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MICROBIOLOGY

Evaluation of the feasibility for replacing sheep blood with human blood in culture media used in microbiological diagnostics

CARLA F. MIGUEL & CHRISTIANE C. PEREIRA

Abstract: The present study aimed to suggest the replacement of animal blood with human blood in culture media, involving alternative methods and ethical considerations, such as animal welfare, in addition to potential laboratory cost reduction. Characteristics of growth and hemolysis development were compared in different culture media, using both sheep blood and human blood. Blood types from the ABO blood group system were tested, and commercially acquired sheep blood agar was used for comparison. Bacteria of the genus *Streptococcus* spp., *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli* were tested. It was observed that growth in media with type A and O positive blood showed closer similarities to those performed in agar with sheep blood. Depending on the bacterial species, the results were either more positive or not, with faster-growing and less demanding bacteria showing better results than, for example, *S. pneumoniae*, which demonstrated difficulty in the growth process and hemolysis generation in human blood agar. The research suggests that in some situations, sheep blood could be replaced, especially when the goal is growth and isolation, but may not be as suitable when the objective is to analyze hemolysis or when the studied species is demanding.

Key words: Culture medium, hemolysis, human blood agar, microbiological diagnosis, sheep blood.

INTRODUCTION

One of the primary procedures in microbiology laboratories is the use of culture media for the growth of microorganisms, with the purpose of identification and diagnosis. Culture media are chemical preparations that can be applied towards different objectives depending on the sample that is inoculated into them. They are of utmost importance for laboratories since they provide the water and nutrients necessary for the growth of bacteria (Niederstebruch et al. 2017). One of the most used culture media in diagnostic laboratories is blood agar, a non-selective medium enriched with blood, which can be derived from sheep, horse, or rabbit (Murray et al. 2020).

It can be observed that currently, various scientific researches rely on methods involving experimenting on animals in order to improve human's life quality. However, environmental and animal preservation are indisputably important factors to be considered in these studies, given that the procedures used can be uncomfortable for animals. Animals are subjected to invasive methods that can lead to contamination and infections (Vicente & Costa 2014).

Moreover, scientific alternatives that replace using animals in experiments can provide relevant and scientifically valid results, potentially reducing or even eliminating the

need for animal blood. Additionally, there are biological differences between species that can lead to results not directly applicable to humans, limiting the use of animals in experiments (Guimarães et al. 2016).

Furthermore, it is also worth noting the costs incurred by clinical microbiology laboratories that use animal blood to produce culture media for microorganism growth. In 2005, Freitas pointed out that a small clinical analysis laboratory conducting diagnostic cultures, has an average cost of approximately R$ 670.65 for the total inputs used in manual cultures within a month. This amount solely represents the cost of results obtained from the use of sheep blood agar and chocolate agar. The average cost per patient for each analysis is R$ 4.91. Nowadays, it is anticipated that these values may be even higher.

Therefore, research about replacing sheep blood with human blood aims for practicality and cost-efficiency in laboratories, while taking into account ethical considerations. It also seeks results equal or similar to those obtained with the use of animal blood in culture media, while still considering the quality and safety of microbiological diagnostics (Leal et al. 2006). For these reasons, it is of great importance to research the feasibility of replacing sheep blood with human blood for the production of blood agar culture media.

The project aimed to analyze the feasibility of substituting sheep blood with human blood in the preparation of blood agar, used in clinical analysis laboratories, undergraduate practical classes, and scientific research. Additionally, the project aimed to observe the growth of certain bacteria regularly cultured on this culture medium and their respective hemolysis, comparing them to the gold standard, sheep blood agar, and testing different types/groups of human blood for possible differences in hemolysis and bacterial growth.

MATERIALS AND METHODS

The research was conducted in the laboratories of Centro Universitário Salesiano - Unisales, in Vitória - ES, between August 2022 and July 2023. For the purpose of obtaining authorization to collect blood from voluntary human donors, the project was submitted to the research ethics committee (CEP) through the Plataforma Brasil, receiving approval and CAAE under the number 62806322.2.0000.5068.

