Original Article

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Microbiological Profile of Deep Tissue and Bone Tissue in Diabetic Foot Osteomyelitis

Microbiology Section

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ABSTRACT

Introduction: Osteomyelitis occurs by contiguous spread or direct inoculation of bacteria into bone from contiguous soft tissue infection or chronic overlying open wound. The common etiological agents in diabetic foot infections include *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*. Specimen of choice in diabetic foot osteomyelitis is bone biopsy and deep tissue.

Aim: Isolation and identification of the bacteria from deep tissue and bone tissue obtained from diabetic foot osteomyelitis. To study the antibiotic susceptibility pattern of the isolated bacteria, to study the concordance of bone biopsy and deep tissue culture in the diagnosis of diabetic foot osteomyelitis and to study the biofilm formation in these pathogens.

Materials and Methods: Descriptive study was conducted in the Department of Microbiology, Kasturba Medical College, Mangaluru for a period of six months from December 2016- May 2017. All the deep tissue and bone tissue samples of diabetic foot ulcer patients received in the microbiology department were processed.

Results: The study included 54 bone tissue and 33 deep tissue specimens. In 31 cases, both bone and deep tissue were studied. Concordance in culture was observed in 22/31(70.96%) cases. The isolation rates of Gram negative and Gram positive organisms were 71.3% and 28.7%. The common isolates were *S.aureus*, *Proteus* spp., *E.coli*, *Enterobacter* spp., *Pseudomonas* spp. The rate of Methicillin Resistant *Staphylococcus Aureus* (MRSA) and Clindamycin resistance in *S. aureus* were 41% and 38%. Extended Spectrum beta lactamases (ESBL) production was seen in 27.27% of *E.coli* and *Klebsiella* spp. The rates of resistance to amikacin, ciprofloxacin and carbapenem among Gram negative bacilli were 28.5%, 23.5% and 15.58% respectively.

Conclusion: The bone biopsy along with deep tissue specimen taken simultaneously would increase the accuracy of detecting the bacterial isolate and to provide effective management. The study of antibiotic susceptibility is necessary to reduce the net effect of the increasing severity of infections. Bone biopsy culture can be substituted by deep tissue samples taken during amputation or debridement.

Keywords: Antibiotic resistance, Bacteria, Biofilm, Biopsy

INTRODUCTION

Osteomyelitis is described as an infection of the bone [1]. It is generally categorized as acute or chronic based on histopathologic results, rather than duration of the infection. Acute osteomyelitis is related with inflammatory bone changes caused by pathogenic bacteria and symptoms generally present within two weeks after infection [2]. Chronic osteomyelitis does not result from acute hematogenous seeding; it usually occurs by contiguous spread or direct inoculation of bacteria into bone from contiguous soft tissue infection or a chronic overlying open wound and has been present for several weeks [2].

Diabetic foot osteomyelitis is difficult to manage. Surgical resection of infected tissue is the traditional approach. There is improving evidence to substantiate conservative management. Conservative management contains antibiotic therapy with or without surgery [3]. Ulcer swab cultures and deep tissue cultures are not always reliable as there is chance of these specimens getting contaminated by superficial normal flora. The gold standard for isolating the causative organism in these cases would be bone biopsy. Bone biopsy culture is not readily available, expensive and has a possibility of harmful effects, therefore, it has been substituted by wound culture or by deep samples taken during surgery such as amputation or debridement of foot lesions [4].

The most common pathogens isolated in osteomyelitis depend on the patient's age. *Staphylococcus aureus* is the most common pathogen followed by Group A *Streptococcus*. *Streptococcus pneumoniae* and *Kingella kingae* are pathogens responsible for acute and chronic hematogenous osteomyelitis in adults and children. In newborn, Group B *Streptococcus* is the primary cause of infection. In more chronic cases, contiguous infection is caused by *Staphylococcus epidermidis, Pseudomonas aeruginosa*, *Serratia marcescens* and *Escherichia coli*. Fungal and mycobacterial infections also have been reported in immunocompromised individuals [5].

A study showed that biofilm production is not necessary to cause sustained infections, but biofilms are difficult to eliminate and thus need a special attention. It facilitates resistance to antimicrobials and host phagocytic clearance.

There have been reports of microbial profile of deep tissue and bone tissue. The literature has very few reports on the concordance between the bone and deep tissue cultures in management of diabetic foot osteomyelitis. Hence, the aim of the study was to analyse the concordance of deep tissue culture and bone biopsy culture in diabetic foot ulcer patients with underlying osteomyelitis and to study the biofilm formation among the pathogens.

MATERIALS AND METHODS

The present descriptive study was conducted at Department of Microbiology, Kasturba Medical College hospital, during the period December 2016-May 2017. The study has obtained clearance from the Institutional Ethics Committee.

Inclusion criteria: All the deep tissue and bone tissue received from diabetic patients (Non repetitive).

