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ORIGINAL ARTICLE

COMPARISON OF ANTIBIOTIC SUSCEPTIBILITY TEST ON NUTRIENT AGAR AND MUELLER HINTON'S AGAR

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Abstract

Background: Antibiotic resistance is caused by overuse and misuse of antimicrobial agents and is found out by an antibiotic susceptibility test (AST). For that, Mueller Hinton agar (MHA) is the recommended medium, however, when unavailable, nutrient agar can also be used.

Objective: To compare the efficiency of nutrient agar and Mueller Hinton's agar in determining the zones of inhibition during antibiotic susceptibility test.

Material and Methods: This descriptive study was carried out in 2 months at Khyber Medical University (IPDM, KMU), Peshawar. 10 preserved samples of Streptococcus mutants isolates were cultured by Kirby Bauer method, using nutrient agar and MHA. Antibiotic discs containing amoxicillin, doxycycline, and clindamycin were applied. Post incubation, zones of inhibition (ZOI) of all drugs were checked and compared for nutrient agar and MHA. The results were analyzed using IBMS SPSS, version 22.

Results: Paired T test was performed that showed the mean ZOI for amoxicillin on nutrient agar and MHA as 14.06 and 13.89, respectively. (T value= 1.4, P value =0.19) For doxycycline, ZOI on nutrient agar and MHA were 17.78 and 17.89, respectively. (T value= -1, P value= 0.34). Whereas, clindamycin had mean ZOI on nutrient agar and MHA as 14.50 and 14.56. (T value= -1, P value= 0.35).

Conclusion: Paired T test was performed that showed the mean ZOI for amoxicillin on nutrient agar and MHA as 14.06 and 13.89, respectively. (T value= 1.4, P value =0.19) For doxycycline, ZOI on nutrient agar and MHA were 17.78 and 17.89, respectively. (T value= -1, P value= 0.34). Whereas, clindamycin had mean ZOI on nutrient agar and MHA as 14.50 and 14.56. (T value= -1, P value= 0.35)

Keywords: Antibiotic Susceptibility test, Mueller Hinton's agar, Nutrient Agar, Kirby Bauer method, Antibiotic Resistance .
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Introduction:

Ever since the discovery of penicillin in 1928 by Flemming, antibiotics have been widely used in treating infections caused by bacterial invasion.[1] The term antibiotics is derived from antibiose by Vuillemin that means to eliminate harmful substances particularly microorganisms. Waksman defined antibiotics as chemical substances that are synthesized by microorganisms to inhibit bacterial growth and cause its death.[2] They are the first choice of drugs when treating infectious diseases. From the 1930s to the 1960s, many new antibiotics had been discovered. This period is known as the 'Golden Age of Antibiotics'.[3] These medications act against bacteria by inhibiting the synthesis of cell wall, protein or DNA. They may also disrupt the cell membrane integrity, or inhibit the metabolic pathways. Antibiotics are used in a variety of sectors including human health, where it may be used as therapeutic agents or prophylactic agents.

However, overuse and misuse of antimicrobial agents have paved the way to antibiotic resistance. The emergence of multidrug resistance (MDR) in pathogenic organisms has been one of the major causes of increased morbidity.[4] According to a report in 2019 by the Centers for Disease Control (CDC), two million morbid cases, including 23,000 fatal cases, are caused by antibiotic-resistant pathogens in

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the United States, every year.[5] In 2024, the impact was found to have increased by 20%, which could likely be due to the pandemic of COVID-19. [6]

A bacterial cell employs a variety of mechanisms to resist the effect of antibiotics. The cell membrane of gram negative bacteria containsporin channels that only permit the passage of specific antibiotics like B-lactams and quinolones.By reducing the number of these channels, entry of these antibiotics into the bacterial cell can be inhibited, thus causing resistance.[7] Moreover, there are efflux pumps present in bacterial cell membrane. These pumps take the drugs out of the cell before they have reached their target. Hence, these pumps also contribute to the development of resistance.[8] Furthermore, bacterial cell is also capable of altering the structure of an antibiotic drug, which, ultimately resist its binding to the cell membrane. Another mechanism of antibiotic resistance include changes in the ribosomal 30S and 50S subunits. To study the antibiotic resistance patterns of a pathogen, an Antibiotic Susceptibility Test is used that includes the Kirby Bauer method of disc diffusion and minimum inhibitory concentration (MIC) methods. [10]An Antibiotic Susceptibility Test (AST) is a laboratory technique employed to check if a certain dose of antibiotic is effective against a specific bacterium. A well isolated culture is necessary for these techniques. For this, a particular growth media such as Mueller Hinton's agar (MHA) is used as the recommended media. Bacterial suspension is inoculated onto the plates containing MHA media, and antibiotic discs are added on it. After incubating it, minimum zones of inhibition are measured and compared with CLSI (Clinical and Laboratory Standards Institute) guidelines. However, in case of unavailability of MHA agar, alternate media must be reached for.

