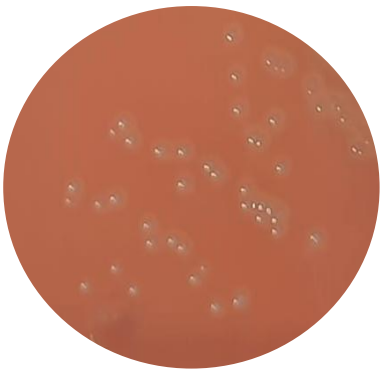
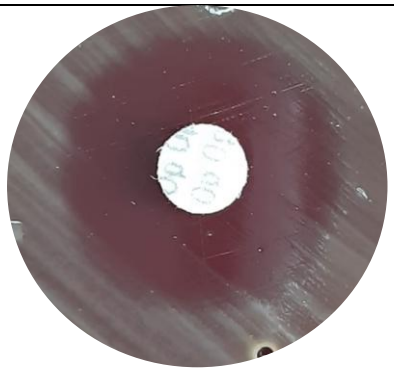
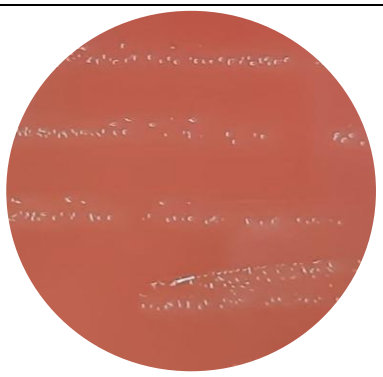
Bacterial growth near the P disk was observed and the zone of inhibition was measured. Using a 6 mm, 5 µg disk, a zone of inhibition of 14 mm or greater indicates sensitivity and allows for presumptive identification of pneumococci. The diameter of the zone was measured by holding the calibrated instrument like zone scales (HiMedia-PW297) over the centre of the surface of Optochin disk.

To perform antimicrobial susceptibility test (AST):

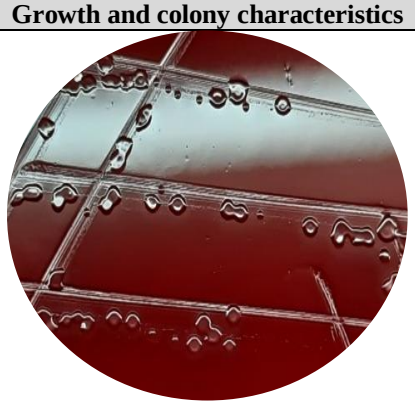
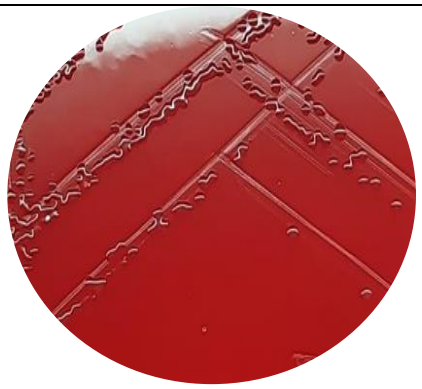
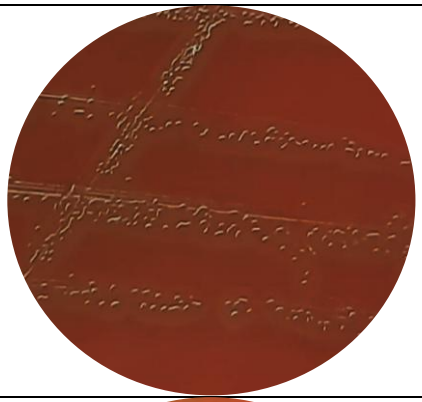
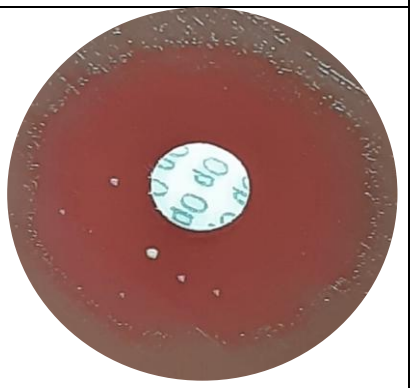
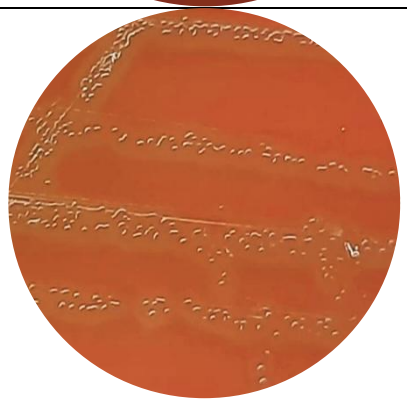
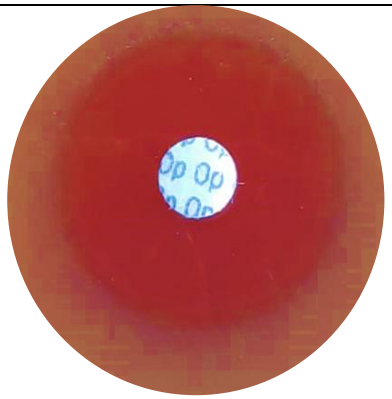
After incubation, the diameters of zone of inhibition were measured by using calibrated zone scales (HiMedia-PW297).