Donors were selected and entered the study only after signing the informed consent form (ICF). The following conditions were followed for volunteer recruitment: being in the age range of 18 to 42 years, having good health conditions, and not using medications that could affect blood cells. In total, 10 volunteers met all the requirements and participated in the donation.

Standardizations and protocol assembly were carried out based on other similar articles and modules and manuals from the Brazilian Health Regulatory Agency - ANVISA (ANVISA 2004, 2010, 2020, Oktari et al. 2019, Russell et al. 2006, Niederstebruch et al. 2017, Niyomdecha et al. 2016).

The human blood samples tested included blood types A, B, AB, and O, with Rh-positive factor, and in the case of type O, Rh-negative donors were also used. Human blood was collected in two tubes, each containing 3.65ml, with 3.2% sodium citrate anticoagulant. After venipuncture in the volunteer, blood typing was performed through capillary blood sampling, using a set of reagents (Lorne Laboratories Limited/ Kovalent Do Brasil LTDA) to confirm the donor's blood group. Blood collections were carried out and processed by the authors and undergraduate research students.

Two groups of tests were conducted. The first test included all the mentioned blood types, while in the second test, there was a reduction in blood types, retaining only A+ and O+ types. The exclusion of B+, AB+, and O- types occurred due to the observation of slow growth and hemolysis that only occurred after a minimum of 72 hours of incubation, which is not common in clinical practice. In other words, with 24-48 hours of incubation, alpha and beta hemolysis would be interpreted as gamma hemolysis.

For comparison purposes, Columbia sheep blood agar 5% purchased in ready-made plates manufactured by Plast Labor Indústria e Comércio de Equipamentos Hospitalar e Laboratório LTDA was used as the gold standard. The medium contains sheep red cell supplementation at 50 ml/L, a mixture of peptones at 23.0 g/L, starch at 1.0 g/L, sodium chloride at 5.0 g/L, and agar at 12.0 g/L. Due to the presence of intact red blood cells, the medium was stored at a temperature of 2 to 15°C, as per the manufacturer's instructions.

The production of the culture medium in the laboratory with human blood followed the recommendations of the manual of the ANVISA (2004) and was carried out immediately after venipuncture, enriching the agar with 5% citrated human blood. Columbia Agar Base medium, manufactured by Kasvi Importação e Distribuição de Produtos Para Laboratórios LTDA, was used, consisting of pancreatic digest of casein at 10.0 g/L, peptic digest of meat at 5.0 g/L, yeast extract at 5.0 g/L, sodium chloride at 5.0 g/L, pancreatic digest of heart at 3.0 g/L, cornstarch at 1.0 g/L, and agar at 13.0 g/L. The choice of this base was made in an attempt to closely approximate the composition of the sheep blood agar culture medium that was purchased and used as the gold standard for comparison. Subsequently, preparation was carried out following the manufacturer's instructions, and after dissolution, the medium was sterilized using an autoclave (Phoenix Indústria e Comércio De Equipamentos Científicos LTDA) and then cooled to 47°C using a water bath (Nova Ética Produtos e Equipamentos Científicos LTDA). Once the ideal temperature was reached, 5 ml of human blood were added to 100 ml of agar, homogenized, and poured into sterile plates in a controlled environment through a laminar flow chamber (Quimis Aparelhos Científicos LTDA).

The bacterial strains used for seeding and growth and hemolysis verification were from the Coleção de Cultura Cefar Diagnóstica (CCCD), including *Staphylococcus aureus* CCCD S008, *Streptococcus* species *S. pyogenes* CCCD S012, *Escherichia coli* CCCD E004, and *Enterococcus faecalis* CCCD E002. Another strain used was *S. pneumoniae*, isolated from a clinical sputum sample (HUCAM - Hospital Universitário Cassiano Antônio Moraes). These species were chosen because they cause different hemolysis in blood agar culture and due to the frequent clinical use of the hemolysis test for the identification of diseases caused by these bacteria.

