A Study on correlation between the drug resistance and biofilm production among the GNB isolated from blood

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Abstract

Introduction: Now days many drug resistant bacteria has been isolated from bacteraemia cases. And these bacteria threaten the patient's life with associated biofilm production around the implants like Intravenous catheters, Venflons, etc.

Aim: to identify the prevalence of drug resistance among GNB (Gram Negative Bacteria) isolated from blood culture and to check their ability to produce biofilm by invitro method.

Materials and Methods: A total of 365 blood samples were collected and processed from clinically diagnosed septicaemia patients. These samples were processed to isolate the bacteria with ABST by using standard CLSI guidelines. Biofilm production was identified by Tube method, Congo red agar method and Tissue culture plate method.

Result: Out of 365, 62 (17%) blood samples were isolated with gram negative bacilli. Out of 62 isolates, 17 (27.4%) were identified as *Escherichia coli*, 13 (21%) were identified to be *Acinetobacter spp*, 12 (19.3%) were *Pseudomonas spp*, 08 (13%) were *Salmonella spp*, 06 (9.6%) were isolated as *Klebsiella spp. Citobacter spp* and *Enterobacter spp* were isolated with 03 (4.8%) each. Among these 62 isolates, 28 (45.2%) were identified to be either ESBL or MDR isolates, 18 (29%) isolates produces biofilm by all three methods. Among the biofilm producers, (55.55%) were found to be the drug resistance bacteria.

Conclusion: Result of this Blood culture reveals the increased rates of drug resistance bacteria which were due to improper use of antibiotics. And the major drug resistant bacteria are associated to produce Biofilm which makes the treatment to be more challenging.

Key words: Biofilm production, Septicaemia, ESBL & MDR isolates, Drug resistance

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Introduction

Most of the lifesaving procedures may also produce some complications by introducing pathogens into the blood stream. As blood acts as enrichment for those pathogens, they may grow rapidly and threaten the host, this is due to lack of host's immunity in hospitalized patients. After 1950's the rate of blood stream infection caused by the members of enterobacteriacea and other gram negative bacteria was increased. Majority of the blood stream infections are hospital acquired. The major genus of gram negative bacilli causing this blood stream infection are *E.coli, Enterobacter, Klebsiella, Citrobacter, salmonella, Acinetobacter, pseudomonas,* etc.[(1)]. Early detection and appropriate treatment is necessary in these cases. Many isolate shows lower susceptiblity to common drugs. Now days, ESBL (Extended Spectrum β-Lactamases) and MBL (Metallo β-Lactamases) strains have been isolated in large number. The emergence and possible spread of carbapenem-resistant Enterobacteriaceae isolates in the province would represent an additional step in the wrong direction that might be enhanced by misuse of carpabenems, as observed a few years ago in some Asian countries[(9)]. Some of these infections may be drug resistance by producing "the biofilm". Biofilm is nothing but an aggregate of bacteria in and around the prosthetics by producing an alginate substance[(2)]. These bacteria grow inside this protective layer and constantly released into blood stream. These bacteria can evade from the host immune response and also from the antibiotic treatment by masking themselves inside the biofilm.

Materials and Methods

A total of 365 blood samples were received in the microbiology laboratory from various wards including intensive care units of Vinayaka Missions Medical College, Karaikal. These samples were processed and analysed results were recorded and findings were noted.

Study Period: Mar-2013 to May-2014

Sample: 5ml and 2ml of intravenous blood sample were collected aseptically from adult and paediatric cases respectively introduced into Biphasic Culture medium.

Culture: Aseptically collected venous blood was inoculated in Biphasic medium (brain heart infusion broth with brain heart infusion agar slant). In enteric fever suspected cases, the sample was also inoculated in biphasic medium (macconkey

broth with macconkey agar slant). Inoculated samples were incubated for about 24 hrs at 37^0C. These blood culture bottles were inspected daily and the results were noted. If no change occurs in the medium, it is noted as negative and the bottles were tilted 90^0 then reverse it, so that the broth will covers the entire slant, and is then reincubated. This process is continued for 7 days to give the negative report. Positive result was noted by the presence of turbidity and gas production and sometimes by the haemolysis. By the next day, the colonies produced on the slant also indicate the positive result. Broth from the positive blood culture bottles were processed with standard methods. And the organisms were isolated as pure culture and they are identified by the colony morphology and standard biochemical tests[3]. Their antibiotic sensitivity patterns were noted by disk diffusion method according to CLSI guidelines and drug resistance strains were identified[15].

Detection of Boi-film Production

Isolates from the blood culture were processed by three different methods to detect the biolfilm producing ability.