This research was designed to evaluate the effectiveness of another growth media such as nutrient agar for AST. Nutrient agar is a non-selective growth media used for cultivation of non-fastidious bacteria that constitutes peptone, beef extract, and agar, along with some vitamins

Material and Method:

Setting and duration: This descriptive study was carried out in 2 months in the year 2024 at Khyber Medical University (IPDM, KMU), Peshawar

Study design: This descriptive study was carried out at the Institute of Pathology and Diagnostic Medicine, Khyber Medical University (IPDM, KMU), Peshawar. This research took almost two months to complete.

Ethical Approval:

Ethical clearance for the study was obtained from the Ethical Review Board of Khyber Medical University (KMU). Ethical approval number: KMU/IPDM/IEC/2024/21

Data collection:

A total of ten preserved samples of *Streptococcus mutans* isolates were taken. They were each cultured initially on nutrient agar plates and confirmed based on morphology. The Kirby Bauer method was followed by using two separate media for the antibiotic susceptibility test. Nutrient agar and Mueller Hinton's agar (MHA) were prepared by following the manufacturer's instructions and poured into culture plates. After solidification, each sample of *Streptococcus mutans* was cultured on Nutrient agar plates and Muller Hinton's agar plates separately in a biosafety cabinet. Antibiotic discs containing amoxicillin (10 µg), doxycycline (30 µg), and clindamycin (2 µg) were applied to each streaked plate. They were then, kept aerobically in an incubator at 37 degrees Celsius for 48 hours. Post incubation, zones of inhibition of all three drugs were checked and compared for nutrient agar and MHA.

Statistical analysis: The results were analyzed using IBMS SPSS software version 22.

Inclusion criteria:

Preserved laboratory isolates of *Streptococcus mutans*

Isolates showing adequate growth on both nutrient agar and Mueller–Hinton agar

Isolates suitable for antibiotic susceptibility testing by the Kirby–Bauer method

Exclusion criteria:

Contaminated bacterial cultures

Isolates showing insufficient or no growth on either medium

Duplicate or damaged samples

Data analysis:

Data were entered and analyzed using IBM SPSS version 22. Descriptive statistics were calculated for zone of inhibition values. Paired t-test was applied to compare the mean zones of inhibition between nutrient agar and Mueller–Hinton agar for each antibiotic. A p-value of ≤0.05 was considered statistically significant.

The zone of inhibition observed for each sample of *Streptococcus mutans* cultured on nutrient agar was compared to those cultured on MHA. (figure:1)

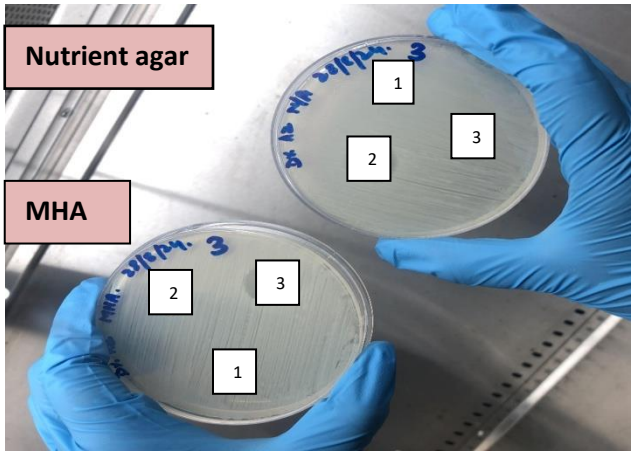

Figure 1: shows the Antibiotic Susceptibility Test done by using Nutrient Agar and Mueller Hinton's Agar

Paired T-test was performed in SPSS version 21, the results of which are depicted in Table no 1.

