Original Research Article

A study on prevalence of Methicillin resistant staphylococcus aureus (MRSA) & Extended spectrum beta lactamase (ESBL) among isolates of Diabetic foot ulcers patients

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Abstract

Aim: The present investigation mainly study to identify the prevalence of Methicillin resistant staphylococcus aureus (MRSA), Extended spectrum beta lactamase (ESBL) and inducible clindamycin resistance in Methicillin resistant Staphylococcus aureus (MRSA) among isolates. **Methodology:** Amongst 100 diabetic cases included in the present study most of them were in 5th and 6th decade, showing ulcers chiefly in right foot. Males were affected primarily than females in the ratio of 2:1. The study carried out in the Department of Microbiology at Sri Venkateswaraa Medical College and research centre from June 2012 to December 2014. **Results:** Among the gram negative bacilli 21 were found to be ESBL producer by phenotype and those positive was compared with gold standard method PCR for the genes TEM and SHV showed 7 and 6 positive respectively. Those gram negative bacilli showing resistance for Imipenems were detected for Metallo-beta lactamase (MBL) and carabapenemase enzyme production of which 5 and 1 were positive by phenotypic method. Those 5 positive strains of MBL were compared with gold standard method PCR for VIM gene. Only one showed positive for VIM gene. **Conclusion:** The prevalence rate of MRSA was found to be high. ESBL and MBL producing gram negative bacterial species have a significant impact on the clinical outcome and efforts to control outbreaks of such infections. Indiscriminate use of third generation cephalosporins to treat gram negative bacterial infections is partly responsible for the emergence of resistance to beta-lactam antibiotics. Infection control measures are required to prevent the spread and reduce emergence of resistance.

Keywords: MRSA, Diabetic foot ulcers, MBL, beta-lactam antibiotics, Resistance.

Introduction

Diabetic foot is one of the most feared complications of diabetes and is the leading cause of hospitalization in diabetic patients. Diabetic patients have a lifetime risk as high as 25% for developing foot ulceration. Every year more than a million diabetic patients require limb amputation[1].

Many studies have reported on the bacteriology of Diabetic Foot Infections (DFIs) over the past 25 years, but the results have been varied and often contradictory. Mostly, the diabetic foot infections are mixed bacterial infections and a proper management of these infections requires an appropriate antibiotic selection, based on the culture and the antimicrobial susceptibility.The common aerobic organisms encountered are *Staphylococcus aureus, Proteus species, Pseudomonas, E. coli, Klebsiella species* and*coagulase negative staphylococci*etc[2]. Since 1940, penicillin was the first Beta lactam antibiotic of choice for many years. Bacteria were so inventive that they developed many mechanisms to escape the action of antibiotics and became resistant.

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Methicillin resistance first appeared among nosocomial isolates of *Staphylococcus aureus* in 1961[3].The incidence of methicillin resistant *Staphylococcus aureus* (MRSA) in India ranges from 30 to 70%[4,5]in diabetic foot.The increasing association of multi-drug resistant (MDR) pathogens with diabetic foot ulcers further compounds the challenge faced by the physician or the surgeon in treating diabetic ulcers without resorting to amputation [6]. Infection with MDR pathogens is also responsible for the increased duration of hospitalisation, cost of management, morbidity and mortality of the diabetic patients. Appropriate selection of antibiotics based on the antibiograms of the isolates from the lesions is most critical for the proper management of these infections.

So, this study was performed to determine the common etiological agents of diabetic foot infections in a tertiary hospital and their prevalence of MDR pathogens in patients with diabetic foot infections was also studied.

Materials and methods

This prospective study was conducted at the Department of Microbiology at Sri Venkestwara Medical College and Hospital over a period of $1^1/_2$ years from June 2013 to decemeber2014.A total number of 100pus and wound samples were collected from diabetic patients attending surgery Outpatient and inpatient department. Samples were collected using sterile swabs after adopting aseptic measures from all outpatient and inpatient. These samples were processed for aerobic culture and antibiotic susceptibility testing during this study period.

Inclusion criteria

- Includes both type I and type II Diabetes mellitus
- Age above 20 years of both sex

Exclusion criteria

- Patients on antibiotics treatment
- Individuals with non-diabetic ulcers.

