Biofilm Producing *Pseudomonas aeruginosa* in Patients with Lower Respiratory Tract Infections

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ABSTRACT

Objectives: To determine the prevalence of Gram-negative bacteria in lower respiratory tract infections and study the biofilm producing *Pseudomonas aeruginosa.*

Methods: This study was conducted at B & B Hospital Pvt. Ltd., Lalitpur, Nepal from February to September 2018. The samples were collected from the patients (n=420) with signs and symptoms suggestive of LRTIs. The isolated organisms were identified and antimicrobial sensitivity was performed. Among all the isolates, *P. aeruginosa* isolates were subjected for biofilm detection by microtitre plate method.

Results: Out of 420 specimens, 90 (21.6%) were culture positive. *Klebsiella pneumoniae* (42.9%) was found to be the predominant organism with higher rate of resistance to antibiotics. A total of 25 isolates of *P. aeruginosa* were isolated among which 15 (60%) were biofilm producers. Biofilm-producing isolates of *P. aeruginosa* were found more resistant to the tested antibiotics.

Conclusion: Gram-negative bacteria were found to be the predominant etiological agents in causing the LRTIs; *K. pneumoniae,* being the most commonly isolated bacteria. Most *P. aeruginosa* were capable of producing the biofilm. The biofilm producers were more resistant to the antibiotics. The biofilm may help increase the resistivity nature of the bacteria.

Key words: LRTI, Gram-negative bacteria, biofilm, antibiotic resistance, MDR

INTRODUCTION

Lower respiratory tract infection is one of the major health problems, being the leading cause of morbidity and mortality in many developing countries (Prajapati and Talsania 2011; Jacobs et al. 2009). The etiology, signs, and symptoms of respiratory infections vary with age, sex, season, immune status of an individual (Mishra et al. 2012). The etiologies of respiratory infections play a significant role in the diagnostic procedure. A variety of organisms are usually involved in LRTIs, Gram-negative bacteria being the most common bacteria (Goel et al. 2009; Kumari et al. 2007). *P. aeruginosa* is a commonly isolated pathogen from LRTIs (Samad et al. 2017). The microbiological investigations play important role in minimizing the complications in LRTIs. The increasing antimicrobial resistance among respiratory pathogens including *P. aeruginosa,* as it adds up the economic burden for the treatment process (Ahmed et al. 2013). *P. aeruginosa* colonizes and produces the biofilm in the respiratory tract of patients with impaired host defense mechanisms (Bentzmann et al. 1996). Biofilm formation is an important mechanism for the survival of *P. aeruginosa* which acts as the protection factor of bacteria against the host immune system and antibiotic therapy, thereby rendering the antibiotic resistance and favoring the chronicity of the infection (Lima et al. 2018).

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METHODS

This study was conducted at B & B Hospital Pvt. Ltd., Gwarko, Lalitpur in collaboration with GoldenGate International College, Kathmandu, Nepal from February to September 2018. A total of 420 non-duplicate respiratory specimens including expectorated sputum, suction tube, tracheostomy tube and pleural fluid from all patients with symptoms of LRTIs were studied. The samples which were not collected and labeled properly and with visible contaminations were excluded. The samples were inoculated onto Mac Conkey Agar (MA), Blood Agar (BA) and Chocolate Agar (CA). The MA and BA have been incubated aerobically at 37°C for 24-48 hours while CA plates were incubated in a candle jar at 37°C for 24-48 hours (Bhatta et al. 2019). The bacterial isolates were identified by standard microbiological procedures including microscopy, colony morphology and biochemical tests as described by the American Society of Microbiology (ASM). Antibiotic Susceptibility tests of the bacterial isolates were performed by Modified Kirby-Bauer Disk Diffusion technique using Mueller Hinton Agar (CLSI 2015). Among all the isolates, *P. aeruginosa* isolates were subjected to biofilm detection by the microtiter plate method.

