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A Study on Prevalence and Antibiotic Susceptibility Pattern of Vancomycin Intermediate and Resistant Staphylococcus Aureus in Clinical Specimen in a Tertiary Care Hospital and Detection of their MIC Values by E-test.

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Abstract: *With the increasing incidence of Methicillin Resistant Staphylococcus Aureus(MRSA), Vancomycin Intermediate Staphylococcus Aureus(VISA) & Vancomycin Resistant Staphylococcus Aureus (VRSA) strains now a days. The study was conducted to find out the magnitude of vancomycin resistance in both MRSA and MSSA and antibiotic susceptibility pattern of those isolates in a tertiary care hospital, Jaipur between January 2017 to June 2018.In this cross sectional study,287 Staphylococcus aureus were isolated and identified from various clinical specimens collected from different departments of the hospital.Among these 287 strains,146 were found to be MRSA and 141 MSSA. Subsequently ,the antimicrobial susceptibility test was performed by Kirby Bauer disc diffusion method as per CLSI guidelines. All strains found resistant to vancomycin by disc diffusion method were again grown on BHI-VSA(Brain heart infusion-Vancomycin screen agar) and also recruited to E- Test for confirmation of resistance. Minimum Inhibitory Concentration (MIC) of ≤2 were considered as VSSA,4-8 as VISA.MIC of ≥16 as VRSA. Vancomycin resistance was seen in 9 isolates of S. aureus by disc diffusion method. Among them neither was found to be VISA nor VRSA when confirmed with E-Test with all having MIC <2 .Though there was no incidence of VRSA but MIC of 1.5µg/ml in our study rang an alarm to the infection control committee of this tertiary care hospital.*

Keywords: *MIC, S.aureus, VISA, VRSA, Vancomycin Screen Agar*

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I. Introduction

Staphylococcus aureus is one of the most notorious and important human pathogen that has long been recognized for its ability to cause serious and invasive diseases. Staphylococcus aureus is the most pathogenic member of Staphylococcus genus. This microbe is ubiquitous in nature which resides in the human as a commensally. Some of the commoner infection caused by Staph. aureus include Pyogenic infection (eg. Folliculitis impetigo, breast abscess, post operative wound infection, osteomylitis, septic arthritis, bronchopneumonia, lungs abscess, empyema). Disseminated infection such as Septicemia. Toxic shock syndrome (TSS), staphylococcal scalded skin syndrome(SSSS), staphylococcal food poisoning.[1] A wide range of antibiotics are used to treat the staphylococcal infections including penicillin, cephalosporin, macrolide, fluroquinolone and glycopeptide group of antibiotics.[3] In early 90s,the major treatment available to combat this organism was Penicillin and all isolates were sensitive to penicillin. In the 1960s, a new semisynthetic penicillinase-resistant antimicrobial drugs *methicillin* (formerly named as celbenine) and oxacillin were developed to treat staphylococcal infections caused by β-lactamase-production.[4] But at present, some S. aureus strains also show resistant to methicillin.[2]

Methicillin-resistant Staphylococcus aureus (MRSA)[5] has been recognized as one of the major pathogens in both hospital and community settings. The first case of MRSA was isolated way back in 1961.Since then, there has been an escalating rate of infections caused by MRSA worldwide resulting in increased mortality and morbidity statistics. In India, the prevalence of nosocomial infections caused by MRSA varies between 20 and 40%.*Vancomycin***,** a glycopeptides antibiotics had been considered to be the "gold standard antibiotic" and the drug of choice over the last 3 decades[6]

Apart from vancomycin ,other effective drugs as linezolid and teticolanin are also widely used. Injudicious and infrequent use of vancomycin even in methicillin sensitive Staphylococcal infection has resulted in emergence of the strains with higher vancomycin MIC.[7] At present few vancomycin resistant S.*aureus* strains have been reported. Initially in early twenties,only VISA (Vancomycin intermediate S. *aureus*) strains

were reported by CDC but even now in India, there is emergence of S.aureus strain with higher vancomycin MIC.

