Detection of Metallo-β-Lactamases and Carbapenemase Producing *Pseudomonas aeruginosa* Isolates from Burn Wound Infection

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ABSTRACT

Objective: The study aims to detect carbapenemase producing *P. aeruginosa* isolated from burn wounds and confirm MBL production by Imipenem-Combined disk method.

Method: A total of 310 non-repeated clinical specimens including tissues, pus aspirates, and wound swabs were processed using standard microbiological procedure. Each identified isolate of *P. aeruginosa* was subjected to *in vitro* antibiotic susceptibility test by using modified Kirby-Bauer disc diffusion method. Two imipenem (10µg) disks were placed on the surface of the agar plate in which one of them was added with 5µl of 0.5M EDTA solution. The result was interpreted after 18 hours of incubation at 37°C by comparing the inhibition zone of imipenem and imipenem-EDTA disks. The increase in inhibition zone by ≥7mm with imipenem-EDTA disks than imipenem alone was considered as MBL Positive. Similarly, for detecting carbapenemase Modified Carbapenem Inactivation Method (mCIM) was used.

Results: *P. aeruginosa* was found to be the predominant organism (13.99%). Among 20 *P. aeruginosa* isolates resistant to imipenem and meropenem, 20% were found to be carbapenemase producer by mCIM assay and 15% were found to be MBL producers by Imipenem-Combined disk method. High percentage of MBL producing isolates of *P. aeruginosa* were found resistant towards tested antibiotics.

Conclusion: This study reports that the clinical isolates of *Pseudomonas aeruginosa* have the ability to produce MBL. The increasing and rapid spread of *P. aeruginosa,* as well as the rate of drug resistance among the isolates, was found to be a worrisome situation.

Keywords: Burn wound infections, Antibiotic susceptibility test, Carbapenemase, Metallo-Beta-Lacatmase (MBL), CLSI

INTRODUCTION

Burns are more persistent cause of infection when compared with surgical wounds. This is because of prolonged hospital stay and larger area involved. In addition, burns provide an appropriate location for bacterial multiplication (Aghnihotri et al. 2004). The predominating organisms that cause burn wound infections in any burn treatment facility change over time. Initially, after the injury Gram positive bacteria inhabit the burn wound (Barret and Herndon 2003) and later Gram-negative bacteria also rapidly colonize the burn wound surface (Wysocki 2002).

Pseudomonas aeruginosa is accountable for serious hospital acquired infections, chiefly in burn patients (Sheikh et al. 2014). Although, a wide range of antibiotics are used for its treatment, the bacterium is

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intrinsically resistant to many antibiotics due to which the therapeutic options for treating serious infections are severely restricted. The resistance to antibacterials is developed either via mutational processes that alter the expression or through the acquisition of resistance genes on mobile genetic elements (i.e., plasmids) and/or function of chromosomally encoded mechanisms. Hence, a global health issue has arisen because of increasing number of multidrug-resistant (MDR) *P. aeruginosa* (Adachi. 2009; Madigan et al. 2012).

Carbapenems are the drug of choice used for treating infections caused by *Pseudomonas aeruginosa*, producing cephalosporinase, AmpC or extended-spectrum β-lactamases (Zavascki et al. 2010). However, the development of carbapenem-resistant *P. aeruginosa* has threatened on the use of carbapenem in the management of its infections. The most frequent reason behind resistant to carbapenem in *P. aeruginosa* is attributed to impermeability through alteration or loss of the porin OprD, increased expression of an efflux pump, or the production of class B metallo-β-lactamases (MBLs) (Kateete et al. 2016).

Production of metallo-beta-lactamase (MBL) enzyme has been one of the major causes of carbapenam resistance in *P. aeruginosa*. Metallo- β-lactamase mediates resistance to β-lactams by cleaving the amide bond of the β- lactam ring. MBLs can be divided into two groups, one that are chromosomally mediated and the other encoded by transferable genes (Walsh et al. 2005). The six different types of mobile MBLs, namely, IMP, VIM, SPM, GIM, SIM and AIM are known so far and the mechanism of hydrolysis varies from one kind to another (Gupta, 2008). *P. aeruginosa* is predominantly known to produce IMP and VIM type MBL (Khosravi and Mihani. 2008). The IMP and VIM genes responsible for MBL production are horizontally transferable via plasmids and can rapidly spread to other bacteria (Zubair et al. 2011).