III. Result

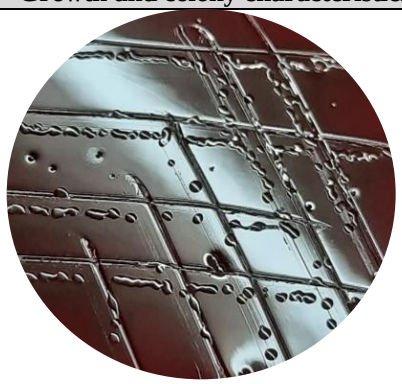
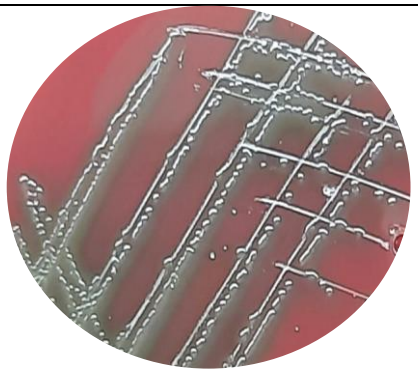
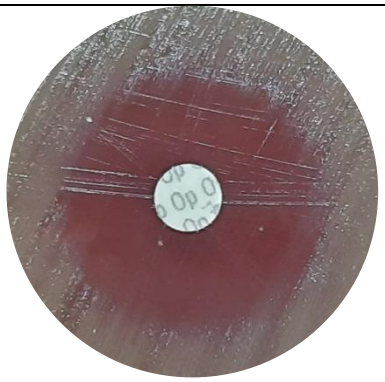
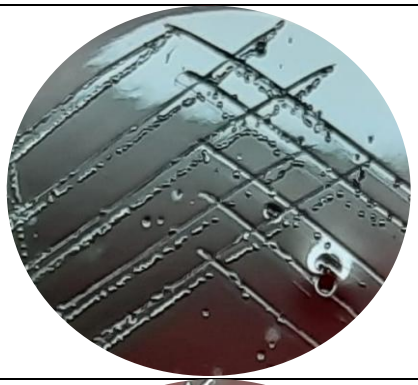
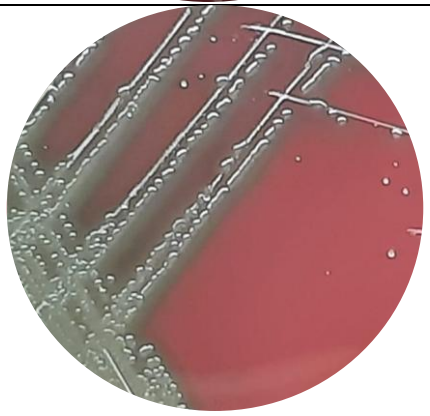
1-A. Nutrient Agar with Human Blood

Medium and Blood used	Incubation condition	Results	
		Growth and colony characteristics	Optochin test
NA+HuB (without wash)	In candle jar*		
	At ambient air		
NA+HuB (with wash)	In candle jar		
	At ambient air		

