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Original Research Article

Invitro susceptibility of Imipenem by E-test in different conditions of Methicillin resistant *Staphylococcus aureus*

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ABSTRACT

MRSA infections have been confined to health care centres for decades, which is recognised as one of the major pathogen in Hospital as well as community settings. MRSA infections are associated with high morbidity and mortality. However, nowadays MRSA infections are increasingly seen in young healthy individuals with no exposure to health care centres, ie;CA-MRSA infections are seen increasingly. 50% to 70% of Staphylococcus aureus isolates submitted to area labs are methicillin resistant. MRSA is a major threat to patient safety. There are antibiotics that can kill MRSA germs. MRSA are resistant to most of the beta lactam antibiotics, not only to beta lactam but also to several other agents which is used for treatment. Imipenem is the most important antibiotic of Carbapenem, it is bactericidal for most aerobic and anaerobic pathogenic bacteria except MRSA. But in several studies its action against MRSA was shown. Here in this study, invitro susceptibility testing indicated that Imipenem is active against MRSA at different conditions, ie; at a physiological pH 7.4 & pH 5.5 an abscess condition and at two temperature 370c and 300c .Since the methicillin resistance is enhanced at 300c, the MIC of Imipenem detected at two temperature. Imipenem has a low molecular weight so it has a better penetration effect, than vancomycin and Daptomycin which is used for the treatment of MRSA. In, invitro study Imipenem spread in the medium more rapidly, and kill bacteria. So in MIC testing by Epsilometri test there is wide zone will be seen.

Context: Imipenem is the most important antibiotic of Carbapenem, it is bactericidal.

Aims: To detect the in-vitro susceptibility of Imipenem by E test in different conditions of Methicillin Resistant Staphylococcus aureus.

Settings and Design: Malabar Institute of Medical Sciences, Calicut; Randomized controlled trial.

Materials and Methods: Sample collection, Confirmation of MRSA by latex agglutination and cefoxitin e-test, detection of MIC of Imipenem by E-test at different conditions of MRSA, Detection of combination effect of Imipenem and Daptomycin against MRSA by E-test.

Statistical analysis used: paired sample t test

1. Introduction

Staphylococcus aureus is a facultative anaerobic or aerobic Gram positive coccal bacterium, frequently found as part of the normal skin flora on the skin of humans which leads to localised superficial self-limiting infections and was the common cause of nosocomial infections.[1] Methicillin Resistant Staphylococcus aureus [MRSA] is a strain of Staphylococcus aureus, which is responsible for several difficult to treat infections in humans. MRSA isolates are resistant virtually to all beta-lactam antibiotics that usually used to treat these organisms due to the low affinity of PBP2a for these drugs.[2] MRSA can cause infections such as boils, wound infections, infected decubitus ulcers, severe

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sepsis, septic shock, cellulitis, abscess etc. If not treated immediately, MRSA infections can infect the heart and lungs and cause pneumonia or even death.[3] MRSA are mainly 2 categories, Hospital Acquired MRSA[HA-MRSA] and Community Acquired MRSA[CA-MRSA].Both CA-MRSA and HA-MRSA become wide spread.[4]

MRSA is of special concern because it is resistant not only to methicillin, oxacillin and nafcillin but also to all other beta lactams including cephalosporin, carbapenem and aztreonam. All MRSA strains contain a mecA gene and regulatory sequences that encode for production of a low-affinity penicillin binding protein [PBP-2][0]. Because of the increasing importance of staphylococcal infections, the limited therapeutic options and economic concerns alternate agents to treat staphylococcal infections are needed.

The common antibiotics that may still work include Vancomycin, Daptomycin, Clindamycin, Doxycycline, Linezolid, Trimethoprime-Sulfamethoxazole combination etc. Now certain cases reported the creeping resistance of vancomycin against MRSA.[5] Imipenem is one of the important drugs of Carbapenem group.[6] Since MRSA shows resistance to Imipenem, but several studies shows its efficacy against MRSA.[7] Action of Imipenem against MRSA are detected in different conditions ie; at a physiological pH7.4 and at an acidic pH 5.5 that is in case of an abscess condition where the pus has an acidic pH. An abscess is a collection of pus that has accumulated in a cavity formed by the tissue in which the pus resides due to an infectious process usually caused by bacteria.[8] pH of pus was almost always acidic –centering in 4.5 to 5.5 range. Presumably the build-up of acid came about because most bacteria convert their foods into acid by products. It was noted that Imipenem induces autolysis in MRSA, which is stronger in mec A strains than in isogenic strains lacking mec A.[9] In both MSSA and MRSA PBP1,2,3 and 4 are present.PBP4 may play an important role in the effect of Imipenem against MRSA because the compound having strong affinity to PBP4.[10] The induction of low affinity PBP in MRSA occurs to a larger extent when the bacteria grown at 30^0c rather than at 37^0c, which condition known to favours the expression of methicillin resistance.[11,12]

