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Biofilm Production by the Organisms Causing Osteomyelitis in a Tertiary Care Hospital

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ABSTRACT

Background: Progressive destruction of bone and formation of sequestra are characteristics of Osteomyelitis (OSM). OSM mostly affects the growing ends of long bones and it is more common in the lower extremity at metaphysis of femur and proximal end of tibia. Chronic bacterial infections could be rationalized in terms of their biofilm etiology. These infections of prostheses and fixation devices could develop over months or years, with few signs of inflammation. **Aims and Objective:** To study the biofilm production by the organisms causing osteomyelitis and to compare the biofilm production methods. **Methods:** specimens were collected from 115 diagnosed patients of osteomyelitis. Samples were collected before start of antibiotics and processed by standard microbiological techniques. All the organisms isolated showing increased resistance to commonly available antibiotics by Kirby-Bauer disc diffusion method, were subjected to biofilm detection methods such as Microtiter plate method (MTP), Tube method (TM), Congo red agar (CRA) method. **Results:** *S. aureus* (41.38%) was the most common organism isolated, followed by *K. pneumoniae* (17.24%), *A. baumannii* (11.21%), *P. aeruginosa* (8.62%). *S. aureus* isolates of OSM were maximally found to be resistant to Penicillin followed by Rifampicin and Cefoxitin. Biofilm producers were isolated in cases of osteomyelitis. MTP detected more number of biofilm producers than TM and CRA method. **Conclusion:** Maximum biofilm producing isolates in OSM were of *S. aureus*. MTP detected more number of biofilm producers, than TM and CRA method.

Keywords: Biofilm, Microtitre plate method, Osteomyelitis, *Staphylococcus aureus*.

INTRODUCTION

The term osteomyelitis (OSM) was first used by the French surgeon Edouard Chassaignac in 1852, who defined the disease as an inflammatory process accompanied by bone destruction and caused by an infecting microorganism.[1] Progressive destruction of bone and the formation of sequestra are characteristics of this diseases.[2] The clinical manifestation and the natural history of OSM depend on several factors like age of patients, site of infection, virulence of infecting organism and the patients resistance.[3] OSM mostly affects the growing ends of long bones and it is more common in the lower extremity at metaphysis of femur and proximal end of tibia.[4] Thus, because of the changes in the manifestations, epidemiology, and etiological agents, it is important to make a precise microbiological diagnosis. When Gristina and Costerton applied the biofilm hypothesis to device-related orthopaedic infections as early as 1984, it became abundantly clear that the clinical "earmarks" of these chronic bacterial infections could be rationalized in terms of their biofilm etiology. Like all biofilm infections, these infections of prostheses and fixation devices could develop over months or years, with few signs of inflammation, and they usually remained localized to the immediate vicinity of the colonized prosthesis.[5]

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Aims and Objectives

1. To study the biofilm production by the organisms causing osteomyelitis.
2. To compare the methods used for biofilm production.

MATERIALS AND METHODS

The study was carried out in department of Microbiology at a tertiary care hospital over a period of 2 years. Pus and bone aspirate collected from all clinically diagnosed and operated cases of osteomyelitis were included in the study.

After receiving approval from Institutional ethics committee, specimens were collected from clinically

and radiologically diagnosed 115 patients of osteomyelitis, attending out patient department and/or admitted to wards of the hospital.

Cases were classified as acute and chronic osteomyelitis. All the samples were collected preferably before start of antibiotics. Specimens were processed for isolation of aerobic bacteria, mycobacteria and fungi.[6,7] Pus sample was collected and processed by standard microbiological techniques.[7] Antimicrobial susceptibility of all bacterial isolates was done. Each isolate was subjected to antimicrobial susceptibility test as per CLSI 2014 guidelines by Kirby-Bauer disk diffusion technique.[8,9] All the organisms isolated from the samples and those showing increased resistance to commonly available antibiotics by Kirby-Bauer disc diffusion method, were subjected to biofilm detection methods. Isolates were identified by standard microbiological procedures.