(1= Amoxicillin, 2= Doxycycline, 3= Clindamycin)

Table 1:Comparison of Zone of Inhibition for Amoxicillin, Doxycycline, and Clindamycin on Nutrient Agar and MHA

Drug	Media used	Mean zone of inhibition (mm)	Standard deviation	Mean difference	T value	P value
Amoxicillin	Nutrient Agar	14.06	6.2	0.17	1.4	0.19
	MHA	13.89	5.9			
Doxycycline	Nutrient agar	17.78	3.6	-0.1	-1	0.34
	MHA	17.89	3.8			
Clindamycin	Nutrient agar	14.50	5.5	-0.2	-1	0.35
	MHA	14.56	5.6			

Table no: 1 shows us that the mean zone of inhibition for amoxicillin on nutrient agar and MHA was 14.06 and 13.89, with a standard deviation of 6.2 and 5.9, respectively. A small insignificant difference of 0.17 was observed between the two media. (T value= 1.4, P value =0.19)

For doxycycline, the mean zone of inhibition on nutrient agar was 17.78, with a standard deviation of 3.6. On MHA, it was 17.89 with a standard deviation of 3.8. This gave us a mean difference of -0.1, which was also found to be insignificant. (T value= -1, P value= 0.34)

Clindamycin had the mean zone of inhibition on nutrient agar and MHA as 14.50 and 14.56, with standard deviations of 5.5 and 5.6, respectively. A small insignificant difference of -0.2 was noted between the two. (T value= -1, P value= 0.35)

This data is also represented in a bar graph. Refer to figure no: 2 given below.

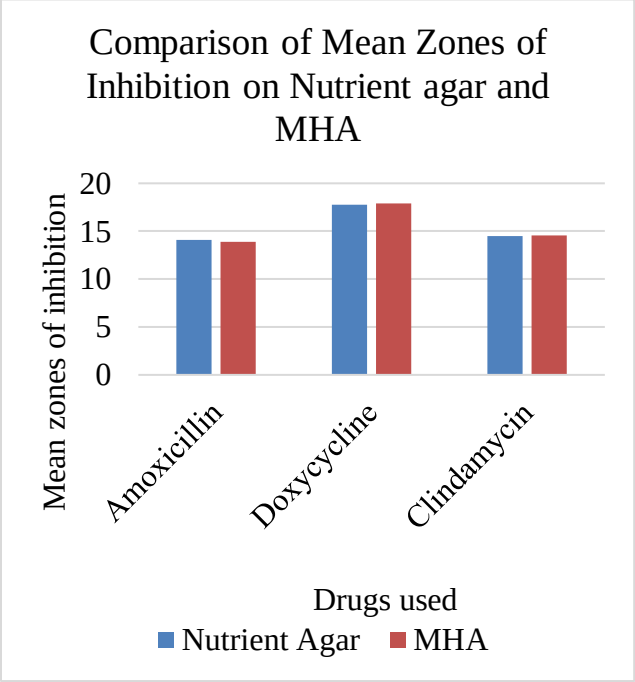

Figure 2: Graphical representation of mean zonesof inhibition on Nutrient agar and MHA.

Discussion:

Antibiotic resistance is a growing global threat especially when it comes to the management of infectious diseases. The overuse and misuse of antibiotics in therapeutics and prophylactic treatments have led to the development of antimicrobial resistance (AMR). AMR is a phenomenon where pathogenic organisms such as bacteria, viruses, fungi, and parasites evolve and become resistant or non-responsive to medications that once successfully treated them. According to Institute for Health Metrics and Evaluation (IHME), 21.36 million people died in 2021due to sepsis, and 4.71 million people out of these suffered from drug resistant infections. It is expected that 39 million people will die from antimicrobial resistant (AMR) if there has been no significant intervention. These staggering statistical values underlines the growing burden caused by AMR worldwide.

In 2024, Niels Hoiby studied microenvironment in antibiotic susceptibility testing in Department of Clinical Microbiology, Denmark. It was found that during AST, bacterial cell transition from planktonic to biofilm mode of growth, which results in the formation of the inhibition zone. The kinetics of the interaction of antibiotics with bacteria are both when antibiotics are administered in a patient, and when AST is performed.[13] Hence, AST is a well-known method used to determine which antibiotics are effective for treating a specific disease.

