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A Study to Comparison of MIC of Linezolid on MRSA by Micro Broth Dilution and E Strip Method in Teaching Hospital, Karnataka, India

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A B S T R A C T

Although for severe MRSA infections, vancomycin is described as the first-line intravenous drug, vancomycin-resistant and intermediate isolates of *Staphylococcus aureus* (VRSA & VISA) have been increasingly reported throughout the world. The therapeutic and life-saving option for VRSA and VISA infections is linezolid. Objective of our study was to assess Minimum inhibitory concentration of linezolid on MRSA by micro broth dilution and E strip method. One hundred fifty isolates from clinical samples were processed. Colonies suggestive of *Staphylococcus aureus* were identified as MRSA using Cefoxitin (30µg) discasper Clinical and Laboratory Standards Institute (CLSI) guidelines. MRSA Positive isolates were tested for Minimum inhibitory concentration of linezolid by E Strip and Micro broth dilution method. In our study out of 150 isolates of *Staphylococcus aureus*, 65(43.3%) were MRSA and 85(56.7%) were MSSA. A total of 65 MRSA isolates were tested for MIC by Estrip and Micro broth dilution, which showed 100% sensitivity for linezolid. In the present study, did not come across any resistant strain. In the study total of 65 isolates were tested, According to MIC of linezolid by E Strip which majority (36.9%) had MIC <2µg/ml followed by 30.8% with MIC<1µg/ml, 23% with MIC <4µg/ml and 9.2% with MIC <0.5µg/ml. In comparison to our study approximately similar MIC values were noted in the study conducted by Stefan Riedel *et al.*, showing 65.1% with MIC <1µg/ml, 27.9% with MIC <2µg/ml, 4.7% with MIC <0.5µg/ml and 2.3% with MIC <4µg/ml.According to MIC of linezolid by micro broth dilution majority 63.1% of the isolates were having MIC <1µg/ml, followed by 26.1% with MIC <2µg/ml, 6.1% with MIC <4µg/ml and 4.7% with MIC <0.5µg/ml.

Introduction

MRSA strains are critical public health threats because they cause hospital-acquired infections that can be difficult and expensive to treat. Many MRSA strains are susceptible only to vancomycin, and this antibiotic is used extensively to treat patients infected with these organisms. Hence, there is also concern that MRSA may serve as a reservoir of organisms that may give rise to vancomycin-resistant strains that could not be killed by available antibiotics.[1] SOver the past two decades, vancomycin has been considered the

antibiotic of choice for MRSA infections.[2] Recent reports describing the therapeutic failure of vancomycin for MRSA infection have aroused considerable concern regarding the emergence of MRSA strains for which there will be no effective therapy.[2]

Several hospital MRSA clones have become multidrug resistant, and have reduced susceptibility to vancomycin.[3]Although for severe MRSA infections, vancomycin is described as the first-line intravenous drug, vancomycin-resistant and intermediate isolates of *Staphylococcus aureus*(VRSA & VISA) have been increasingly reported throughout the world. The therapeutic and life-saving option for VRSA and VISA infections is linezolid.[4]

Linezolid is the first antimicrobial of oxazolidinone group available since 2000. It is the only antibiotic available as an oral formulation for resistant *Staphylococcus* infection.[5]

It is effective in skin and soft tissue infections, nosocomial pneumonias including VAP, infective endocarditis and MRSA meningitis. It is also effective in eradication of both nasal and throat colonization of MRSA.[6]Linezolid acts by inhibiting bacterial protein synthesis through binding to the peptidyltransferasecentre (PTC) of the 50S ribosomal subunit.[5]

To date, the following mechanisms responsible for linezolid resistance have been reported in clinical isolates of *S. aureus*:

(i) Mutations in the domain V region of one or more of the five or six copies of the 23S rRNA gene[7](ii) Acquisition of the plasmid-mediated ribosomal methyltransfer-asecfrgene[8]and (iii) Deletions or mutations in the ribosomal protein L3 of the PTC. Additional mutations in domain V of the 23S rRNA genes and substitutions in ribosomal protein L4 of the PTC are also reported in laboratory-derived linezolid-resistant *S. aureus*strains.[9]

The first case of linezolid-resistant staphylococci appeared within 1 year after linezolid was approved for therapeutic use[10]although linezolid resistance in *Staphylococcus aureus* is uncommon, emergence has been shown from some parts of the world From India, first case report of linezolid resistance was published in 2011 from Kashmir.[11]

Objective of our study was to assess Minimum inhibitory concentration of linezolidon MRSA by micro broth dilution and E strip method.

