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A COMPARATIVE STUDY BETWEEN AGAR DILUTION METHOD AND E-TEST STRIP METHOD FOR THE DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) TO CONFIRM VRSA AMONG MRSA

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ABSTRACT

BACKGROUND: The rise of Vancomycin-Resistant Staphylococcus aureus (VRSA) among Methicillin-Resistant Staphylococcus aureus (MRSA) strains poses a significant challenge in the medical field. Glycopeptides such as vancomycin are frequently the antibiotics of choice for the treatment of infections caused by methicillin resistant Staphylococcus aureus (MRSA). For the last few years incidence of vancomycin intermediate S. aureus and vancomycin resistant S. aureus (VISA and VRSA respectively) has been increasing in various parts of the world. Accurate estimation of the Minimum Inhibitory Concentration (MIC) is essential for confirming VRSA and guiding appropriate therapy. This study compares the accuracy and usefulness of two popular MIC determination techniques: agar dilution and the E-test strip method.

AIM AND OBJECTIVE: A comparative study between agar dilution method and e-test strip method for the determination of minimum inhibitory concentration (MIC) to confirm VRSA among MRSA. **MATERIAL AND METHODS:** This was a observational Cross sectional study carried out in the Department of Microbiology at Government Medical College, Kota Rajasthan. The samples were processed immediately reaching the lab, in case of delay the samples were refrigerated at 40C. A total of 185 clinical isolates of MRSA were screened out of 384 Staphylococcus aureus isolates which were tested for vancomycin MICs using both agar dilution and E-test strip methods according to the CLSI guidelines.

RESULTS: In the present study Vancomycin screen agar method of the 185 clinical isolates of MRSA was studies out of which 6 (3.24%) were detected as VRSA. Out of the total 185 MRSA isolates there were 02 (1.08%) found to be VRSA, 03 (1.62%) were observed as VISA. The incidence of VRSA was found to be 1.62%. There were 180 (97.30%) found to be VSSA by both agar dilution method and E-test strip method. Compared MIC values of the 185 MRSA determined by both agar dilution method and E-test strip method. In the present study the Agar dilution method was considered as the gold standard for MIC determination. It provides precise and reproducible results but is labor-intensive and time-consuming. E-test strip method offers a moreconvenient and faster alternative. It is easy to perform and interpret but may have variability in results compared to the agar dilution method. **CONCLUSION:** Both agar dilution method and E-test strip method are useful in measurement of MIC values. There is slightly variation in MIC values determined by both agar dilution method and E-test strip method. E-test strip method is easy to perform as compare to agar dilution method. Agar dilution method is a time consuming and labor-intensive but is a satisfactory gold standard method for MIC determination. The selection of the appropriate MIC determination method is vital for the optimal clinical treatment of VRSA infection

KEYWORDS: VRSA, MRSA, MIC determination, agar dilution, E-test strip, vancomycin resistance.

1. INTRODUCTION

In contemporary medical practice, one of the most important concerns is the increasing prevalence of antibiotic resistance among bacterial infections. MRSA, which stands for methicillin-resistant Staphylococcus aureus, has been a tough obstacle for a considerable amount of time owing to the fact that it is resistant to a number of medications, including methicillin. Treatment regimens have become even more challenging as a result of the appearance of vancomycin-resistant *Staphylococcus aureus* (VRSA) in more recent times. Due to the fact that vancomycin has been the foundation for treating MRSA infections, the development of resistance to this antibiotic greatly restricts the treatment choices available and highlights the need of using precise diagnostic methods to identify the presence of VRSA infection [1,2].

The measurement of the Minimum Inhibitory Concentration (MIC) is an essential step in the process of the classification of bacterial isolates as either antibiotic-resistant or antibiotic-susceptible. Agardilution and the E-test strip technique are two prominent procedures that are used to determine the minimum inhibitory concentration (MIC). In the agar dilution technique, which is widely regarded as the gold standard, different quantities of an antibiotic are added to an agar medium, and then the growthof bacteria is evaluated. The approach in question is labor-intensive and time-consuming, despite the fact that it is reliable. The E-test strip technique, on the other hand, provides a more convenient and speedier option. This approach involves applying a gradient of antibiotic concentrations to a strip that is then put on an infected agar plate. The result is a minimum inhibitory concentration (MIC) measurement at the intersection of the bacterial growth inhibition ellipse [1].

In spite of the fact that both approaches are designed to precisely identify the MIC, differences between them might have an effect on therapeutic judgments. The incorrect categorization of VRSA may result in the selection of improper treatments, the subsequent spread of resistant strains, and unfavorable consequences for patients. For this reason, it is very necessary to analyze and contrast the performance of different approaches in order to guarantee accurate MIC determination.

An Overview of the Problem of Antibiotic Resistance The problem of antibiotic resistance has developed into a threat to the health of people all over the world, putting at risk the effectiveness of medications that were formerly able to cure bacterial diseases with remarkable consistency. A broad variety of medical treatments that depend on efficient antibiotic prophylaxis are made more difficult as a result of this expanding danger, which undermines the basic successes of modern medicine. Infections that were once controllable are now possibly fatal. Several processes, which bacteria may exploit individually or in combination, are responsible for the development of antibiotic resistance. Bacteria create enzymes that breakdown or change antibiotics, making them useless. Enzymatic degradation is a key method that bacteria use to accomplish this. Beta-lactamase enzymes are a good example since they have the ability to degrade beta-lactam medicines like penicillin. Alterations to target sites are another approach. bacterium have the ability to change certain places inside their cells that antibiotics target, which may result in a reduction in the drug's ability to attach to the bacterium. The mecA gene, which produces an altered penicillin-binding protein (PBP2a) with a decreased affinity for beta-lactam antibiotics, is a good example of this. MRSA is a particularly good example of this [3].

Expulsion of antibiotics from the cell before they can reach their targets is made possible by efflux pumps, which are another kind of resistance mechanism. This allows bacteria to impart resistance to numerous antibiotics at the same time. Modifying their cell membranes in order to prevent antibiotics from entering their cells is another way that bacteria might have decreased permeability. Porin proteins, which regulate the movement of chemicals into and out of the bacterial cell, are often altered in order to accomplish this goal. Biofilms are complex communities that are encased in a protective extracellular matrix. These biofilms prevent antibiotics from penetrating the bacteria and shield them from both the immunological response of the host and antimicrobial drugs. Biofilms are formed by some bacteria [4].

Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant *Staphylococcus aureus* (VRSA) are two varieties of resistant bacteria that are among the most worrying. A substantial contributor to morbidity and death on a global scale, methicillin-resistant *Staphylococcus aureus* (MRSA) was primarily a hospital-acquired illness but has now expanded to community settings. Because methicillin is not effective against MRSA, which is resistant to all beta-lactam antibiotics, it is necessary to use other therapies such as vancomycin. On the other hand, the appearance of vancomycin-resistant strains of the bacterium VRSA has caused the medical community to express widespread concern. The vanA gene, which modifies the terminal amino acid residues of peptidoglycan precursors and thereby reduces the binding affinity of vancomycin, is one of the resistance mechanisms that VRSA strains have developed. Increased death rates, longer hospital stays, and greater medical expenditures are all consequences of the worldwide expansion of antibiotic-resistant bacteria, which presents a significant risk to public health. This places a greater strain on healthcare systems since resistant infections make the treatment of common infectious illnesses more difficult. Further complicating the situation is the fact that the pipeline for new antibiotics is not sufficient enough to keep up with the fast development of resistance. The development of efficient treatment options and the reduction of the impact of these resistant strains requires a thorough understanding of the processes and prevalence of these strains. The promotion of antibiotic stewardship, which ensures the prudent use of antibiotics in both healthcare and agriculture, the enhancement of global surveillance systems to monitor the spread of resistant strains, the investment in the research and development of new antibiotics and alternative therapies such as bacteriophages and immunotherapies, the improvement of infection control practices in healthcare settings, and the education of the general public on the significance of responsible antibiotic use and the problems that are associated with antibiotic resistance are all important strategies [5,6].

In order to guarantee the continuous effectiveness of antimicrobial drugs and to protect the health of people all over the world, it is necessary to take a coordinated and multidisciplinary approach to the issue of antibiotic resistance. It is vital to have a comprehensive understanding of the complicated processes and the prevalence of resistance infections across the world in order to successfully address this catastrophe.

Considering the Importance of Vancomycin in the Treatment of MRSA

As an antibiotic, vancomycin has been the antibiotic of choice for treating MRSA infections for a very long time. This is especially true in situations when other antibiotics are ineffective owing to resistance. Because of its effectiveness in inhibiting the production of cell walls by *Staphylococcus aureus*, it has become an important component in the treatment of severe infection caused by MRSA. On the other hand, the appearance of strains of *Staphylococcus aureus* that are resistant to vancomycin (VRSA) represents a considerable risk to this last security measure. Both the binding affinity and efficiency of vancomycin are decreased as a result of the acquisition of resistance mechanisms by VRSA strains. One such mechanism is the vanA gene, which alters the peptidoglycan precursors found in the bacterial cell wall. Not only does this resistance make treatment regimens more difficult to follow, often requiring the use of alternatives that are either less effective or more hazardous, but it also highlights the critical necessity for accurate diagnostic procedures. When it comes to preventing treatment failures and limiting the development of highly resistant strains, it is essential to have accurate diagnosis and control of VRSA [7]. . Because of this, it is very necessary for clinical settings to develop and apply dependable diagnostic methods for measuring the Minimum Inhibitory Concentration (MIC) of vancomycin. This is necessary in order to guarantee that appropriate treatment interventions are carried out and to reduce the negative effect that VRSA has on public health [8-10].

The Importance of Finding an Accurate MIC

The measurement of the Minimum Inhibitory Concentration (MIC) is a critical step in clinical microbiology. It is necessary for the classification of bacterial isolates as either antibiotic-resistant or antibiotic-susceptible. Because they directly inform therapeutic choices on the selection of suitable antimicrobial medicines, accurate MIC values are very important. This helps to ensure that patients get the most effective therapy possible. Because medicines like vancomycin are essential for the management of MRSA infections, determining the minimum inhibitory concentration (MIC) with precision is even more

important. Considering that vancomycin is a primary therapy for methicillin-resistant *Staphylococcus aureus* (MRSA), it is essential to correctly determine the point at which the antibiotic is no longer able to prevent the development of bacteria, which is an indication of resistance. Detecting strains of vancomycin-resistant *Staphylococcus aureus* (VRSA) requires a level of accuracy that is very difficult to achieve. The incorrect categorization of resistance as a consequence of faulty MIC values may result in poor therapeutic decisions, which in turn can lead to treatment failures, prolonged infections, and an increased risk of transmission. In addition, as a result of correct calculation of the MIC, doctors are able to modify therapeutic interventions accordingly, hence optimizing doses and choosing alternative therapies when they arerequired. Therefore, an accurate minimum inhibitory concentration (MIC) test is an essential component of efficient antimicrobial stewardship. This test helps to ensure that current antibiotics continue to be effectivewhile also preventing the spread of resistance strains [11].

METHODOLOGY

Research Design: The research design for this study was observational and descriptive, employing a cross-sectional approach. This design allows for the collection of data at a single point in time, without intervention or manipulation of variables. It is suitable for investigating the prevalence and characteristics of *Staphylococcus aureus*, including methicillin-resistant strains, within a specified population.

Data Collection and Procedure

Place of Study: The study was conducted in the Department of Microbiology, Government Medical College, Kota, Rajasthan, India.

Duration of Study: Data collection took place over a period of three years, starting from September 7, 2019, to September 6, 2022, following approval from the Departmental Research Committee (DRC).

Sample Size Determination- The sample size was calculated using a statistical formula based on the prevalence rate of vancomycin-resistant *Staphylococcus aureus* (VRSA) among various clinical samples. An average prevalence rate of 20% was used, resulting in a calculated sample size of 384.

The Ethical Letter: The Ethical clearance was duly obtained from the Institutional Medical College of GMC, Kota.

Specimen Collection and Processing- *Staphylococcus aureus* isolated from various clinical samples, including pus, urine, sputum, blood, throat swab, and pleural fluid, were processed according to standard protocols in the bacteriology lab of the Department of Microbiology.

Identification of the Organism: Isolated *Staphylococcus aureus* underwent microscopic examination, subculture, and manual identification tests including Gram staining, nutrient agar, blood agar, and mannitol salt agar. Biochemical tests such as the catalase and coagulase tests were also conducted for the identification.