Daptomycin a cyclic lipopeptide with activity against most Gram positive pathogens, including MRSA. It appears to be more rapidly bactericidal than vancomycin.[13] In the last years development of new anti-microbial agents targeted to treat MRSA infections, such as Daptomycin, has lead to SSaccess antimicrobial activity of such drugs in combinations particularly Beta-lactams.[14,15]

2. Aim

To detect the in-vitro susceptibility of Imipenem by E test in different conditions of Methicillin Resistant Staphylococcus aureus.

3. Objectives

1. To detect Minimum inhibitory concentration of Imipenem at pH 7.4 at 30 &37^oc in MRSA by E test method.
2. To detect MIC of Imipenem at pH 5.5 at 30 &37^0c in MRSA by Etest method.
3. To detect MIC of Daptomycin in combination with Imipenem in MRSA by Etest method.ss
4. To detect MIC of Daptomycin without Imipenem in MRSA by E test method.

4. Materials and Methods

The study was conducted at the Department of Clinical Microbiology Laboratory of Malabar Institute of Medical Sciences, Calicut, for a period of three months starting from 9^{th} April to 10^{th} July 2012.

4.1. Collection of samples

From 6875 vaious clinical samples consecutive Staphylococcus aureus were isolated from the Microbiology Laboratory were used for this study. Both invasive and non-invasive samples collected according to recommended standard procedures.

4.2. Identification and Isolation of bacteria

1. Thin smear was prepared from the samples collected ,and do Grams stain
2. The gram positive cocci with cluster formation alone were further analysed.
3. Then the sample was cultured on Blood agar and Macconkey agar.
4. After overnight incubation at 37^0c the plates were examined for any growth.
5. If growth was obtained the colony characteristics were defined according to their size, transparency, elevation, and haemolytic properties.
6. Gram stain was performed with each type of colony obtained on Blood agar and MacConkey agar
7. From Gram stain, if Gram positive cocci was observed, a Catalase test was performed to rule out Staphylococcus group.
8. With Catalase positive organisms, Coagulase test was performed to see if it belongs to coagulase positive Staphylococcus aureus, Coagulase negative Staphylococci were excluded from the study.

4.3. Confirmation of methicillin resistant staphylococcus aureus

4.3.1. Detection of MRSA by latex agglutination test

1. A loopful of colonies from blood agar was suspended in 200μLI of extraction reagent[0.1ml NaOH] and boiled for 3minute.

2. Cooled and then add extraction reagent 2[0.5m KH2PO4], and mix well.
3. This mixture was centrifuged and 50μ L of the supernatant was added to each of the two circles of the test slide.
4. Anti-penicillin binding protein 2a monoclonal antibody sensitized latex [50μl] was added to one of the circles and 50μl of negative control latex5P was added to the other circle.
5. The presence of agglutination was visually evaluated after 3minute
6. Staphylococcus aureus ATCC33591[positive control] and ATCC25923[Negative control]were included as controls in the test.
7. Strains showing positive agglutination test was taken for the study.

4.3.2. Cefoxitin Epsilometri test[E-test] for detection of MRSA

1. Suspension of the test organism in sterile saline was prepared
2. Turbidity was adjusted to 1McFarland standard
3. Lawn culture of the bacterial suspension was inoculated into Muller Hinton agar media using sterile swab.
4. Apply cefoxitin E strip to the inoculated agar surface with the MIC scale facing upwards.
5. Then the plate was incubated at 37^0c for overnight.
6. After incubation read the MIC where the inhibition ellipse intersects the MIC scale.
7. MIC value of ≤4 was considered as susceptible to methicillin ie;MSSA
8. MIC value >4 was considered as resistant to methicillin ie;MRSA.

With the confirmed MRSA strains MIC of Imipenem was detected.

4.4. Detection of mic of imipenem at pH7.4 and 5.5 AT 30 and 37^0C by e –test method against MRSA

Preparation of muller hinton agar with pH 5.5

1. It was prepared by dissolving 38gm of MHA powder in 1000ml distilled water.
2. The pH of the medium was adjusted to 5.5 by adjusting it with 1N HCL and NaOH.
3. Then media is sterilized, poured, and solidify and keet in refrigerator. Before use plate was dried.