After separating the strains for testing, inoculations were made with bacterial suspensions in Brain Heart Infusion Broth (BHI) medium manufactured by Kasvi Importação e Distribuição de Produtos Para Laboratórios LTDA, a nutrient broth that accelerates and aids their growth and development on solid medium, as per the ANVISA (2004) manual. The inoculated samples were then incubated for 24 hours at 37°C in an incubator (Nova Ética Produtos e Equipamentos Científicos LTDA). The inoculation procedure followed the manual prescribed by ANVISA (2010), module 4, using the exhaustion seeding method with sterile disposable loops of 10µl (Cral Artigos Para Laboratório LTDA).

After seeding the blood agar plates with bacterial suspension in BHI, the plates were incubated at 37°C. For facultative anaerobic bacteria (*S. pneumoniae*), an anaerobic jar

connected to a vacuum pump was used. The first reading of results was performed after 24 hours of incubation, and the second reading was conducted after 48 hours of incubation.

The growth of each strain, colony appearance, and types of hemolysis caused in the culture media with sheep and human blood were analyzed after each incubation period, following the ANVISA (2004) manual. The interpretation of results was done as follows: the presence of a transparent halo around the colonies indicated total lysis, indicating beta-hemolysis; the presence of a greenish halo around the colonies indicated partial lysis, indicating alpha-hemolysis; the absence of a halo around the colony indicated no lysis, indicating gamma-hemolysis.

RESULTS AND DISCUSSION

In the first set of tests, all the mentioned blood types were used, and the initial bacteria used for these tests were *Staphylococcus aureus* and *Streptococcus pyogenes*. Regarding growth of both species, it was observed that it was the same for all tested human blood types, including colony size, appearance, and colors similar to those of sheep blood. However, the hemolysis observed in media with blood types of AB, B, Rh-positive, and O, Rh-negative was not as acceptable when compared to bacterial growth in sheep blood medium.

In culture media with blood types B+, AB+, and O-, slow growth was observed, and hemolysis generation occurred only after a minimum of 72 hours, which is not common in clinical practice. Therefore, it is understood that with 24-48 hours of growth, alpha and beta hemolysis would be interpreted as gamma. Additionally, A+ and O+ blood types are the most common and frequently found in Brazil, which would facilitate their use in clinical routine (Beiguelman 2003 cited in Santos et al. 2017). For these reasons, only A+ and O+ blood types were tested in the second round of tests.

In the first round of tests, *S. pyogenes* displayed a significant size, colonies with consistent colors, and standard size for the tested blood types, including those excluded in the second test. However, concerning the incubation time, it was observed that in human blood types of AB+, B+, and O, the species initiated its growth at 48 hours, demonstrating the need for an average of 72 hours for the formation of a hemolysis considered adequate for species identification (Figure 1).

In the first test, *S. aureus* displayed growth after 48 hours of incubation. The observed morphology was considered good, with colonies characteristic of the strain in question, a rounded shape, and whitish colonies. However, despite the good morphology presented, hemolysis was not demonstrated on human blood agar for blood types of AB+, O-, and B+ (Figure 2).

In the second set of tests, blood types A and O, Rh-positive, and sheep blood were used for comparison. Additional bacterial species were introduced in this second set of tests to expand the study, including *Streptococcus pneumoniae*, *Escherichia coli*, and *Enterococcus faecalis*. The growth results obtained from the second test were satisfactory; however, for some species, a longer time was required to visualize the expected hemolysis pattern in agar with human blood.

Regarding the growth of *Escherichia coli*, it was considered the same for all three different blood types, and colonies of the species achieved good development within 24 hours of incubation. Morphological analysis revealed that on sheep blood agar, the colony appeared to be whitish and creamier. The species presented in all three blood types with shiny colonies, creamy consistency, rounded shape, and irregular edges.