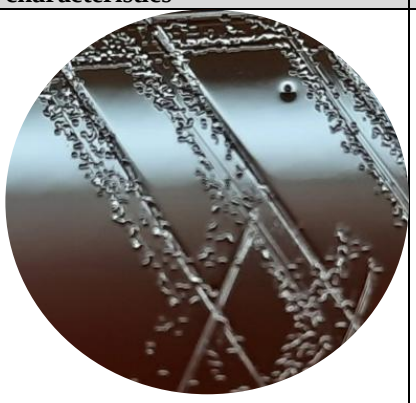
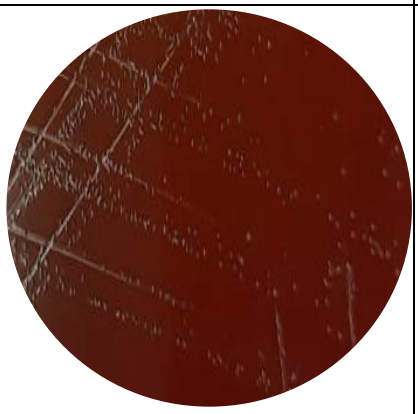
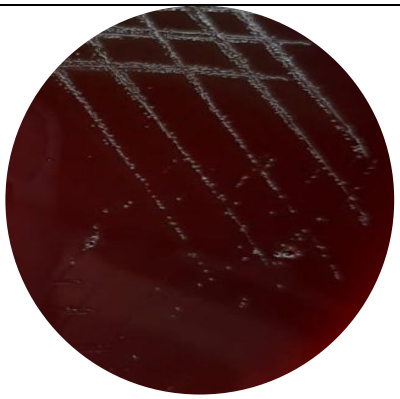
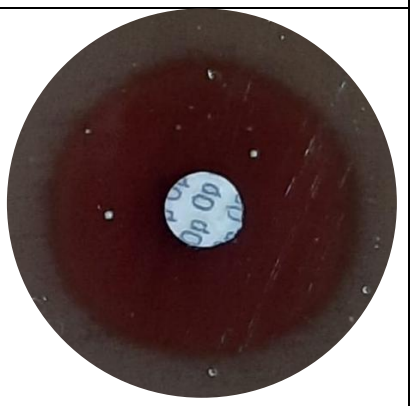
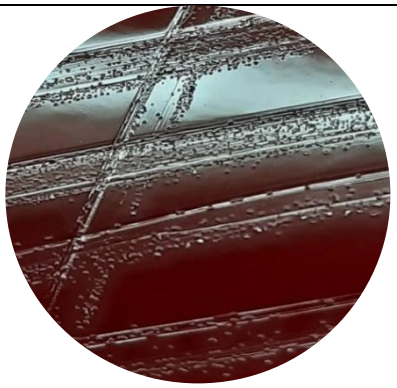
1-B. Nutrient Agar with Sheep Blood

Medium and Blood used	Incubation condition	Results	
		Growth and colony characteristics	Optochin test
NA+ShB (Without wash)	In candle jar		
	At ambient air		
NA+ShB (With wash)	In candle jar		
	At ambient air		