This higher vancomycin MIC has been attributed by increased thickness of cell wall as in case of Vancomycin Intermediate Staphylococcus aureus.[8] Vancomycin resistance is acquired by mutation and thickening of cell wall due to accumulation of excess amounts of peptidoglycan.[9]

Keeping the above points in view and also that documented reports of VISA and VRSA in India including Rajasthan are very few ,the present study was planned to find out the prevalence of vancomycin intermediate Staphylococcus aureus and vancomycin resistant staphylococcus aureus among isolates of Staphylococcus aureus in various clinical specimens along with their antibiotic sensitivity pattern so as to guide the clinicians of our hospital to select appropriate antimicrobial agents and also to make them aware ,that if inappropriate use of vancomycin is continued it may lead to impending public health disaster. There fore the present study is designed to find out the prevalence of vancomycin intermediate and resistant Staphylococcus aureus in clinical specimen and to determine the antibiotic susceptibility pattern of vancomycin intermediate and resistant Staphylococcus aureus and to measure the MIC values of vancomycin intermediate and resistant Staphylococcus aureus by E-test.

II. Material And Methods

The present study was conducted in the Department of Microbiology, NIMS Jaipur during the year 2017-2018. A total of 287 non- duplicate Staphylococcus. aureus isolates from various clinical specimens [pus, wound or vaginal swabs, blood, body fluids (csf, pleural fluid, ascitic fluid) urine, sputum, ET secretion etc. were included in the study. Isolates from both in-patients and out-patients were considered. Institutional Ethical clearance was obtained. Data regarding age, sex, etc was obtained from the requisition form submitted to microbiology Department, NIMS, Jaipur.

2.1 Isolation & Identification, of Staphylococcus aureus[10,11]

Smears were prepared from pus, wound swab, sediments of body fluids and respiratory samples, stained by Gram staining and examined for presence of inflammatory cells, epithelial cells and the type of microbial flora. As soon the samples received in the laboratory, Streak culture method was employed for sample inoculation on Blood agar, Nutrient agar. Culture plates were incubated at 37^{o} C aerobically for 24-48 hours .Plates were observed for typical colony characteristics of Staphylococcus aureus on Blood agar (β haemolysis) and Nutrient agar (yellow pigment).For plates with mixed culture growth, sub culture was done on blood agar and incubated for 18-24 hours aerobically at 37^{o}C. Sub culture was also done for Staphylococci colonies more than 48 hours old, where ever required. Gram's staining was performed from representative colonies, and observed for Gram positive cocci in clusters, under oil immersion microscope. Catalase test was performed from Nutrient agar plate. This test is positive for Staphylococcus aureus and differentiates from Streptococci. Slide coagulase test was employed to detect presence of clumping. factor (bound coagulase) which was indicated by prompt clumping in positive isolates. Irrespective of result of slide coagulase test, tube coagulase test was done for presence of coagulase enzyme (Free coagulase). Staphylococcus aureus is positive. For all Staphylococcus aureus strains mannitol fermentation was observed on Mannitol salt agar.

2.2 Antibiotic Sensitivity testing of Staphylococcus aureus[12]

Antibiotic susceptibility to a panel of drugs was tested using (Modified Kirby Bauer's method. Antibiotic discs procured commercially [Hi-media Laboratories, Mumbai] and were placed on inoculated MHA plates using forceps. Plates were incubated at 35±2PC for 18-24 hours. Zone of inhibition of all the antibiotics were measured with scale in reflected light against a black background, to the nearest mm. Interpretation was done according to the guidelines of Clinical Laboratory Standards Institute (2012). All Staphylococcus aureus isolates were subjected to predetermined panel of antibiotics which includes cefoxitin[30ug], Penicillin [10µgm], Cotrimoxazole [1.5/23.75µgm], Erythromycin[15µgm], Ciprofloxacin [5µgm], Clindamycin [2 µgm], Gentamycin [10 µgm], Levofloxacin [5 µgm], Tetracycline [30 µgm], Vancomycin [30 µgm]. Staphylococcus aureus ATCC 25923 was used as control. Cefoxitin disc (30 µgm) was used along with other antibiotics, to detect methicillin resistant isolates as it is a potent inducer of mec A gene mediated resistance[12].

2.3 Detection of Vancomycin resistance

Following 3 methods were used and comparison between modified Kirby -Bauer disc diffusion method using 30μg Vancomycin disc and BHI vancomycin screen agar (6 µg/ml) was done keeping in view E-test as gold standard for detection of vancomycin resistance.