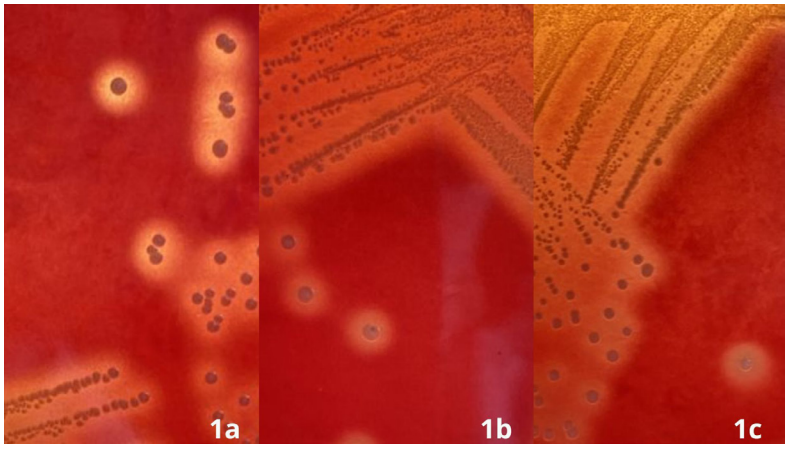

Figure 1. ***S. pyogenes*** **in human blood type AB+ (1a), human blood type O- (1b), and human blood type B+ (1c) after 72 hours of incubation. Despite the demonstration of hemolysis, there was slow growth in the respective blood types shown.**

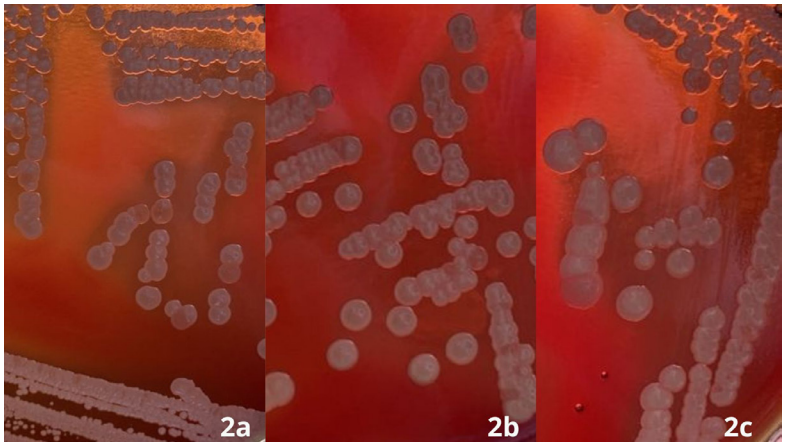

Figure 2. ***S. aureus*** **seeded on human blood agar for blood types of AB+ (2a), O- (2b), and B+ (2c). Growth observed in these specific blood types after 48 hours of incubation, with no hemolysis observed in these tested human blood types.**

Hemolysis was not observed in any of the three types of culture media (Figure 3).

The species *Streptococcus pyogenes* exhibited good growth in all three different blood types; however, it was observed that the incubation time required for colony visualization on agar with human blood was longer, and better results were obtained after 48 hours. The colonies analyzed in the tests using human blood and sheep blood agar exhibited the same morphological characteristics, with circular colonies of small diameter, smooth and shiny surfaces, and a creamy consistency. As expected, the characteristic of this species, beta-hemolysis was observed in all blood types (Figure 4).

Streptococcus pneumoniae, also known as pneumococo, exhibited a different growth pattern compared to other species due to its facultative anaerobic nature. Due to being a species with higher nutrient requirements, oxygen presence, and growth time, it was observed that the optimal incubation time for better results with human blood was 48 hours. Hemolysis results on sheep blood agar were already visible after 24 hours, but hemolysis became more pronounced after 48 hours. Colonies displayed distinct characteristics in some aspects, such as greater opacity on sheep blood agar. However, it was noted that the colonies on all agar types were matte and of similar sizes. The characteristic morphology of this species, with flattened and umbilicated colony appearance due to autolysis, was not observed. The characteristic alpha-hemolysis of pneumococo was visible in all three blood types, although it appeared to be less pronounced on