4.4.1. Procedure

1. Imipenem E strip was allowed to come to room temperature before opening the container.
2. Prepare the inoculum using confirmed MRSA strains in saline and turbidity was adjusted to 1McFarland standard'
3. After dipping the swab in inoculum, press out extra fluid from the swab before streaking to avoid excess inoculums.
4. Lawn culture of bacterial suspension was done evenly in three planes onto the surface of MHA plate with pH5.5 and pH7.4.
5. Apply Imipenem E-strip to both MHA plate having pH 5.5 and 7.4 with MIC scale facing upwards.
6. Plates were incubated at 37^0c and 30^0c for overnight.
7. After incubation, read the MIC were the inhibition ellipse intersects the MIC scale.
8. An MIC value of≥16 μ gm/ml was taken as resistant;8 μ gm/ml taken as intermediate, and ≤4 μ gm/ml was taken as susceptible[CLSI2012]

4.5. Detection of mic of daptomycin without imipenem in mrsa by e- test

1. Daptomycin E strip was placed on to the inoculated MHA plate with pH7.4
2. Plates were incubated at 37^0c overnight.
3. After the incubation read the MIC value of MRSA aginst Daptomycin.
4. MIC values of >1ug/ml was taken as resistant and ≤1ug/ml was taken as susceptible[CLSI 2012]
5. Detection of mic of daptomycin in combination with imipenem for mrsa by e-test

4.6. Imipenem incorporated MHA plate preparation

MHA plate was prepared and pH was adjusted to 7.4.It was then sterilized by autoclaving at 121^0c for 20minutes and cooled .After cooling incorporate Imipenem powder, the subclinical concentration of Imipenem for MRSA ie;0.032 μ gm/ml in media.Then mix well and pour media into petriplates, solidify, and stored in refrigerator.

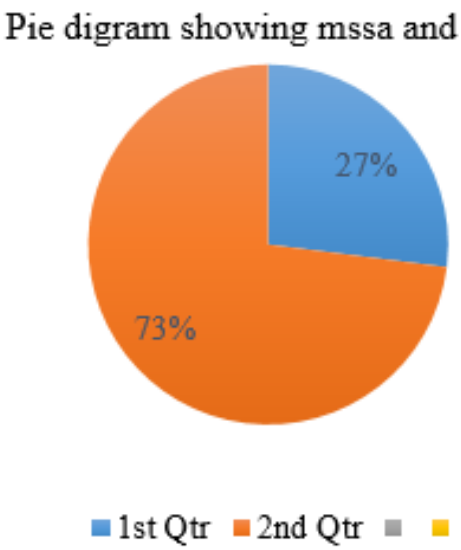
4.6.1. Procedure

1. Lawn culture the bacterial suspension into the prepared MHA plate incorporated with Imipenem, using sterile cotton swab.
2. Apply Daptomycin E-strip to the agar surface ,then incubate at 37^0c for overnight.
3. After incubation read the MIC where the inhibition ellipse intersects the MIC scale.
4. MIC values of >1 μ g/ml was taken as resistant and ≤1 μ g/ml was taken as susceptible[CLSI]

5. Observation and Results

Out of 6875 various clinical samples 110 consecutive Staphylococcus aureus were isolated from the Clinical Microbiology Laboratory of Malabar Institute of Medical Sciences, Calicut were used for this study. In which invasive and non-invasive samples are involved. In which 40 strains are confirmed as MRSA, by PBP2a latex agglutination test

and Cefoxitin E-test method.

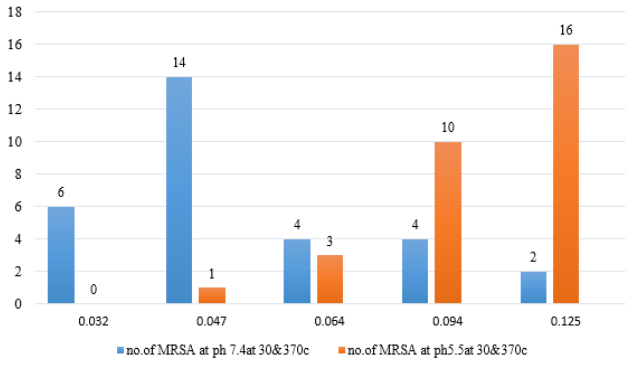


Graph 1: MRSA-27% & MSSA-73%

Results of mic of imipenem for mrsa at Ph7.4 and 5.5 at 30&370c by E-Test.

