Biofilm Production in Carbapenem Resistant Isolates from Chronic Wound Infections

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ABSTRACT

Biofilms are communities of microorganisms covered with extracellular polymeric substances. Such biofilm phenotype makes the microorganism resistant to antibiotics and plays a role in wound chronicity. This results in prolonged hospital stays in ICU, greater cost, and increased mortality. **Methods:** *Pus swabs (59) were collected from a tertiary care hospital near Chennai were processed and identified using standard procedure followed by antibiotic susceptibility testing and identification of carbapenem resistance by Modified Hodge test as per CLSI guidelines. The biofilm formation was tested using plastic microtiter plate method.* **Results:** *Out of 59 pus swabs, 51 yielded growth with 69 isolates and 8 yielded no growth. Among the 69 isolates, 51 were GNB and 18 were GPC. Biofilm detection was noted in 84.31% (43/51) GNB isolates with 0.1% crystal violet whereas 100% (51/51) showed biofilm positive with 0.1% safranin. About 74.50% (38/51) isolates of GNB were carbapenem resistant by screening with disk diffusion method. Only 24% (6/25) of GNB isolates among Enterobacteriaceae were positive by Modified Hodge test method.* **Conclusion:** *The result shows the association of biofilm production among carbapenem resistant isolates obtained from chronic wound infections.*

Keywords: Biofilms, carbapenem, wound infection

INTRODUCTION

Biofilms are micro-communities formed by combination of same or different microorganisms and cover within a matrix of extracellular polymeric substances (EPS) [1]. According to the National institute of health, 80% of microbial infections in the human are biofilm mediated infections [2]. The ability of a microorganism to form biofilm is an important virulence factor and cause persistent infections [3]. In biofilm mode of growth, microorganisms escape host defences and show poor response to antibiotics. Such biofilm phenotype paves way for the emergence of multi-drug resistance organisms and result in treatment failure.

Multi drug resistant organisms (MDRO) is a challenging problem throughout the world and its origin is primarily nosocomial [4]. The mainstay of therapy for MDR GNB is carbapenem, a beta lactam group of antibiotics with broad spectrum of activity. The emergence of new carbapenamases producing organisms is the current challenge for treatment of MDR GNB.

The potential association of biofilms in the pathology of chronic wounds has been demonstrated to play a role in wound chronicity [5]. According to Indian epidemiological data, chronic wound was reported as 4.5 per 1000 population [6]. The number of chronic wounds will increase worldwide due to the increase in lifestyle diseases, such as diabetes, obesity, and cardiovascular diseases. The chronic wound infection includes diabetic foot ulcer (DFU), pressure ulcer and venous leg ulcer. Diabetic foot ulcer is highly prevalent among the world and cause impaired healing, leading to osteomyelitis, bacteraemia and sepsis. Treatment failure for diabetic foot ulcer may ultimately lead to limb amputation. It was reported as >25% of persons with diabetes have the risk to develop foot ulceration

during their life time. Roughly 14% to 24% of people with a lower extremity ulcer eventually suffer an amputation [7]. Diabetes and, therefore, diabetic foot ulcer complications are growing at double digit rates and have the potential of becoming even a more devastating epidemic.

Thus, the presence of chronic wound infection with MDR organism along with biofilm phenotype pose a significant burden to health care system. This results in prolonged hospital stays in ICU, greater cost, and increased mortality. Therefore, the present study is designed to identify carbapenem resistant isolates and their ability to form biofilm from chronic wound infection.

MATERIALS AND METHODS

A total of 59 pus swabs were collected from a tertiary care hospital near Chennai during the period of January 2014 to December 2014 after obtaining institute ethical clearance. The collected pus swabs were processed and isolates were identified using standard procedure [8].

All the isolates were subjected to antibiotic susceptibility testing on Mueller Hinton Agar (MHA) plates by Kirby-Bauer disk diffusion method using antibiotic disc (Himedia, Mumbai) as per CLSI guidelines [9]. The GNB isolates belonging to Enterobacteriaceae resistant to carbapenem were subjected to phenotypic confirmation by Modified Hodge test [10].