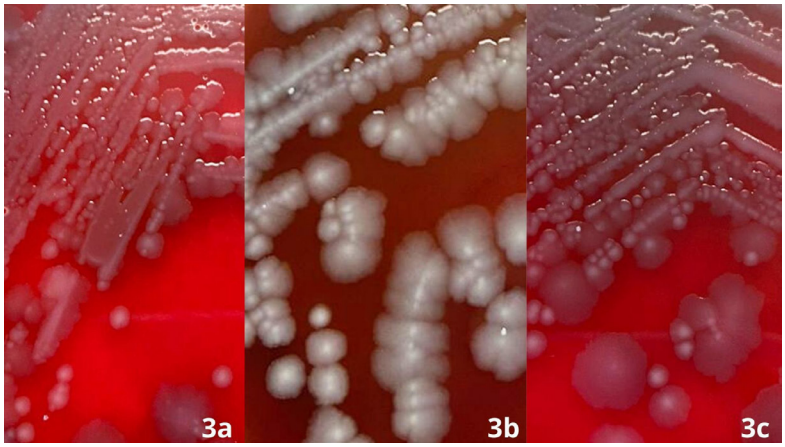

Figure 3. ***E. coli*** **seeded on human blood agar type O+ (3a), sheep blood agar (3b), and human blood agar type A+ (3c). Growth observed after 24 hours of incubation. Morphologically similar colonies are observed in all three images, as well as the absence of hemolysis in all three growths.**

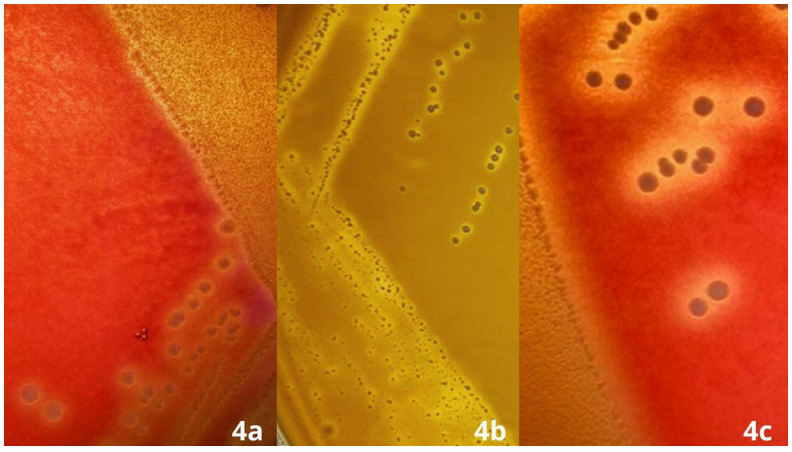

Figure 4. ***S. pyogenes*** **respectively on human blood agar type O+ (4a), sheep blood agar (4b), and human blood agar type A+ (4c), growth recorded after 48 hours of incubation. Observation of beta-hemolysis in all blood types.**

human blood agar compared to sheep blood agar (Figure 5).

Being a facultative anaerobic species, *S. pneumoniae* growth becomes more specific and demanding than other bacteria used in the study. It is believed that this fact contributed to the difficulty in visualizing alpha-hemolysis on human blood agar compared to sheep blood agar.

The species *Enterococcus faecalis* managed to achieve good growth with numerous colonies in all three different blood types. However, in terms of incubation time, human blood required 48 hours to exhibit considerable hemolysis for a proper diagnosis. The colonies analyzed on all three blood types were shiny, small in diameter, well-rounded, and creamy in consistency. An important observation is that the hemolysis patterns were different, with alpha-hemolysis on sheep blood and beta-hemolysis on human blood (Figure 6).

For *Enterococcus faecalis,* the differentiated hemolysis patterns in human blood, presenting as alpha-hemolysis or no hemolysis in sheep blood, and beta-hemolysis when using human, horse, or rabbit blood, have been previously documented in the literature (Trabulsi & Alterthum 2008). Therefore, the results of the current research corroborate and confirm previous studies on the growth of this species.

