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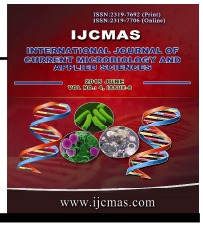

Original Research Article

Comparative evaluation of biofilm production in Multidrug resistant and sensitive Gram negative clinical isolates

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A B S T R A C T

Microbial biofilms have been associated with a variety of persistent infections which respond poorly to conventional antibiotic therapy. These biofilms help in the spread of antibiotic resistant traits in nosocomial pathogens by increasing mutational rates by exchange of genes which are responsible for antibiotic resistance. The present study was carried out to compare and evaluate Biofilm production among drug resistant and drug sensitive Gram Negative isolates. A total of 180 Gram negative bacterial isolates from various clinical samples were included in the study. The identification and antibiotic susceptibility testing of all the isolates was done as per the standard laboratory protocol. Biofilm production was detected by Congo red Agar method and Air liquid interface assay. Among the total 180 isolates *Escherichia coli* 38%, *Klebsiella spp 22%*, *Proteus spp 14%*, *Pseudomonas spp 13% etc*. Considering the antibiotic susceptibility pattern 53% of the gram negative isolates were multidrug resistant. Among MDR strains, 54% of isolates were found to be biofilm producers, which was only 27% among drug sensitive bacilli. The standard in vitro antibiotic susceptibility tests are not predictive of the therapeutic outcome of biofilm associated infections. Due to which the overall cost and hospital stay are increased in biofilm associated infections. Hence detection of biofilms for all the isolates on a routine basis will help to choose concomitant measures in preventing the development of drug resistance and a check on nosocomial infections.

Introduction

Biofilms has emerged as a component of socio-microbiology which is a cooperative existence among microbes either inter species or intraspecies. A microbiologist preferred for many years studies of bacterial pathology focused primarily on the planktonic state and so far there is an emphasis that bacteria and their effects were studied as pure and homogenous cultures only. The view has altered now due to polymicrobial communities with beneficial social behaviour of cross species protection and enhanced virulence among microbes (Donlan R M, 2002). Hence viewing bacteria from the perspective of multicellular behaviour is in essence altering our view of microbiology to regard them as communities rather than single

celled. Naturally bacteria exists in one of the two types of population, planktonic i.e, non interacting free swimming single cells and sessile as an aggregated unit of interactive bacteria attached to a surface or interfaces within the confines of a biomass. A biofilm is a surface associated mono or poly microbial colony, where cells adhere to each other and also to the surface, that is embedded in a self produced glycocalyx or extracellular polymeric matrix, which protects the microorganisms from hosts immune system and antimicrobial therapy (Costerton J W et al, 1999).

The formation of biofilm is a cyclic process which requires physical, chemical, environmental and biological processes (Danese P N et al, 2000). It mainly consists of several steps like deposition of the microbes followed by adhesion and anchorage to the biotic tissue epithelia or abiotic device surfaces. Optimum osmolarity, iron availability, carbon source, oxygen tension, temperature, moisture etc are the other contributing factors (Davies DG,1998). Later growth, multiplication and dissemination to the other sites takes place. Given the sufficient resources a biofilm can develop readily on any surface or interfaces. Bacteria within the biofilms differ both in behaviour and in phenotypic form from the planktonic bacteria. Conventional clinical microbiology can detect only the planktonic forms which are entirely different from the enclosed sessile forms within the biomass (Bordi, et al, 2011).

The failure of antimicrobial agents to treat biofilms has been attributed to a variety of mechanisms. Organisms encapsulated in the matrix grow more slowly and the antibiotic cannot act effectively on the slow growers. This is due to the fact that encapsulated populations have a decreased nutrient and oxygen supply resulting in a static metabolic rate where antibiotic binding proteins are poorly expressed and as a consequence low antibiotic susceptibility (O'Toole, et al, 2000). This may lead to a less susceptible genotype selecting a resistant population. The exopolysaccharide matrix acts as a barrier and prevents the diffusion of antibiotic molecules into the deeper layers of the film which is the extrinsic resistance mechanism. Bacteria within the biofilm develop interbacterial communication and transfer genetic information and plasmids due to the close proximity. Hence the population can survive the antibiotic concentration of about 1000 times higher than needed to eradicate planktonic bacteria of the same species (Lewis K, 2007).

Biofilm is the predominant mode of growth of bacteria and this plays a central role in pathogenesis of chronic, persistent, relapsing infections and emergence of virulent multidrug resistant traits among pathogens (Parsek M R, 2012). The first recorded observation concerning a biofilm was by given by Antonie Von leuwenhoeck (1632-1723) who observed animalcules in his own dental plaque sample through his primitive microscope in 1684. According to a public statement from the National Institute of Health, more than 65% of all microbial infections are biofilm associated (Amy Proal, 2008).