In 2017, Carbapenem-resistant *P. aeruginosa* (CRPA) is ranked as the second most critical-priority bacterium among 20 antimicrobial-resistant bacterial species in a survey conducted by World Health Organization on multi-country antibiotic resistance (Tacconelli et al. 2018). Moreover, infection with MBL producing organism such as *P. aeruginosa* is associated with higher rates of mortality, morbidity and health care cost, especially due to inadequate empirical therapy (Picao et al. 2008; Kaleem et al. 2010). Although, there have been reports and studies involving increasing drug resistance in burn patients worldwide, the information regarding the etiology and management of burn wound infections is limited in developing countries like Nepal. Hence, the prime focus of this study is to determine proportion of MBL and carbapenemase in *P. aeruginosa* isolated from burn wound which if taken into consideration either during empirical therapy or pathogen directed therapy can substantially reduce health care associated cost and more importantly the emergence and spread of resistance itself.

MATERIALS AND METHODS

This cross sectional hospital based prospective study was conducted in between June 2018 and December 2018 in microbiological laboratory of Nepal Cleft and Burn Center, Kirtipur Kathmandu. A total of 310 non-repeated clinical specimens including tissues, pus aspirates, and wound swabs were processed using standard microbiological procedure during the study period. The samples of patients not listed as burn victims were excluded from the study.

All the samples were collected by experienced medical personnel using standard microbiological procedures. Wound swabs were collected by using sterile cotton-wool swab taking special care to avoid the contamination by the commensal organism. Pus aspirate samples were either collected in a sterile syringe or by using a sterile cotton-wool swab. The cotton-wool swabs were then placed back into sterile tubes and capped. Tissue culture samples were collected with special care avoid contamination by commensal organisms and placed in a sterile container. Pus aspirate from the wounds was collected with the help of sterile syringe and contamination by the commensal organism was prevented with special care (Cheesebrough M 2018). After proper labeling, the samples were transported to the microbiology laboratory promptly.

The wound swabs and pus aspirate were directly inoculated into Blood Agar (BA) and Macconkey Agar (MA) with the help of a sterile loop. The inoculated agar plates were incubated aerobically overnight at 37°C. The tissue culture from burn wounds was first aseptically removed from the container and inoculated into Brain heart Infusion (BHI) broth and incubated aerobically overnight at 37°C. This was followed by subculture on BA and MA (HiMedia). Standard

microbiological procedures were followed for the identification of the isolates as described in Bergey's manual of systemic bacteriology

Each identified isolate of *P. aeruginosa* was subjected to *in vitro* antibiotic susceptibility test by modified Kirby-Bauer disc diffusion method as recommended by CLSI guidelines on Muller Hinton Agar (CLSI. 2018). Commercially available antibiotic discs of HiMedia were used that includes amikacin (30 µg), gentamycin (10 µg), ciprofloxacin (5 µg), cefepime (5 µg), piperacillin/tazobactam (100/10 µg), cefoperazone/sublactam (75/30 µg), meropenem (10 µg), imipenem (10 µg), doxycycline (30 µg), ceftazidime (30 µg), polymyxin B (10 µg), colistin (CL). MDR isolates were detected based on their resistance to two or more antibiotics (Cheesbrough. 2006; CLSI. 2018)

Detection of metallo-beta-lactamase production by Combined Disk (CD) assay

Following the standard procedures, the test organism was inoculated on MHA plate as recommended by the CLSI guidelines. Two imipenem (10µg) disks were placed on the surface of the agar plate in which one of them was added with 5µl of 0.5M EDTA solution. The result was interpreted after 18 hours of aerobic incubation at 37°C by comparing the inhibition zone of imipenem and imipenem-EDTA disks. The increase in inhibition zone by ≥7mm with imipenem-EDTA disks than imipenem alone was considered as MBL Positive (Yong et al. 2002).