All the isolates were subjected to biofilm formation by using plastic microtiter plate as described by Stepanovic et al. [11]. The biofilm formation on microtiter plate were detected using 0.1% crystal violet and safranin separately and then the optical density (O.D) of each well was measured at 490 nm using automated Multiskan ELISA reader (Bio rad, France). The tests were carried out in triplicate and the results were averaged. The cut-off O.D. was calculated as follows:

Cut off O.D. (O.D.c)=Mean+3 Standard Deviation of Negative control.

Positive control=Average

Sample=Average

No biofilm=O.D. less than O.D.c

Weak biofilm=O.D. less than 2 O.D.c., but greater than O.D.c

Moderate biofilm =O.D. less than 4 O.D.c., but greater than 2 O.D.c

Strong biofilm=O.D. greater than 4 O.D.c

RESULTS

Out of 59 pus swabs, 51 yielded growth and 8 yielded no growth by conventional method after 18 h to 24 h of aerobic incubation. Out of the 51 specimens with growth, single isolate was obtained from 36 specimens and more than one isolate from 15 specimens. Two types of organism were isolated from 12 specimens and 3 types from 3 specimens.

The total numbers of isolates obtained from 51 samples were 69. Among the 69 isolates, 51 were GNB and 18 were GPC.

The GNB isolates include *Escherichia coli* (n=8), *Klebsiella pneumoniae* (n=6), *Enterobacter* spp (n=3), *Proteus* spp (n=8), *Pseudomonas* spp (n=13) and *Acinetobacter* spp (n=13). GPC isolates include *Staphylococcus aureus* (n=4), Coagulase Negative *Staphylococci* (CONS) (n=6), *Streptococcus* spp (n=5) and *Enterococcus* spp (n=3) (Table 1).

Table 1 Distribution of microorganisms from chronic wound infection

Type of chronic wound ulcers (Number of specimen=59)	Number of specimen with growth			Number of specimen without growth
	No. of isolate from each specimen	GNB (N=51)	GPC (N=18)	
Venous leg ulcers (8)	3 Single	*Escherichia coli* (1) *Klebsiella pneumoniae* (1) *Proteus* spp (1)	-	2
	3 Mixed (6)	*Escherichia coli* (1) *Enterobacter* spp (1) *Pseudomonas* spp (2)	*Staphylococcus aureus* (1) *CONS* (1)	

Pressure ulcer (20)	14 Single	*Escherichia coli* (1) *Klebsiella pneumoniae* (1) *Proteus* spp (1) *Pseudomonas* spp (3) *Acinetobacter* spp (4)	*Staphylococcus aureus* (1) *CONS* (1) *Enterococcus* spp (1) *Streptococcus* spp (1)	2
	2 Mixed (4)	*Escherichia coli* (1) *Klebsiella pneumoniae* (1) *Enterobacter* spp (1) *Pseudomonas* spp (1)	-	
	2 Mixed (6)	*Klebsiella pneumoniae* (1) *Proteus* spp (1) *Acinetobacter* spp (1)	*Staphylococcus aureus* (1) *Enterococcus* spp (1) *Streptococcus* spp (1)	
Diabetic foot ulcer (24)	18 Single	*Escherichia coli* (3) *Klebsiella pneumoniae* (2) *Enterobacter* spp (1) *Proteus* spp (2) *Pseudomonas* spp (4) *Acinetobacter* spp (2)	*Staphylococcus aureus* (1) *CONS* (2) *Streptococcus* spp (1)	0
	5 Mixed (10)	*Escherichia coli* (1) *Proteus* spp (3) *Pseudomonas* spp (2) *Acinetobacter* spp (4)	-	
	1 Mixed (3)	*Pseudomonas* spp (1) *Acinetobacter* spp (1)	*CONS* (1)	
Traumatic ulcer (4)	2 Mixed (4)	*Acinetobacter* spp (1)	*CONS* (1) *Enterococcus* spp (1) *Streptococcus* spp (1)	2
Arterial ulcers (3)	1 Single	-	*Streptococcus* spp (1)	2

The results of Microtiter plate assay for detection of quantitative biofilm formation was compared by staining with 0.1% crystal violet and 0.1% safranin (Tables 2 and 3). Quantitative detection in 51 GNB isolates with 0.1% crystal violet showed 84.31% (43/51) biofilm positive whereas 100% (51/51) showed biofilm positive with 0.1% safranin. In crystal-violet staining, 13/51 isolates (25.49%) showed strong biofilm positive, 13/51 isolate (25.49%) showed moderate biofilm positive and 17/51 isolates (33.33%) were weak biofilm positive.