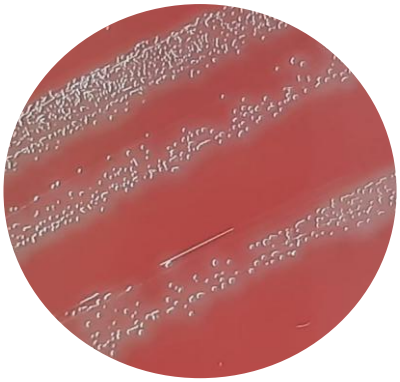
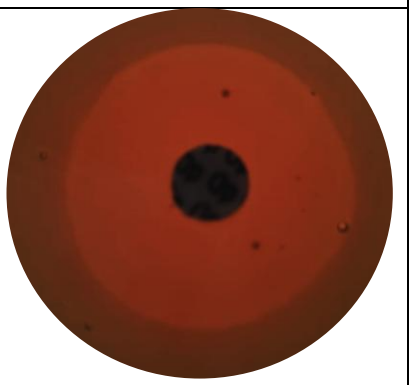
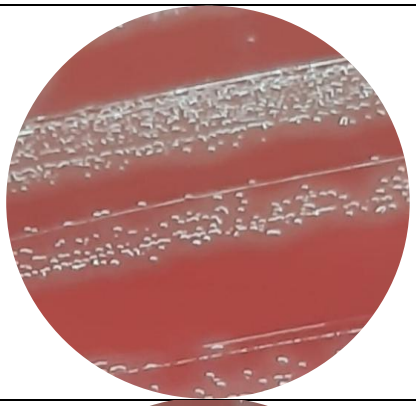
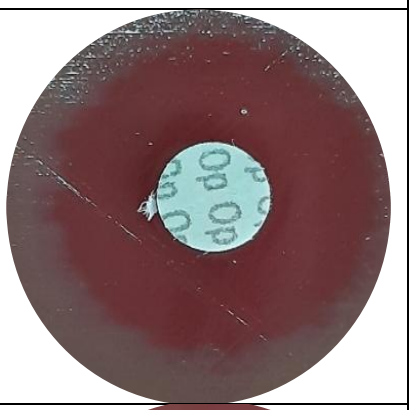
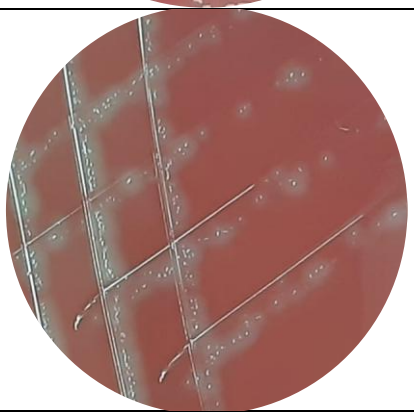
1-C. Tryptic Soya Agar (TSA) with Human Blood

Medium and Blood used	Incubation condition	Results	
		Growth and colony characteristics	Optochin test
TSA+HuB (without wash)	In candle jar		
	At ambient air		
TSA+HuB (with wash)	In candle jar		
	At ambient air		

1-D. Tryptic Soya Agar (TSA) with Sheep Blood

Medium and Blood used	Incubation condition	Results	
		Growth and colony characteristics	Optochin test
TSA+ShB (without wash)	In candle jar		
	At ambient air		
TSA+ShB (with wash)	In candle jar		
	At ambient air		

1-E. Blood Agar Base (BAB) with Human Blood

Medium and Blood used	Incubation condition	Results	
		Growth and colony characteristics	Optochin test
BAB+HuB (without wash)	In candle jar		
	At ambient air		
BAB+HuB (with wash)	In candle jar		
	At ambient air		

1-F. Blood Agar Base (BAB) with Sheep Blood

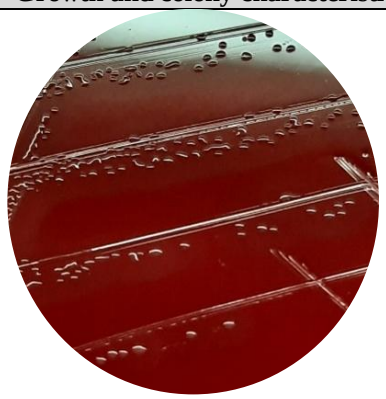
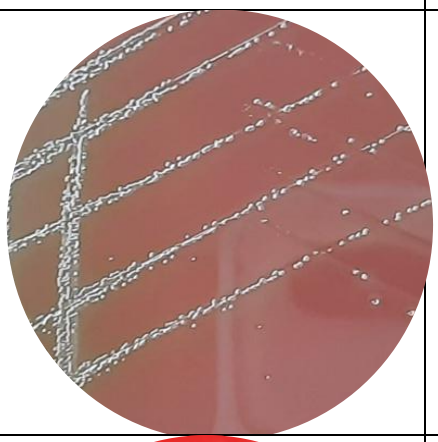
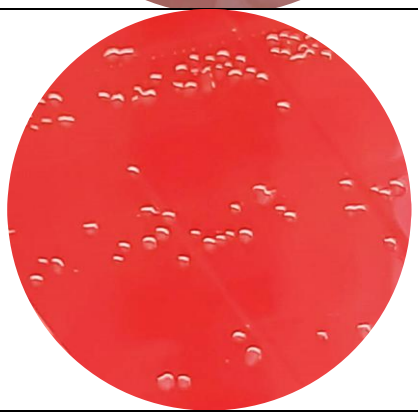
Medium and Blood used	Incubation condition	Results	
		Growth and colony characteristics	Optochin test
BAB+ShB (without wash)	In candle jar		
	At ambient air		
BAB+ShB (with wash)	In candle jar		
	At ambient air		