MIC of Imipenem [ug/ml]	No. of MRSA for MIC ofImipenem at pH 7.4 at 30&37^0 c	No. of MRSA for MIC ofImipenem at pH 5.5 at 30&37^0 c
0.032	6	0
0.047	14	1
0.064	4	3
0.094	4	10
0.125	2	16

The table shows that MIC of Imipenem at different phi e; at 7.4 and 5.5 at 30&37^{0}c for MRSA strains shows in vitro susceptibility to Imipenem, there is no variation of MIC at the two temperatures.



Graph 2: Result of Imipenem at pH7.4&5.5 at 30&37^{0}c by E test

The paired sample t test on average between number of MRSA for MIC of Imipenem at pH7.4 at 30&37^{0}c and number of MRSA for MIC of Imipenem at pH5.5at 30&37^{0}c

	Mean	N	Std.Deviation	Std.Error Mean
Imipenem at pH7.4	.05773	30	.026297	.004801
Imipenem at pH5.5	.10597	30	.0234881	.004287

Mean diference	Std.error mean	p-value
-0.0482	0.0040	.000

The above tables give the details of paired sample t test for testing the significance of average MIC of Imipenem at pH7.4 at 30 &37^{0}c and MIC of Imipenem at pH5.5 at 30&37^{0}c.From the second table we have the p value for the test is 0.000;it shows that the average MIC of Imipenem at pH 7.4 at 30&37^{0}c and MIC of Imipenem at pH5.5 at 30&37^{0}c is significant at 5%level. From the first table we can see that the means are 0.05773 and 0.10597 and there is a significant increase in the average MIC of Imipenem at pH 7.4 at 30&37^{0}c to average MIC of Imipenem at pH5.5 at 30&37^{0}c.

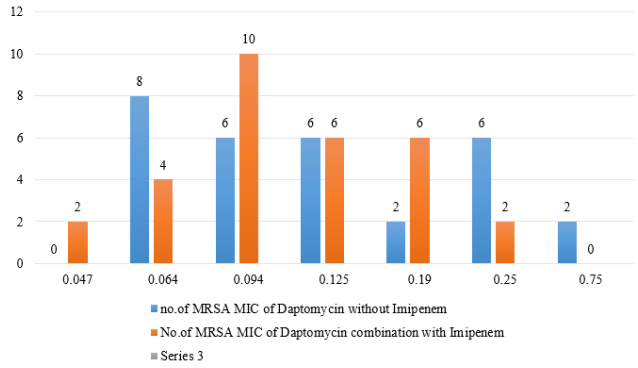
Results of mic of daptomycin with imipenem and without imipenem in MRSA

MIC of Daptomycin[ugm/ml]	No.of MRSA for MIC of Daptomycin without Imipenem	No.of MRSA for MIC of Daptomycin in combination with Imipenem
0.047	-	2
0.064	8	4
0.094	6	10
0.125	6	6
0.19	2	6
0.25	6	2
0.75	2	-

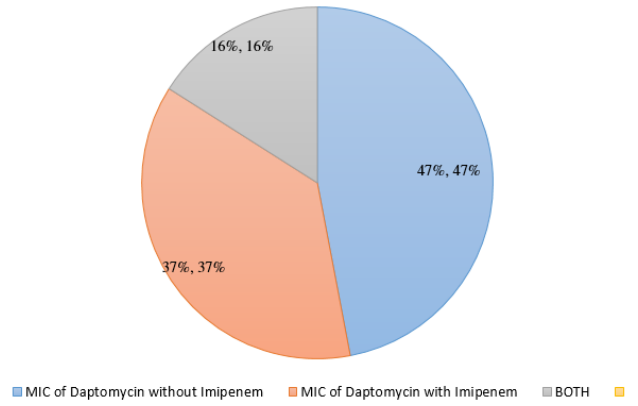
From the observations it was found 11 MRSA isolates have that Daptomycin MIC less at combination with Imipenem than without Imipenem ie; 37%,5 isolates of MRSA had equal MIC for Daptomycin with and without Imipenem combination,ie; 16% ;remaining 14 isolates have Daptomycin less without Imipenem than with Imipenem ie; 47%.