Modified Carbapenem Inactivation method (mCIM)

A 1-µl loop full of test organism from an overnight agar plate was transferred to a tube containing 2 ml of trypticase soy broth (TSB) and the suspension was vortexed followed by addition of 10-µg meropenem disk to the suspension. The suspension was incubated at 35 °C for four hours at ambient air. Prior to completion of four-hour incubation, a 0.5 McFarland suspension of *E. coli* ATCC 25922 was prepared and inoculated onto MHA plate following Modified Kirby-Bauer Disk Diffusion Method. The meropenem disc was removed from TSB suspension after complete four-hour incubation with the help of a 10-µl loop, taking care to remove excess liquid from the disk. The freshly removed meropenem disc was immediately placed on the MHA plate that has been inoculated with *E. coli* ATCC 25922. The plate was incubated at 35 °C in ambient air overnight. The zone of inhibition around the meropenem disc was measured and interpreted. If the zone of inhibition was measured to be 6-15mm, the test organism was Carbapenemase positive, if the zone of inhibition was measured to be greater or equal to 19mm, the test organism was carbapenemase negative. And if the zone of inhibition was measured to be 16-18mm, the test organism was considered intermediate (CLSI 2018).

RESULT

Out of 310 non-repeated clinical samples collected, 72.58% showed significant bacterial growth. Overall 336 isolates were isolated from the culture positive samples of which 134 (59.56%) showed mono-microbial bacterial growth while 91 (40.44%) showed poly-microbial bacterial growth of the cases. The isolation of Gram positive and Gram negative bacteria were 26.49% and 73.51%, respectively.

Among the Gram positive bacteria, *S. aureus* (11.01%) was most commonly isolated followed by CoNS (8.93%), whereas for Gram negative bacteria 13.99% of isolates were *P. aeruginosa* followed by *K. pneumoniae* (12.8%) and *Acinetobacter calcoaceticus-baumannii* complex (10.12%).

Table 1: Gram positive isolates among samples

Gram Positive isolates	Wound Swab	Tissue culture	Pus aspirate	Total
	No. (%)	No. (%)	No. (%)	No. (%)
S. aureus	33 (9.82)	4 (1.19)	0 (0)	37 (11.01)
E. faecalis	11 (3.27)	11 (3.27)	0 (0)	22 (6.55)
CoNS	27 (8.04)	2 (0.60)	1 (0.30)	30 (8.93)
Total	71 (21.13)	17 (5.06)	1 (0.30)	89 (26.49)

Table 2: Gram negative isolates among samples

Gram Negative isolates	Wound Swab	Tissue culture	Pus aspirate	Total
	No. (%)	No. (%)	No. (%)	No. (%)
A. lwoffi	5 (1.49)	7 (2.08)	0 (0)	12 (3.57)
ACB complex	26 (7.74)	7 (2.08)	1 (0.30)	34 (10.12)
E. coli	22 (6.55)	11 (3.27)	0 (0)	33 (9.82)
C. koseri	22 (6.55)	9 (2.68)	0 (0)	31 (9.23)
E. aerogenes	14 (4.17)	5 (1.49)	1 (0.30)	20 (5.95)
E. cloacae	5 (1.49)	2 (0.60)	0 (0)	7 (2.08)
K. oxytoca	2 (0.60)	1 (0.30)	0 (0)	3 (0.89)
K. pneumoniae	30 (8.93)	13 (3.87)	0 (0)	43 (12.8)
P. mirabilis	5 (1.49)	3 (0.89)	0 (0)	8 (2.38)
P. vulgaris	3 (0.89)	1 (0.30)	0 (0)	4 (1.19)
S. marcescens	4 (1.19)	1 (0.30)	0 (0)	5 (1.49)
P. aeruginosa	37 (11.01)	10 (2.98)	0 (0)	47 (13.99)
Total	175 (52.08)	70 (20.83)	2 (0.60)	247 (73.51)

Table 3: Mono-microbial and poly-microbial bacterial growth

Samples	Mono-microbial growth	Poly-microbial growth
	No. (%)	No. (%)
Wound Swab	102 (45.33)	66 (29.33)
Tissue Culture	29 (12.89)	25 (11.11)
Pus aspirate	3 (1.33)	0 (0)
Total	**134 (59.56)**	**91 (40.44)**

P. aeruginosa showed high resistant rate towards doxycycline (91.49%) followed by ciprofloxacin (82.98%) and gentamycin (82.98%). All isolates of the bacteria were susceptible to colistin. Among the 47 *P. aeruginosa* isolates, 82.97% were found to be MDR.