Figure 1. Growth and colony characteristics of *S. pneumoniae* ATCC® 49619™ on different bacteriological media

All the different types of BA plates were evaluated for physical appearance. In the physical observation the degree of color intensity and opacity were examined. BA plates prepared from TSA with HuB and ShB without washing showed the darkest red color and highest opacity [as shown in Figure 1-C and 1-D] whereas the BA plates prepared from NA and BAB with HuB and ShB without washing showed the less darkness and opacity [as shown in Figure 1-A, 1-B, 1-E and 1-F]. BA plates prepared from NA and BAB with washed HuB and ShB showed very light red color and opacity as compared to unwashed blood [as shown in Figure 1-A, 1-B, 1-E and 1-F].

In the microbial growth analysis, *S. pneumonaie* ATCC® 49619™, *Streptococcus dysgalactiae subsp. equisimilis* and *S. aureus* were evaluated on different types of BA plates for their growth and colony characteristics and hemolysis pattern. Significant differences in colony morphology, size of colony and hemolytic pattern were observed as shown in below Figure 1.

Hemolytic patterns could be easier to read on washed blood than the unwashed blood. In addition, clearer and more accurate hemolytic patterns and wider hemolytic zones were observed when incubated in candle jar *i.e.,* CO_2 enriched environment. Hemolytic pattern was difficult to observe with BA plates prepared from TSA [as shown in Figure 1-C and 1-D] due to dark coloration of TSA medium. BA plates prepared from NA & BAB with ShB showed the most obvious and accurate hemolytic patterns when compared to BA plates prepared from NA & BAB with HuB [as shown in Figure 1-B and 1-F]. We also found that washing of blood prior to addition in the media made the lighter color of BA as compared to use of unwashed blood. Lighter color of media leads to wider zone of hemolysis and poor growth and colony characteristics (very small pinpoint colonies) as compared to unwashed blood [as shown in Figure 1].

Growth and colony characteristics of *Streptococcus pneumoniae* ATCC® 49619™ on different blood agar media:

Moderate to good growth with dome shaped and more mucoid colonies without any hemolysis on BA plates prepared from NA and TSA with unwashed HuB when incubated at ambient air was observed [as shown in Figure 1-A and 1-C].

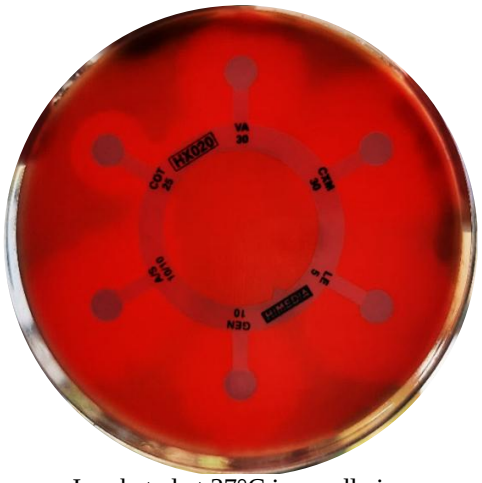
Luxuriant growth of flat with a central depression (doughnut shape) colony with α-hemolysis developed on BA plates prepared from NA and TSA with unwashed ShB irrespective of incubation condition [as shown in Figure 1-B and 1-D].

α-hemolysis is very clear on the BA plates prepared from NA with washed HuB and ShB irrespective of presence of CO_2 during incubation time [Figure 1-A and 1-B]. It was difficult to observe α-hemolysis on BA plates prepared from TSA with and without washing of HuB and ShB when incubated at 37 °C in candle jar due to dark coloration of medium [Figure 1-C and 1-D]. Luxuriant growth with medium size α-hemolytic colonies having typical doughnut shape appearance were grown on the BA plates prepared from BAB with washed and unwashed HuB and ShB irrespective of presence of CO_2 during incubation period [as shown in Figure 1-E and 1-F]. We observed that washing of blood resulted in impairment of microbial growth as very small pin point bacterial colonies grown on BA prepared from washed blood as compared to medium sized colonies grown on BA prepared from unwashed blood.

