Indonesian Journal of Medical Laboratory Science and Technology

Open Access

p-ISSN 2684-6748 | e-ISSN 2656-9825

IJMLST

The Potential Use of EDTA as an Alternative to Defibrination in Preparing Blood Agar Plates with Human AB Blood Type on *Staphylococcus aureus* Culture

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Received: February 6th, 2021
Revised: March 8th, 2021
Accepted: March 31th, 2021
Published: April 28th, 2021

DOI: 10.33086/ijmlst.v3i1.1923

Abstract

Blood Agar Plates (BAP) are composed of blood as one of the compositions. Sheep's blood is usually used, but since it is difficult to be obtained, human AB blood type was used as an alternative. In preparing BAP, blood is defibrinated to lyse the blood clotting factors. Blood clots can also be prevented by adding anticoagulants, such as ethylenediaminetetraacetic acid (EDTA). This study aims to investigate the potential use of EDTA as a substitute for defibrination in preparing BAP with human AB blood type. This study employed a completely randomized design with true experimental method using *Staphylococcus aureus* as the sample. The parameters were the number of colonies, types of hemolysis, and hemolysis zone. The results showed that the *S. aureus* grown on BAP with EDTA-human AB blood type was 64 colonies (mean), produced β-hemolytic pattern, and 6 mm hemolytic zone. In contrast, the *S. aureus* grown on BAP with defibrinated human AB blood type showed 82 colonies (mean), β-hemolytic pattern, and 5 mm hemolytic zone. There were significant differences in the number of colonies ($0.000 < \alpha$) and hemolytic zones ($0.02 < \alpha$). However, there was no difference in the hemolysis type (both treatments produced β-hemolysis). EDTA was possible to be used as a substitute for defibrination in preparing BAP to assess the hemolysis type of *S. aureus*, but it might not be able to be used as a benchmark for counting the number of colonies and determining the hemolysis zone of *S. aureus*.

Keywords

AB Blood Type, Blood Agar Plates, Defibrination, EDTA, *Staphylococcus aureus*

INTRODUCTION

Blood Agar Plates (BAP) are universal culture media for growing various bacteria and also can be used as a differential medium to differentiate hemolytic bacteria from non-hemolytic bacteria. In general, BAP contains defibrinated mammalian blood (1). Defibrination refers to the process of removing clotting factors from blood to allow the perfect mixture of blood and medium. Blood clots formation can also be prevented by adding anticoagulants, such as ethylenediaminetetraacetic acid (EDTA) (2). EDTA is frequently used since it is unable to distort blood cells (3).

BAP are also used to differentiate pathogenic bacteria based on the hemolytic patterns (4). Standard BAP are commonly prepared using sheep's blood. However, since ensuring the sterility of sheep venous blood after the collection process is challenging, human A, B, O, or AB blood types can be used as alternatives (1). BAP with human AB blood type offers easier identification of *Staphylococcus aureus* due to its wider hemolytic zone when compared to BAP with other blood types (1).

S. aureus can induce red blood cells (RBC) lysis by four hemolysis types (α, β, γ, and δ). Hemolysis is observed on the appearance of a clear zone around the bacterial colony on the BAP. Hemolysis is caused by a toxin called hemolysin. It is one of the virulence factor of *S. aureus*. It also determines the virulence factor of coagulase positive staphylococci (CPS) and coagulase negative staphylococci (CNS) (5). It plays a role in bacterial invasion and also helps in disengaging from immune response (6).

EDTA has long served as an anticoagulant with competitive advantages as opposed to other anticoagulants. Its major advantage is that it does not promote blood cells distortion and hence an excellent option for most hematological tests (3). To the best of our knowledge, the use of EDTA as a substitute for defibrination in preparing BAP with human AB blood type as culture media for the growth of *S. aureus* has never been conducted. Based on this basis, this study aims to investigate the potential use of EDTA for such usage.