Detection of biofilm by microtiter plate method

Microtiter plate culture as described by Christensen (Christensen, 1989) was performed to detect biofilm formation by the *P. aeruginosa* isolates. The suspension of *P. aeruginosa* isolates was prepared in Tryptic Soya Broth (TSB) supplemented with 1% glucose. The suspension was diluted at 1:100 with fresh TSB. Then 200 µl of suspension was loaded into wells of 96 well sterile flat-bottom polystyrene micro-titer plate. A set of 3 such microtitre plates were prepared. *P. aeruginosa* strain PA01 and TSB with 1% glucose were used as the positive and negative control respectively. The micro-titer plate with bacterial suspensions was then incubated at 37°C for 24 hours. After incubation, the suspension was removed by gentle tapping and each well was washed with 200 µl of Phosphate Buffer System (pH 7.3) four times. Subsequently, 2% sodium acetate was used for fixation followed by staining with 100µl of 0.1% crystal violet. Excess stain was removed by washing the plate with de-ionized water and dried. The microtiter plate was then rinsed with 0.2 ml of ethanol-acetone (80:20 v/v). The ELISA reader was used to obtain the absorbance at a wavelength of 570 nm. The value of optical densities for each isolate was calculated from the average of three wells. The value was compared to the optical density of the negative control (ODc). The isolates were classified based on mean optical densities (Stepanovic et al. 2000):

OD ≤ ODc (≤0.658): non-biofilm producer

ODc < OD ≤ 2× ODc (>0.658-1.361): weak biofilm producer

2× ODc < OD ≤ 4 × ODc (>1.316-2.632): moderate biofilm producer

4× ODc < OD (>2.632): strong biofilm producer

RESULTS

Culture of samples

A total of 420 specimens including 349 sputum, 31 pleural fluid, 28 suction tip, and 12 tracheostomy specimens were processed. Among them, 90 specimens showed positive culture including 68 (75.65%), 1 (1.19%), 13 (14.26%) and 8 (8.9%) from sputum, pleural fluid, suction tube and tracheostomy tip respectively (Figure 1). The male inpatients showed the highest culture positive (56 ; 62.3%) while female outpatients showed the least culture positive (2; 2.2%).

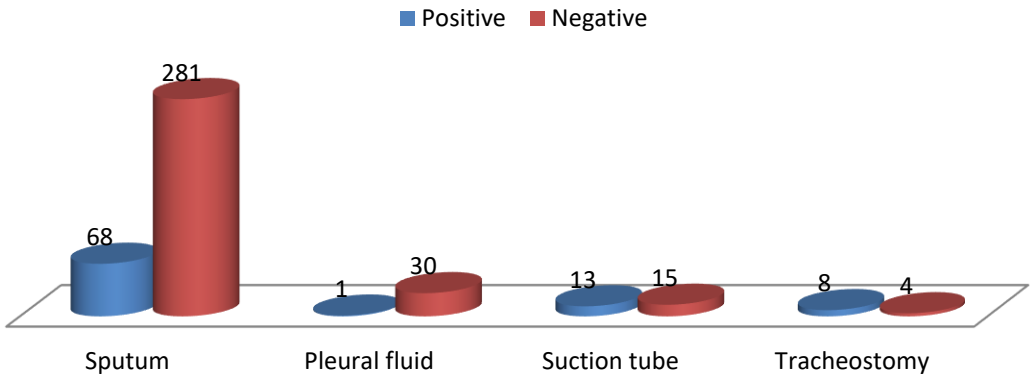

Figure 1: Barchart showing culture positivity in different specimens

Monomicrobial and polymicrobial growth

Out of 90 culture positive samples, a single bacterium was isolated from each of 66 (73.33%) specimens whereas polymicrobial infection was detected in 24 (26.67%) specimens; including 11 (45.83%), 1 (4.16%), 7 (29.17%), and 5 (20.84%) from sputum, pleural fluid, suction tube and tracheostomy tube respectively (Figure 2).

Distribution of different organisms in clinical samples

A total of 114 Gram-negative bacteria were isolated from 90 culture-positive specimens. *K. pneumoniae* was the most predominant bacteria (n=49, 42.9%), followed by *P. aeruginosa* (n=25, 21.9%) (figure 3).