With this back ground the present study was undertaken to analyze the prevalence of multidrug resistant Gram negative isolates from various clinical samples and to compare and evaluate Biofilm production among drug resistant and drug sensitive Gram Negative clinical isolates.

Materials and Methods

This prospective study was carried over a period of 9 months, from December, 2013 to August, 2014 at Gandhi Medical college Hospital, a tertiary care centre, Andhra

Pradesh, India. A total of 180 Gram negative bacterial isolates from various clinical samples like blood, urine, sputum, pus and sterile fluids were included in the study.

Processing of samples and identification of isolates was done as per the standard bacteriological techniques. The antibiotic susceptibility testing of all the isolates was performed by Kirby bauer disc diffusion method on Mueller Hinton Agar as per CLSI guidelines (M100- S 23, 2013). The representative antibiotics among all classes were used for sensitivity testing. Bacterial isolates that demonstrated resistance to at least one agent each, in three antibiotic classes were considered as Multi Drug resistant (CDC MDRO/CDI, module, 2014).

All the 180 gram Negative bacilli were evaluated for the phenotypic detection of Biofilm production by Congo red agar method (Freeman D J et al, 1989) and Air liquid interface assay (Constantin OE, 2009). ATCC *Pseudomonas aeruginosa* PAO 1 and ATCC 27853 *Pseudomonas aeruginosa* were used as positive and negative controls respectively (Laverty G et al, 2012 and Perez L RR et al, 2011).

In Congo red agar method the isolates were inoculated on Congo red agar media (Hi media laboratories, Mumbai) and incubated at 37 ^{0} C for 18-24 hours. Biofilm producer bacteria exhibited black crystalline, dry, rough colonies whereas Non biofilm producers showed smooth, reddish coloured colonies. A 5 point colour reference scale was applied for the quantification of biofilm production. Biofilm producers were regarded as BB – Bright black or OB – Opaque black. Anything between pink, red to Bordeaux was taken as non biofilm producer strains.

In air liquid interface assay method, 25 ml of sterile nutrient broth with 1% glucose in a 50 ml Falcon tube was inoculated with 0.5ml of test bacterial suspension containing about 10^{4} CFU/ml. A sterile micro glass slide was immersed into it and incubated at 37°C in upright position without shaking. Lid was loosely fitted for aeration inside the tube. The tubes were observed daily for a maximum of 9 days visually for the turbidity at the air liquid interface and also at the edges of the immersed glass slide. Any turbidity seen either at the junction, edges of the immersed glass slide or deposit at the bottom, then the slides were gently pulled out by holding the edges with a sterile forceps and placed on a blotting paper, heat fixed and stained with an aqueous solution of Congo red for 3 minutes, washed with water and left for air drying. The slides were then examined under a compound microscope under 10X then 40X followed by 100 X.Tubes or slides that were negative for biofilm after 9 days were discarded. (Sara Marti et al, 2011, Oana Emilia Constantin, 2009).

A biofilm positive slide observed at 10X as clumps of material, at 40X showed net like structure with pores and fibres and at100X observation of bacterial cells associated with exopolysaccharide fibres and embedded microcolonies were noted. Negative slides under microscope does not reveal an adherent matrix morphology only the scattered planktonic cells which were heat fixed were observed. The exopolysaccharide matrix adherent only at the junction of air liquid was not important but it should be accompanied with the bacteria and biofilm matrix adhering all throughout the immersed portion of the slide also. Only turbidity at the junction can also be due to the drying of the media because of the longer incubation required which can lead to a false positive result (Caiazza and O' Toole , 2004).

Result and Discussion

Distribution of the total 180 Gram Negative bacilli were *Escherichia coli* 68 (38%), *Klebsiella spp* 40 (22%), *Proteus spp* 26 (14%), *Pseudomonas spp* 24(13%), *Acinetobacter spp* 9(5%), *Citrobacter spp* 8 (4.5%), *Enterobacter spp* 5 (3%) from the clinical samples, of which 76 (42%) were urine, 48 (27%) pus, 23 (13%) sputum, 19(10%) blood, 14 (8%) were sterile fluids (Table 1). *Escherichia coli* was the predominant isolate followed by *Klebsiella spp* mostly isolated from urine samples. On evaluation of drug resistant and drug sensitivity pattern of 180 gram negative isolates, 95 (53%) were multidrug resistant and 85 (47%) were drug sensitive (Table 2). Considering the biofilm formation of the isolates, it was found that 74(41%) were biofilm producers and 106(59%) were non biofilm producers (Table.3). On comparative evaluation of biofilm production and drug susceptibility pattern of gram negative isolates it was observed that among the drug resistant population, 54% were biofilm positive and 46% were biofilm negative. Whereas 27% were biofilm producers and 73% were biofilm non producers among drug sensitive isolates(Table 4) indicating drug resistance and biofilm production are directly proportional, which obtained a significant P value (< 0.005) by Chi square method.