Exclusion criteria: Superficial wound infections and samples from non-diabetic patients.

Bone and deep tissue sample were transferred aseptically into 500 µL of Brain Heart Infusion (BHI) broth containing sterile glass beads, capped tight and vortexed until the tissue was homogenized [6]. The homogenized tissue was then used for Gram stain and cultured on chocolate agar, 5% sheep blood agar, MacConkeys agar and incubated at 37°C for 24 hours for the isolation of the pathogen. Identification, antibiotic susceptibility testing and percentage of concordance with regard to organisms grown in both the cultures were assessed. The antibiotic susceptibility testing was done by using the modified Kirby-Bauer disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2017) [7]. MRSA detection was done by Cefoxitin (30 µg) disk diffusion method [7]. ESBL production in *Klebsiella* spp. and *E.coli* was detected by the screening and confirmatory tests recommended by CLSI guidelines. The test was done using both cefotaxime (30 µg) and ceftazidime (30 µg) alone and in combination with clavulanic acid i.e., cefotaxime-plus-clavulanate (30 µg+10 µg) and ceftazidime-plus-clavulanate (30 µg+10 µg). A ≥3 and 5 mm increase in the inhibition zone diameter for cefotaxime and ceftazidime antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was considered as positive result for ESBL production and was interpreted as a phenotypic evidence for ESBL production. The positive control used was *Klebsiella pneumoniae* ATCC 700603 [7]. The strain was procured from Himedia laboratories, Mumbai.

Biofilm formation: Growth from chocolate agar was taken in 5 ml of sterile BHI and incubated overnight followed by a 1:100 dilution in BHI. Then, 100 µl of diluted broth was incubated at 37°C over night in commercially available pre-sterilized, polystyrene, round-bottomed 96-well microtitre plate for biofilm production. The microtitre plate was washed with distilled water [8].

Crystal Violet Assay: After washing with distilled water, microtitre plate was stained with 120 µL of 0.1% aqueous crystal violet solution and incubated at room temperature for 15 minutes. Afterwards, each well was washed four times with sterile distilled water and blot dry; immediately destained with 125 µL of 95% methanol, incubated for 15 minutes at room temperature. After destaining, 100 µL of destaining solution was transferred to a new well and the destaining solution was measured spectrophotometrically with ELISA reader at 570 nm [8].

RESULTS

Total of 54 bone tissue, 33 deep tissue specimens and in 31 cases both bone and deep tissue were included in the study. Concordance in culture was observed in 22/31(70.96%) cases. A total of 94 organisms, 41(39.4%) from bone tissue, 31(29.8%) from deep tissue and 22 (21%) from bone and deep tissue were isolated. Among which 27 (28.7%) were Gram positive and 67 (71.3%) were Gram negative organisms. The distribution of Gram positive and Gram negative isolates are depicted in [Table/Fig-1-3] respectively.

Organism	Bone Tissue	Deep Tissue	Bone and Deep tissue	Total
Gram Positive	7 (17.07%)	10 (32.25%)	10 (45.45%)	27
Gram Negative	34 (82.9%)	21 (67.74%)	12 (54.54%)	67
Total	41	31	22	94

[Table/Fig-1]: Types of organisms from bone and deep tissue.

Staphylococcus aureus is the common isolate among the Gram positive organisms (70.37%).

Among the Gram negative isolates, predominant were Enterobacteriaceae members like *E.coli, Klebsiella* spp, *Enterobacter* spp. and *Proteus* spp. (67%) followed by *Pseudomonas* spp. and other Non fermenters (33%).

Gram positive isolates	*Staphylococcus* spp.	*Enterococcus* spp.	*Streptococcus* spp.	Diphtheroids	Total
Bone tissue	6 (31.5%)	1 (20%)	0	0	7
Deep tissue	5 (26.31%)	2 (40%)	1 (100%)	2 (100%)	10
Bone and deep tissue	8 (42.1%)	2 (40%)	0	0	10
Total	19	5	1	2	27

[Table/Fig-2]: Gram positive isolates in bone and deep tissue.

Gram Negative Isolates	*E.coli*	*Klebsiella* spp.	*Enterobacter* spp.	*Proteus spp*	Pseudomonas + Non Fermenters	Total
Bone tissue	7 (58.3%)	5 (62.5%)	2 (28.57%)	8 (44.44%)	12 (54.54%)	34
Deep tissue	5 (41.66%)	3 (37.5%)	2 (28.57%)	6 (33.33%)	5 (22.72%)	21
Bone and deep tissue	0	0	3 (42.85%)	4 (22.22%)	5 (22.72%)	12
Total	12	8	7	18	22	67

[Table/Fig-3]: Gram negative isolates in bone and deep tissue.

The rate of MRSA and Clindamycin resistance in *S. aureus* was 41% and 38%. All the *S. aureus* strains were sensitive to Linezolid and Vancomycin.