Resistance pattern of Gram-negative respiratory pathogens

The highest number of *K. pneumoniae* was resistant to ceftriaxone (93.8%) followed by cefepime (87.8%). Similarly, the highest number of *Acinetobacter* spp. was resistant to ceftriaxone and cefepime (87%).

The majority *of P. aeruginosa* isolates showed the highest resistance to cefeperazone/sulbactum followed by piperacillin/tazobactum. A total of 11 *P. aeruginosa* isolates was found to be the multidrug resistant (MDR).

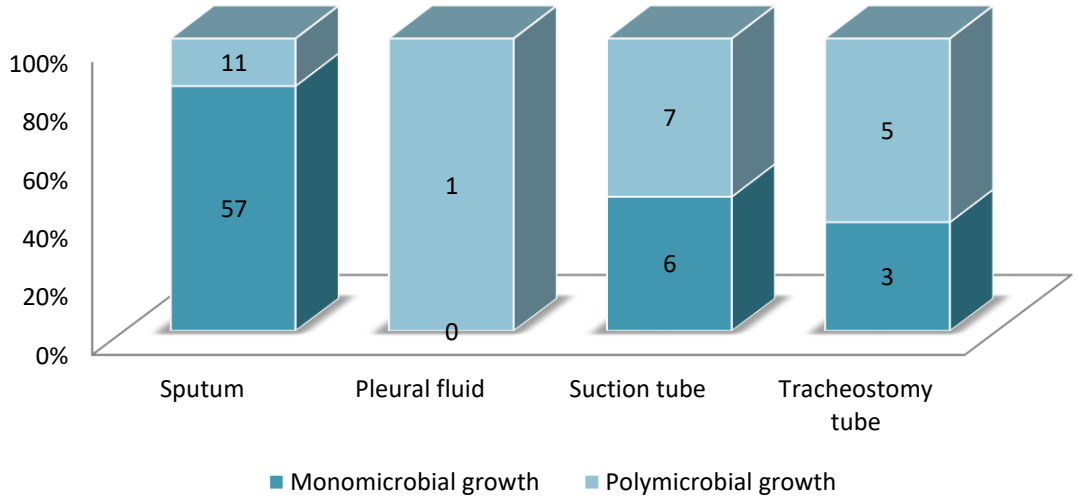

Figure 2: Barchart showing monomicrobial and polymicrobial growth in different specimens

Table 1: Distribution of bacterial isolates

Organism	Number of isolates (%)
K. pneumoniae	49 (42.98)
P. aeruginosa	25 (21.92)
E. coli	18 (15.8)
Acinetobacter spp.	16 (14.03)
K. oxytoca	2 (1.75)
P. vulgaris	1 (0.88)
P. mirabilis	1 (0.88)
C. freundii	1 (0.88)
H. influenzae	1 (0.88)
Total	**114**

Table 2: Resistance pattern of Gram-negative respiratory pathogens

Antibiotics	*K. pneumoniae* (%)	*P. aeruginosa* (%)	*E. coli* (%)	*Acinetobacter* (%)	*K. oxytoca* (%)
Amikacin	73.4	32	55.5	50	50
Gentamicin	71	28	50	50	100
Ciprofloxacin	69.4	28	77.8	31.3	50
Ceftriaxone	93.8	-	83.3	87	100
Ceftazidime	-	28	-	-	-
Cefepime	87.8	48	88.9	87	100
Imipenem	79.5	44	61.7	50	50
Meropenem	75.5	48	50.6	43	50
Piperacillin/Tazobactum	77.5	56	55.5	56	100
Cefeperazone/Sulbactum	75.5	68	61.1	56.	100
Colistin	0	0	0	0	0

Biofilm producers among *Pseudomonas aeruginosa*

Among the 25 *P. aeruginosa* isolates, 15 isolates were found to be a biofilm producer; out of which 2 (13.33%) were strong, 4 (26.67%) were moderate and 9 (60%) were weak biofilm producer. The higher biofilm producers were isolated from sputum specimens (n=11; 73.33%) followed by suction tube (n=3; 20%) and tracheostomy tube (n=1; 6.67%) (Table3). Similarly, 14 isolates from the inpatients and 1 isolate from the outpatient were biofilm producers (Figure 4).