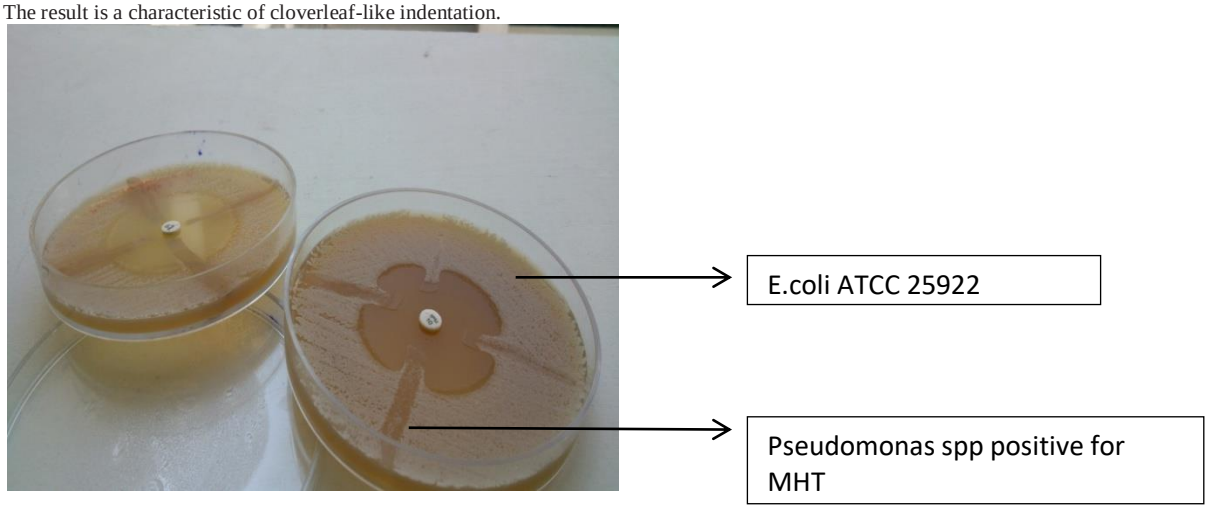
Patients of both genders who were clinically confirmed by physician, to have diabetes mellitus were evaluated and the data was collected using the help of questionnaire. Informed consent was obtained from the patient before collecting the sample. Institutional ethical committee clearance was obtained before commencement of the study.

Phenotypic detection of carbapenemase

Modified Hodge test for the detection of enzyme carabapenemase [7]:

Productionof Carbapenemaseenzyme was detected by streaking a lawn of the 1:10 dilution of *E.coli ATCC 25922* to a Mueller Hinton agar plate and allow to dry for 3–5 minutes. 10 µg meropenem disc was placed in the center of the test area. In a straight line organism was streaked from the edge of the disk to the edge of the plate. Up to four organisms can be tested on the same plate with one drug. It was incubate overnight at 35°C ± 2°C in ambient air for 16–24 hours. The test isolate was said to be positive when it showed a characteristic cloverleaf-like indentation. This is due to produces of enzyme which allows the growth of a carbapenem susceptible strain *(E.coli ATCC 25922)* towards a carbapenem disc[4-8].

The result is a characteristic of cloverleaf-like indentation.



Phenotypic detection of metallo beta lactamase

Disc potentiation method:

A 0.5 M EDTA impregnated ceftazidime (CTZ) disc and also a plain CTZ disc was placed on a lawn culture of the isolate and kept for 16-18hrs of incubation at 35°C. Standard ATCC strains were also tested in parallel. An increase in zone size of 7mm and above was considered positive for MBL production.

Polymerase chain reaction

Polymerase chain reaction (PCR) is a molecular method which is used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.They are used for the diagnostic of DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing) and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers are short DNA sequences complementary to the target region along with a DNA polymerase for the specific target gene amplification.