Table 2 Biofilm formation of GNB using microtiter plate method by crystal violet (0.1%) and safranin (0.1%) staining

GNB Isolates	Crystal Violet (0.1%)			Safranin (0.1%)		
	Strong (%)	Moderate (%)	Weak (%)	Strong (%)	Moderate (%)	Weak (%)
Escherichia coli (n=8)	2 (25)	4 (50)	2 (25)	1 (12.5)	3 (37.5)	4 (50)
Klebsiella pneumoniae (n=6)	2 (33.33)	1 (16.66)	3 (50)	2 (33.33)	1 (16.66)	3 (50)
Enterobacter spp (n=3)	2 (66.66)	0	1 (33.33)	1 (33.33)	2 (66.66)	0
Proteus spp (n=8)	2 (25)	3 (37.5)	3 (37.5)	2 (25)	4 (50)	2 (25)
Pseudomonas spp (n=13)	2 (15.38)	3 (23.07)	3 (23.07)	3 (23.07)	5 (38.46)	4 (30.76)
Acinetobacter spp (n=13)	3 (23.07)	2 (15.38)	5 (38.46)	2 (15.38)	6 (46.15)	5 (38.46)
Total (51)	13 (25.49)	13 (25.49)	17 (33.33)	11 (21.56)	21 (41.17)	19 (37.25)
Cut off value for (1) Crystal violet: Strong= ≥ 0.72, Moderate=0.36-0.72, Weak=0.18-0.36 (2) Safranin: Strong= ≥ 0.83, Moderate=0.4-0.83, Weak=0.18-0.4						

Table 3 Biofilm formation of GPC using microtiter plate method by crystal violet (0.1%) and safranin (0.1%) staining

GPC Isolates	Crystal Violet (0.1%)			Safranin (0.1%)		
	Strong (%)	Moderate (%)	Weak (%)	Strong (%)	Moderate (%)	Weak (%)
Staphylococcus aureus (n=4)	1 (25)	2 (50)	1 (25)	1 (25)	2 (50)	1 (25)
CONS (n=6)	2 (33.33)	2 (33.33)	1 (16.66)	1 (16.66)	3 (50)	2 (33.33)
Streptococcus spp (n=5)	0	2 (40)	2 (40)	1 (20)	3 (60)	1 (20)
Enterococcus spp (n=3)	0	2 (66.66)	1 (33.33)	0	0	2 (66.66)
Total (18)	3 (16.66)	8 (44.44)	5 (27.77)	3 (16.66)	8 (44.44)	6 (33.33)
Cut off value for (1) Crystal Violet: Strong= ≥0.72, Moderate=0.36-0.72, Weak=0.18-0.36 (2) Safranin: Strong= ≥0.83, Moderate=0.4-0.83, Weak=0.18-0.4						

In safranin staining, 11/51 isolates (21.56%) showed strong biofilm positive, 21/51 isolates (41.17%) showed moderate biofilm positive and 19/51 isolates (37.25%) showed weak biofilm positive.

Quantitative detection for biofilm in 18 GPC isolates with 0.1% crystal violet showed 88.88% (16/18) biofilm positive whereas 94.44% (17/18) showed biofilm positive with 0.1% safranin (Figure 1). In crystal-violet staining, 3/18 isolates (16.66%) showed strong biofilm positive, 8/18 isolates (44.44%) showed moderate biofilm positive and 5/18 isolates (27.77%) were weak biofilm positive.

In safranin staining, 3/18 isolates (16.66%) showed strong biofilm positive, 8/18 isolates (44.44%) showed moderate biofilm positive and 6/18 isolates (33.33%) showed weak biofilm positive.