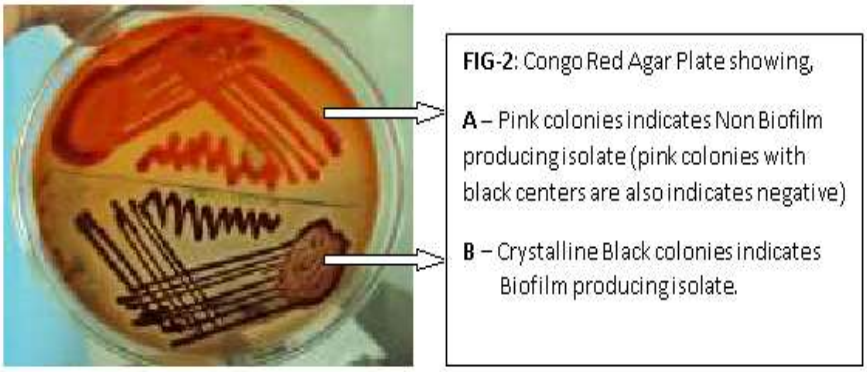
1. Tube method
2. Congo red agar method
3. Tissue culture plate method

These are the three different methods followed by Christensen et al[4]. These three methods are standardised by using *Staphylococcus aureus* (ATCC 35984) as a known biofilm producer and *Staphylococcus epidermidis* (ATTC 12228) as a known non biofilm producer.

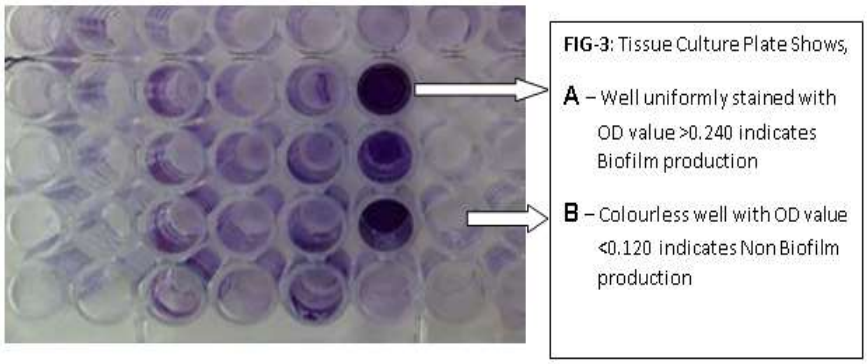
1. **Tube Method:** Round bottom test tube with TSB glu (Trypticase soy broth with 1% glucose) was inoculated with loopful of colonies from plates of primary isolates and incubated at 37^0C for 24 hrs. Then the broth from the tubes were discarded and washed with PBS (phosphate buffer saline of pH 7.3) and kept for dying. With 0.1% Crystal violet, tubes were stained and washed with distilled water and dried by keeping it in inverted position. Then the tubes were observed for violet colour stained layer at the wall and bottom, which indicates the biofilm production positive.

Fig. 1: **Tube method showing, A – No visible violet colour indicates non Biofilm producing isolate B & C – Wall and bottom of tubes showing visible violet colour indicates Biofilm producing isolates**

2. **Congo Red Agar Method:** Congo red dye was prepared separately as concentrated aqueous solution and added to other constituents of media, and was sterilized by autoclave. Congo Red Agar Plates were inoculated from the primary isolates and incubated aerobically at 37^0C for 24 to 48 hours. Formation of dry crystalline black colonies indicates positive result for biofilm production. Pink colonies indicates the non-biofilm producers, eventhough occasional darkening at the centers of colonies were produced. Intermediate result was recorded if darkening of the colonies without dry crystalline consistency.