Community living among various microbes leading to enhanced virulence and cross species antimicrobial resistance is a challenge to the medical fraternity. Biofilm formation is a well known pathogenic mechanism, where bacteria are successful at colonization and persistence over their free living planktonic counterparts because of active cell division and recruitment of secondary invader pathogens (Hans curt Flemming et al, 2011). Present study was undertaken on various Gram negative clinical isolates from various samples to detect the biofilm formation by two different phenotypic methods and to analyse biofilm production with respect to the multi drug resistance of the isolates.

The key factors contributing to the pathogenicity associated with biofilms are the production of EPS and the adhering capacity of the microbes (Adilson Oliveira et al, 2010). These two phenomenon were addressed in this study by the Congo red agar method which detects the polysaccharide matrix production by the bacteria (Khalid Mahmood Hammadi and AfafAbdulrahman Yousif, 2014) and Air liquid interface assay which , not only shows the adhering capability but also the adherent clumps of microbes embedded in slime (Andrew J Spiers et al, 2003) . The Congo red agar method was sensitive, rapid and facilitates the visual chromatic evaluation of biofilm production. It also supports multiplication and growth of Gram negative bacilli and other bacteria on account of its non inhibitory nature and yielded a viable growth (Mariana NS et al , 2009).It was also practical to carry out this method on a routine basis as it was easy and economical. The Air liquid interface assay provides a simple system for microscopic analysis of biofilm production over a time range. The advantage is both the adherent potential of matrix and the embedded bacterial cells are visualized after appropriate staining technique.

The present study mentioned the distribution of Gram Negative bacilli among clinical samples (Table 1) where *Escherichia coli* was the predominant pathogen with 38% , followed by *Klebsiella spp* 22% and *Proteus spp, Pseudomonas spp* with 14.5% and 13 % respectively. Similar results are reported by Stephen E Mshana et

al (2009) with 33.7% of *E.coli*, 24% of *Klebsiella spp* and 12% of *Pseudomonas spp*, whereas *Proteus spp* is only 8.2% in their study. Also Jaggi et al (2012) isolated 43.9% *E.coli*, followed by *Kelbsiella spp* (19.7%), *Pseudomonas spp* (15%). Jayaprada et al (2012) showed similar findings of *E.coli* 31.8% and *Klebsiella spp* 23.6% in their study. Isolation of *Acinetobacter spp* is only 5% in this study, but it is reported as 10.3% by Stephen E Mshana et al (2009), 9.9 % by Jaggi et al (2012) and 17.8% by Jayaprada et al (2012).

On evaluating the drug susceptibility pattern of the gram negative isolates, our study showed 32.6% of *E.coli* and 25% of *Klebsiella spp* were multidrug resistant. Followed by 16.8% of *Pseudomonas spp*, 13.6% of *Proteus spp* and 7.3% of *Acinetobacter spp* (Table 2). A high drug resistant pattern of 63.7% for *Kelbsiella spp* is reported by Stephen E Mshana et al (2009), followed by 24.4% for *E.coli*, 17.7% for *Acinetobacter spp*, 6.4% for *Proteus spp*. A high multidrug resistance is reported by Jaggi et al (2012), where 57.9% are *Klebsiella spp* and 54.2% are *E.coli*, this may be due to including both ESBL and MDR isolates in their study. Over all the wide spread use of broad spectrum antibiotics leading to selective survival advantage of pathogen is the common reason for multi drug resistance among the bacteria.

On analyzing the biofilm production among the Gram negative isolates, 66.6% were *Acinetobacter spp*, 58.3% were *Pseudomonas spp*, 50% *Citrobacter spp*, followed by 45 % *Klebsiella spp*, 40 % *Enterobacter spp*, 38.4% *Proteus spp*, 29.4% *E.coli* (Table 3). Relatively high percentages of biofilm production observed with *Acinetobacter spp, Citrobacter spp and Enterobater spp* was may be due to their less number in this study.

Table.1 Details of the Gram Negative isolates based on clinical samples

Gram negative bacilli	Urine (n=76)	Pus (n=48)	Sputum (n=23)	Blood (n=19)	Sterile fluids (n=14)	Total (n=180)
Escherichia coli	30(39.5%)	16(33.3%)	13(56.5%)	05(26.3%)	04(28.5%)	68(38%)
Klebsiellaspp	22(29%)	06(12.5%)	04(17.4%)	06(31.6%)	02(14.3%)	40(22%)
Proteus spp	06(7.9%)	11(22.9%)	01(4.3%)	04(21%)	04(28.5%)	26(14.5%)
Pseudomonas spp	09(11.8%)	08(16.6%)	03(13%)	02(10.5%)	02(14.3%)	24(13%)
Acinetobacterspp	02(2.6%)	03(6.3%)	-	02(10.5%)	02(14.3%)	09(5%)
Citrobacterspp	04(5.3%)	03(6.3%)	01(4.3%)	-	-	08(4.5%)
Enterobacterspp	03(3.9%)	01(2.1%)	01(4.3%)	-	-	05(3%)
Total	76(42%)	48(27%)	23(13%)	19(10%)	14(8%)	180