Detection of MRSA and VRSA: Methicillin-resistant *Staphylococcus aureus* (MRSA) was detected using the cefoxitin disk diffusion method, while the presence of vancomycin-resistant strains (VRSA) was determined by using vancomycin agar screen plates. MIC determination to confirm VRSA was performed by using the agar dilution method and E-test strip test.

Statistical Analysis

Statistical analysis may include calculations of prevalence rates, descriptive statistics of sample characteristics, and comparisons of antimicrobial susceptibility patterns among different clinical samples. Additionally, the correlation between demographic variables and antibiotic resistance profiles may be analyzed using appropriate statistical tests. The significance level for statistical tests may be set at $p < 0.05$.

Vancomycin Screen Agar Method [12] **:**

Vancomycin Agar Screen test was utilized to screen *Staphylococcus aureus* strains for resistance to vancomycin. For the isolates, which gave positive result on vancomycin agar screen test, were tested for MIC determination by using agar dilution method and E-test.

Preparation of Vancomycin Agar Screen plates:

i.) Medium used for vancomycin agar screen plates was Brain Heart Infusion (BHI) agar supplemented with 6 µg/ml vancomycin.

ii.) For the preparation of 100 ml of BHI agar plates containing 6 µg/ml vancomycin, 1 vial of aliquoted 600 µl of the 1 mg/ml vancomycin stock was taken out from deep freezer and add in 100 ml of autoclaved warm BHI media.

iii.) Immediately after adding antibiotic stock solution, it was mix slowly and pour approx. 5 plates.

"The following formula was used for making 6 µg/ml vancomycin BHI agar plate from 1 mg/ml stock solution (1000 µg/ml)".

$$C1\ V1 = C2\ V2$$

C1 = Concentration of vancomycin stock solution.
V1= Volume taken from stock solution for making 6 µg/ml vancomycin BHI agar.
C2= Concentration of vancomycin BHI agar.
V2= Volume of the BHI agar.

$$C1\ V1 = C2\ V2$$
$$1000\ \mu g/ml\ V1 = 6\ \mu g/ml \times 100\ ml$$
$$V1 = 600\ \mu l$$

Inoculum preparation:

i.) Fresh culture of the strain was used to be prepared a suspension of tested strain equivalent to 0.5 Mc Farland standard.

ii.) Standardized inoculum was prepared of 0.5 McFarland turbidity standard (approx. $1.5x10^8$ CFU/ml) by using the direct colony suspension method. 3-5 well isolated colonies of the same morphological type were taken from 18–24-hour culture plate and prepare saline suspensions in tubes containing sterile saline.

Plate Inoculation:

i.) The suspension was inoculated by using a micropipette to spot a 10 µl drop (final conc. 10^6cfu/ml) on the surface of the BHI agar plate containing 6 µg/ml vancomycin.

ii.) Square grid template was prepared to spot approx. 13 – 14 test strains and 2 QC strains in one plate.

iii.) Incubation conditions: The plates were incubated at 35±2°C for 18-24 hrs in an inverted position.

Interpretation and Reporting:

Presence of more than one colony of the strain or light film of growth was interpreted as reduced susceptibility to vancomycin.

MIC determination of Vancomycin:

MIC determination methods recommended by CLSI and CDC for VRSA are agar dilution method and E-test.

A. Agar Dilution Method[13]: -

I. Introduction:

i.) The agar dilution method was utilized to determine MIC.

ii.) Determination of minimum inhibitory concentration (MIC) of vancomycin for *S. aureus* isolates which grew on vancomycin agar screen plates were identified by agar dilution method.

iii.) CLSI recommends agar dilution method for the detection of MIC for VRSA.

II. Preparation of Vancomycin Hydrochloride stock solution:

i.) First step was to prepare the 1 mg/mL stock solution of vancomycin hydrochloride.

ii.) Use of the following formula to determine the amount of powder needed for a standard solution:

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Conc. (µg/mL)}}{\text{Potency (µg/mg)}}$$

Example: To prepare 100 mL of a stock solution containing 5120 µg/mL concentration with antimicrobial powder that has a potency of 950 µg/mg.

The amount of vancomycin hydrochloride powder was calculated as follows:

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Conc.(µg/mL)}}{\text{Potency (µg/mg)}}$$

$$\text{Weight (mg)} = \frac{100\ \text{(mL)} \times 5120\ \text{(µg/mL)}}{950\ \text{(µg/mg)}}$$

Weight (mg) = 538.95 mg

Therefore, dissolve 538.95 mg of antimicrobial powder in 100 mL of diluent.

iii.) Prepare each aliquots containing 1-2 ml of 1 mg/ml stock solution of vancomycin hydrochloride.

iv.) Label each vial of aliquoted 1 mg/ml stock solution as **"Working Stock of Vancomycin".**

III. Preparation of vancomycin MIC plates:

For making vancomycin MIC plates of different concentrations (0.5-256 µg/ml) dilution of the stock solution was made according to the given table 1.

Table 1: Dilution of Vancomycin for MIC plates.

Step	Concentration (µg/mL)	Source	Vol. (mL)	Diluent (mL)	Intermediate Concentration (µg/mL)	Final Conc. At 1:10 dilution in Agar (µg/mL)
1	5120	Stock	2	2	2560	256
2	5120	Stock	1	3	1280	128
3	5120	Stock	1	7	640	64
4	640	Step 3	2	2	320	32
5	640	Step 3	1	3	160	16
6	640	Step 3	1	7	80	8
7	80	Step 6	2	2	40	4
8	80	Step 6	1	3	20	2
9	80	Step 6	1	7	10	1
10	10	Step 9	2	2	5	0.5
11	10	Step 9	1	3	2.5	0.25
12	10	Step 9	1	7	1.25	0.125

Readings were interpreted according to recent CLSI guideline: -

- MIC ≤2 mg/l for vancomycin-susceptible *S. aureus* (VSSA).
- MIC of 4-8 mg/l for vancomycin-intermediate *S. aureus* (VISA).
- MIC ≥16 mg/l for vancomycin-resistant *S. aureus* (VRSA).