Antibiotic resistance pattern of biofilm producing and non-producing *P. aeruginosa*

The overall antimicrobial resistance patterns of *P. aeruginosa* showed that biofilm producers were more resistant than biofilm non-producers. The highest number of biofilm-producing *P. aeruginosa* was found to be resistant to Cefeperazone/Sulbactum. Similarly, the highest number of biofilm non-producers was found to be resistant to Cefepime.

Biofilm and MDR

Out of 15 biofilm producing *P. aeruginosa,* 8 (53.3%) were MDR while among 10 biofilm negative isolates, 3 (30%) were MDR.

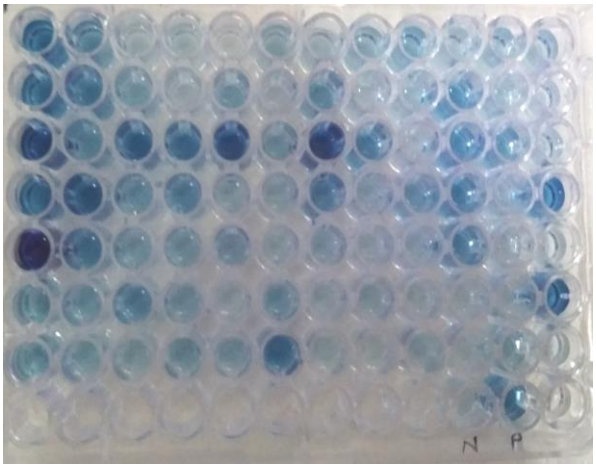

Figure 4: Detection of biofilm by Microtiter Plate method (H_{10}- Negative Control, H_{11}- Positive Control)

Table 3: Biofilm producing *P. aeruginosa*

Specimens	Number of biofilm producer (n=15)			
	Strong (%)	**Moderate (%)**	**Weak (%)**	**Total (%)**
Sputum	2	2	7	11 (73.33)
Suction tip	-	2	1	3 (20)
Tracheostomy tube	-	-	1	1 (6.67)
Total	**2 (13.33)**	**4 (26.67)**	**9 (60)**	**15 (100)**

Table 4: Antibiotic resistance pattern of biofilm producing and non-producing *P. aeruginosa*

Antibiotics	Resistance pattern	
	Biofilm producer (%)	Biofilm non-producer (%)
Amikacin	8 (53.3)	2 (20)
Cefepime	7 (46.7)	6 (60)
Ciprofloxacin	8 (53.3)	5 (50)
Gentamicin	5 (33.3)	2 (20)
Imipenem	6 (40)	3 (30)
Meropenem	7 (46.7)	3 (30)
Piperacillin/tazobactum	6 (40)	4 (40)
Cefeperazone/Sulbactum	10 (66)	5 (50)
Colistin	0	0
Ceftazidime	5 (33.3)	3(30)

DISCUSSION

Gram-negative bacteria were the major pathogens isolated from lower respiratory tract infections and *K. pneumoniae* was the most predominant one. The increased rate of resistance of those pathogens to routinely used antibiotics were obsereved in our study. This situation shows a huge problem in management of LRTIs caused by such bacteria pathogens. In addition, we also reported most of *P. aeruginosa* isolated from the patients with LRTIs to have the ability to produce biofilm. The increased resistance was observed among biofilm producers as compared to biofilm non-producers which might add more challenge in antimicrobial therapy to treat the infections.

The prevalence of LRTI was found to be 21.4% which was similar to the study by Nepal et al. 2018(24.6%). However, the rate of culture positivity was higher in the studies by Mishra et al. 2012 (44.4%) and Ieven et al. 2018 (59%). The prior use of antibiotics, exclusion of viral and other atypical bacteria in this study may have resulted in a lower prevalence rate of LRTIs (Ahmed et al. 2018; Nepal et al. 2018). The suction tube culture showed the highest rate (66.67%) which was supported by the study conducted by Nepal et al. 2018, at Kathmandu Model Hospital. Kathmandu with 100% culture positivity. The culture positivity was relatively higher in the specimens from inpatients (92.3%) than outpatients (6.7%). A similar result was obtained in the study by Khan et al. 2015. The hospitalized patients with long-term stay and who are under medication and steroids are susceptible to LRTIs due to weaker immune status (Guzek et al. 2014). The surgical manipulations, intubations also furnish the suitable environment for opportunistic bacteria to cause LRTIs (Bajpai et al. 2013).