Molecular method for dna extraction

Culture broth was prepared by inoculating a pure culture of the organism into the nutrient broth and incubated overnight at 37ºc

DNA purification procedure

Before starting purification reaction water bath was set
And Elution Buffer was warmed upto 56 ºc

↓

2ml of culture broth was centrifuged
at 8000 rpm for 5 min until pellet is formed

↓

Supernatant was discarded

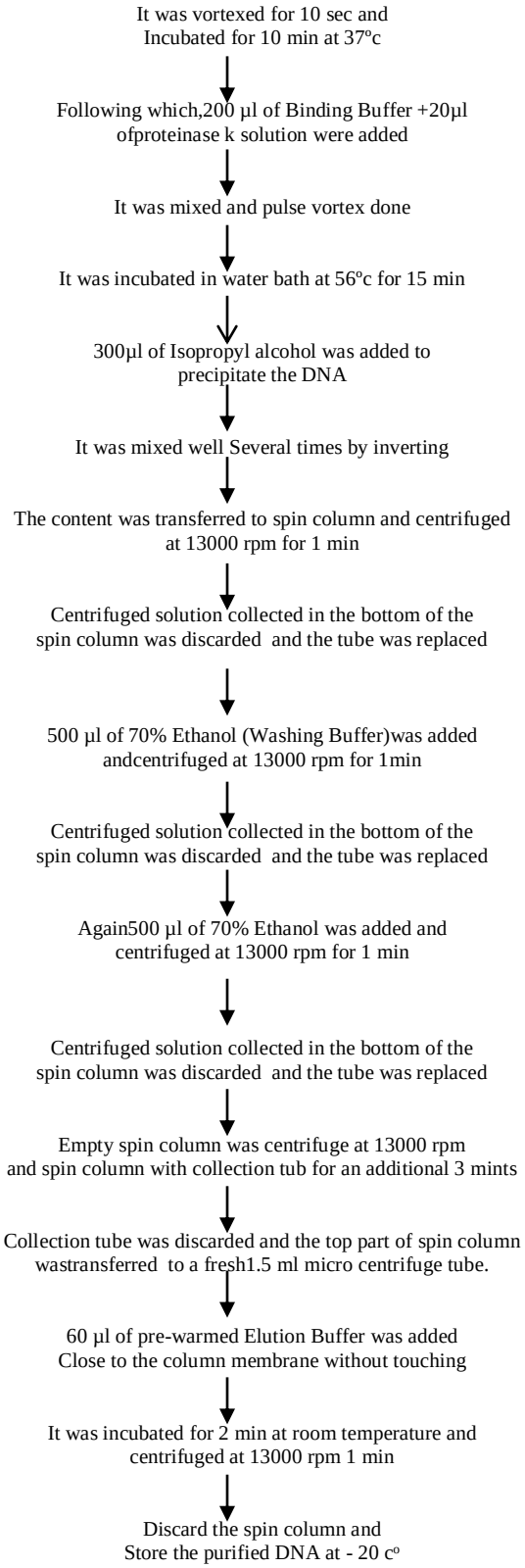
↓

The pellet was dissolved by Vortex

↓

200µl Phosphate Buffer /TE +180 µl of Digestion Buffer
+ 20 µl of lysozyme to the centrifuge column were added

↓



The PCR was done to detect ESBL for SHV and TEM group of genes

- Equipment's required:

1. Micropipettes

2. Thermo cycler for PCR
3. Electrophoresis apparatus
4. Ultraviolet trans illuminator

➢ 2x TANK BUFFER PREPARATION

Calculation:

2xrequired volume Buffer

= 300 ml = 6 ml

50 x stock Buffer 50x

For example: 6ml 50x Buffer + 294 ml distilled water

* Less than 500 ml make 2x

* More than 1000 ml make 1x

➢ Master Mix:

1. Taq buffer 1x conc
2. dNTP 0.2mm
3. Primer (forwards and reverse)0.3mm
4. $MgCl_2$

➢ 15 µl master mix with 5 µl of DNA samples was added in each PCR tube

➢ PCR tubes were kept in the thermo cycler and following settings were made

Steps		Temperature	Timing
1	INITIATION	95ºc	5 min
2	DENATURATION	95ºc	45 sec
3	ANNEALING	58ºc	45 sec
4	EXTENSION	72ºc	30 sec
5	FINAL EXTRACTION	72ºc	3min

Cycle was repeated from the step 2 to step 4 for 35 time and PCR products was obtained and stored at 4°c in deep freezer.

Agarose gel preparation

For 50 ml of 1x buffer 1 gmagarose powder was added. It was mixed well and heated until it dissolved and gave a clear consistency. Following which 5µl of ethidium Bromide was added. The comb was set in electrophoresis tray and the gel content was poured into the electrophoresis tray upto the thickness of 0.5 to 0.9 cm. After the gel was solidified it is put into tank buffer solutiontris EDTA in DNA free water. Following which comb was removed.