B. E-test Strip Method:

I. Introduction:

i.) Another method for the Determination of minimum inhibitory concentration (MIC) of vancomycin for *S. aureus* isolates which grew on vancomycin agar screen plates was done by E-test.

ii.) CDC recommends E-test method for the detection of MIC for VRSA.

II. Procedure:

i.) In this test inoculum suspensions of 0.5 McFarland standard were prepared.

ii.) Dip a sterile cotton swab to the inoculum suspension and carefully streak the entire surface Muller-Hinton agar evenly in three directions.

iii.) Allow excess moisture to be fully absorbed and ensured that the surface of Muller-Hinton agar was completely dry before applying E-test strips.

iv.) An E-test strip containing a concentration gradient of Vancomycin ranging from 0.016 - 256 µg/ml was used to check the susceptibility.

v.) E-test strip was applied on to the agar surface with the MIC scale facing upwards. This was done by using forceps.

vi.) It was ensured that the whole strips were in complete contact with the agar surface.

vii.) Plates were incubated in an inverted position at 37℃ for overnight incubation.

Fig.1 E-test strip method.

Interpretation of MIC values: -
Readings were interpreted according to recent CDC guideline:

- MIC≤2 µg/ml for vancomycin-susceptible *S. aureus* (VSSA).
- MIC of 4-8 µg/ml for vancomycin-intermediate *S. aureus* (VISA).
- MIC≥16 µg/ml for vancomycin-resistant *S. aureus* (VRSA) [14].

Advantages and Disadvantages:

Agar Dilution Method: Highly accurate, suitable for research settings, but requires extensive resources and trained staff.

E-test Strip Method: Practical for clinical use, faster results, but may be less precise than the agar dilution method.

Sr. No.	Age group	*S. aureus* No. (%)	Male No. (%)	Female No. (%)
1	0-10	15 (3.91)	8 (53.33)	7 (46.67)
2	11-20	21 (5.47)	11 (52.38)	10 (47.62)
3	21-30	70 (18.23)	38 (54.29)	32 (45.71)
4	31-40	67 (17.45)	33 (49.25)	34 (50.75)
5	41-50	57 (14.84)	29 (50.88)	28 (49.12)
6	51-60	48 (12.50)	26 (54.17)	22 (45.83)
7	61-70	63 (16.41)	36 (57.14)	27 (42.86)
8	71-80	43 (11.20)	14 (32.56)	29 (67.44)
	Total	384 (100)	195 (50.78)	189 (49.22)

RESULT

In the present study a total of 384 *Staphylococcus aureus* isolates were collected from different clinical samples. These isolates were from patients admitted to Maharao Bhim Singh Hospital (MBSH) and New Medical College Hospital (NMCH), as well as from out-patients at both hospitals of Government Medical College in Kota, Rajasthan, India.

Table 2: *Staphylococcus aureus* segregation by age and sex (n = 384).

In the current study out of the 384 *S. aureus* isolates, 70 (18.23%) belonged to (21-30) age group followed by 67 (17.45%) from (31-40) age group, 63 (16.41%) from (61-70) age group, 57 (14.84%) from (41-50) age group, 48 (12.50%) from (51-60) age group, 43 (11.20%) from (71-80) age group, 21 (5.47%) from (11-20) age group and least in the age group 15 (3.91%) from (0-10) age group.

Among 195 *S. aureus* isolated the ratio of male patients observed that 38 belonged to (21-30) age group followed by 36 from (61-70) age group, 33 from (31-40) age group, 29 from (41-50) age group, 26 from (51-60) age group, 14 from (71-80) age group, 11 from (11-20) age group, 8 from (0-10) age group.

Among 189 *S. aureus* isolated from female patients, 34 belonged to (31-40) age group followed by 32 from (21-30) age group, 29 from (71-80) age group, 28 from (41-50) age group, 27 from (61-70) age group, 22 from (51-60) age group, 10 from (11-20) age group, 7 from (0-10) age group.

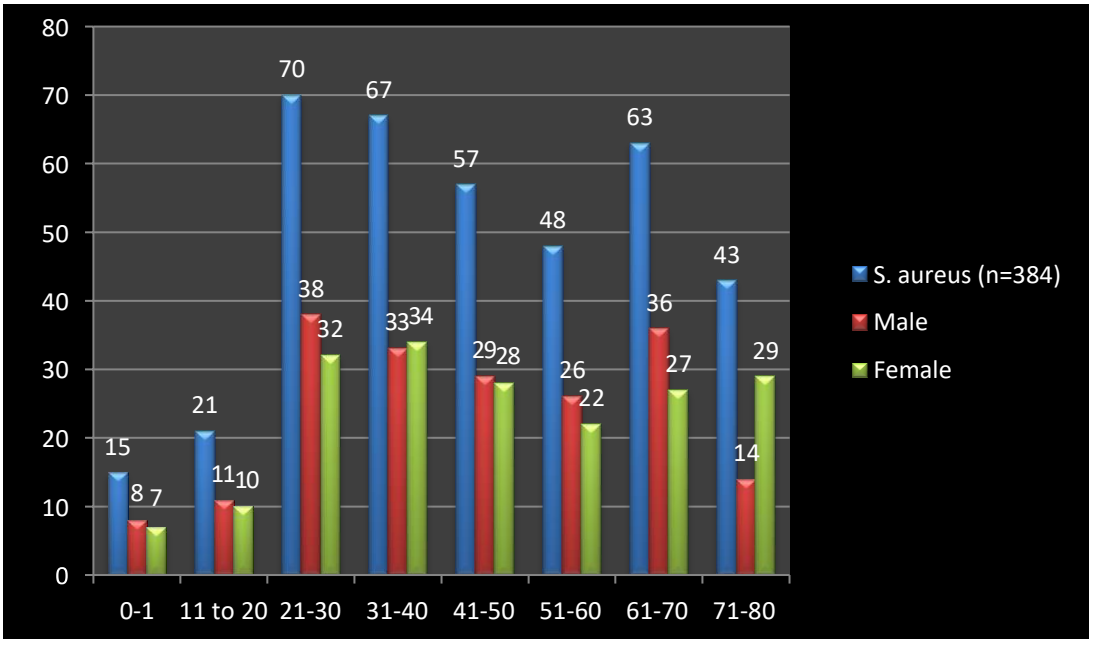

Fig. 2: Staphylococcus aureus age and sex distribution (n = 384).