We found that Zone of Inhibition (ZoI) in antimicrobial susceptibility test was very clear and sharp when the BA plates incubated in candle jar, this is because the presence of CO_2 enhances the hemolysis process. So, result interpretation will be easier and clearer due to greenish coloration of medium (α-hemolysis) [as shown in Figure 2]. On the other hand, we noted that ZoI is decreased on the BA plates when incubated in candle jar as compared to ambient air. The same is applicable for the Optochin test [as shown in Figure 1].

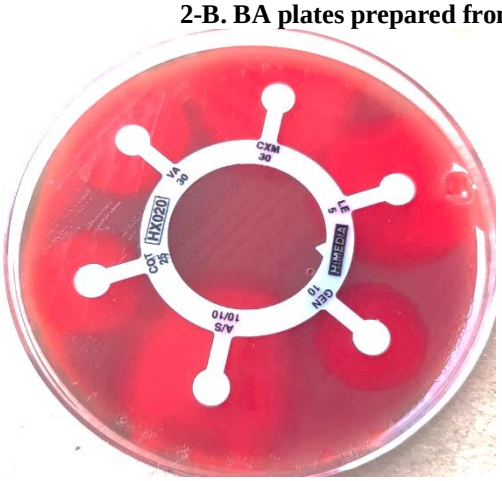
2-A. BA plates prepared from BAB with HuB without wash

Incubated at 37°C in ambient air



Incubated at 37°C in candle jar

2-B. BA plates prepared from BAB with ShB without wash



Incubated at 37°C in ambient air

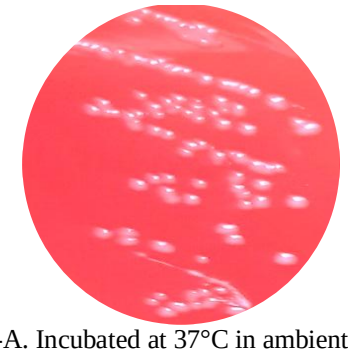
Incubated at 37°C in candle jar

Figure 2. Antimicrobial susceptibility test results of *S. pneumoniae* ATCC® 49619™ on BA prepared from BAB. *2-A.* HuB without wash and *2-B.* ShB without wash

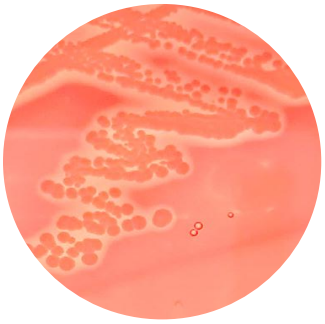
Growth and colony characteristics of *Streptococcus dysgalactiae subsp. equisimilis* on different blood agar media:

Streptococcus dysgalactiae subsp. equisimilis gave no hemolysis on all BA plates prepared from NA, TSA and BAB with unwashed HuB and ShB and when incubated at ambient air. Whereas the same organism gave clear cut β-hemolysis on all the BA plates prepared from NA, TSA and BAB with unwashed HuB and ShB but when incubated in candle jar. One of such result with BA plate prepared from BAB and unwashed HuB was shown in Figure 3.

This *Streptococcus dysgalactiae subsp. equisimilis* gave very clear β-hemolysis on all the BA plates prepared from washed ShB and HuB, no matters whether they were incubated in candle jar or at ambient air.



3-A. Incubated at 37°C in ambient air



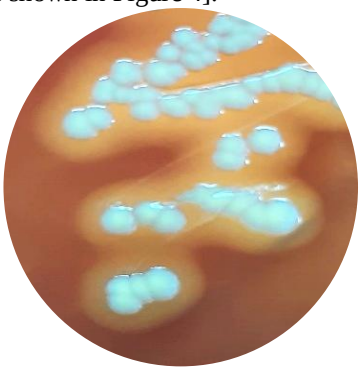
3-B. Incubated at 37°C in candle jar

Figure 3. Growth and colony characteristics of *Streptococcus dysgalactiae subsp. equisimilis* on BA prepared from BAB and HuB without washing

Growth and colony characteristics of *Staphylococcus aureus* on different blood agars media:

Luxuriant growth of big golden yellow color pigmented colonies of *S. aureus* developed on all the BA plates (NA, TSA and BAB) prepared from unwashed as well as washed HuB and ShB. *S. aureus* gave no hemolysis on all the BA plates prepared NA & TSA from unwashed HuB and ShB when incubated at ambient air. Whereas the same organism gave clear cut β-hemolysis on all the BA plates (NA, TSA and BAB) prepared from unwashed HuB & ShB but when incubated in candle jar [as shown in Figure 4].