Staphylococcus aureus grew adequately and well in all types of blood agar with large quantities of colonies, which is a good result. Regarding the incubation time, growth was achieved in 24 hours in all three different blood types, but the expected colony size for

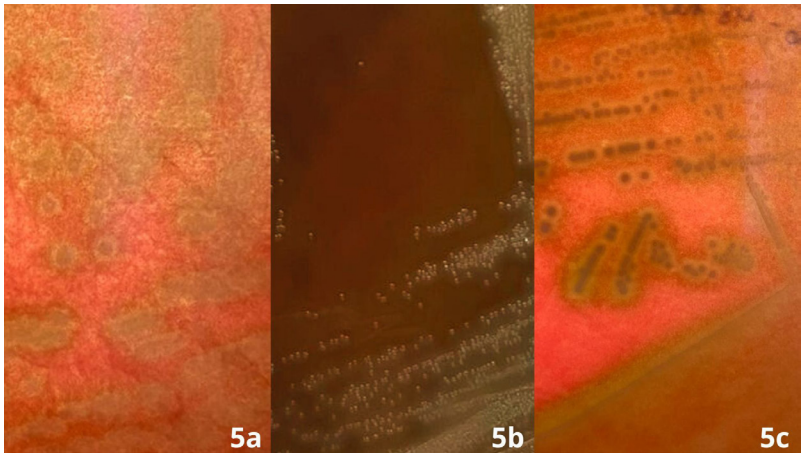

Figure 5. ***S. pneumoniae*** **respectively on human blood agar type O+ (5a) with 48 hours of incubation, sheep blood agar (5b) with 24 hours of growth, and human blood agar type A+ (5c) with 48 hours of growth. It is observed that the hemolysis presented in the three different blood types is alpha-hemolysis.**

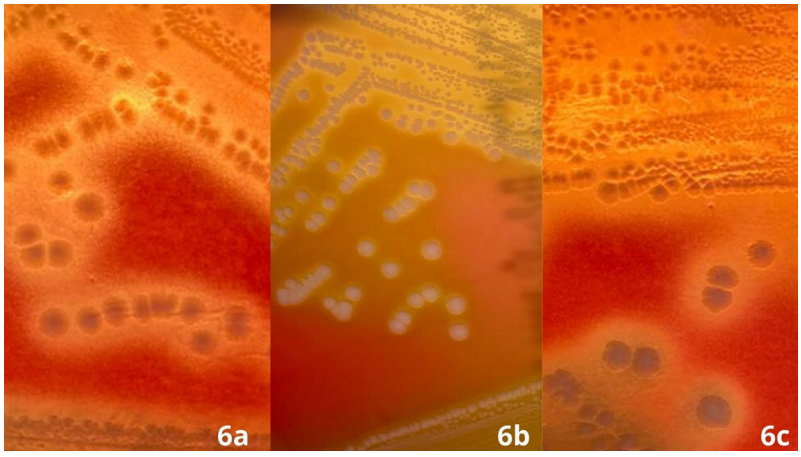

Figure 6. ***E. faecalis*** **respectively on human blood agar type O+ (6a) with 48 hours of growth; sheep blood agar (6b) with 24 hours of growth; and human blood agar type A+ (6c) with 48 hours of growth. It is observed that sheep blood agar displays alpha-hemolysis, while both types of human blood agar exhibit beta-hemolysis.**

human blood was only reached after 48 hours of incubation. In terms of morphological aspects, the results were similar in all three cases, with colonies appearing shiny, creamy, and golden yellow. As for the expected hemolysis, beta-hemolysis was observed in sheep blood and human blood type A, but no hemolysis occurred in human blood type O (Figure 7).

Despite not observing hemolysis on human blood agar type O+, AB+, B+ and O- the growth was quite significant and valid. Therefore, it can be suggested that, for this species, human blood agar may serve for isolation but not as the primary diagnostic medium in clinical practice with hemolysis observation. Other tests such as Gram staining, catalase, and coagulase are recommended for confirming the identification of *S. aureus* in such cases.

Analyzing the presented results, it can be concluded that the use of blood agar supplemented with citrated human blood can be an alternative for clinical identification of bacteria like *E. coli*, *S. pyogenes*, *E. faecalis*, and *S. aureus*. This is because these are simpler bacteria with easy growth adaptation to culture media, demonstrating hemolysis of some of the species on the tested blood agar.