All the GNB isolates (n=51) were screened for carbapenem resistance by disk diffusion method using Imipenem (10 µg), Ertapenem (10 µg) and Meropenem (10 µg) and phenotypic confirmation by Modified Hodge test was also performed.
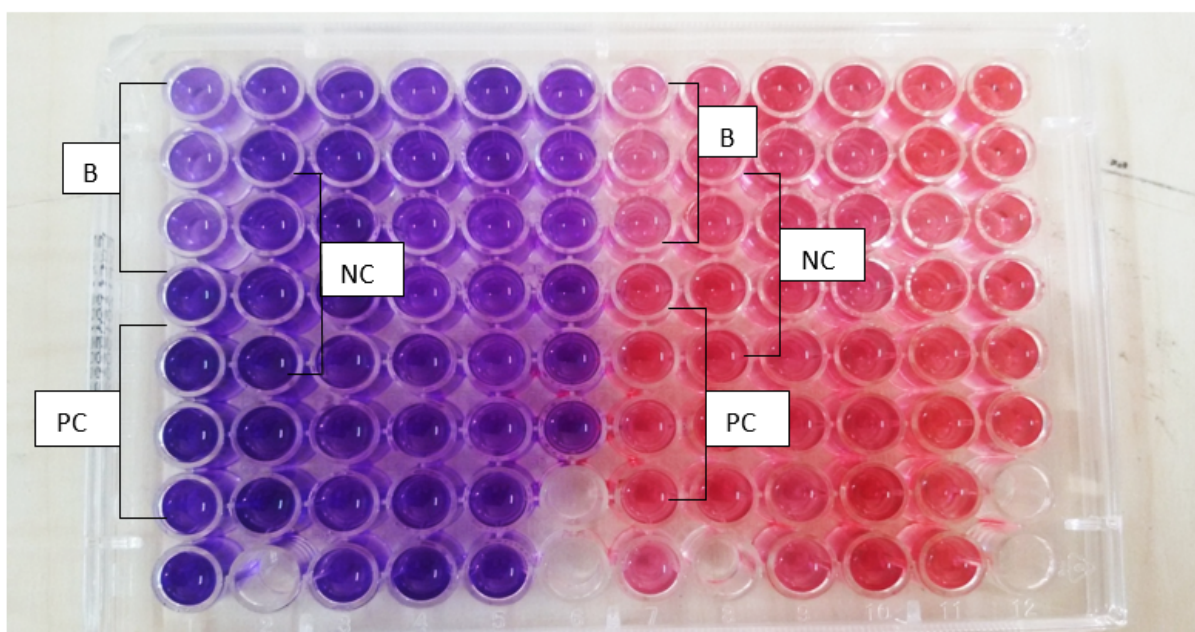

Figure 1 Microtiter plate showing biofilm formation by 0.1% crystal violet (left) and 0.1% safranin staining (right)

B=Blank, PC=Positive control, NC=Negative control; From A3-F6; A9-F12 were test isolates.
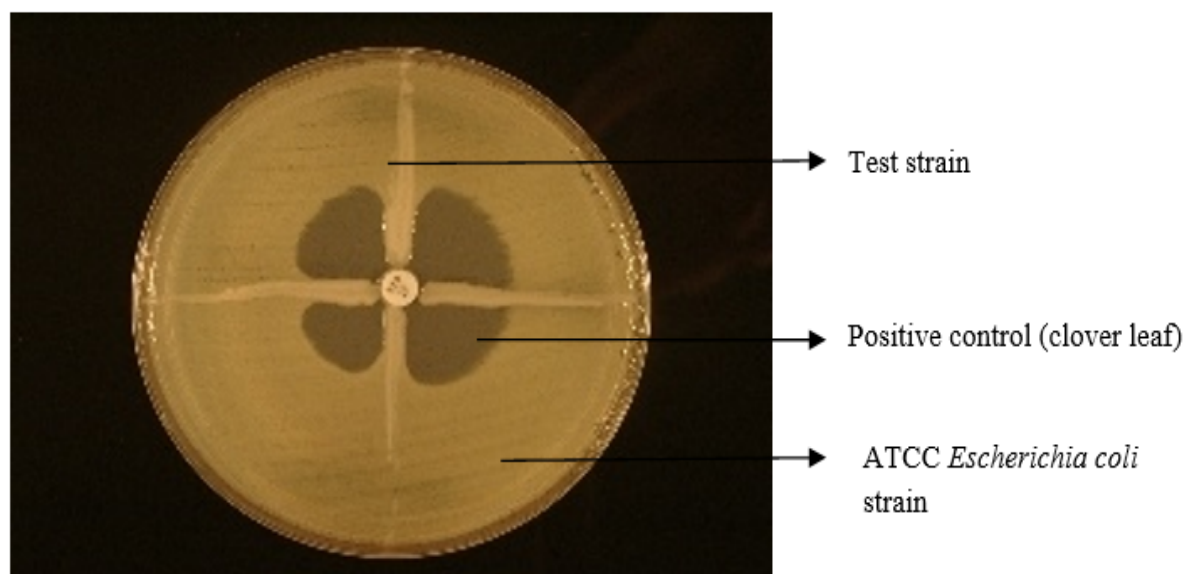