4-A. Incubated at 37°C in ambient air



4-B. Incubated at 37°C in candle jar

Figure 4. Growth and colony characteristics of *Staphylococcus aureus* on BA prepared from BAB and HuB without washing

This *S. aureus* gave very clear β-hemolysis on all the BA plates prepared from washed HuB and ShB, no matters whether they were incubated in candle jar or at ambient air. So, it is concluded that presence of CO_2 enriched environment as well as washing of blood enhances the production of hemolysin.

IV. Discussion

This research investigated the most suitable medium for preparation of BA for the easy and accurate diagnosis of *S. pneumoniae* in routine microbiology laboratory. It was found that the physical appearance, including intensity of color and opacity of washed and unwashed HuB and ShB at the same blood concentration were different with different types of media used to prepare BA.

We found that BA plates prepared from TSA has a dark red and turbid appearance which interfered in result interpretation of hemolysis pattern as well as AST and Optochin test. Whereas the BA plates prepared from NA and BAB have the physical appearance just like the BA plates available on commercial basis. But if we compare NA and BAB, then luxuriant growth along with other typical colony characteristics were observed on BA plates prepared from BAB. Presence of CO_2 enhances the growth characteristic and hemolysis irrespective of washed and unwashed HuB and ShB.

From our data, we recommend that BA prepared from BAB and HuB incubated in candle jar can give results equivalent to BA plates prepared from ShB, and it also support bacterial growth that results in true and clear hemolysis which is very important for accurate diagnosis of human pathogens mainly *S. pneumoniae* in microbiological laboratory. In addition to this, we also recommend to incubate the BA plates at 37°C in CO_2 incubator or in candle jar to get the true hemolysis pattern.

Our observations that washing of blood can make reading of hemolysis very easy are similar to those reported by Niyomdecha *et al.,* 2016, who also noted that washing of blood enhances the hemolytic patterns.[1] But growth of organisms is suppressed as compared to unwashed blood [1]. *Streptococcus spp.* used in this study displayed decrease in the size of colony and size of the hemolytic zone and/or sharpness of the zone edge on the different BA plates when prepared from washed HuB and ShB. Our findings that ShB can enhance the growth and size of colony as compared to HuB were supported by the findings of Russell *et al.,* (2006) but inconsistent with Egwuata *et al.,* (2014) who reported that no significant difference due to the use of ShB & HuB [6, 19]. Colonies of *S. pneumoniae* were dome shape and more mucoid when incubated at ambient air compared to colonies on all different types of BA were flat with a central depression when incubated in candle jar *i.e.*, CO_2 enriched atmosphere. CO_2 enriched atmosphere also enhances the hemolytic enzyme production. Maximum growth of *S. pneumoniae*, *Streptococcus dysgalactiae subsp. equisimilis* and *S. aureus* with best typical colony characteristics like doughnut shape appearance with prominent α-hemolysis (in case of *S. pneumoniae*), β-hemolysis (in case of *Streptococcus dysgalactiae subsp. equisimilis*) and β-hemolysis along with golden yellow pigments were observed on BA prepared from BAB with washed and unwashed HuB and ShB with CO_2 enriched environment during incubation period.

V. Conclusion

The described findings have profound implications for developing countries where it is difficult to procure ShB to prepare BA. So, an easily applicable BA can now be prepared from BAB with HuB for easy and accurate diagnosis of *S. pneumoniae*. As mentioned before, BA containing animal blood is not a feasible option in many developing countries. On the other hand, experts have so far considered HuB unsuitable for the preparation of blood agar. Our studies show that BA prepared from BAB and citrated HuB when incubated in candle jar is an acceptable alternative for the isolation and identification of *Streptococcus pneumoniae*.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.
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References