2.3.1 Disc diffusion by Modified Kirby Bauer's Method[13]

It was performed using Vancomycin 30p.g disc. The diameter of-zone of inhibition was measured and interpreted according to CLSI guidelines 2007 [13]. B.BHI vancomycin screen agar[12] BHI-VSA (Hi-Media, India) plates containing 6m/m1 vancomycin were prepared. Inoculum suspensions were prepared by selecting colonies from subcultured colonies on nutrient agar plates. The colonies were transferred to sterile saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard. Using a micropipette, spot of 10-µL drop onto agar surface was done and were incubated aerobically at 35±2°Cfor 24 hrs[14]. Any growth is examined carefully with transmitted light.

2.3.2 Determination of Minimum Inhibitory Concentration (MIC) values[15]

The MIC value of vancomycin was determined by E-test [Epsilometer-test]. Inoculum suspensions were prepared by selecting colonies from overnight growth on nutrient agar plates. The colonies were transferred to sterile saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard and a lawn culture was prepared by pouring the growth suspension on the surface of the BHI agar plate. After drying the surface for half an hour, the E-strips were placed over the surface and incubated over night at 35°C.The plates were read only when sufficient growth was seen and the MIC values were recorded where the ellipse intersects the MIC scale on the strip. If the ellipse intersects the strip in between 2 dilutions MIC was recorded as the value which is nearest to the intersection. For classifying isolates of Staphylococcus aureus with reduced susceptibility to vancomycin based on the laboratory breakpoint published by the clinical and laboratory standards institute [CLSI guidelines][12]

Vancomycin sensitive Staphylococcus aureus [VSSA]: ≤2 µg/ml.
Vancomycin intermediate Staphylococcus aureus [VISA]: 4-8 µg/ml.
Vancomycin resistant Staphylococcus aureus [VRSA]: ≥16 µg/ml.

III. Observation And Results

During the study period, a total of 287 non duplicate Staphylococcus aureus isolates were obtained from variation clinical specimens. Methicillin resistance was detected in 146 (51%) of the total isolates. Isolates that were sensitive to methicillin were 141 (49%) in 287strains.

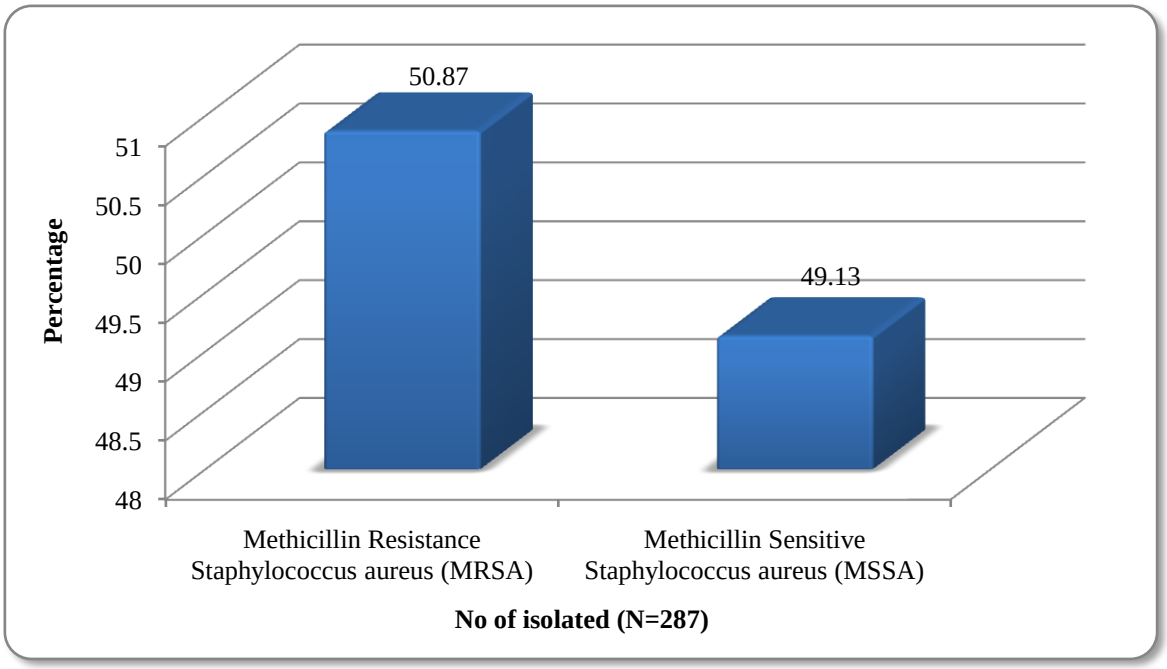

Fig. 1: Prevalence of MRSA and MSSA in total of 287 Staphylococcus aureus isolates