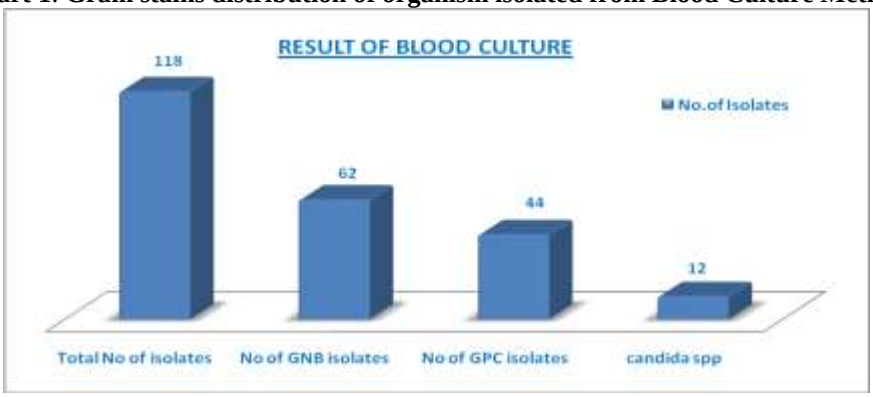


3. **Tissue Culture Plate Method:** Broth discarded from tube method was diluted by 1in100 with fresh medium. 0.2 ml aliquots of the diluted culture was transferred to Individual wells of sterile polystyrene, 96 well-flat bottom tissue culture plates (Tarson Kolkata, India) and one rows were filled with fresh medium, which serves as control to check sterility and non-specific binding of media. These plates were incubated at 37°C for about 18 hours and 24 hours. After incubation, broth from each well was removed by tapping the plates gently. Then wells were washed with 0.2 mL of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. Repeat washing procedure for about four times. Sodium acetate (2%) was added, to fix the Biofilms formed by adherent 'sessile' organisms in the wells and crystal violet (0.1% w/v) was added to each well for staining the biofilm layer. All the wells were washed with deionised water and kept for drying. Biofilm formed by the adherent cells were uniformly stained with crystal violet. Optical density (OD) of the wells were taken by using micro ELISA auto reader (model: RT0140215RBK, Robonik) at wavelength of 570 nm (OD570 nm). These OD value indicates the strength of biofilm layer and was compared with the negative control. OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values.



Result

Chart 1: **Gram stains distribution of organism isolated from Blood Culture Method**



GNB – Gram Negative Bacilli; GPC – Gram Positive Cocci

Chart 2: **GNB isolates obtained from Blood Culture Method**

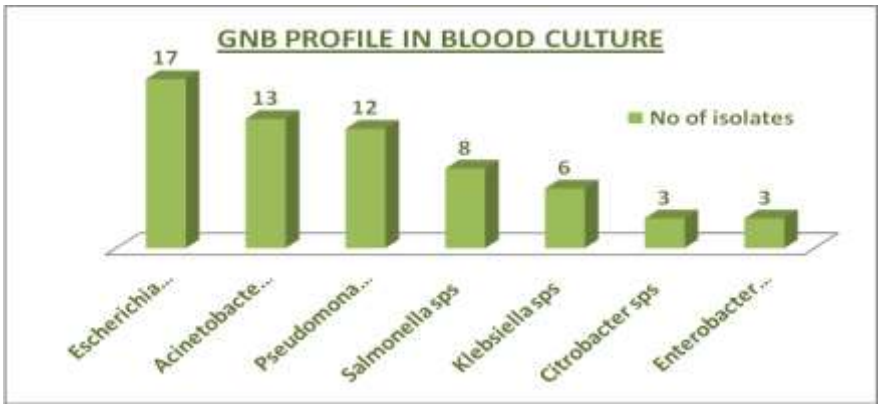


Chart 3: **Resistant isolates obtained from Blood Culture Method**

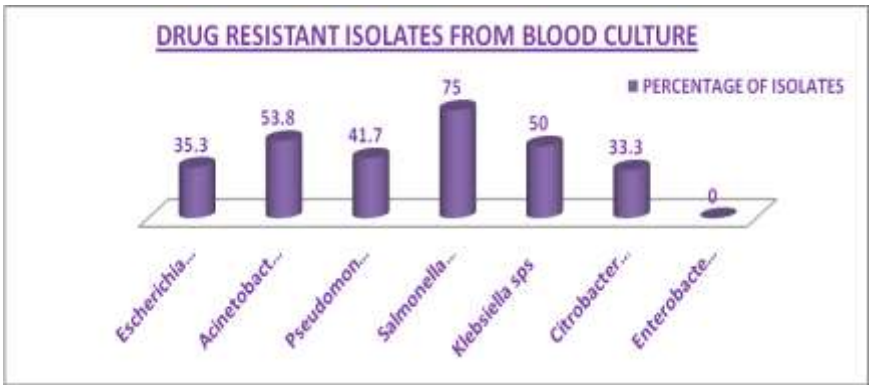
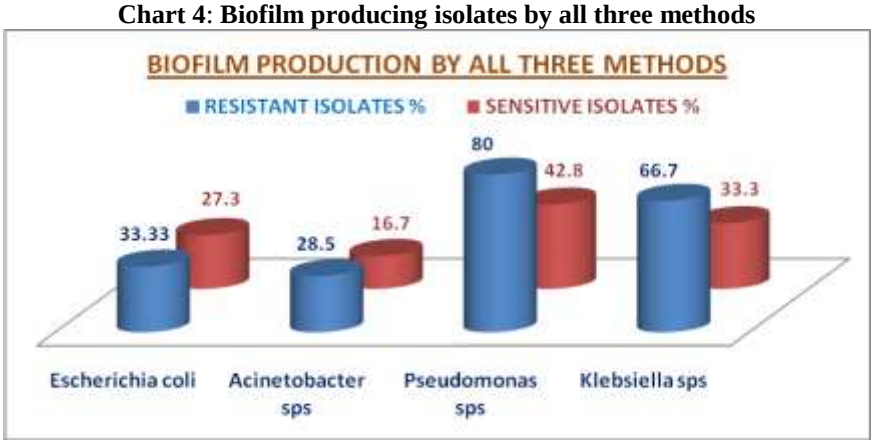