Gel document:(UV-Trans illuminator)

This a system used to look for migrated DNA strand after electrophoresis based on their molecular weight. The DNA molecules in the gel can be stained to make them visible. DNA may be visualized using ethidium bromide which is intercalated into DNA and fluoresce under ultraviolet light.

Results

Table 1: Distribution of ESBL among gram negative bacilli by phenotypic method

Organism	Total no isolates	Double disc approximation test	Disc diffusion method
E.coli	7	6	7
Klebsiella spp	6	4	6
Proteus spp	1	1	1
Pseudomonas spp	7	5	7
total	21	16	21

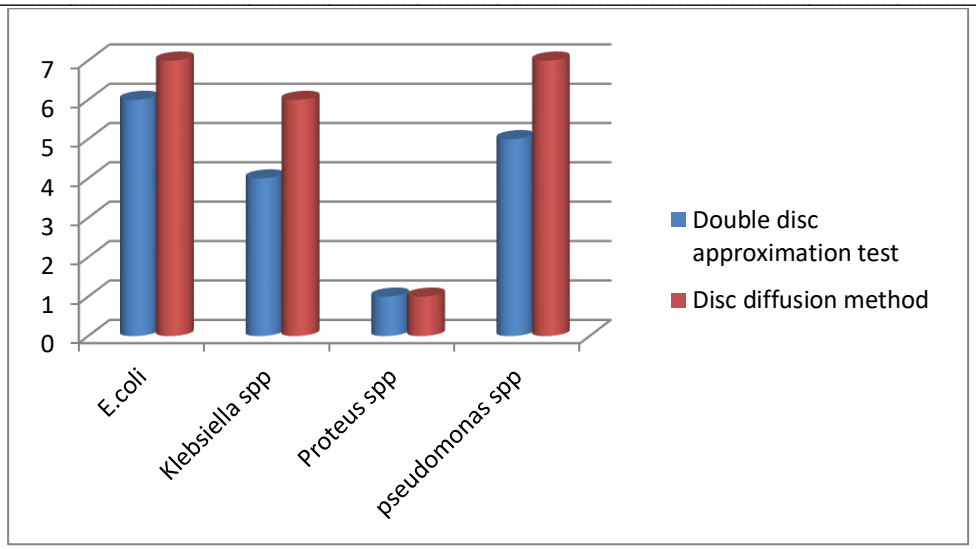


Fig 1: Distribution of ESBL among gram negative bacilli by phenotypic method

Table 1 and Fig 1 shows the distribution of ESBL among gram negative bacilli. Among the isolate Pseudomonas spp and E.coli were found to be more prevalent followed by Klebsiella spp and Proteus spp.

Table 2: Detection of ESBL among gram negative bacilli by genotypic method

Organism positive for ESBL by phenotypically	No. of isolates	Genotypic detection of SHV gene	Genotypic detection of TEM gene	Percentage
E.coli	7	3	2	71.4%
Klebsiella spp	6	1	2	50%
Pseudomonas spp	7	2	2	57.1%
Proteus spp	1	1	-	100%
total	21	7 (20%)	6 (18%)	61.9%