Table.2 Drug susceptibility pattern of the isolates

Gram negative bacilli	Multi drug resistant	Drug sensitive	Total
Escherichia coli	31(32.6%)	37(43.5%)	68
Klebsiellaspp	24(25%)	16(18.8%)	40
Proteus spp	13(13.6%)	13(15%)	26
Pseudomonas spp	16(16.8%)	08(9.4%)	24
Acinetobacterspp	07(7.3%)	02(2.3%)	09
Citrobacterspp	02(2%)	06(7%)	08
Enterobacterspp	02(2%)	03(3.5%)	05
Total	95(53%)	85(47%)	180

Table.3 Distribution isolates based on Biofilm production

Gram negative bacilli	Biofilm positive	Biofilm Negative
Escherichia coli (n=68)	20(27%)	48(45%)
Klebsiellaspp (n=40)	18(24.3%)	22 (20.8%)
Proteus spp (n=26)	10(13.5%)	16 (15%)
Pseudomonas spp (n=24)	14(18.9%)	10 (9.4%)
Acinetobacterspp (n=9)	06(8%)	03 (2.8%)
Citrobacterspp (n=8)	04(5.4%)	04 (3.8%)
Enterobacterspp (n=5)	02(2.7%)	03(2.8%)
Total (n=180)	74 (41%)	106 (59%)

Table.4 Comparative evaluation of Biofilm production in Drug Resistant and Sensitive gram negative isolates

	Biofilm positive	Biofilm negative	Total
Drug resistant	51 (54%)	44 (46%)	95 (53%)
Drug sensitive	23 (27%)	62 (73%)	85 (47%)
Total	74 (41%)	106 (59%)	180

But Pramodhini Subramanian et al (2012) reported 73.9% of *Citrobacter spp* and 50% of *Enterobacter spp* as biofilm producers. Zubair M et al and Afreenish Hassan et al (2011) reported in their study on diabetic ulcers that *E.coli*was the predominant biofilm producer with 42% and 46% respectively. Carlos J Sanchez et al (2013) reported *Pseudomonas spp* as the highest biofilm producer with 83% . Bacteria having the ability to produce biofilms lead to long term persistence, promote colonization and increased rate of infections. Biofilm producing clinical isolates tend to survive on implanted medical devices and also on tissue wounds, cystic fibrosis, dental lesions, necrotizing fasciitis, osteomyelitis, otitis media and so on (Ercan Pinar et al, 2008).

Looking into the comparative evaluation of the prevalence of biofilm production and drug susceptibility pattern of Gram negative bacilli our study revealed that 54% of the drug resistant isolates were biofilm positive. But in case of drug sensitive isolates biofilm production was only 27%. Similar findings of 53.75% multi drug resistance is shown by Vishwajeet Bardoloiet al (2014), 68% by

Mahabubul Islam Majumder et al (2014)and as high as 90% of multi drug resistance is reported by Pramodhini Subramanian et al (2012), also around 95% of resistance among *Acinetobacter spp* by Deepa et al (2012). Factors like slow growth, presence of exopolysaccharide matrix slow the diffusion of antibiotics leading to the changes in the physicochemical properties of the drug due to the time lapse are implicated (Thien-Fah C Mah et al, 2001). Other mechanisms are alteration in the outer membrane porin proteins, decrease permeability of the drug to the target site and genetic mechanisms include the exchange of gene and plasmid at a faster rate coding for drug resistance and higher rate of mutations due to the close proximity of microbes (Silpi Basak et al, 2013).

In conclusion, increasing burden of biofilm production and drug resistance among the routine clinical isolates is alarming as this leads to persistent chronic infections which is a challenge to infection control practices in health care settings. Establishing an association between drug resistance and biofilms has generated immense speculation and knowledge about the nature of biofilms and the means to diagnose biofilm related infections (Rose cooper et al, 2010) .Though extensive studies have been carried out on various aspects of biofilms, however with regard to the enumeration in a basic clinical laboratory, very little practical information is available.

Overall the present study demonstrated a high propensity among the Gram negative bacteria to form biofilm and a significant association of biofilms with multiple drug resistance. An understanding of the drug resistance associated with biofilms is gained in the development of new and more effective treatment modalities that not only attempt to eradicate bacteria but also affect the biofilm architecture may prove to be more effective in improving the patients outcome in the near future.

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