Materials and Methods

Study Setting

The study was carried in Department of microbiology,MandyaInstitute of medical Sciences, Mandya, Karnataka

Study design

Cross sectional study

Study period

January 2017 to December 2017

Sample size

150 Samples

Source of data

All isolates of *Staphylococcus aureus* from various clinical samples like urine, pus, sputum, blood and other body fluid received in microbiology laboratory, Mandya institute of medical science, Mandya.

Method of collection of data

The collected samples were inoculated onto MacConkey agar, Blood agar, Mannitol salt agar &incubated at 37°c for 24-48 hours. Yellow colonies from MSA were sub cultured on Nutrient agar. *Staphylococcus aureus* were identified and confirmed by Catalase test, Gram's staining and Coagulase test (Slide and tube) with respective controls using golden yellow colonies from Nutrient agar. A total of 150 isolates of *Staphylococcus aureus* thus obtained were screened for MRSA.

MRSA Positive isolates were tested for Minimum inhibitory concentration of linezolid by E Strip and Micro broth dilution method.

E-Strips

Requirements

E-test strips, McFarland standard 0.5, Forceps, Small sterile cotton swabs, wicker hams card (White paper with black lines), peptone broth, 4 ml volumes in tubes, Mueller Hinton II agar plates.[12]

Prepare inoculum

Remove the E strip from freezer (-20 ^{o}C)at least 30 minutes before required. With a loop, touch the top of 3 or 4 colonies & transfer to a tube of peptone water. Emulsify the inoculum on the inside of the tube to avoid lumps, incubate for 1-2hours at 37^{o}C.Compare turbidity to that in 0.5 McFarland standard by Nephlometry or Wickerhams card. Adjust turbidity of inoculum to match the standard.[12]

Inoculate agar plate

Lawn the MHA plate within 15 minutes of preparing the adjusted inoculum. Dip a sterile cotton swab into the inoculum and pulling out, slightly rotate the swab several times against the inside of the tube above the fluid level to remove excess liquid. Streak the swab over the entire surface of the agar plate. Rotate the plate approximately 60º, then repeat streaking. Complete the inoculation by running the swab around the rim of the agar.[12]

Apply E strips

Open E-test package by cutting along the broken line. If strips stick together, they may be pulled apart by handling the section marked E. Do not touch any other area of the strip Apply strips to agar surface using forceps (or E-test applicator if available). Place the strip with the 'E end' at the edge of the plate and with the scale visible. Do not press E-strip. Within 60 seconds E-strip will be absorbed and firmly adhere to the agar surface.[12]

Results interpretation

On day 2 Read MIC at the point where ellipse intersects the scale. If a MIC value between two-fold dilutions is seen, always round up to the highest value. Remember to read the MIC value at complete inhibition of all growth including isolated colonies.If the intersect differs on either side of the strip, read the MIC as the greater value. Ignore any growth at the edge of the strip.When the growth occurs along the entire strips, report the MIC as>the highest values on the MIC strips. When the inhibition ellipse is below the strip report the MIC <the lowest values on the MIC strips.[12]

To check purity of inoculum

Transfer inoculum from the tube onto a nutrient agar plate using a 10 µl loop. Incubate plates at 35^{o}C for 16 to 20 hours in ambient air. Compare the growth with AST plate having E strip.

Quality control

S.aureus ATCC 29213

Micro broth dilution

Requirements

Micro titre tray, mirror, graduated pipettes, multichannel pipettes, Micro tips, wicker hams card Mueller Hinton broth, Mc Farland standard solution.[12]

Standardisation of inoculum

From a pure culture plate, pick material from at least 3-4 colonies. Suspend in 4ml peptone water, Incubate at 37°C for 1-2 hours. Adjust to McFarland 0.5 using (Nephelometer) or Compare visually with the McFarland 0.5 standard using wicker hams card. McFarland 0.5 has approximately 10^8 CFU/ml, the McFarland matched inoculum has to be diluted further 100 times to get $5x10^4$ CFU/ml.