Microtiter plate method[10]

This quantitative test described by Christensen et al,[11] is considered the gold-standard method for biofilm detection.[12] Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37ºC for 24 hours. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates (Sigma-Aldrich, Costar, USA) were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37ºC for 24 hours. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic et al.[13]

Interpretation of biofilm production

Average OD value	Biofilm production
≥ ODc / ODc <~≤ 2x ODc	Non / weak
2x ODc < ~≤ 4x ODc	Moderate
>4x ODc	Strong

Tube method[10]

Described by Christensen et al,[14] this is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 hours. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times.

Congo Red Agar method[10]

Freeman et al,[15] had described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium. CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar No.1,10 g/L and Congo Red indicator 8 g/L. First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121ºC for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55ºC. CRA plates were inoculated with test organisms and incubated at 37ºC for 24 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.[16] The experiment was performed in triplicate and repeated three times.

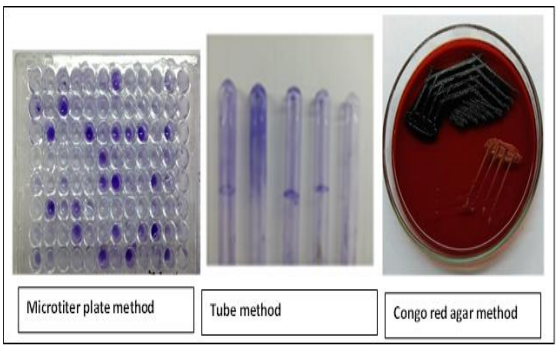

Image 1: The methods of Biofilm production.

RESULTS

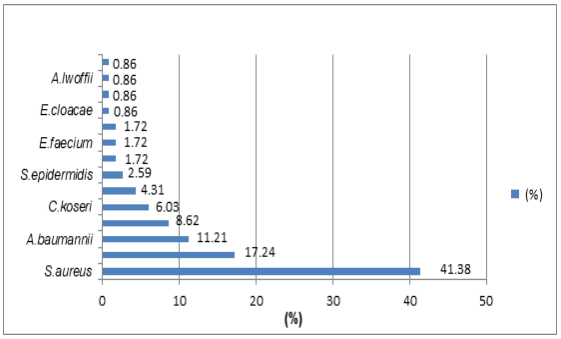

Figure 1: Graphical representation of organisms causing osteomyelitis.

The present study was carried out in 115 clinically diagnosed patients of Osteomyelitis (OSM) from August 2014 to July 2016. In 101 samples, 116 organisms were isolated and in 14 samples no organism was isolated. Representation of individual pathogen of Osteomyelitis is shown in [Figure 1].

[Figure 1] shows that, among the total number of organisms isolated, *S. aureus* (41.38%) was the most common, followed by *K. pneumoniae* (17.24%), *A. baumannii* (11.21%), *P. aeruginosa* (8.62%).

S. aureus isolates of OSM were maximally found to be resistant to Penicillin (97.92%) followed by Rifampicin (58.25%) and Cefoxitin (52.08%). Out of 48 isolates of *S. aureus*, 37.50% were MRSA, 6.25% were ICR, 14.58% were MRSA + ICR.

Klebsiella pneumonia isolates were 100% resistant to Ampicillin, *Citrobacter koseri* isolates showed 100% resistance to Amoxycillin - Clavulanate.

In this study, 25 isolates were of Non fermenter. All the isolates of *A. baumannii* were resistant to Ceftazidime, Cefotaxime and Cefipime. P. aeruginosa was maximally resistant to Cefipime (70%), followed by Ceftazidime, Piperacillin - Tazobactam, Gentamicin and Amikacin. *A. lwoffii* was found to be resistant to Ceftazidime, Cefotaxime, Cefipime, Piperacillin, Piperacillin - Tazobactam, Aztreonam, Imipenem.

Table 1: Biofilm producing organisms in osteomyelitis.