Since 1944, disk diffusion method, also known as the Kirby-Bauer technique, is the most widely used method for performing antibiotic susceptibility test. This technique plays a crucial role in guiding the physicians about which antibiotics to use for treating a disease for a specific patient. Several guidelines and protocols have been developed for standardization of disk diffusion methods, including specifications for the types of culture media used for AST.Previously bacterial inoculated agar plates are impregnated with antibacterial discs and allowed to incubate at an optimum temperature (usually 35-37 degrees Celsius) for the required amount of time. Subsequently, the diameter of zone of inhibition is measured.[15] Obtained results, then, should be analyzed according to already given guidelines by organizations like the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[16]Moreover, an American study states that while developing an antibiogram, which shows us the cumulative report of the effectiveness of a wide range of antibiotics used for a specific treatment, small differences of a few millimeters in the zones of inhibition should be ignored when deciding if a bacteria is resistant, susceptible or intermediate susceptible. This helps to reduce variability and increases the reliability of the results, which ultimately benefits the treatment outcomes of a patient.

Disc diffusion method for conducting the Antibiotic Susceptibility Test has several advantages, that include ease of performance, low cost and its wide application on various bacteria and a number of antibacterial agents. [18] This method was introduced by Kirby Bauer who gave forth the protocol for utilizing this technique, which inculcates the usage of Mueller Hinton's agar (MHA) as a recommended media for carrying out the AST.[19]

However, when Mueller Hinton's agar is not available, or in case of limited budget, nutrient agar, being an all-purpose media, could also be used for carrying out the AST

Our study shows that Nutrient agar and Mueller Hinton's agar both had the same results when the antibiotic susceptibility test was being carried out. The difference obtained between the mean zones of inhibition was small and insignificant, owing to the T values and P values. Hence, we can either use Nutrient agar or Mueller Hinton agar for finding the antibiotic susceptibility and resistance patterns.

However, according to Mohamed S M Nassar, MHA is the standard medium that should be used for finding the AST and nutrient agar gives multiple errors, making it unreliable.[20] This contradicts our study.

In 2022, Aryal S performed an experiment and found out that Muller Hinton's agar has a loose consistency that results in better diffusion of antibiotics, creating true zones of antibiotics.[21]

On the contrary, a Canadian study used the agar dilution method to find the minimum inhibitory concentration of antibiotics, where antimicrobial substances were directly put into nutrient agar.[22] This was done as nutrient agar is a non-selective media and it supports the growth of various microbes. Hence, it aligns with our study where nutrient agar gave favorable results while determining the antibiotic susceptibility test.

Moreover, Niederstebruch claimed that under controlled conditions, Standard Nutrient Agar 1 (StNA1) could also be used to for developing zones of inhibition and interpretation of antibiogram of Beta hemolytic *Streptococci* in comparison with Müller-Hinton agar that was supplemented with 5 % blood, which was termed blood agar.[23]

Another Australian study by Catherine Satzke compared MHA supplemented with citrated human or citrated sheep blood with routinely used Mueller-Hinton agar supplemented with defibrinated sheep blood in determining the AST for *Streptococcus pneumonia*, and it was found that these alternatatives did not perform that the same levels and should not be used for the AST.[24]

Further advancement and research is being carried out to set research priorities and identify research gaps. World Health Organization (WHO) has encourage research and investment for better understanding of AMR dynamics and also to facilitate policy translation for reduction of the burden and consequences caused by AMR.[25]

Nonetheless, even though Mueller Hinton's Agar is the preferred medium for finding AST, it is possible to use nutrient agar instead of MHA in case of limited resources or budget constraints. This will, in turn, lowers the total expenses of the study as well.

Limitations

However, this research employed only three antibiotics, including amoxicillin, clindamycin and doxycycline, against an alpha hemolytic gram positive bacteria that was *Streptococcus mutans.* Limitations of this study includes requiring to check the effectiveness of using these two media for bacteria other than *Streptococcus mutans* and for a wider range of antibiotics.

Conclusion:

It can be concluded that although Mueller-Hinton Agar is the recommended medium for antimicrobial susceptibility testing (AST), nutrient agar can be used as an alternative in case of the unavailability of MHA.

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