Table 3: Distribution of *S. aureus* isolated from different clinical samples.

Sr. No.	Clinical Specimens	Number of *Staphylococcus aureus*	Percentage (%)
1	Pus	215	56
2	Urine	77	20
3	Sputum	50	13
4	Blood	27	7
5	Throat swab	8	2
6	Pleural fluid	7	2
	Total	**384**	**100**

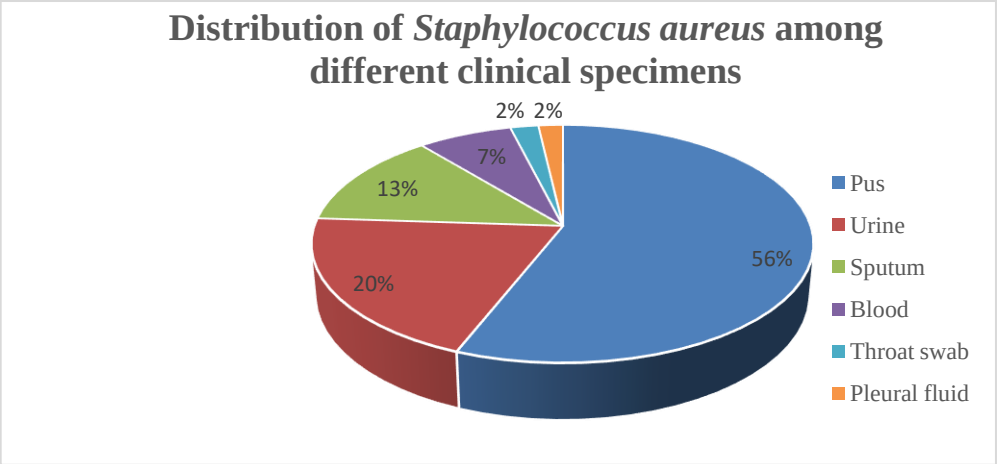

Fig 4: Distribution of *Staphylococcal aureus* among different clinical specimens.

Table 4: Distribution of *S. aureus* among MRSA and MSSA.

Staphylococcus aureus	**384**	**100%**
MRSA	185	48.18%
MSSA	199	51.82%

Among 384 *staphylococcus aureus*, 185 (48.18%) were methicillin resistant and 199 (51.82%) were methicillin sensitive.

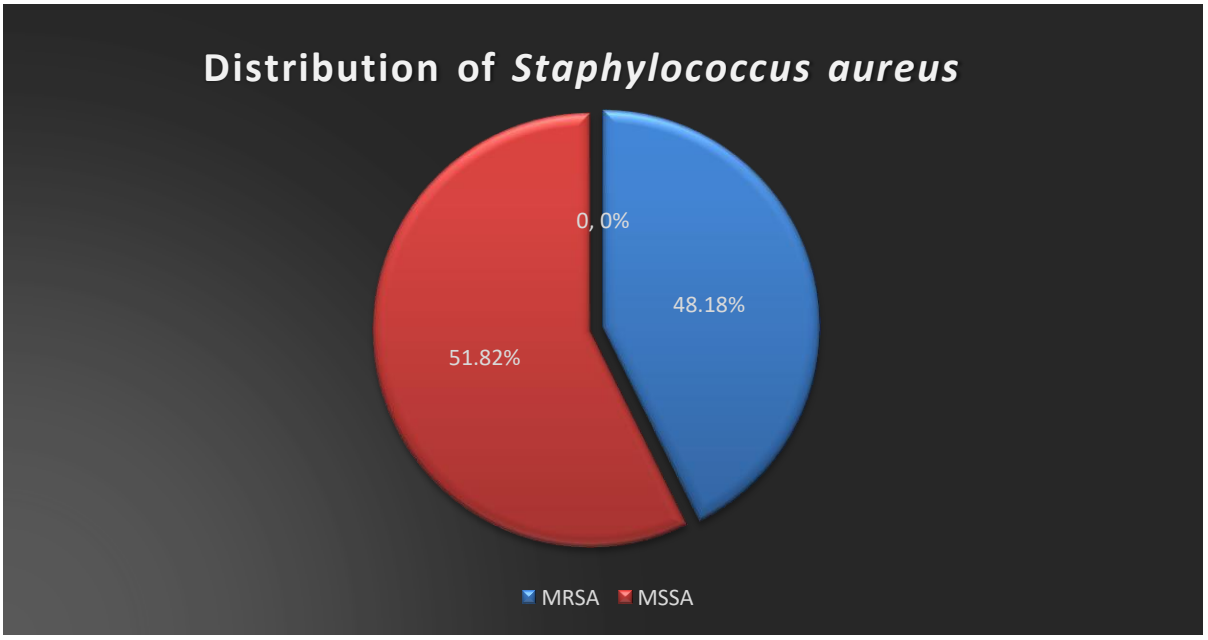

Fig 4: Distribution of *Staphylococcal aureus* among MRSA and MSSA.

Table 5: The vancomycin screen agar technique of 6 µg/ml* MIC, *was used to identify VRSA among MRSA isolates.

MRSA **No, %**	**VRSA** **No. %**	**VSSA** **No. %**
185 (100%)	06 (3.24%)	179 (96.76%)

The agar dilution method and the E-test strip method were used to confirm the presence of 6 (3.24%) VRSA isolates out of 185 MRSA discovered by the vancomycin screen agar method.