3.1 Vancomycin Resistance

Three phenotypic methods employed in detection of Vancomycin resistance,

3.1.1 Disc Diffusion method

In a total of 287 S.aureus stains, 9 (3%) isolates had shown resistance towards Vancomycin in Disc diffusion.

3.1.2 BHI Vancomycin screen agar (6 ugtn/ ml)

None of the isolates grew on So no resistance is.reported by this method.

3.1.3 Epsilometer [E-testl for Vancomycin

It was considered as Gold Standard. MIC values of all the 287 isolates fall in between range of≤ 2µg /ml, which is category of sensitive. All of the 9 isolates, which were resistant by Vancomycin disc diffusion, are in range of sensitive MIC.

Table No 1: Detection of Vancomycin resistance by different phenotypic methods

Methods	VISA	VRSA	VSSA	Total
Vancomycin disc diffusion (30 ug/disc)	NA	9	278	287
Vancomycin Screen Agar(6ug/ml)	00	00	287	287
Vancomycin E Test	00	00	287	287

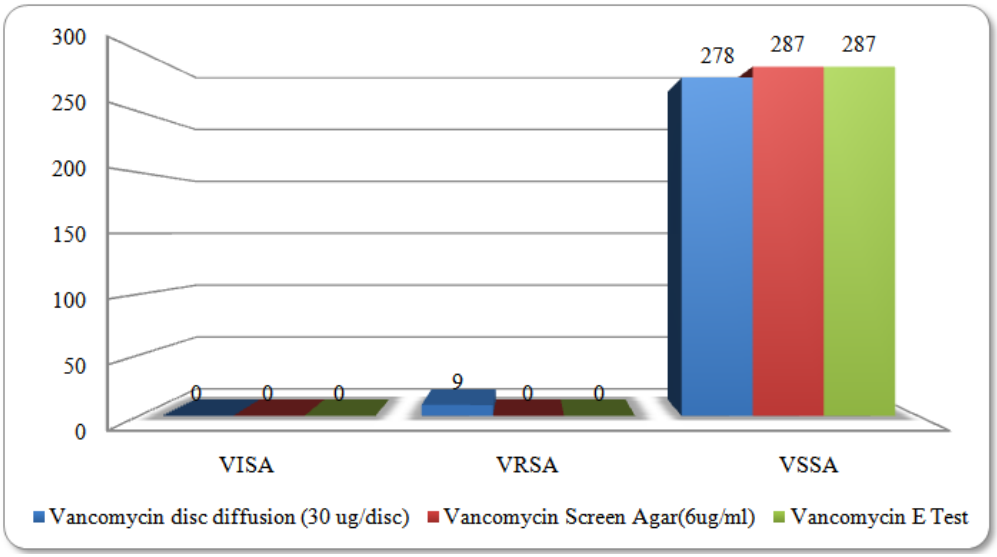

Fig. 2: Detection of Vancomycin resistance by different phenotypic methods

Table No 2: Distribution of the cases according to MIC Values (ugm/ml) against vancomycin

MIC Values (ugm/ml)	Total	
	No	%
0-0.5	169	58.89
>0.5-1	112	39.02
>1-1.5	6	2.09
Total	287	100.00