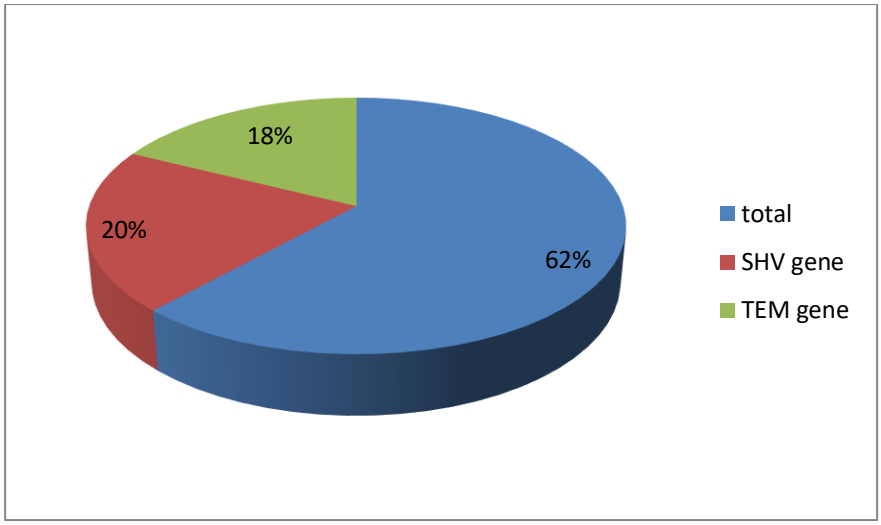
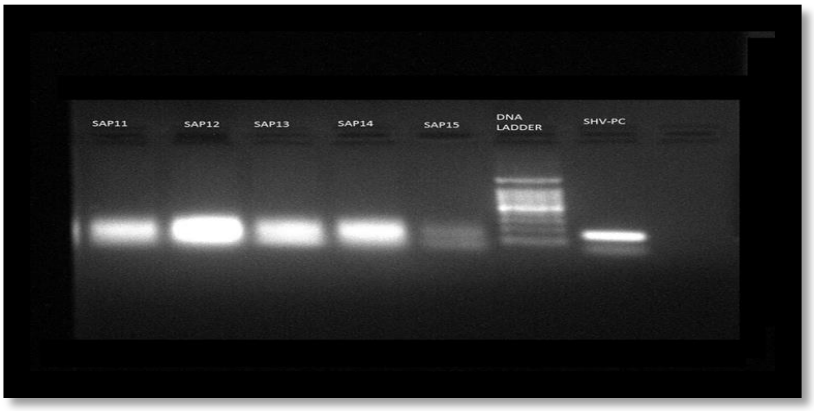


Fig 2:Detection of ESBL among gram negative bacilli by genotypic method

Table 2 and Fig 2 shows detection of ESBL by genotypic method among gram negative bacilli in diabetic foot ulcer. Among the 21 isolates, 7 (20%) were positive for SHV gene and 6 (18%) for TEM gene.

SHV gene



TEM gene

Table 3: Detection of Metallo-betalactamase among gram negative bacilli

Organism isolated	No.of MBL producer	Percentage
E.coli	1	16%
Klebsiella spp	1	17%
Acinetobacterspp	3	50%
Pseudomonas spp	1	17%

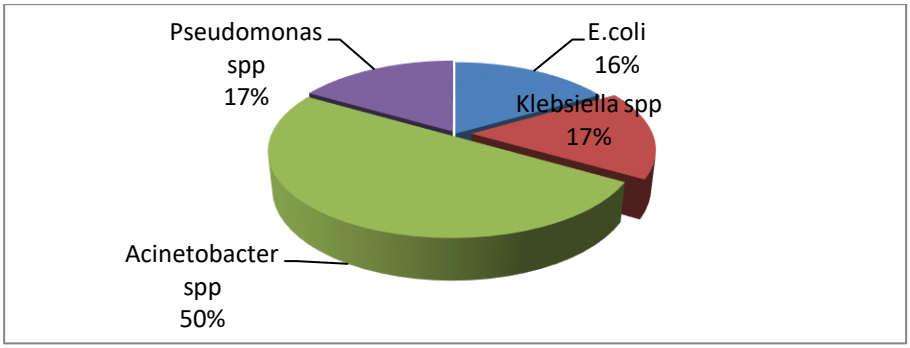
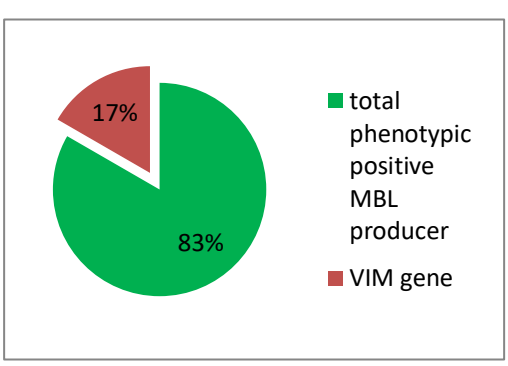


Fig 3: Detection of Metallo-betalactamase among gram negative bacilli

Table 3 and Fig 3 shows the detection of Metallo-betalactamaseamong gram negative bacilli in diabetic foot. Among the isolates 50% were acinetobacter species following 17% were Pseudomonas species and Klebsiella species and 16% in E.coli.