MATERIALS AND METHODS

The materials were Blood Agar Base (Merck) media, human AB blood type, EDTA vacutainer tube, pure culture of *S. aureus*, NaCl 0.9%, and a 0.5 McFarland solution. A completely randomized design was employed in this study. The *S. aureus* pure culture was obtained from the Microbiology Laboratory collection (STIKes Hutama Abdi Husada Tulungagung, Indonesia). Human AB blood type was collected from sterilized venous blood. For the control group, the blood was defibrinated manually using marbles, while for the experimental group, it was directly collected

using an EDTA vacutainer tube (1.8 mg/mL blood). After the collection, the blood was prepared for making BAP. *S. aureus* was inoculated on NaCl 0.9% and the turbidity was matched to a 0.5 McFarland solution for density comparison of a bacterial suspension with 1.5 x 10^8 CFU/mL. T streak method was used to inoculate *S. aureus* from NaCl 0.9% on BAP. *S. aureus* was incubated for 48 hours, then the type of hemolysis was observed by measuring the hemolytic zone. The *S. aureus* colonies were also counted. The results were analyzed using MANOVA with 0.05 significance level.

RESULTS

The colonies of *S. aureus* were round, small, smooth, convex, and white after 48 hours of incubation on the BAP with EDTA-human AB blood type. In contrast, when grown on BAP with defibrinated human AB blood type, *S. aureus* colonies were medium, smooth, convex, and white. The growth of *S. aureus* on the BAP, both with EDTA-human AB blood type and defibrinated human AB blood type, was observed based on three parameters: a) the number of colonies; b) zones of hemolysis; and c) types of hemolysis (Table 1). Analysis using MANOVA for number of colonies and hemolytic zones showed that the P-values were 0.000 and 0.02, respectively.

Table 1. The growth of *S. aureus* on the BAP with EDTA-human AB blood type and defibrinated human AB blood type.

Blood Type	Number of Colonies			Hemolytic Zones (mm)			Hemolysis Types (α, β, γ, or δ)		
	1	2	3	1	2	3	1	2	3
EDTA-human AB Blood Type	62	65	65	6	6	6	β	β	β
Defibrinated Human AB Blood Type	82	84	80	5	5	5	β	β	β

DISCUSSION

Carbohydrates in blood are the main sources of nutrition to support bacterial growth. Blood can boost the growth of certain bacteria (7). Defibrination is only intended to remove blood clotting factors in erythrocytes rather than to eliminate the nutritional content (4). The mean number of *S. aureus* colonies grown on BAP both with EDTA-human AB blood type and defibrinated human AB blood type is depicted in Figure 1.

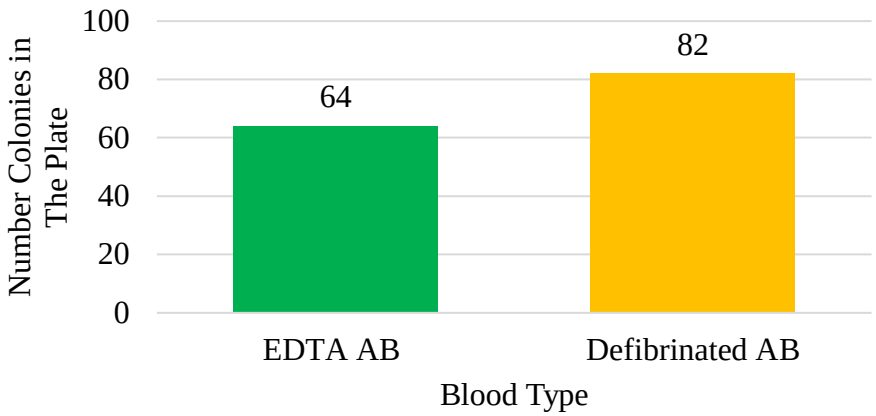

Figure 1. The mean number of *S. aureus* colonies grown on BAP both with EDTA-human AB blood type and defibrinated human AB blood type.