Paired sample t test on average MIC between the combination of Daptomycin with Imipenem and without Imipenem

The above tables the details of paired sample sample t-test for testing the significance of average MIC between



Graph 3: Results of mic of daptomycin combination with and without imipenem against MRSA



Graph 4: Results of MIC of Daptomycin combination with and without Imipenem

	Mean	N	Std.deviation	Std.error Mean
Daptomycin without Imipenem	0.17353	30	0.171033	0.031226
Daptomycin with Imipenem	0.12267	30	0.056659	0.010345

Mean Difference	Std.Error Mean	P-value
.0508	.0229	0.034

the Daptomycin without Imipenem and Daptomycin with Imipenem. From the second table we have the p value for the test is 0.034, which is less than 0.05; it shows that the average MIC between the Daptomycin without Imipenem and with Imipenem is not much significant at 5% level. From the first table we can see that, the means are 0.17353 and 0.12267 and there is a slight significant increase in the average MIC between the Daptomycin without and Daptomycin with Imipenem.

6. Discussion

The present study was carried out at MIMS, Calicut. The aim of the study was to detect the invitro susceptibility of

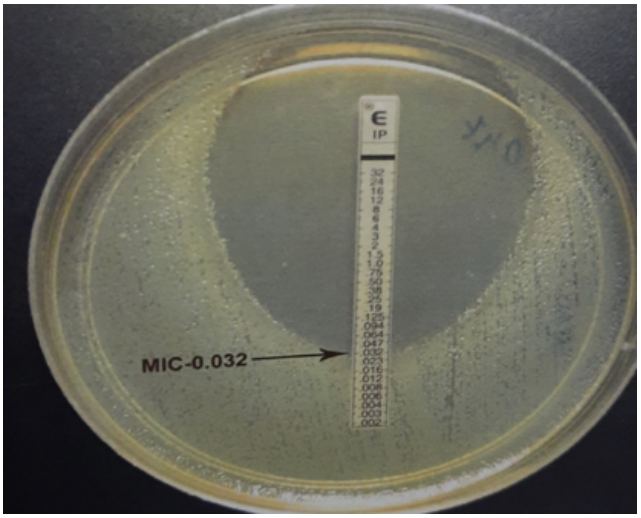


Fig. 1: MIC of Imipenem at Ph7.4

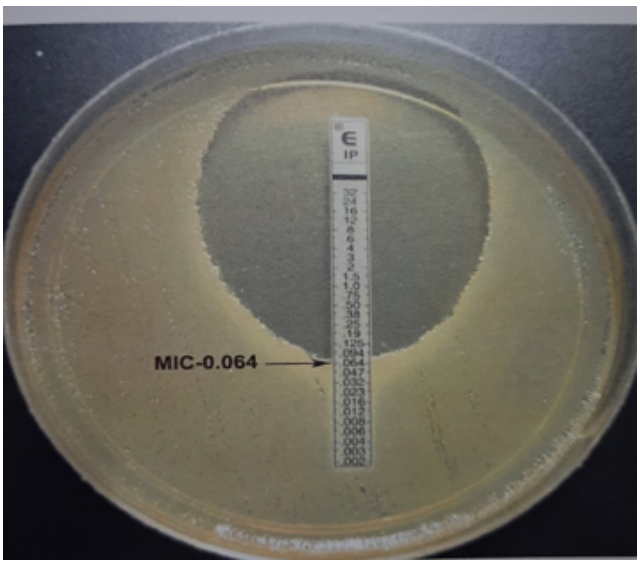


Fig. 2: MIC of Imipenem at pH5.5

Imipenem by E test in different conditions of MRSA,and a combination effect of Imipenem with Daptomycin.

Our results are generally in keeping with a study obtained by Takano.T et al.[16] That study shows superior invitro activity of Carbapenems over anti-MRSA and some related antimicrobial agents for CA-MRSA but not for HA-MRSA. Among the carbapenems examined, CA-MRSA strains were most susceptible to Imipenem [MIC-0.12MICROGM/ML].

Our results are agreement with those results obtained by Shannon.K and et al.[17] This study shows Imipenem-cilastatin efficacy against MRSA and MSSA, and proved its activity against both the organism isolated from different clinical specimens.