Table 4: Detection of Metallo-betalactamase among phenotypical positive gram negative bacilli by genotypic method

Organism positive for MBL by phenotypic method	No. of isolates	Genotypic detection of VIM gene
E.coli	1	-
Klebsiella species	1	-
Acinetobacter species	3	1
Pseudomonas species	1	-



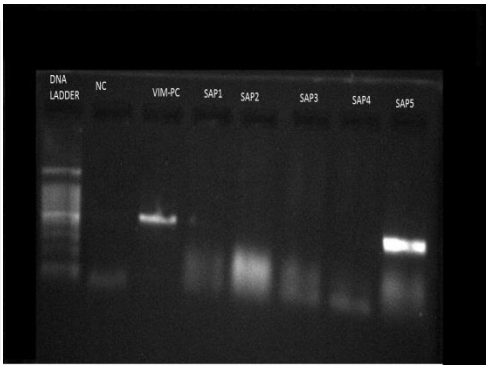


Fig 4: Detection of Metallo-betalactamase among phenotypical positive gram negative bacilli by genotypic method

Table 4 and Fig 4 shows the detection of Metallo betalactamase among phenotypical positive gram negative bacilli by genotypic method. Among the 5 (83%) of isolates, 1(17%) isolates was positive for VIM gene by genotypic method.

Table 5: Detection of carbapenamase among gram negative bacilli by phenotypic method

Organism	No. of Imipenem resistance isolates	MHT positive
Pseudomonas species	1	1
E.coli	1	-
Klebsiella species	1	-
Acinetobacter species	3	-

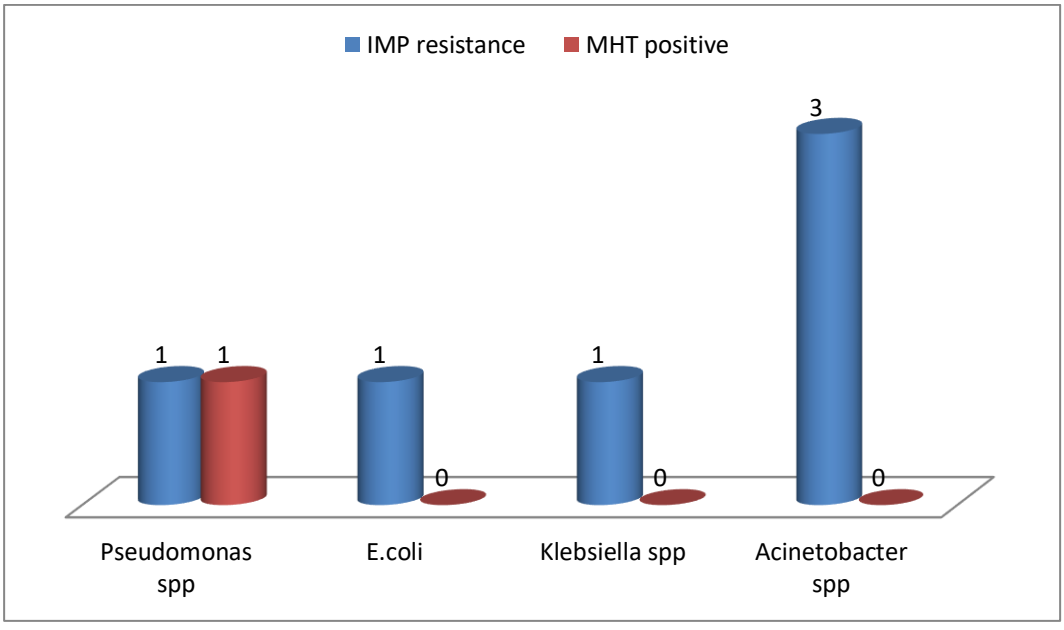


Fig 5: Detection of carbapenamase among gram negative bacilli by phenotypic method

Table 5 and Fig 5 shows the detection of carabapenemase among gram negative bacilli. Those gram negative bacilli showing imipenem resistance were detected for enzyme carabapenemase by Modified Hodge Test (MHT).