The P-value of number of colonies was 0.000, indicating that there was a significant difference between the number of *S. aureus* colonies that grow on BAP with EDTA-human AB blood type and BAP with defibrinated human AB blood type. The number of *S. aureus* colonies grown on BAP with defibrinated human AB blood type was higher than grown on BAP with EDTA-human AB blood type. This occurs since EDTA has inhibitory power, although it is very low.

In a previous study, the addition of EDTA to egg white lysozyme extract reduced the number of *S. aureus* and *Salmonella typhosa* colonies (8). Na-EDTA combined with nisin at certain concentrations and pH also inhibited the growth of Gram-positive and Gram-negative bacteria (9). Combination of EDTA/tromethamine and oxytetracycline did not significantly affect *Staphylococcus hominis*, whereas EDTA/tromethamine with several other antibiotics had low inhibitory effects on other antibiotic-resistant bacteria (10). EDTA is able to inhibit the growth of *S. aureus* because it can damage the membrane of bacterial cells. Bacterial cell membranes consist of various types of lipids. The types of lipid in each bacteria (even in one species) are different. They differ due to environmental conditions (11). EDTA is believed to have a detergent-like mechanism to the chelating mechanism because the solution of the tetrasodium salt in EDTA can dissolve grease (12,13,14). This alkalinity, however, does not occur in low concentrations (15). This study underlines the abundance of *S. aureus* colonies which were able to grow on BAP containing EDTA since there was only a small amount of EDTA in the blood (1.8 mg/mL blood). The concentration used in this study is extensevily used in clinical laboratories for chemical blood assay.

The mean of *S. aureus* hemolytic zones grown on BAP both with EDTA-human AB blood type and defibrinated human AB blood type is depicted in Figure 2.

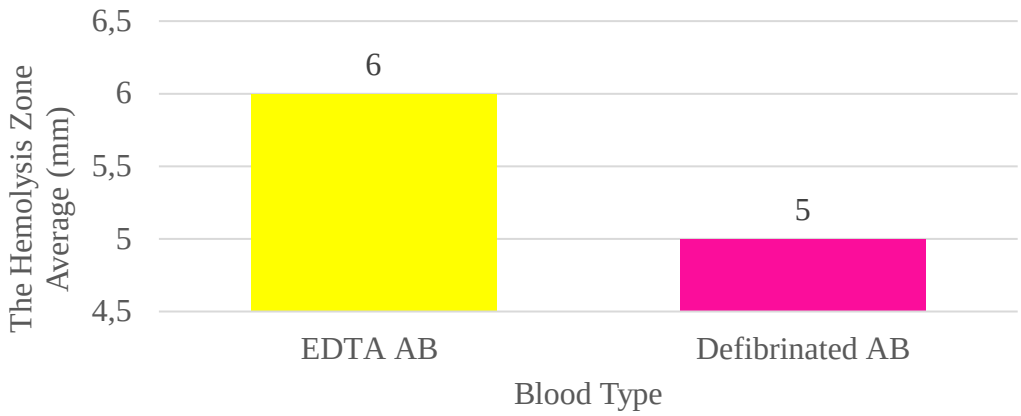

Figure 2. The mean of *S. aureus* hemolytic zones grown on BAP both with EDTA-human AB blood type and defibrinated human AB blood type.

S. aureus colonies grown on BAP with EDTA-human AB blood type showed wider hemolytic zones compared to those grown on BAP with defibrinated human AB blood type. The complete hemolytic zones could only be observed after 48 hours of incubation. A 24-hours incubation did not display any hemolytic zones on all plates. Bacterial colony characteristics on culture media after 48 hours of incubation are easier to distinguish (16). Hemolytic zones are the clear areas around the bacterial colonies that grow on BAP. They are determined by lifting the agar plates to a light source coming from behind (transmitted light), and then the diameter is measured by a ruler in millimeters (17).

The P-value of hemolytic zones was 0.02, indicating that there was a significant difference between *S. aureus* colonies grown on BAP with EDTA-human AB blood type and those grown on BAP with defibrinated human AB blood type. *S. aureus* colonies on BAP with defibrinated human AB blood type grew more than those on BAP with EDTA-human AB blood type. *S. aureus* was able to grow and produce hemolytic zones in both BAP with EDTA-human AB blood type and BAP with defibrinated human AB blood type. Previous study showed that *S. aureus* can grow well in BAP with all of the human blood types (A, B, AB, and O) (1). This is because the morphology and nutrient content in the blood are consistent (18).