The rate of ESBL production was 27.27% in *E.coli, Klebsiella* spp. and *Proteus mirabilis*. The antibiotic resistance rates are shown in [Table/Fig-4,5] respectively.

Amikacin	28.5%
Carbapenems	15%
Cefoperazone sulbactam	19%
Ceftazidime	99%
Ciprofloxacin	23.5%
Piperacillin tazobactam	20%

[Table/Fig-4]: Antibiotic resistance rates among the Gram negative pathogens.

Ciprofloxacin	34%
Clindamycin	38%
Cloxacillin	41%
Cotrimoxazole	36%
Erythromycin	49%
Gentamicin	44%
Linezolid	0%
Teicoplanin	0

[Table/Fig-5]: Antibiotic resistance rates among *Staphylococcus aureus*.

Total of 94 clinical isolates were subjected to biofilm production, 47 of the isolates were biofilm positive (45%), among them 22 isolates were from bone tissue and 18 isolates were from deep tissue, 7 isolates from both bone and deep tissue.

Among the 27 Gram positive isolates, *S. aureus* showed highest biofilm production (13/19) followed by *Enterococcus* spp. (1/5). Out of *S. aureus* (13) biofilm producers, only 6 were MRSA and 4 isolates were clindamycin resistant.

Among 67 Gram negative isolates, *Pseudomonas* spp. and Non fermenters showed highest biofilm formation (17/22), followed by *Proteus* spp. (7/18), *Klebsiella* spp.(5/8), *Enterobacter* spp. (3/7) and *E. coli* (1/12). Out of which 15 isolates showed multidrug resistance (resistant to 3 or more classes of antimicrobials).

DISCUSSION

The diagnosis of diabetic foot osteomyelitis requires high index of suspicion. The culture of bone specimen is the gold standard for conclusive microbiological diagnosis in osteomyelitis. It is not widely accepted due to its invasiveness and the possibility of worsening of peripheral vascular disease and neuropathy [9]. Previous studies have shown an overall concordance percentage between bone and swab culture in patients with suspected diabetic foot osteomyelitis as 22.5% [10]. In a previous study, bone and soft tissue cultures were identical in 49% of the cases [11]. Our study showed a concordance of 22/31(70.96%) in bone tissue and deep tissue cultures which shows that a deep tissue culture can help to isolate the true pathogen in 70% of cases without taking a bone biopsy. The concordance between the cultures results of deep tissue and of bone biopsy specimens was found to be similar in both Gram positive and Gram negative isolates.

Staphylococcus aureus is by far the most commonly involved organism reported. It elaborates a range of extracellular and cell-associated factors contributing to its virulence. In our study, Gram negative bacilli were the predominant pathogens (74.03%) which show that the Gram negative flora is equally important in diabetic osteomyelitis. Previous studies have shown similar findings in diabetic osteomyelitis with *Pseudomonas* spp. and *E. coli* as predominant pathogens [12].

A high rate of antibiotic resistance is recorded in our study. MRSA rate was 41% and ESBL production was seen in 27.27% of *E.coli* and *Klebsiella* spp. The rate of resistance to amikacin, ciprofloxacin and carbapenem among GNB were 28.5%, 23.5% and 15.58% respectively. The above fact indicates the increasing rate of drug resistance in pathogens causing osteomyelitis which may lead to difficulty in treatment [13].

The rate of biofilm formation was more in *S. aureus* (76.4%) and *Pseudomonas* spp. (77.2%) compared to the previous studies. The rates of biofilm production reported in earlier studies were *S. aureus*: 20% and *Pseudomonas* spp.: 26.5% [14].

Osteomyelitis complicates approximately 10–20% of foot ulcers in individuals with diabetes mellitus. The management of these cases is debatable. The drawbacks of non surgical approach with antibiotics include remission rates of 60%, long duration of therapy and antibiotic resistance. Surgical approach is also associated with high recurrence and difficulty in distinguishing infected and non infected bone during surgery. Bone biopsy helps in the accurate identification of pathogens in infected bone, thus ensuring an appropriate regimen targeting the pathogen.

In the antibiotic era, chronic osteomyelitis remains a difficult task to treat and has a high rate of relapse after successful treatment and these relapses are usually due to bacterial evasion of host defenses by formation of biofilm. Hence due to these concerns clinicians usually treat chronic osteomyelitis with antibiotic therapy that is parenteral, high dose, and prolonged and antibiotic resistance may be an additional factor for treatment failure.

LIMITATION

Limitations of the study were the clinical outcome in the patients was not noted.

CONCLUSION

This study has elucidated the fact that bone biopsy along with deep tissue specimen taken simultaneously increases the accuracy of detecting the bacterial isolate and providing effective management. Deep tissue cultures have shown a concordance of 70% with bone tissue. The knowledge of the antibiogram of the invasive isolates is necessary to reduce the net effect of the increasing severity of infections and economic burden.

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