Discussion

In our study, among the 37 gram positive isolates 27 were *Staphylococcus aureus* and 10 were coagulase negative *Staphylococcus*. They were all uniformly susceptible to Linezolid and about 81% to Vancomycin for *Staphylococcus aureus* and 50% for *CONS*. Moreover 66.6% of them were MRSA. This shows a high prevalence of MRSA comparatively. But similar prevalence was seen in the study conducted by Sivaraman Umadeviet al. The prevelance

rate in other studies on diabetic foots were reported only 10 - 44% such as Ravisekhar Gadepalli et al[9]

Those *Staphylococcus aureus* showing phenotypic resistance to methicillin were confirmed by Polymerase Chain Reaction (PCR),of which 15 were positive for mecA gene.So this implies that the detection of MRSA can be done in a simpler method by using a Cefoxitin disc in routine laboratory where molecular methods are not available. All the *Staphylococcus* isolates were checked for induced clindamycin resistance.Out of which 3 (8.1%) *Staphylococcus aureus* isolates were positive for D test.

In our study among the enterobacteriaceaeisolates, *Klebsiella species* was common isolate (15.1%) followed by *E.coli* (12.6%) and *Proteus species* (6.3%). *Citrobacter freundii*was the least common isolates. A similar study was seen in *Klebsiella pneumonia* (59.7%) and *E.coli* (40.29%).. But in Anandi et al (2004) shows *Proteus mirabilis, E.coli, Pseudomonas aeruginosa, Klebsiella species* and *Enterococcus species* were isolated in decreasing order. Among the non enterobacteriaceae *Pseudomonas spp* (20.2%) was common isolate followed by *Acinetobacterspp* was the least common isolate (6.3%). In study conducted by A. Ravi shekar et al shows *Pseudomonas spp* 16% and *Acinetobacterspp* 2.4%. According Sivaram et al study *Pseudomonas spp* was 17%.

The antibiotic susceptibility pattern of the gram negative isolates increase resistance to ampicillin, amoxyclav, co-triamoxazole among the members of enterobacteriaceae. All the members of enterobacteriaceae were uniformly sensitive to gentamicin, amikacin and ciprofloxacin. Similar sensitivity was seen in the study conducted by Anandi et al. some recent study showed resistance even to those drugs in Ravisekar Gadepalli et al[9]In non-fermentors, about 93% of *Pseudomonas species* were sensitive to imipenem and 56.2% were sensitive to ciprofloxacin and piperacillin+tazobactam.About 40% of *Acinetobacter species* were sensitive to Imipenem followed by 20% to Co-trimoxazole, cefotaxime, cefatriazone, cefaperazone+sulbactam, amikacin, Gentamicin, piperacillin+tazobactam. Similar sensitivity pattern was seen in the study conducted bySivaraman Umadevi et al [8]. In the study conducted by Ektabansal et al in 2009, Pseudomonas spp. Shows 100% sensitive to imipenem.

In our study among the gram negative bacilli, 21 were found to be ESBL producer by phenotype and those positives was compared with gold standard method PCR for the genes TEM and SHV which were 7 and 6 positives respectively. Because of the cost constrains;only the phenotypically positive ESBL isolates were confirmed by PCR. There multiple genes responsible for ESBL production, so those strains showing negative for TEM and SHV many also be positive for other genes responsible. Instudy conducted by Mohammad Zubair et al shows ESBLs 81.9% were found to be positive out of 127 samples for the bla gene, of which blaCTX–M showed 81.8% positivity, followed by blaTEM (50%) and blaSHV (46.9%).

In our study those gram negative bacilli showing resistance for Imipenems were detected for Metallo-beta lactamase (MBL) and carabapenemase enzyme production, of which 5 and 1 were positive by phenotypic method. Those 5 positive strains of MBL were compared with gold standard method PCR for VIM gene. Only one showed positive for VIM gene[10-14]

Conclusion

Among the gram negative bacilli ESBL shows prevalence of about 42.8% and 12.2% for MBL production by phenotypic method. Out of which 26.5% shows positive by PCR for TEM and SHV gene and 2% shows positive for VIM gene respectively. The prevalence rate of MRSA was found to be high. ESBL and MBL producing gram negative bacterial species have a significant impact on the clinical outcome and efforts to control outbreaks of such infections. Indiscriminate use of third generation cephalosporins to treat gram negative bacterial infections is partly responsible for the emergence of resistance to beta-lactam antibiotics. Infection control measures are required to prevent the spread and reduce emergence of resistance.

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