The suspension should be used for inoculation within 15 minutes to avoid further growth.[12] Antibiotic solution was diluted with sterile normal saline with different dilutions starting from 0.5, 1, 2, 4,8,16,32µl.

Procedure

The microtitre trays were inoculated with 50 µl of the inoculum suspension using a multi-channel pipette each well contains approximately 2.5 x 10^4 cells. 50 µl of antibiotic solution were added to the well-marked with different dilutions respectively. Plates were sealed and incubated at 37ºC for 18-22 hrs.

The incubation time is extremely important to obtain reliable end points. Quality control strains are run in parallel to the test strains.[12]

On day 2 Check growth in the positive control wells and no growth in negative control well. MIC is read as the lowest concentration without visible growth. Growth observed from micro titre plate were further cross verified by spot inoculation. Growth from micro titre plate were inoculated onto nutrient agar plate and incubated at 37ºC for overnight.[12]

On day 3 Results of micro broth dilution by unaided eye were compared with spot inoculation. Note: the MIC is determined from two fold dilutions of the antimicrobial agent. Be aware that "the true" MIC can be anywhere between the observed MIC and the dilution step below. Interpretation of MIC is done according to CLSI guidelines.

Statistical analysis

Data was entered into Microsoft excel data sheet and was analysed using SPSS 22 version software. Categorical data was represented in the form of Frequencies and proportions.

Results and Discussion

In the present study, 150 *Staphylococcus aureus* were screened for MRSA. Out of 150 isolates, 65 (43.3%) were MRSA and 85 (56.7%) were MSSA.

Out of 65 MRSA isolates, males have higher prevalence of 67.7% compared to females showing 32.3%.Out of 85 MSSA isolates, females have higher prevalence of 45.9% compared to males showing 54.1%.

Majority of MRSA isolated were in the age group < 14 yrs showing 80%, followed by age group >60 yrs showing 52%,age group between 31-60yrs showing 41.1 % and least 33.3% between 15-30 yrs.

Linezolid is an oxazolidionone derivative, used to treat infections caused by MRSA. MRSA is a pathogen that can result in wide spectrum of infections ranging from simple wound infection to life threatening illnesses because of which antimicrobial susceptibility testing and treating MRSA in very important. First case of linezolid resistance was detected in July 2004.

In our study we collected 150 isolates of *Staphylococcus aureus* and screened for MRSA, out of which 65 found to be MRSA. In the Present study, Out of 83(55.3%) males and 67(44.6%) females screened from 150 isolates, 44(67.7%) MRSA were isolated from males and 21(32.3%) were isolated from females.

In comparison with our study, the study conducted by Nadia aslam *et al.,*[13] shows majority of MRSA were isolated from male patients with 65.2% and female with 34.8%. Out of 65 MRSA, majority of isolates were isolated from age group less than 14 years with 80% followed by age group more than 60 years with 52% in comparison with Shakya *et al.,* [13] showing 79% below 14yrs and 48% above 60 yrs.

Similar findings were observed in a study conducted by Rachel J Gorwiz *et al.,* [14]showing72% below 14yrs and 47% above 60 yrs. In our study we have isolated MRSA from four different clinical samples at a time. Majority of the studies have taken from wound infections or pus sample received at their laboratory.

Out of 65 MRSA isolates majority were isolated from pus samples 48(73.8%), followed by sputum 11(16.9%) and urine 6(9.3%).Study conducted by Ali Khalid *et al.,* [15] shows similar distribution of MRSA isolates among the clinical samples. 60% isolated from pus samples, followed by 24% from sputum and endotracheal secretion, and 10% from urine samples.

Determination of minimum inhibitory concentration of linezolid by E Strip

In the present study total of 65 isolates were tested, out of which majority (36.9%) had MIC <2µg/ml followed by 30.8% with MIC<1µg/ml, 23% with MIC <4µg/ml and 9.2% with MIC <0.5µg/ml.

In comparison to our study approximately similar MIC values were noted in the study conducted by Stefan Riedel *et al.,* showing 65.1% with MIC <1µg/ml, 27.9% with MIC <2µg/ml, 4.7% with MIC <0.5µg/ml and 2.3% with MIC <4µg/ml. In the study conducted by J.L Kutti *et al.,* [16] showing 52.5% with MIC<1µg/ml, followed by 42.5% with MIC<0.5µg/ml, 2.5% with MIC<2µg/ml and MIC<4µg/ml. Study conducted by Curtis G Gemmel *et al.,* [17] shows all the isolates tested were having MIC value below 0.5µg/ml.