Gram positive group	No. of isolates	Biofilm production (%)
S. aureus	48	09 (18.75)
S. epidermidis	03	02 (66.67)
E. faecium	02	01 (50)
Enterobacteriaceae isolates		
K. pneumoniae	20	00
C. koseri	07	02 (28.57)
E. coli	05	01 (20)
S. Typhi	02	00
P. mirabilis	01	01 (100)
E. cloacae	01	00
Non Fermenter group		
A. baumannii	13	01 (7.69)
P. aeruginosa	10	00
A. lwoffii	01	00
S. maltophilia	01	00
Fungal pathogens		
Candida albicans	02	00
Total	116	17 (14.66)

[Table 1] shows that, 17(14.66%) biofilm producers were isolated in cases of osteomyelitis.

Table 2: Methods of Biofilm detection (n=17)

Methods	Positive (%)	Negative (%)	p-value
Microtiter plate method (MTP)	17 (100)	00	0.000006
Tube method (TM)	8 (47.06)	9 (52.94)	
Congo red agar method (CRA)	3 (17.65)	14 (82.35)	

[Table 2] shows that, out of 116 organisms isolated from cases of OSM, 17 (14.66%) isolates were biofilm producers. Microtiter plate method (MTP) detected more number of biofilm producers than tube method (TM) and congo red agar (CRA) method. This shows that microtiter method is the better method than the other two method and it is statistically significant ($p < 0.05$).

Statistical Analysis

Data which collected in the questionnaire were entered and analysed in Epi Info software version 7.2.[17] Categorical data was analysed by means of mean, standard deviation and quantitative data by proportion and percentage. The group differences were tested using chi-square, or others depending on the type of variable. p- value < 0.05 was considered to derive a level of significance.

DISCUSSION

Osteomyelitis is an invasive and hard-treatable infectious disease conducted to periosteum and cortex of osteon by haversion canals and it will cause necrosis, if periosteum is infected. Microorganisms avoid the host defence and antibiotics through a multiplicity of mechanisms including surviving in a dormant state inside osteoblasts, developing a biofilm, and acquiring a very slow metabolic rate.[18]

Congo red agar showed a low correlation with the tube method and microtitre plate method. Similar results have been reported by Hassan A et al (2011),[10] they also evaluated these three different methods. The microtitre plate method was highly satisfactory in terms of biofilm-positive phenotype detection. The authors concluded that the microtitre plate method was the most sensitive and accurate method showing good reproducibility for the detection of biofilm formation.

Out of 116 isolates, biofilm production was seen in 17 (14.66%) isolates as shown in [Table 1]. These isolates showed biofilm production by microtitre plate method (100%), tube method (47.06%) and by congo red agar method (17.65%) as shown in table-2. Mathur T et al (2006),[12] found 53.8% clinically significant CONS to be biofilm producers by MTP, 41.4% by TM and 5.17% by CRA method. Taj Y et al (2012),[19] found that *S. aureus* showed 54.8% biofilm production by TM, 3.47% by CRA method. Deka N (2014),[20] observed isolates showing biofilm production by MTP (83%), TM (57%) and CRA (20%). Hassan A (2011),[10] observed biofilm production by MTP (64.7%), TM (49%)

It is thus concluded that even though microtitre plate method is laborious, it is the method which should be followed for testing biofilm production. Tube and congo red agar method, though easy to perform, were found to be less sensitive and many strains might be reported as false negative. Present study found variable amount of biofilm production in various species as shown in [Table 1]. Out of 17

isolates of biofilm producers in osteomyelitis, maximum biofilm production was seen in *S. aureus*.

CONCLUSION

Osteomyelitis is one of the most frequent bacterial infections, and of the primary reasons for the prescription of antibiotics.

Out of the total isolates, 17 (14.66%) were biofilm producers. Maximum biofilm producing isolates were of *S. aureus*.

Microtiter plate method detected more number, than Tube method and Congo red agar method for biofilm production.

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