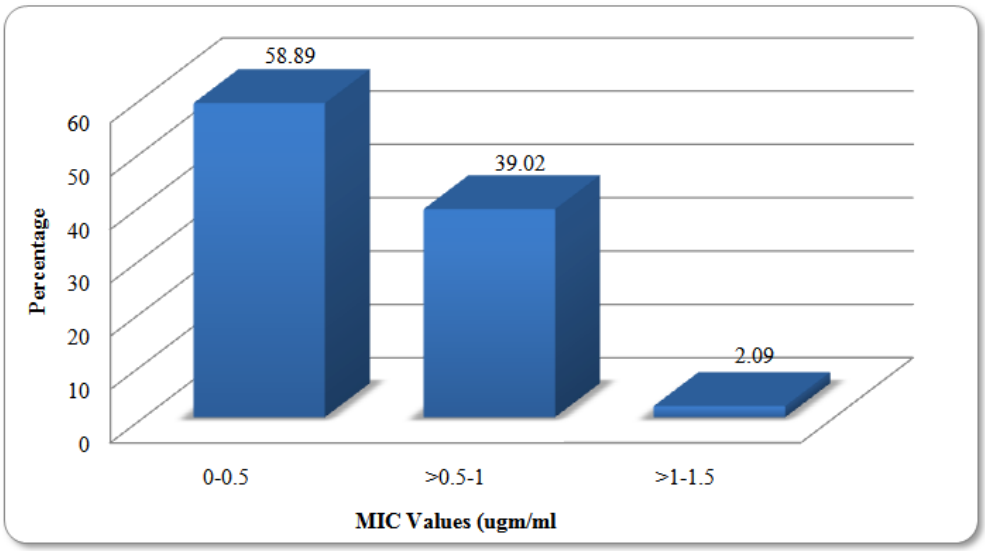

Fig.3: Distribution of the cases according to MIC Values (ugm/ml) against vancomycin

3.2 Antibiogram

All 287 Staphylococcus aureus strains were tested for antibiotic susceptibility pattern against a panel of predefined antibiotics, which was described earlier.

Staphylococcus aureus showed 88% resistance to Pencillin G, followed by 51% resistance to Cefoxitin, 43% each to Co-trimaxazole and Erythromycin, 66% to Ciprofloxacin, 31% to Clindamycin, 16% to Gentarnycin and 28% to Levofloxacin and 3% each for both Tetracycline and Vancomycin.

IV. Discussion

Staphylococcus aureus is a major human pathogen and is one of the commonest causative agent of Community and Hospital acquired infections[11]. The treatment of Staphylococcus aureus infection has become problematic because of emergence of resistance to Penicillin, Methicillin, Vancomycin and many other antibiotics, by acquiring several resistance mechanisms. Increased antimicrobial resistance for such an organism is, therefore a cause of concern.

In the past few decades MRSA has emerged as an important nosocomial pathogen worldwide. In India, prevalence rate varies from 30-85% in different parts and has now become endemic.[16,17,18] A multicentric study done in India involving 17 tertiary care Hospitals reported MRSA prevalence of 41% in 2008-2009.

MRSA is of serious therapeutic, concern not only due to its resistance to Methicillin, but also because of resistance to many other antimicrobials that are used on regular basis in Hospitals. Therefore, the most reliable and sustained therapeutic agerit against methicillin-resiStant Staphylococcus aureus (MRSA) strains is Vancomycin[19]. There is still controversy in clinicians regarding the outcome of Vancomycin treatment in MRSA. Increasing prevalence of MRSA, lead to the extensive use of vancomycin. This inturn lead to the decreased susceptibility to Vancomycin all over the World including India, this was soon followed by strains of Staphylococcus aureus that were totally resistant to vancomycin[20,21]. Such resistance resulted in serious clinical and public health consequences because currently few licenced alternatives are available. to treat vancomycin resistant Staphylococcus aureus infections.

Thus the present study was undertaken to determine the current status of Vancomycin susceptibility in our Hospital setup. to compare the Vancomycin disc diffusion test with BHI- VSA [6µgm/ml] for detecting Vanomycin resistance considering E-Test as gold standard and also to determine the antibiotic susceptibility pattern of these isolates.

In the present study a total of 287 non-duplicate staphylococcus strains were isolated from various clinical specimens. Among all these samples highest isolation was from pus 175 (61%). Harcharan Singh et al in Udaipur (65%)[22], Manu Chaudhary et al in H.P (63%)[23] and Ankur Goyal et at in Agra (66,03%) [24], also reported the highest isolation of Staphylococcus. aureus from pus.

