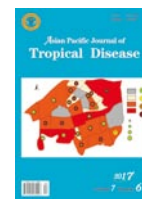


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Biofilm formation and antimicrobial resistance in *Klebsiella pneumoniae* isolated from patients visiting a tertiary care center of Nepal

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ABSTRACT

Objective: To evaluate the biofilm forming ability of *Klebsiella pneumoniae* (*K. pneumoniae*) isolates recovered from patients visiting a tertiary care center of Nepal and to determine their antimicrobial resistance with special reference to extended spectrum β -lactamase (ESBL) production.

Methods: A total 60 phenotypically identified clinical isolates were included in this study. The production of biofilm was detected by tissue culture plate method and their antimicrobial susceptibility was determined by standard microbiological methods.

Results: Among the total isolates, 44 (73.3%) isolates were found to be biofilm producers. The biofilm positive strains exhibited higher rate of antimicrobial resistance compared to the biofilm negative strains. Multi-drug resistance was seen in 39/60 (65%) isolates of which 36 (92.3%) were biofilm producers and 3 (7.7%) were biofilm non-producers. Similarly, ESBL production was detected in 26/60 (43.3%) isolates. Among ESBL producers, 24 (92.3%) were biofilm producers and the rest 2 (7.7%) were non-producers.

Conclusions: Biofilm formation in clinical isolates of *K. pneumoniae* is very high with varying degrees of resistance to all commonly used antimicrobial agents. The biofilm positive strains are more resistant to antimicrobial agents than the biofilm negative strains. Therefore, it is recommended to identify biofilm producing *K. pneumoniae* for the effective use of antimicrobial agent.

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is a Gram-negative, encapsulated, ubiquitous opportunistic pathogen of the family Enterobacteriaceae. The bacterium is implicated in a spectrum of community-acquired and nosocomial infections such as pneumonia, meningitis, sepsis, urinary tract infection, device-associated and

surgical wound site infections[1]. In the past, it was reported to cause serious infections primarily in immunocompromised individuals, but due to emergence and spread of virulent strains in the recent years, both the healthy and immunodeficient individuals are susceptible to its infections[2]. Every year millions of people around the world are affected by these bacteria with a high mortality rate[3].

K. pneumoniae produces a number of virulence factors that contribute to pathogenesis, including a thick polysaccharide capsule, fimbrial and non-fimbrial adhesins and siderophores[4]. Moreover, pathogenicity and chronicity of *K. pneumoniae* infections are increased by its biofilm forming ability[5]. The biofilm is an assembly of microbial cells that are irreversibly associated with a surface and enclosed in an exopolysaccharide matrix[6]. Bacteria growing in the biofilms are phenotypically distinct from planktonic organisms, including increased resistance to host immune defenses and to antimicrobial compounds[6]. The molecular nature of this resistance has not been fully elucidated. However, the resistance could be due to the slowly growing state of the cells in the deeper

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#These authors contributed equally to this work.

layers of thick biofilms, which have less access to antibiotics and nutrients, and to the impaired diffusion of antimicrobial molecules within the biofilms[6]. There is increased horizontal gene transmission in biofilms, with high plasmid transfer rates, which aggravates the problem of resistance[7].

The emergence of antibiotic-resistant strains of *K. pneumoniae* has become a cause of concern because extended-spectrum β -lactamases (ESBL) and carbapenemase producing strains have been isolated frequently from most of the part of the world[8]. ESBLs are plasmid-mediated enzymes that are responsible for resistance to all penicillins and cephalosporins, including the sulbactam and clavulanic acid combinations and monobactams such as aztreonam[9]. ESBL production is frequently associated with multi-drug resistance to antibiotics limiting the therapeutic options. Therefore, knowledge regarding the biofilm formation and antimicrobial resistance is very crucial for proper management of *K. pneumoniae* related infections.

In this study, we aimed to evaluate the biofilm forming abilities of the clinical isolates of *K. pneumoniae* and determine their antimicrobial resistance with special reference to ESBL production.