Chart 4: **Biofilm producing isolates by all three methods**



Discussion

According to Khanal, Sharma and Roy et al, the positivity in blood culture have been reported as 44%, 33.9% and 16.4% respectively[6,7,8]. In our study, the positivity in blood culture is 32.3%. The major reason for the variation in blood culture positivity is due to the prior usage of broad spectrum antibiotic by patients as self medication or by the counter sold medications, before reaching the tertiary care hospitals. In this study, among the total of 118 isolates the incidence of Gram Positive organism including *Candida spp* was 47.5% and the Gram negative bacilli was about 52.5%. The profile of 62 Gram Negative Bacterial isolates are as follows; 17(27.4%) isolates were *Eschericia coli*, which is the commonly available organism that can infects the immunosuppressed patients followed by 13(21%) isolates of *Acinetobacter spp*. *Pseudomonas spp* were isolated with 12(19.3%) isolates, since this organism is resistant to chemical disinfectants and can cause major hospital acquired infections. About 8(13%) isolates were Salmonella spp followed by 6 (9.6%) were *Klebsiella spp* and 3(4.8%) isolates were obtained from each of *Citrobacter spp* and *Enterobacter spp*. Similar result was obtained by Usha Arora et al in 2007[1].

On seeing the drug resistant nature of these isolates, it is found that nearly half of the isolates were found to be resistant to routine antibiotics. About 35.3% of *Eschericia coli*, 33.3% of *Citrobacter spp* and 50%

of *Klebsiella spp* were found to be ESBL producers and also resistant to multiple drugs as said in previous study by Sanghamithra Data et al[11]. Coming to non-fermenters, it is found that, 53.8% of *Acinetobacter spp* and 41.7% of *Pseudomonas spp* were found to be MBL producers as well as multiple drug resistant isolates. The major problem regarding the increased rate of drug resistant is due to improper antibiotic policy and also lack of awareness among public towards antibiotic therapy. Stopping even some of the inappropriate and unnecessary use of antibiotics in people and animals would help greatly in slowing down the spread of resistant bacteria[12]. But in case of Enteric fever, patients were undergone with routine antibiotic as well as higher antibiotics without proper diagnosis. Hence, the rate of drug resistance in Salmonella sps is quite high recording about 75%.

In this study, the biofilm producing ability among Gram Negative Bacilli are identified as follows; the major organism is identified as *Pseudomonas spp* with 80% in resistant strains and 42.8% in sensitive strains. The second major organism is *Klebsiella spp* with 66.7% of resistant and 33.3% sensitive strains. This is followed by *Eschericia coli* with 33.33% of resistant and 27.3% of sensitive strains and *Acinetobacter spp* with 28.5% of resistant and 16.7% of sensitive isolates were recorded to be the biofilm producers. On concentrating this, it is clearly identified that the major biofilm producers are capsulated organism and also alginate producers. According to the study of Lee et al., there is a strong relation between biofilm formation and β-lactamases production[5]. The second major problem regarding the drug resistance is may be due to the consequence of biofilm development. It is said that the ability of *Acinetobacter spp* to transfer genes horizontally might also enhance within these micro communities facilitating the spread of antibiotic resistance[5]. In our study, it's to be noted that, most of the biofilm producers are also resistant to routine antibiotics and also to multiple drugs.

Conclusion

In these present days, it is a must to create awareness among peoples about the importance of the tertiary care hospitals in identifying the infection and recommendation of appropriate antibiotic therapy. This will reduces the rate of drug resistance among the bacteria. The implants introduced in bacteremia patients may helps the bacteria to produce biofilm around them. This will makes the treatment much more worsen than before. Hence, proper usage of implants should be followed and care should be taken to prevent biofilm production. And this is must to identify that, whether resistant strains can produce biofilm or viceversa, which may help us to handle the situation much better.

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