Figure 2 Modified Hodge test

About 74.50% (38/51) isolates of GNB were positive for carbapenem resistant by screening with disk diffusion method. Only 25% (6/24) of GNB isolates among Enterobacteriaceae were positive by Modified Hodge test method

(Table 4 and Figure 2) whereas non-fermenters were not subjected to Modified Hodge test. All the GNB isolates positive for carbapenem resistance by Modified Hodge test were also positive for biofilm formation.

Table 4 Comparison of screening test and phenotypic method for carbapenem resistant with biofilm

GNB (n)	Disc diffusion n (%)	Modified Hodge test=n (%)	Biofilm=n
Escherichia coli (n=8)	8 (100)	1 (12.50)	1 (Strong)
Klebsiella pneumoniae (n=6)	5 (83.33)	2 (33.33)	1 (Moderate) 1 (Weak)
Enterobacter spp (n=3)	3 (100)	2 (66.66)	1 (Strong) 1 (Weak)
Proteus spp (n=8)	5 (62.50)	1 (12.50)	1 (Weak)
Pseudomonas spp (n=13)	10 (76.92)	-	1 (Strong) 2 (Moderate) 2 (Weak)
Acinetobacter spp (n=13)	7 (53.84)	-	1 (Strong) 3 (Moderate) 2 (Weak)
Total - 51	38 (74.50)	6 (24)	4 (Strong) 6 (Moderate) 7 (Weak)

DISCUSSION

The pertinent problem of wound infection was greatly influenced by their ability to provide suitable niche for microbial communities to form biofilm. This biofilm formation could attribute for chronicity of the wound and risk of developing multi drug-resistant strains of pathogenic bacteria.

In this study, a total of 69 isolates were recovered out of 51 pus swabs and 13.55% (8/59) of pus swabs showed no growth. The reason for no growth was mainly due to the collection of specimen with surface swabs whereas, some studies have demonstrated the deep tissue biopsies as superior for culturing [12]. In addition, conventional techniques based on culturing non-fastidious, aerobic microorganisms do not support the growth of anaerobes.

The severity of wound infection was related to polymicrobial growth whereas, monomicrobial growth was frequent in mild infections. Of the 51 pus swabs with growth, 70.58% (36/51) were monomicrobial and 29.41% (15/51) were polymicrobial in our study. Polymicrobial nature of chronic wound was observed in some studies [13,14] whereas, few studies have demonstrated majority of the isolates as monomicrobial [15,16]. In recent years, using molecular techniques such as PCR and metagenomics could able to demonstrate the complex ecology of chronic wounds [17,18].

In the present study, Gram negative bacilli were predominately isolated (n=51) than Gram positive cocci (n=18). Among the Gram-negative bacilli, predominantly non-fermenters such as *Pseudomonas aerogunisa* (25.49%; 13/51) and *Acinetobacter* species (25.49%; 13/51) were isolated followed by members of Enterobacteriaceae such as *Escherichia coli* (15.68%; 8/51), *Proteus* spp (15.68%; 8/51), *Klebsiella pneumoniae* (11.76%; 6/51) and *Enterobacter* spp (5.88%; 3/51). Of the Gram-positive cocci, *CONS* (33.33%; 6/18), *Streptococcus* spp (27.77%; 5/18), *Staphylococcus aureus* (22.22%; 4/18) and *Enterococcus* spp (16.66; 3/18) were isolated. Similar findings were also observed in few studies [19].

Bacterial adherence is an essential step in the development of biofilms both invitro and in vivo. In the present study, Quantitative microtiter assay was done to demonstrate the biofilm production in chronic wound isolates. Among the two different staining methods used for biofilm detection, safranin staining (100%; 51/51) was found highly sensitive than crystal violet staining (84.31%; 43/51).