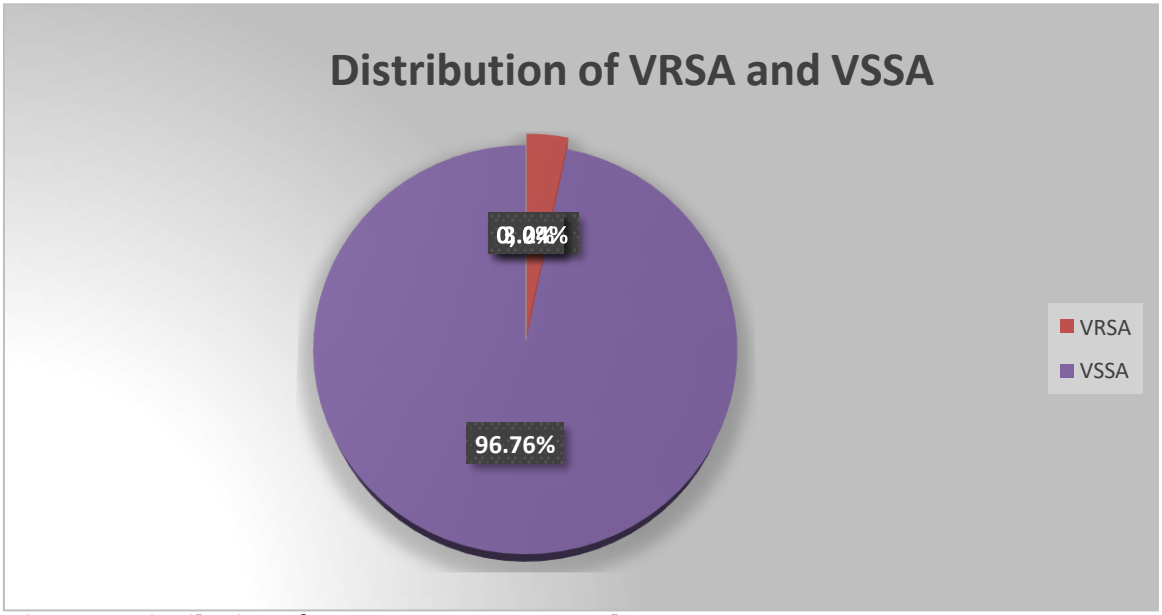

Figure 5: Distribution of MRSA among VRSA and VSSA.

Table 6: MIC determination by agar dilution method and E-test strip method, a comparative study.

MIC (µg/ml)	Agar dilution	%	E-test	%
0.5	8	4.32	2	1.08
0.75	-	-	3	1.62
1	93	50.27	68	36.76
1.5	-	-	22	11.89
2	79	42.70	85	45.95
3	-	-	0	0
4	2	1.08	2	1.08
6	-	-	0	0
8	1	0.54	1	0.54
12	-	-	0	0
16	2	1.08	2	1.08
32	00	0	00	0
Total	185	100	185	100

Agar dilution method:

Out of 185 MRSA 8 (4.32%) MRSA shows 0.5 µg/ml MIC by agar dilution method followed by 93 (50.27%) MRSA shows 1 µg/ml MIC, 79 (42.70%) MRSA shows 2 µg/ml MIC, 2 (1.08%) MRSA shows 4 µg/ml MIC, 1 (0.54%) MRSA shows 8 µg/ml MIC, 2 (1.08%) MRSA shows 16 µg/ml MIC and 00 (0%) MRSA shows 32 µg/ml MIC.

E-test strip Method:

Of the 185 MRSA isolates 2 (1.08%) MRSA shows 0.5 µg/ml MIC by E-test strip method followed by 3 (1.62%) MRSA shows 0.75 µg/ml MIC, 68 (36.76%) MRSA shows 1 µg/ml MIC, 22 (11.89%) MRSA shows 1.5 µg/ml MIC, 85 (45.95%) MRSA shows 2 µg/ml MIC, 2 (1.08%) MRSA shows 4 µg/ml MIC, 1 (0.54%) MRSA shows 8 µg/ml MIC, 2 (1.08%) MRSA shows 16 µg/ml MIC and 0 (0%) MRSA shows 3, 6, 12, 32 µg/ml MIC.

Table 7: The Agar dilution method and the E-test strip method were used to determine the VRSA, VISA, and VSSA isolates based on their MIC values.

MIC By	VRSA No. (%)	VISA No. (%)	VSSA No. (%)
Agar dilution method	02 (1.08)	03 (1.62)	180 (97.30)
E-test strip method	02 (1.08)	03 (1.62)	180 (97.30)

MRSA isolates were subjected to MIC determination by the agar dilution method and E-test strip method. Of the 185 MRSA isolates 02 isolates were identified as VRSA and 03 isolates were identified as VISA by both methods.

DISCUSSION

Infections due to Staphylococci are the global health problem due to increase drug resistance developed by this organism [15] It accounts for 30% of hospital acquired infections while around 50% of blood stream infections reported Staphylococcus as the main organism to be isolated on culture [16, 17]. The threat on public health posed by *Staphylococcus aureus* is a matter of concern for WHO due to its increased virulence and resistance patterns.

The increase in isolates of *S.aureus* with resistance to methicillin and decreased susceptibility to vancomycin has created concern for development of new antistaphylococcal agents that kills resistant mutants. Emergence of VRSA/VISA may be due to buildings of selective pressure of vancomycin.

In a recent scenario vancomycin is the treatment choice for MRSA (Methicillin Resistant *Staphylococcus aureus*) infections. However, it has resulted in the evolution of vancomycin intermediate *Staphylococcus aureus* (VISA) and (vancomycin resistant *Staphylococcus aureus* (VRSA).

In this present study, there were total 384 isolates of *Staphylococcus aureus***,** out of which maximum isolates were observed in the age group of 21-30 years (18.23%) followed by 31-40 years (17.45%). Our results were comparable to the study by Mandal M *et al* and Goyal A *et al* which showed high prevalence in the age groups of 21-30 years (36.11%) and 20-40 years (51%) respectively. However, in the study by Kaur K *et al* the prevalence was high in the age group ranging from 16-40 years (43.2%) which was in constrast to the present study. This could be due to wider margin of age range selected in their study (Table-8).

Table 8: Comparison of age-based prevalence of *Staphylococcus aureus* isolates with other studies

Present study (N=384)	Kaur K *et al* (N=162) [15]	Goyal A *et al* (N=379) [16]	Mandal M *et al* (N=108) [17]
21-30 years (70/18.23%)	16-40 years (70/43.2%)	20-40 years (193.29/51%)	21-30 years (39/36.11%)
31-40 years (67/17.45%)	-	0-20 years (98.54/26%)	11-20 years (23/21.29%)

In the study of Kaur K *et al*, there were 162 isolates of *Staphylococcus aureus*, of which 85 (52.4%) were obtained from females while 44 (47.5%) were obtained from male patients. The higher number of isolates was obtained from male patients which was similar to our study. In contrast, a study from Bihar conducted by Mandal M *et al*and a study conducted in Nepal by Adhikari R *et al* reported high prevalence of *Staphylococcus aureus* isolates in females compared to males (Table-9).