Table 4: Antibiotic susceptibility profile of *P. aeruginosa* (n= 47)

Antibiotics	Sensitive	Resistant
	No. (%)	No. (%)
Amikacin	9 (19.15)	38 (80.85)
Gentamycin	8 (17.02)	39 (82.98)
Ciprofloxacin	8 (17.02)	39 (82.98)
Cefepime	12 (25.53)	35 (74.47)
Piperacillin/ Tazobactam	30 (63.83)	17 (36.17)
Cefoperazone/ Sublactam	12 (25.53)	35 (74.47)
Meropenem	21 (44.68)	26 (55.32)
Imipenem	27 (59.57)	20 (40.43)
Doxycycline	4 (8.51)	43 (91.49)
Ceftazidime	11 (23.4)	36 (76.60)
Colistin	47 (100)	0 (0)

Among the 20 imipenem and meropenem resistant *P. aeruginosa* isolates, 4 (20%) isolates were detected to be carbapenemase producers by modified Carbapem Inhibition Method (mCIM) while 3 (15%) isolates were detected to be MBL producers by using Combined Disk (CD) test.

Both MBL and carbapenemase producing *P. aeruginosa* showed complete resistant towards amikacin, gentamycin, ciprofloxacin, cefepime, doxycycline and ceftazidime, However, MBL producing isolates were 66.67% and 100% resistant for cefoperazone/sublactam and piperacillin/tazobactam respectively whereas that for carbapenemase were 50% and 75%. All the MBL positive isolates were MDR.

Table 5: Antibiogram of carbapenemase, MBL producing and Non-MBL *P. aeruginosa* (n=24)

Antibiotics	Carbapenemase producing	MBL producing	Non- MBL
	Resistant (%)	Resistant (%)	Resistant (%)
Amikacin	4 (100)	3 (100)	17 (94.12)
Gentamycin	4 (100)	3 (100)	17 (100)
Ciprofloxacin	4 (100)	3 (100)	17 (100)
Cefepime	4 (100)	3 (100)	17 (100)
Piperacillin/ Tazobactam	3 (75)	3 (100)	10 (58.82)
Cefoperazone/ Sublactam	2 (50)	2 (66.67)	17 (100)
Doxycycline	4 (100)	3 (100)	17 (100)
Ceftazidime	4 (100)	3 (100)	17 (100)
Colistin	0 (0)	0 (0)	0 (0)

DISCUSSION

Burnt areas are susceptible site for microbial colonization and proliferation within few hours of injury as the trauma and the wound local microenvironment induces immunosuppressant state (Srinivasan et al. 2009; Gonzalez et al. 2016). These organisms may further cause disseminated infection following colonization and it has been estimated that 75% of all deaths in burnt patients were associated with infections. As the etiology of burn wound changes with time, the expanded use of antibiotics leads to the development as well as selection of multidrug resistant (MDR) bacteria which results in treatment failure and intensifies the complications (Srinivasan et al. 2009; Gupta et al. 2019). The Gram negative pathogen *P. aeruginosa* presents the maximum incidence and even becomes predominate among the burn wound pathogens (Gonzalez et al. 2016). Therefore, this study was carried out to investigate the etiology of burn wound with special focus on *P. aeruginosa* and its antibiotic susceptibility pattern. In addition, carbapenem resistant *P. aeruginosa* strains were selected for testing MBL and Carbapenemase production.

In this study, out of 310 samples processed, 225 (72.58%) samples had significant bacterial growth. This culture positivity rate is lower to the other study done which have shown 87.5% and 86.5% growth rate (Rajbahak et al. 2014; Dahag et al. 2018). The lower rate of bacterial isolation in the present study may be due to the differences in the specimen size involved in those studies. Overall, 59.56% and 40.44% samples showed mono-microbial and poly-microbial bacterial growth respectively. This finding is in harmony with the studies by Dahag et al. (2018) and Rajbahek et al. (2014) were mono-microbial (46% and 54.4%) outweighed poly-microbial growth rate (40.5% and 45.6%). However, in a study by Ali et al. (2017), 59.6% of samples were polymicrobial and remaining monomicrobial. The polymicrobial incidence might be because a suitable environment is created by the presence of one microorganism that enables other pathogenic microorganism to colonize the respective niche resulting in the synergistic interaction among pathogens to cause disease (Ali et al. 2017). In addition, several factors of the wound such as formation of excessive devitalised tissue, increased tension in the wound, haematoma and seromas and foreign bodies influence patients to secondary bacterial infections (Bangera et al. 2016).