The male patients were more susceptible to LRTIs with 66.7% culture positivity in this study. The result coincided with the study by *Olugbue et al. 2011.* A higher rate of LRTIs in the male may be attributed to a high incidence of smoking and alcohol consumption (Ziyade et al. 2010). Poly-microbial infection was observed in 26.7% of the specimen. The studies by Mishra et al. 2012, Khan et al. 2015 and Nepal et al 2018 also revealed a lower rate of poly-microbial infection i.e. 9%, 20% and 15.36% respectively. The identification of the polymicrobial infection is very important for treatment strategies since the polymicrobial infection mightn't be managed with an antibiotic.

Among the heterogeneous bacterial etiological agents of LRTIs, member of the Enterobacteriaceae family remains the predominant pathogens. All the isolated bacteria were Gram-negative. *K. pneumoniae* (42.9%) was the most predominant organism followed by *P. aeruginosa* (21.9%) which corresponds to the study by Nepal et al. 2018. Similar results were present in other studies as well (Ahmed et al. 2018; Okesola and Oni 2009). The higher prevalence of *K. pneumoniae* may be due to their ubiquitous presence and their ability to cause nosocomial infections (Paczosa et al. 2016). Among the isolated *P. aeruginosa*, 44% were found to be MDR. Vishwanath et al. 2013 reported 5.7% MDR *P. aeruginosa*. However, Goel *et al*. 2009 found higher rates of MDR *P. aeruginosa* which may be due to the inclusion of specimens from the Intensive Care Unit. The spread of antimicrobial resistance genes among the clinical pathogens, irrational use of antimicrobials, etc. contribute to the development of MDR nature in bacteria (Ahmed et al. 2013)

The increasing rate of MDR among different bacterial pathogens are of great concern since the infections caused by those pathogens might have longer hospital stay with higher morbidity and mortality.

In this study, 60% of *P. aeruginosa* were reported to be biofilm producers which were similar to the study conducted by Yekani et al. 2017. Lima et al. 2018 reported 77 *P. aeruginosa* as the biofilm producer. Among the biofilm producers, the highest producers were isolated from sputum. Similarly, the inpatients harbored the maximum biofilm producers which may be due to the increasing use of invasive diagnostic procedures and patients' association with indwelling devices (Dash et al. 2013). The biofilm-producing *P. aeruginosa* showed greater resistance to cefeperazone/ sulbactum (66%). Other study by Saha et al. 2018 showed the different antibiotic-resistant patterns of biofilm-producing *P. aeruginosa*. The biofilm producers render the inefficient antibiotics treatment thereby promoting chronic infectious diseases (Saha et al. 2018; Sanchez et al. 2013; Alves et al. 2014; Rao et al. 2008). Since biofilm production not only contribute the pathogens to adopt in the different environmental niche but also help them to resist towards many antimicrobial agents. Most of hospital equipments were colonized with such pathogens. Therefore, it is equally important to routinely screen the bacterial pathogens for biofilm production with their antibiogram patterns.

This study couldn't observe biofilm production and compare with antibiogram pattern of all bacteria isolates from LRTIs. As a main objective, we just focus on *P. aeruginosa*. Moreover, due to limited time and budget, the MIC of all antimicrobial agents to *P. aeruginosa* couldn't be performed. The overall results were based on disc diffusion method.

CONCLUSION

This study reveals the predominance of Gram-negative bacteria in LRTIs and their increased rate of resistance to routinely used antibiotics. *K. pneumoniae* was found to be the most predominant organism in LRTI. The study also reported most of *P. aeruginosa* isolated from the patients with LRTIs to have the ability to produce biofilm. The increased resistance was observed among biofilm producers as compared to biofilm non-producers.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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