Hemolysis type of *S. aureus* grown in BAP both with EDTA-human AB blood type and defibrinated human AB blood type are presented in Figure 3. Both treatments showed the same type of hemolysis which was β-hemolysis. In a previous study, *S. aureus* was able to produce β-hemolysis when grown on BAP with the four human blood types (A, B, AB, and O) (1).

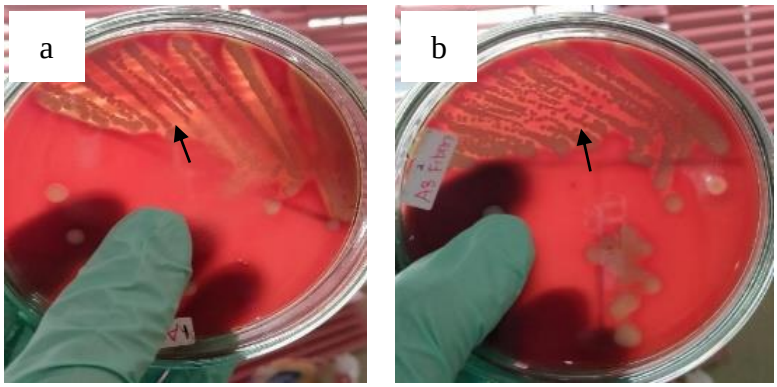

Figure 3. Hemolysis type of *S. aureus* on (a) BAP with EDTA-human AB blood type and (b) with defibrinated human AB blood type.

S. aureus can produce the four types of hemolysis: α, β, γ, and δ. Certain *S. aureus* strains produce β toxin, which is a neutral sphingomyelinase (19). This toxin is produced in large quantities by some strains of *S. aureus* and secreted into culture media as an exotoxin with a molecular weights of 35,000 (20).

β-hemolysis is also referred to as complete hemolysis. β-hemolysin is highly influential on human immunity. Apart from hemolysin, there are other toxins that affect the virulence of *S. aureus* in humans. One of them is Panton-Valentine Leucocidin (PVL), which is a cytotoxin that is highly toxic to human neutrophils (21). Certain strains of *S. aureus* also produce the toxic shock syndrome toxin-1 (TSST-1) which triggers toxic shock syndrome (TSS) (22). Other strains produce additional exoproteins, including TSST-1, staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI), exfoliative toxins (ETA and ETB), and leucocidin (20).

Defibrination requires expertise and timeliness. An error may cause the blood to clot, leading to the imperfection of the media mixture and nutrients damage. EDTA could be used as a substitute for blood defibrination to grow *S. aureus* on BAP since it was able to have *S. aureus* produce the same type of hemolysis in defibrinated blood BAP, but with wider hemolytic zone and less colony growth. Furthermore, EDTA can simplify the process of preparing BAP as defibrination step was eliminated, thereby reducing the error factor in making BAP.

CONCLUSIONS

S. aureus could grow on BAP with EDTA-human AB blood type and defibrinated human AB blood type. EDTA could be used as a substitute for defibrinated blood in the process of making BAP to observe the hemolytic patterns of *S. aureus*, but could not be used as a benchmark for counting the bacterial colonies and measuring the hemolytic zones of *S. aureus*.

AUTHOR CONTRIBUTIONS

Dora Dayu Rahma Turista: conceptualization, methodology, analyzing, writing-reviewing and editing. Eka Puspitasari: data curation, supervision, validation. Fanny Kurnanda: visualization, investigation, validation.

ACKNOWLEDGMENT

Gratitude was expressed to the Departement of Medical Laboratory (STIKes Hutama Abdi Husada Tulungagung, Indonesia) for the support in completing this study.

CONFLICT OF INTEREST

No conflict.

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