Gram positive isolates accounted for 26.49% of samples while 73.51% of the isolates were identified as Gram negative, which is similar to Yousefi-Mashouf and Hashemi (2006). The Gram positive organisms which caused burn wound infections in this study were *S. aureus*, CoNS, *Enterococcus* which is similar to other study by Naqvi et al. (2014). The Gram negative isolates in this study were identified to be *A. lwoffi, Acinetobacter calcoaceticus-baumannii* complex, *E. coli, C. koseri, E. aerogenes, E. cloacae, K. oxytoca, K. pneumoniae, P. mirabilis, P. vulgaris, S. marcescens,* and *P. aeruginosa.* Similar etiology is reported by Altoparlak et al. (2004). The incidence of Gram negative isolates was found to be much more than Gram positive isolates in burn wound infections. As the microbial profile of burn wound infection change over time, Gram positive bacteria inhabits the burn wound for first 48 hours of injury and later, Gram-negative bacteria also rapidly colonize the burn wound surface. (Barret and Herndon.2003; Wysocki 2002).

Among the isolates, *P. aeruginosa* was found to be predominate which accounted for 13.99% of total isolates. This result is in concordance with previous

reports (Agnihotri et al. 2004; Gupta et al. 2019), where the same bacteria is most frequently isolated but is in contrast to other studies which report *S. aureus* as predominant organism (Lesseva and Hadjiiski 1996; Komolafe et al. 2003). The reason that *P. aeruginosa* is most commonly identified in the burn wards may be due to the fact that organism thrives in a moist environment (Atoyebiet al. 1992). Furthermore, it is a ubiquitous microorganism and could affect individual with immunocompromised situation and responsible for nosocomial infections (Lanotte et al. 2004).

In our study, the resistant of *P. aeruginosa* towards antibiotics was alarmingly high. Resistant to piperacillin and tazobactum in our study was 36.17% which is in harmony to 38.6% in a study by Sheikh et al. (2014) but in contrast to 27.8% and 19.45% in the studies by Srinivasan et al. (2009) and Saaiq et al. (2015) respectively. In the studies by Agnihotri et al. (2004) and Sheikh et al. (2014), *P. aeruginosa* showed 53.85% and 55.20% resistant towards amikacin while that for our study was 36.17%. Similarly, 76.60% isolates were resistant for ceftazidime which was higher in comparison to other study which showed 63.72% and 66.80% resistant (Agnihotri et al. 2004; Sheikh et al. 2014). In this study resistance to carbapenem antibiotics- imipenem and meropenem were found to be at 59.57% and 44.68% respectively, while Khosravi et al. (2007) reported slightly lower resistance (41%) for both imipenem and meropenem. However, Coetzee et al. (2013) observed resistance of imipenem and meropenem at 90.2% and 93.4% respectively. This undoubtedly exhibits that the drugs that were previously supposed to be effective in literature against *P. aeruginosa* are becoming more resistant (Chaudhary et al. 2019)

In the present study, the most susceptible antibiotics against *P. aeruginosa* was colistin (100%). Similar results were seen in earlier studies where colistin is sensitive upto 100% (Shanthi and Sekar 2009; Viedma et al. 2012). Selective pressure from the use of antimicrobial agents is the major determinant for the emergence of resistance (Mesaros et al. 2007). This outcome suggests that colistin should keep as the reserved drug to treat MDR isolates.

Among the imipenem and meropenem resistant *P. aeruginosa,* 15% showed MBL production by CD test and 20% showed carbapenem production by mCIM test. In contrast, Saderi et al. (2010) reported that 94% of imipenem resistant isolate were positive by imipenem-CD test while Saderi et al. (2008) showed 39.06% of all isolates were MBLs positive by ceftazidime- CD test. In this study, MBL producing *P. aeruginosa* (by CD test) was completely resistant to most of the antibiotics used except cefoperazone/sulbactam which showed 66.67% resistance while all the isolates were susceptible to colistin. This result is co-relates with the result published by Anvarinejad et al. (2014). All the MBL positive isolates were MDR including resistance to antibiotics prescribed as the first line of treatment-cefepime, ciprofloxacin, amikacin, meropenem, imipenem, piperacillin/tazobactam, and gentamicin which co-relates with Mirsalehian et al. (2017).

CONCLUSION

P. aeruginosa still remains the predominant bacteria isolated from burn wound infection. The high proportion of MBL and carbapenemase producers warrants the detection of MBL and carbapenemase in routine laboratory coupled with rational use of antibiotics inorder to limit the spread of such enzymes producing organism.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests

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