Table 9: Comparison of gender based (Male: M, Female F) prevalence of *Staphylococcus aureus* isolate with other studies

Present Study (N=384)		Kaur K et al (N=162) [15]		Mandal M *et al* (N=108) [17]		Sarrafzadeh F *et al* (N=250) [18]		Adhikari R *et al* (N=95) [19]	
M	F	M	F	M	F	M	F	M	F

195 (50.78%)	185 (49.22%)	85 (52.4%)	44 (47.5%)	37 (34.26%)	71 (65.74%)	188 (75.2%)	62 (24.8%)	41 (43.16%)	54 (56.84%)
Ratio	1.03:1	1.93:1		0.52:1		3.03:1		0.76:1	

In the present study most of the *Staphylococcus aureus* isolates i.e. 215 were obtained from pus samples that accounted for 56% followed by urine samples, the incidence of which were 77 (20%). Like the present study, the number of *Staphylococcus aureus* isolates was high is pus sample in the studies conducted by Usha MG *et al* (126/66.31%), Kaur K et al (96/59.2%) and Nepal N *et al* (42/52.5%) followed by blood (16/8.42%), urine (30/18.5%) and pus (16/41%) respectively. In contrast to our study, the study of Kandel SN *et al* showed higher incidence in urine sample (9/23.1%) followed by pus (8/20.5%). Another, study of Maharjan M *et al* reported higher incidence in wound swab (27/47.4%) followed by pus sample (16/41%) (Table-10).

Table 10: Comparison of prevalence of *Staphylococcus aureus* isolates with other studies according to various clinical specimen

Present study (N=384)	**Usha MG et al N=190 [20]**	**Kandel SN et al (N=39) [21]**	**Kaur K (N=162) [15]**	**Nepal N et al (N=80) [22]**	**Maharjan M *et al* (N=74) [23]**
Pus 215 (56%)	Pus 126 (66.31%)	Urine 9 (23.1%)	Pus and Wound 96 (59.2%)	Pus 42 (52.5%)	Wound swab 27 (47.4%)
Urine 77 (20%)	Blood 16 (8.42%)	Pus 8 (20.5%)	Urine 30 (18.5%)	Wound swab 21 (26.3)	Pus 16 (41%)

The present study reported that, of 384 *Staphylococcus aureus* isolates, 185 (48.18%) were methicillin resistant (i.e. MRSA) while199 (51.82%) were methicillin sensitive (MSSA). In the study of Osman MM *et al*, 25 (41%) of *Staphylococcusaureus* isolates were MRSA while 36 (59%) were MSSA which was similar to our study. Likewise, in the studyof Jayshree N et al, the prevalence of MRSA was 41.38% (12) while the study of Thati V *et al* showed a very high prevalence of MRSA i.e. 79.6% (285) which was higher than that observed in our study (table-11).

Table 11: Comparison of prevalence of MRSA among *Staphylococcus aureus* isolates with other studies

Present Study (N=384)		**Osman MM *et al* N=61 [24]**		**Arora S *et al* N=250 [25]**		**Jayshree *et al* N=29 [26]**		**Thati V *et al* N=358 [27]**	
MSSA	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA
199 (51.82%)	185 (48.18%)	36 (59%)	25 (41%)	135 (58.7%)	115 (41.3%)	17 (58.62%)	12 (41.38%)	73 (20.4%)	285 (79.6%)

In the present study, VRSA and VISA strains among the MRSA isolates were determined using vancomycin screen agar method and was further confirmed by agar dilution method and E-test strip method. Of 185 MRSAisolates vancomycin screen agar method detected 3.24% (3) of VRSA isolates and 96.76% (179) VSSA. The incidence of VRSA strains was documented in this study was lower than that observed in the previous studiesof Thati V *et al* (23/6.42%), Kaur K *et al* (23/27.71%) and Olufunmiso O *et al* (89/33.5%) (Table-12).

Table 12: Comparative determination of VRSA and VSSA among MRSA isolates by using vancomycin screen agar method (MIC = 6 µg/ml) with other studies

Present Study (N=185)		**Kaur K *et al* (N=83) [15]**		**Thati V *et al* (N=358) [27]**		**Olufunmiso O *et al* (N=266) [28]**	
VRSA	VSSA	VRSA	VSSA	VRSA	VSSA	VRSA	VSSA
6 (3.24%)	179 (96.76%	23 (27.71%)	60 (72.28%)	23 (6.42%)	335 (93.57%)	89 (33.5%)	147 (55.26%)

On agar dilution method, it was found that for most of the MRSA isolates (50.27%) were represent the MIC 1 µg/mL while for 0.54% of cases MIC was 8 µg/mL. On E-test strip method, it was found that for most of the MRSA isolates (57.84%), the MIC was 1.5-2 µg/mL while for 0.54% of cases MIC was 8 µg/mL. None of the isolates showed MIC of 32 µg/mL. Previous studies have also determined the MIC of vancomycin as per CLSI guidelines, the comparative analysis of which with the present study is shown in the given table-13.

In the study of Mongy MA *et al*, 96% of MRSA isolates had MIC of 2 µg/mL, while in the study of Mandal M *et al*, 50% of the isolates showed MIC of 2 µg/mL. Mohanty *et al* determined the MIC of vancomycin using E test method. They found that for 40.16% of the MRSA isolates, the MIC was 2 µg/mL. Likewise, Kumari J *et al* and Kaur K *et al* also documented comparable findings to our study. In their study too, the MIC for most of the isolates was ≤2 µg/mL which was in support to the present study. In the study by other investigator Kaur K *et al* recorded 11.7% of VRSA strains with the MIC between 4-8 ug/ml (VISA) and 2.46% had MIC > 16 ug/mL showing complete resistance to vancomycin.

Table 13: MIC determination for vancomycin by agar dilution method and E-test strip method, a comparative study.