Nevertheless, for the isolation and identification of *S. pneumoniae*, the use of human blood is not recommended based on the results obtained in the present study. However, further studies are suggested using a standard strain, similar to the others tested in the research, as the use of a clinical sample strain isolated about two years ago may have influenced the results. Additionally, a new test

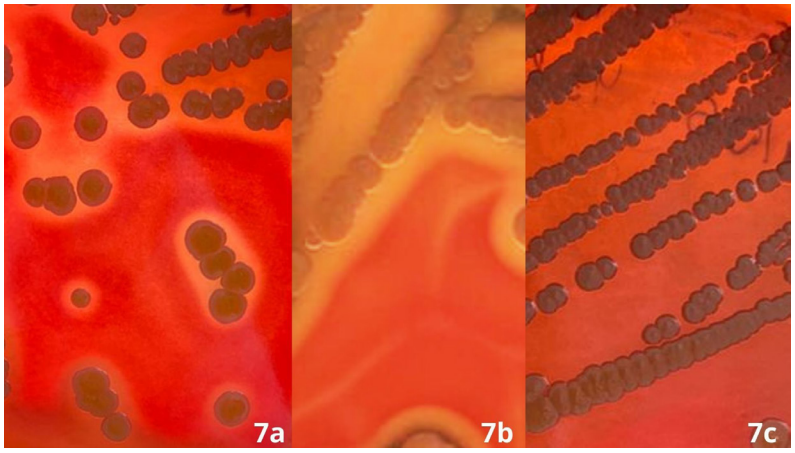

Figure 7. ***S. aureus*** **seeded on human blood agar type A+ (7a), sheep blood agar (7b), and human blood agar type O+ (7c). In sheep blood agar, expected growth is observed after 24 hours, while in human blood agar, it takes 48 hours of incubation. The colonies are morphologically similar, but hemolysis is only presented in sheep blood agar and human blood agar type A+.**

is also suggested by modifying the growth environment, using an anaerobic jar that provides 5% CO2, as the results may differ (Oktari et al. 2019). Considering that the characteristic morphology of this species, with flattened and umbilicated colony appearance due to autolysis, was not observed, it is believed that this result could also be different if the growth environment includes 5% CO2 (Carvalho 2022).

In this study, venous human blood collected in tubes containing citrate as an anticoagulant was used. In other literature publications with the same purpose of validating human blood agar, blood bags are typically used for the test (Russell et al. 2006). Nevertheless, it is believed that the results can be compared since citrate of sodium serves as an anticoagulant in both blood bags and tubes.

In the tests conducted in this study, total human blood was used without separating its constituents, unlike sheep blood used in preparing standard media, where only red blood cells are included. Despite this, it was possible to observe that some species exhibited good hemolysis, but not all. Suggestion for future research would be to test human blood that has been defibrinated, like what is done with sheep blood. However, studies have shown that citrated sheep blood agar offers the same results as defibrinated sheep blood (Russell et al. 2006). This could also be the case with human blood, meaning that defibrinated blood may not be superior for diagnosis compared to blood collected with citrate.

It is believed that the defibrination process may not be practical for most microbiology laboratories, which require faster, easier, and more cost-effective procedures. Considering this reality in clinical laboratories, human blood agar is seen as a viable option for everyday clinical use in some cases, potentially saving costs for the laboratory. As mentioned in the introduction, there is an average cost of supplies used in a microbiology laboratory. The total cost of supplies using agar supplemented with sheep blood is R$ 670,65, a significant expense, especially considering that blood agar is not the sole diagnostic method used in microbiology laboratories.

It should also be considered the use of human blood by laboratories and the associated risk of this process due to potential infectious agents that may be present in these blood samples. Therefore, to avoid increasing the laboratory costs with pre-tests, it is suggested that the use of human blood for culture medium production should originate from blood bags in blood centers that are not in use, as they are already routinely tested after donation.