[1]. Niyomdecha N, Phakamas W, Nana A. Modified Human Blood Agar as Substitute for Sheep Blood Agar in Laboratories of Developing Countries. J Pure Appl Microbiol 2016; 10:1773-9.
[2]. Satzke C, Seduadua A, Chandra R, Carapetis JR, Mulholland EK, Russell FM. Comparison of citrated human blood, citrated sheep blood, and defibrinated sheep blood Mueller Hinton agar preparations for antimicrobial susceptibility testing of *Streptococcus pneumoniae* isolates. J Clin Microbiol 2010; 48: 3770-2.
[3]. Magbojos CR, Aro RS, Caringal MCS, Castillo MM, Llanes DA, Sumaray KD. Preparation of the blood-enriched agar with the use of red cell suspension. Asian Journal of Health 2011;1:259-75.
[4]. Ellen Y, Benjamin AP, Niaz B, Ellen JB. Hair sheep blood, citrated or defibrinated, fulfills all requirements of blood agar for diagnostic microbiology laboratory tests. PLoS ONE 2009;7:1-8.
[5]. Schoenian & Susan. A beginner's guide to raising sheep. 2007. Available: http://www.sheep101.info/201/about.html.
[6]. Russell FM, Biribo SSN, Selvaraj G, Oppedisana F, Warren S, Seduadua A, Mulholland EK, Carapetis JR. As a bacterial culture medium, citrated sheep blood agar is a practical alternative to citrated human blood agar in laboratories of developing countries. J Clin Microbiol 2006; 44: 3346-51.
[7]. Notter DR. Potential for Hair Sheep in the United States. J Animal Sci 2000;77(E-Suppl), 1. doi:10.2527/jas2000.77e-suppl1h.
[8]. Hogman CF, Meryman HT. Storage parameters affecting red blood cell survival and function after transfusion. Transfus Med Rev 1999;4:275-96. doi: 10.1016/s0887-7963(99)80058-3.
[9]. Johnson DR, Kaplan EL, Sramek J, Bicova R, Havlicek J, Havilickova H, Moliova J, Kriz P. Laboratory diagnosis of group A streptococcal infections. World Health Organization, Geneva, Switzerland 1996.
[10]. Gratten M, Battistutta D, Torzillo P, Dixon J, Manning K. Comparison of goat and horse blood as culture medium supplements for isolation and identification of *Haemophilus influenzae* and *Streptococcus pneumoniae* for upper respiratory tract secretions. J Clinc Microbiol 1994;11:2871– 2.
[11]. Niederstebruch N, Sixt D, Benda BI, Banboye N. A suitable blood agar containing human blood especially for the use in laboratories of developing countries. J Infect Dev Ctries 2017;5:399-406. doi:10.3855/jidc.8957.
[12]. Niederstebruch N, Sixt D. Standard Nutrient Agar 1 as a substitute for blood-supplemented Müller–Hinton agar for antibiograms in developing countries. Eur J Clin Microbiol Infect Dis 2012;32:243. DOI 10.1007/s10096-012-1792-6.
[13]. Phillips CA. The effects of citric acid, lactic acid, sodium citrate and sodium lactate, alone and in combination with nisin, on the growth of Arcobacter butzleri. Lett Appl Microbiol *1999;*29:424–8. https://doi.org/10.1046/j.1472-765X.1999.00668.x.
[14]. Young KM, Foegeding PM. Acetic, lactic, and citric acids and pH inhibition of Listeria monocytogenes Scott A and the effect on intracellular pH. J Appl Bacteriol 1993; 74:515–20.
[15]. Anand C, Gordon R, Shaw H, Fonseca K, & Olsen M. Pig and Goat Blood as Substitutes for Sheep Blood in Blood-Supplemented Agar Media. J Clin Microbiol 2000;2:591–4.
[16]. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. Color atlas and textbook of diagnostic microbiology, 5th edn. Lippincott-Raven Publishers, Philadelphia, Pa; 1997. p. 363–393.
[17]. Hoppe JE, Schlagenhauf M. Comparison of Three Kinds of Blood and Two Incubation Atmospheres for Cultivation of Bordetella pertussis on Charcoal Agar. J Clin Microbiol 1989;9:2115-7. http://apps.who.int/iris/bitstream/handle/10665/41879/9241544953_eng.pdf?sequence=1
[18]. Murray PR, Baron EJ. Manual of Clinical Microbiology, 9th edn. Washington, D. C.: ASM Press. 2007.
[19]. Egwuatu TO, Ongunsola FT, Okodugha IM, Jide B, Arewa DG, Osinupebi OA. Effect of blood agar from different animal blood on growth rates and morphology of common pathogenic bacteria. Adv Microbiol 2014;4**:**1237-41.