In the study conducted by Patel *et al.,* [18] and Yuka kitano *et al.,* [19] all the isolates had MIC <2µg/ml. Sader *et al.,* [20], Ali Khalid *et al.,* [15]&YousifTahira *et al.,* [21] showing similar kind of results with MIC< 1µg/ml. Study conducted by Vaishali u Thool *et al.,* [22] shows 8.9% had MIC<4µg/ml in comparison with our study.

Determination of minimum inhibitory concentration of linezolid by micro broth dilution

In our study majority 63.1% of the isolates were having MIC <1µg/ml, followed by 26.1% with MIC <2µg/ml, 6.1% with MIC <4µg/ml and 4.7% with MIC <0.5µg/ml. C Muller Seriyas *et al.,* [21] in his study, he observed that majority 86.7% of isolates were having MIC<0.5µg/ml, followed by 7% with

MIC <2µg/ml, 3.3% with MIC< 4µg/ml and 3% with MIC <1µg/ml. In the study conducted by S K Pillai *et al.,* [7], 50% isolates had MIC value <4µg/ml, followed by 20% with MIC<8µg/ml & MIC<16µg/ml and 10% with MIC<2µg/ml. In the study conducted by Citron *et al.,* and Deborach *et al.,* [23], all isolates had MIC<4µg/ml. In the study conducted by Stefen Riedel *et al.,* [24] and Robert K Flamm *et al.,* [25], shows all the isolates had MIC<2µg/ml. In the study conducted by GulserenALkatas, D F Basri, Nuramira Mohammed *et al.,* [26-28] shows all the isolates had MIC<1µg/ml.

Table.1 Distribution of MRSA and MSSA among the clinical samples

Samples	MRSA (%)	MSSA (%)	Total
Urine	6 (40.0)	9 (60.0)	15
Pus	48 (41.4)	68 (58.6)	116
Sputum	11 (61.1)	7 (38.9)	18
Throat swab	0	1 (100)	01
Total	**65**	**85**	**150**

Out of 150 isolates, 116 (77.3%) were isolated from pus, 18 (12%) from sputum, 15(10%) from urine and 1(0.7%) from throat swab. Out of 65 MRSA, 48 (73.8%) were isolated from pus, 11(16.9%) from sputum, 6 (9.3%) from urine. Out of 85 MSSA, 68(80%) were isolated from pus, 9(10.6%) from urine, 7(8.2%) from sputum and 1(1.2%) from throat swab.

Table.2 Distribution according to MIC of Linezolide by E-strip and Microbroth dilution

METHOD	MIC <0.5µg/ml	MIC <1µg/ml	MIC <2µg/ml	MIC <4µg/ml
Micro broth dilution	4.7%	63.1%	26.1%	6.1%
E-STRIP	9.2%	30.8%	36.9%	23%

MRSA Positive isolates were tested for Minimum inhibitory concentration of linezolid by E Strip and micro broth dilution method.

MIC of linezolide by microbroth dilution

Majority 63.1% of the isolates were having MIC <1µg/ml, followed by 26.1% with MIC <2µg/ml, 6.1% with MIC <4µg/ml and 4.7% with MIC <0.5µg/ml.

MIC of linezolide by E-strip

In our study total of 65 isolates were tested, out of which majority(36.9%) had MIC <2µg/ml followed by 30.8% with MIC <1µg/ml, 23% with MIC <4µg/ml and 9.2% with MIC <0.5µg/ml.

Table.3 Results of MIC of Linezolide by E-strip and Microbroth dilution

Method	MIC	
	Sensitive (%)	Resistant (%)
Microbroth Dilution	65(100)	Nil
E-STRIP	65 (100)	Nil

A total of 65 MRSA isolates were tested for MIC by Estrip and microbrothdilution, which showed 100% sensitivity for linezolid. In our study, we did not come across any resistant strain. In current study, no linezolid resistant strain was isolated. Higher MIC values in the present study shows emerging Linezolid resistance in the hospitals. This emerging linezolid resistance is mainly due to empirical and prolonged therapies, still resistant strains of LRSA is rare.

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