Of the 51 GNB isolates, 25.49% (13/51) were strong biofilm producers and 25.49% (13/51) were moderate biofilm producers. 33.33% (17/51) were weak biofilm producers with crystal violet staining. 15.68% (8/51) GNB did not show biofilm production. 21.56% (11/51) were strong biofilm producers, 41.17% (21/51) were moderate biofilm producers and 37.25% (19/51) were weak biofilm producers with safranin staining.

Of the 18 GPC isolates, 16.66% (3/18) were strong biofilm producers and 44.44% (8/18) were moderate biofilm producers by both crystal violet and safranin staining. 27.77% (5/18) with crystal violet staining and 33.33% (6/18) with safranin staining were weak biofilm producers.

Our findings correlated well with the study from James et al. [20], where he could demonstrate the presence of biofilm in 60% of chronic wounds and only 6% of acute wounds. In one study, 44% of *Acinetobacter* spp isolates from wound infection was biofilm positive and multi-drug resistant [21]. The demonstration of biofilm production by chronic wound isolates was well explained in a study with non-fluid culture sites such as superficial/deep tissue showing a strong correlation of isolates for biofilm formation rather than that of isolates from body fluids such as blood or urine.

The reason suggested was that the adaptation favours the survival of organism in solid tissue making the biofilm eradication a difficult or challenging problem in some infections [22]. The presence of large amounts of biofilms in wound bed acts as a mechanical barrier to cell migration, granulation and re-epithelialization and stimulates a chronic state of inflammation which slows healing [23].

Antibiotic resistance in chronic wound infection is an important public health problem. It was suggested that the chronic wounds were found to be colonized and infected with antibiotic resistant strains [24]. The reason being the frequent use of antibiotics in chronic wound along with complex microflora favours the dissemination of resistant organism to others. In order to treat the emerging multi-drug resistant organisms in alarming rate, the only drug of choice is carbapenems. Due to the increasing resistance to carbapenem group of antibiotics leaves the healthcare system with almost no effective alternative drugs. Detection of carbapenemase producers is essential for rapid adaptation of the antibiotic therapy and isolation of colonized patients in order to prevent the development of nosocomial outbreaks [25].

Currently, for identification of carbapenemase producers, the Gram negative bacterial isolates were screened first by disk diffusion technique to detect the reduced susceptibility to carbapenem followed by phenotypic confirmation for invitro production of carbapenemase by modified Hodge test.

In the present study, 74.50% (38/51) of GNB isolates were resistant to carbapenem by disk diffusion method whereas only 24% (6/25) of Enterobacteriaceae were positive by phenotypic confirmatory method such as Modified Hodge test as recommended by CLSI. Nearly 19 GNB isolates negative by modified Hodge test indicates that the disc diffusion method can detect the carbapenem resistant not only due to carbapenemase enzymes but also by other different resistance mechanisms. It was suggested that the mechanism of reduced permeability to the outer membrane porin proteins with over expression of chromosome or acquired Amp C or ESBL could also lead to carbapenem resistant [26]. In the modified Hodge test, highest prevalence of resistant to carbapenem was seen in *E. coli* followed by *Enterobacter* spp, *Klebsiella pneumoniae* and *Proteus* spp. Other studies have reported that *Klebsiella pneumoniae* showed the highest percentage of resistance to carbapenem [27,28]. Further confirmation is by performing MIC for the carbapenem resistant isolates and detecting the presence of carbapenemase genes by PCR. Among the non-fermenters, 76.92% (10/13) of *Pseudomonas* spp and 53.84% (7/13) of *Acinetobacter* spp were resistant to carbapenem. However, as per CLSI, modified Hodge test is not recommended for non-fermenters to detect carbapenemase production. All the carbapenem resistant isolates showed correlation with biofilm formation.

CONCLUSION

The present study emphasizes the need to detect biofilm phenotype and to screen for carbapenem resistance by modified Hodge test, which is a simple method to detect the carbapenemase among Enterobacteriaceae in resource limited settings. Such detection helps to avoid unnecessary use of broad spectrum antibiotics and thereby prevent treatment failures and development of resistance due to use of this antibiotic.

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