Analyzing the growth time, a crucial factor in clinical diagnosis and result reporting, many bacteria required a longer incubation time for more visible hemolysis in human blood tested. This could be a disadvantage for diagnostic laboratories. One possible explanation is the differences in the constitution of ovine (sheep and lamb) blood compared to human blood. The literature reports that the number of erythrocytes in ovine blood can range from 9.0 to 15 million per microliter of blood, while in human blood, the average value is between 4.5 and 5.1 million per microliter of blood. The average hemoglobin value is the same for both sheep and humans, ranging from 9.0 to 15.0 g/dL for sheep and between 13.2 and 14.9 for humans. Additionally, the lifespan of erythrocytes is similar, with sheep having a lifespan of 125 to 150 days and humans 120 days (Estefania 2018, Silva 2016, Rosenfeld et al. 2019). Furthermore, there is also reported variation in red blood cell size between the two blood types, which could potentially influence bacterial growth in these media (Yeh et al. 2009).

In conclusion, this study shows that for simpler and less demanding bacteria, the future use of agar made with human blood is possible, with types A and O seemingly performing better for growth and hemolysis observation. However, for more demanding bacteria, it may not be recommended. On the other hand, if the procedure aims solely at bacterial growth and isolation, the use of human blood instead of sheep blood is valid.

It could be suggested the conduction of tests to verify bacterial growth in culture media with human blood agar preserved with other anticoagulants different from the citrate used in this study. However, previous research has already shown that procedures using the preservatives EDTA and heparin did not yield satisfactory results, especially when considering the possibility for these substances to inhibit various bacterial species growth. Furthermore, it was also indicated that anticoagulants EDTA and heparin can cause hemolysis in agar containing human blood, which would hinder the interpretation of results (Niederstebruch et al. 2017). Additionally, even if the results were promising with the use of other anticoagulants, the advantage of this replacement would have to be evaluated since blood bags generally contain citrate and with this exchange they would become unavailable, perhaps impacting the cost-effectiveness of laboratories (which is one of the desired advantages).

A suggestion to future projects would be to conduct new tests using donated blood bags as a source of blood, as this would be a more feasible and widely accessible way to produce media in laboratories. However, it is essential to remember that even when using preservatives in blood bags, red blood cells tend to undergo morphological, metabolic, and oxidative changes over time, decreasing the stability of these cells. These changes are known as storage lesions (Frank et al. 2013, Lagerberg et al. 2017).

Depending on the composition of the anticoagulant solutions used in blood bags, the expiration period of these may vary, generally being around 21 days and possibly extending to 35 or 42 days (BRASIL 2015). Therefore, it is recommended that the use of donated blood for the preparation of culture media should be done with blood up to 21 days old, with newer blood being even more ideal to avoid storage lesions, which could compromise the use of media in clinical diagnosis.

The use of defibrinated human blood could also be tested for the production of culture media, making this medium more similar to what is currently produced with sheep blood. However, even if good results are obtained, this

may not be a good alternative when it comes to speed and economy in clinical laboratories.

Finally, it would be important to test other types of base agar to be mixed with blood, as it is possible that the base, regardless of the blood, influences the behavior of bacterial growth.

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CARLA F. MIGUEL[1]
https://orcid.org/0009-0009-8608-6530

CHRISTIANE C. PEREIRA[2]
https://orcid.org/0009-0006-2374-184X

[1]Centro Universitário Salesiano, UniSales, Avenida Vitória, 950, Forte São João, 29017-950 Vitoria, ES, Brazil

[2]Coordenadora da Pós-Graduação e Coordenadora do Curso de Farmácia, Centro Universitário Salesiano, UniSales, Avenida Vitoria, 950, Forte São João, 29017-950 Vitória, ES, Brazil

Correspondence to: **Christiane Curi Pereira**
E-mail: christianecuri@gmail.com

Author contributions

Writing and submission of the project to funding institutions, Assembly of protocols, Preliminary tests, Conducting practical experiments, Discussion of results, Editing: Miguel and Pereira. Quoting of inputs, Collection of human blood, Writing of results: Miguel. Request for purchase of inputs, Final review: Pereira.