In our study 146 (51%) isolates turned out to be MRSA and 141 (49%) as MSSA. from a total of 287 Staphylococcus aureus strains. The prevalence rate of MRSA in our institute is 51%, which is similar to the studies conducted by S.Vidhani and P.L. Mehndiratta et al in 2001[25] showing a prevalence rate of 51.6% and almost comparable -to the study conducted by Majumdar et al in 2001[26] and Assadullah et al in 2003[27] showing 52.9% prevalence rate. The higher rate in their studies may been attributed to the fact that the studies were conducted at a tertiary care multispecialty center with more and more patients coming from periphery and small nursing homes, where injudious use of antibiotics and inadequate infection control policies are prevalent.

In the present study, maximum MRSA were isolated from pus 89(61%), followed by blood 22(15%), respiratory secretions 15(10%), Swabs and body fluids 7(5%) each and least from Urine 6 (4%). This pattern correlates with studies conducted by Vidya Pai et al in 2010[28] and Nitish Kumar Sharma et al 2013[18]. This is due to the reason that Staphylococcus aureus accounts for most of the skin and soft tissue infections, septicemia and also respiratory tract infection.

Comparatively MRSA prevalence was more in males. (71%) than in females (29%) in our study: Similar findings was also reported by Rao BN et al[29] and Abhishek Mewar et al[30]. The increased rate of MRSA infections among males could be due to their more outdoor activities, inturn exposing them to contaminated environment and also compared to females, accidental injuries are more common among men.

Most of the MRSA strains were isolated from 21-30 yrs of age group (ie 23.69%) and in 31-40yrs (ie 17.77%), indicating MRSA infection is more common in working and old age group. The reason for this may be that younger age group are more involved in outdoor activities inturn exposing them to contaminated environment and in older age group it may be due to waning immunity, hormonal abnormalities and co-morbid conditions. Similar pattern of affected age group. was also reported by Ankur Goyal et al in 2013[24].

In the present study all 287 Staphylococcus aureus strains were screened for vancomycin resistance by Vancomycin disc diffusion method [30g/disc] and BHI-Vancomycin screen agar [6µg/ml]. These were further confirmed by Vancomycin E-test. All 287 Staphylococcus aureus isolates had MIC values ≤ 2µg/ml, hence all were sensitive to Vancomycin and were labeled as VSSA according to the CLSI guidelines 2012[12]. Among

these 169(59%) isolates had MIC of < 0.5µg/ml, 112[39%] had MIC of >0.5-1 µg /ml. Only 6[2%] had MIC of >1-1.5 µg /m1 and none of the isolates had MIC values of >1.5 µg /ml.

In our study no VISA. and VRSA found. This may be due to the fact that the community acquired MRSA (CA-MRSA) unlike the hospital acquired MRSA (HA-MRSA) are known to be sensitive to drugs other than vancomycin. Because of its. high cost, vancomycin may not be in use in the peripheral rural setups, thus decreasing the selection pressure for vancomycin resistance[31].

The current study only indicates the tip of iceberg. More and more studies should be undertaken in future to 'monitor the emergence of resistance to these. antibiotics. This also necessitates to find out better treatment policies and also to use cheaper and effective alternative anti - MRSA drugs so as to reduce the antibiotic pressure on vancomycin. Also clinicians should continue to exercise caution in their use of vancomycin in order to preserve this useful antibiotic and prolong its therapeutic usefulness.

V. Conclusion

To conclude, the result of our present study indicated high antibiotic resistance in commonly used antibiotics by MRSA isolates. The increased use of vancomycin drug has worsened the sensitivity.

We should undertake more and more such studies in future to fight against rising menace of antibiotic resistance, Also more research should be done to find better treatment policies, effective and cheaper alternative antibiotics in developing countries like ours. Even the clinical microbiology laboratories must ensure using detection methods with good sensitivity and specificity. We should also undertake more studies to find out the accurate screening method for VISA and VRSA. The findings of the studies should be shared with hospital infection control committee to help in the formulation of infection control polices and also antibiotic policies. So that the primary care givers can use antibiotics rationally.

However, S. aureus with reduced susceptibility was not observed in the present study. So, as a precautionary measure before starting the patient on vancomycin, the clinicians should seek the help of Clinical Microbiologist to determine the MIC of such strains so that emergence of vancomycin resistance can be prevented. All the laboratories should routinely test the MIC of vancomycin for all Staphylococcus aureus infection for appropriate treatment of patients and also for implementation of infection control.

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