Present Study (N=185)		**Mongy MA *et al* N=50 [29]**	**Mandal M *et al* N=32 [17]**	**Mohanty *et al* N=127 [30]**	**Kumari J *et al* N=98 [31]**		**Kaur K *et al* N=162 [15]**
Agar dilution	E-strip	Agar dilution	Agar dilution	E-strip	Agar dilution	E-strip	Agar dilution
0.5 µg/mL: 8 (4.32%)	0.5-0.75 µg/mL: 5 (2.70%)	-	-	0.5-0.75 µg/mL: 0 (0%)	0.5 µg/mL: 11 (11.2%)	0.5-0.75 µg/mL: 1 (1%)	-
1 µg/mL: 93 (50.27%)	1 µg/mL: 68 (36.76%)	-	1 µg/mL: 1 (3.12%)	1 µg/mL: 23 (18.11%)	1 µg/mL: 37 (37.8%)	1 µg/mL: 11 (11.2%)	-
2 µg/mL: 79 (42.70%)	1.5-2 µg/mL: 107 (57.84%)	2 µg/mL: 48 (96%)	2 µg/mL: 16 (50%)	1.5-2 µg/mL: 95 (74.80%)	2 µg/mL: 46 (46.9)	1.5-2 µg/mL: 41 (40.82)	<2 µg/mL: 139 (85.80%)
4 µg/mL: 2 (1.08 %)	4 µg/mL: 2 (1.08 %)	4-8 µg/mL: 49 (98%)	4 µg/mL: 3 (9.37%)	3-4 µg/mL: 9 (7.09%)	4 µg/mL: 4 (4.1%)	3-4 µg/mL: 45 (45.92%)	4-8 µg/mL: 19 (11.7%)
8 µg/mL: 1 (0.54%)	8 µg/mL: 1 (0.54%)	8 µg/mL: 49 (98%)	8 µg/mL: 8 (25%)	-	-	-	
16 µg/mL: 2 (1.08%)	16 µg/mL: 2 (1.08%)	16 µg/mL: 50 (100%)	16 µg/mL: 1 (3.12%)	-	-	-	16 µg/mL: 3 (1.8%)

32 µg/mL: 0	32 µg/mL: 0	32 µg/mL: 50 (100%)	32 µg/mL: 2 (6.25%)	-	-	-	32 µg/mL: 1 (0.6%)
-	-	-	64 µg/mL: 1 (3.12%)	-	-	-	-

By agar dilution method and E-test strip method 2 (1.08%) VRSA and 3 (1.62%) of VISA isolates were detected (Table-7). This result was comparable to the study of Thati V *et al* who documented 1.9% of VRSA strains and 4.46% of VISA strains. In the study of Osman MM *et al*, there was no isolation of any VRSA stains and the overall prevalence of VISA was 12%. They were mostly observed in the patients having underlying conditions such as long-term hospitalization, serious disease and immune-suppressive therapy (Table-14).

Table-14: Comparative determination of VRSA, VISA and VSSA isolates by Agar dilution method and E-test strip method on the basis of MIC values.

Present study N=185			**Thati V *et al* (N=358) [27]**			**Osman MM et a*l* (N=25) [24]**			**Maharjan M *et al* (N=45) [23]**		
VRSA	VISA	VSSA	VRSA	VISA	VSSA	VRSA	VISA	VSSA	VRSA	VISA	VSSA
2 (1.08%)	3 (1.62%)	180 (97.29%)	7 (1.95%)	16 (4.46%)	335 (93.57%)	0	3 (12%)	22 (88%)	5 (11.11%)	15 (33.33%)	25 (55.55%)

Agar dilution technique and E-test strip method both are used to determine the minimum inhibitory concentration (MIC) of vancomycin among MRSA isolates. The comparison research offers useful insights into the efficiency of both of these methods. In each case, there are benefits and disadvantages associated with the different techniques. In clinical settings, the decision of which approach to use may be contingent on the particular needs of accuracy, the availability of resources, and the demand for speedy findings.

The use of these strategies in a variety of situations, as well as the influence that they have on the management and treatment of infections caused by MRSA and VRSA, might be the subject of exploration in further study. For the purpose of effective antibiotic stewardship and infection control, the results highlight the need of accurately determining the minimum inhibitory concentration (MIC).

Vancomycin has been recognised as the first-line treatment for MRSA. Unfortunately, there has been a rise in the use of this antibiotic for various diseases, such as pseudomembranous colitis related to *Clostridium difficile* and coagulase-negative staphylococcal infections in hospitalised patients [32–33]. When this antibiotic was first released in 1858, it was assumed that no resistance would develop because resistance was difficult to produce. In 1997, the first strain of S. aureus with reduced susceptibility to vancomycin was described from Japan [34]. Since then, there has been an increase in the number of cases with both VISA and VRSA (vancomycin-intermediate and vancomycin resistant *S. aureus*). This has triggered off alarms in the medical community as *S. aureus* causes life-threatening infections in hospitalized and non-hospitalized patients [35] as Vancomycin is the main antimicrobial agent available to treat serious infections with MRSA but unfortunately, many nations have lately reported a decline in vancomycin susceptibility of *S. aureus* as well as the isolation of vancomycin-intermediate and resistant *S. aureus* [36].

CONCLUSION

In the present study the Agar dilution method was considered as the gold standard for MIC determination. It provides precise and reproducible results but is labor-intensive and time-consuming. E-test strip method offers a more convenient and faster alternative. It is easy to perform and interpret but may have variability in results compared to the agar dilution method.

The worrisome rise in the prevalence of antibiotic-resistant pathogenic strains of bacteria, particularly *Staphylococcus aureus* resistant to vancomycin is increasing. Vancomycin remains dominated till there is control of resistance to vancomycin or the new antibiotic with effect superior to it are available. However, control of VRSA has possessed challenge as VRSA isolates have shown resistant to several available antibiotics (a condition known as multi drug resistant VRSA). It has caused availability of limited treatment options leading to inadequate antibiotic therapy and increased mortality or morbidity in the afflicted cases. Further, once MRSA or VRSA permanently resides in

environment or hospital settings, it becomes almost impossible to get rid of them. Given the prevalence of antibiotic misuse, it's crucial for authorities to take prompt action to prevent the development of VRSA and VISA strains. Strict

regulations on irrational antibiotic consumption could be an effective solution. Additionally, a statewide surveillance programme is needed to map the vancomycin susceptibility pattern in the country.

Declarations:

Conflicts of interest: There is no any conflict of interest associated with this study

Consent to participate: We have consent to participate.

Consent for publication: We have consent for the publication of this paper.

Authors' contributions: All the authors equally contributed the work.

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