In another study the in-vivo activity of Imipenem against clinical isolates of bacteria obtained from patients at

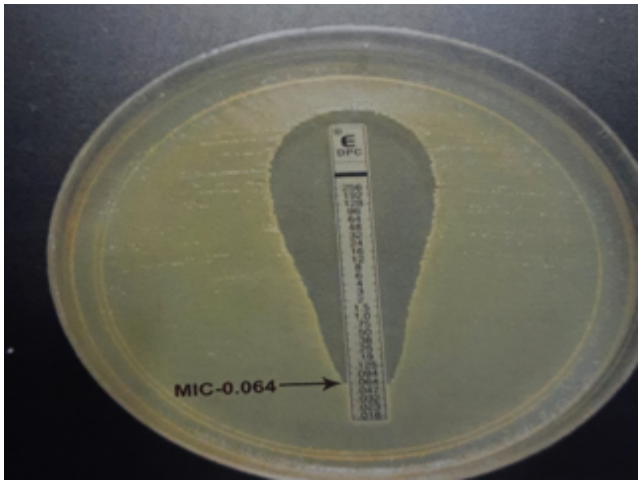


Fig. 3: MIC of Daptomycin without Imipenem.

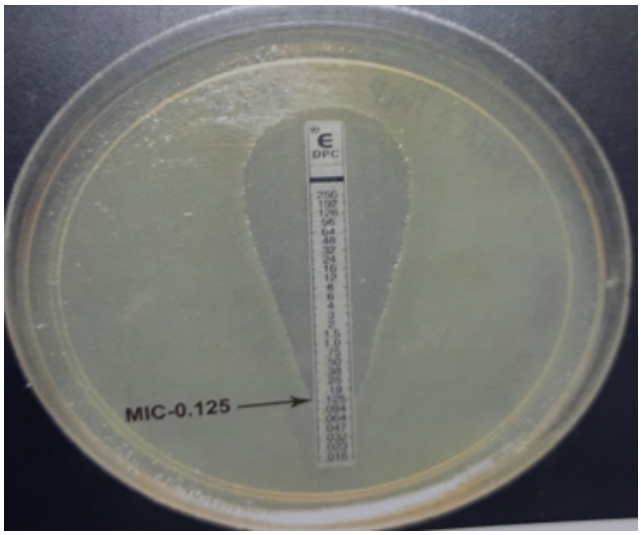


Fig. 4: MIC of Daptomycin combination with Imipenem.

Kaula Lumpur General Hospital were studied.[18] MIC of Imipenem against isolates were determined using an agar dilution method though Imipenem is very active against MSSA, it found to induce resistance in MRSAs. There is also wide disparity in imipenem MIC between MRSA s and MSSAs. Despite this Imipenem has been shown to be an effective agent in the treatment of both MRSA and MSSA infections.

Our results are generally in keeping with also in another study which is the, Enhanced activity of Daptomycin and Vancomycin combined with Imipenem or Oxacillin against CA-MRSA isolates were reported. Imipenem MIC for CA-MRSA values ranged from 0.03-1mg/L. Combination effect of Imipenem with Daptomycin and Vancomycin clearly indicated enhanced inhibition.[19] Here in this study the combination effect of imipenem plus Daptomycin also shows susceptibility results.

Hesitation in recommending Imipenem as an anti staphylococcal, especially against MRSA infections, has been studied based on invitro work. Imipenem act against MRSA by binding preferentially to PBP4 and PBP1 and then to 2 and 3. In this study the MIC of Imipenem in all different conditions were not greatly altered. And different conditions Imipenem shows enhanced inhibition and also in combination Imipenem with Daptomycin has better action against MRSA.

7. Conclusion

MRSA infection are greatly increased nowadays, and are the most common pathogen that cause serious infections in patients. MRSA was resistant to beta lactam antibiotics, including Imipenem .In this study the MIC of Imipenem against MRSA by E test shows a good susceptibility at different conditions involving two pH7.4 ie; physiological condition & at pH 5.5 which is condition of an abscess, and two temperature 37&30^0c because at lower temperature methicillin resistance is enhanced, and also a combination of Imipenem with Daptomycin. Since the Vancomycin the drug of choice for MRSA which has high molecular weight, and which now shows a creeping resistance, ie; MIC now at borderline .In case of endocarditis caused by MRSA vancomycin is not effective ,because its penetrating action into tissue is poor. Because of low molecular weight and good penetration capacity Imipenem can be used for the treatment of MRSA infections. Although this study was small, non-randomized, and in-vivo conditions are not studied, Imipenem appears to be an effective agent against MRSA infections. Further clinical trials will be necessary to establish the efficacy of Imipenem, and its combination with Daptomycin against serious MRSA infections.

8. Conflict of Interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

9. Source of Funding

None.

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