2. Materials and methods

A cross-sectional study was conducted in the Department of Microbiology, Chitwan Medical College and Teaching Hospital (a 600-bed tertiary care hospital) located in Central Nepal. A total of 60 randomly selected, phenotypically identified (as per the standard microbiological methods) clinical isolates of *K. pneumoniae* recovered from both out-patient departments (OPDs) and in-patient departments (IPDs) were included in this study. These isolates, obtained from sputum, pus, urine and endotracheal (ET) tube were subjected for the detection of biofilm forming abilities and antimicrobial susceptibility.

2.1. Biofilm detection by tissue culture plate method

Biofilm detection by tissue culture plate method was done as described by Christensen *et al.*[10]. Briefly, a single colony of *K. pneumoniae* was grown overnight at 37 °C in 2 mL of trypticase soy broth. The bacterial culture was then diluted (1:100) with sterile fresh medium. Each well of a 96-well flat-bottomed polystyrene tissue culture plate (3 wells for each strain) was filled with 200 μ L of the diluted culture. Sterile broth was used as a negative control. The plate was incubated for 24 h at 37 °C. The content of each well was discarded carefully and washed gently with phosphate buffer saline in order to remove free floating bacteria. Adherent bacteria were fixed with 99% methanol for 10–15 min. The plates were decanted, allowed to dry and stained for 10 min with 0.1% crystal violet (CV). Excess stain was rinsed off by washing with tap water. Optical density (OD) of stained adherent biofilm was measured by using a micro-ELISA reader (Human) at a wavelength of 570 nm. *K. pneumoniae* 700603 was used as a positive control strain. The interpretation of biofilm production was done as per the criteria of Stepanovic *et al.*[11].

2.2. Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed by the standard disk diffusion technique (modified Kirby-Bauer method) and interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines[12]. *K. pneumoniae* 700603 was used as a control strain.

The isolates were considered multi-drug resistant (MDR) isolates when they showed non-susceptibility to at least one agent in three or more antimicrobial categories based on the guidelines recommended by joint initiative of the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC)[13].

2.3. Detection of ESBL production

2.3.1. Screening by standard disk diffusion method

Screening of ESBL production was done by standard disk diffusion method according to CLSI guidelines using two disks, ceftazidime (30 μ g) and cefotaxime (30 μ g)[12]. An inhibition zone of \leq 22 mm for ceftazidime and \leq 27 mm for cefotaxime indicated a probable ESBL producing strain which was further confirmed by the phenotypic confirmatory tests.

2.3.2. Confirmation by phenotypic tests

The confirmation of ESBL production was done by two methods, namely combined disk method and double disk synergy test.

2.3.2.1. Combined disk method

In this method, confirmation was done following the CLSI guidelines using both ceftazidime (30 μ g) disks and ceftazidime with clavulanate disks[12].

Test organism was inoculated on Mueller–Hinton agar (MHA) plate by lawn culture technique. Antibiotics were brought to room temperature before use. Ceftazidime and ceftazidime with clavulanate disks, each 15–20 mm apart, were placed on the inoculated MHA plates and incubated overnight at 37 °C. Zone of inhibition was measured and zone diameter more than 5 mm in ceftazidime with clavulanate than that in ceftazidime disk alone was considered to be phenotypically confirmed ESBL production.

2.3.2.2. Double disk synergy test

ESBL production was phenotypically further confirmed by double disk synergy test as described by Jarlier *et al.*[14]. Test inoculum was spread by lawn culture on MHA. Antibiotic disks of amoxicillin-clavulanate (20/10 μ g) (augmentin) was placed at the center and three different antibiotics including cefotaxime, ceftazidime and aztreonam were placed at distance 15–20 mm (center to center). The plates were incubated overnight at 37 °C. Distance was maintained properly in order to accurately detect the synergy. Any distortion or increase in the zone of inhibition of three antibiotic disks towards the augmentin disk was considered as positive for the ESBL production. *K. pneumoniae* 700603 was used as a control strain for a positive ESBL production and *Escherichia coli* 25922 was used as a negative control for the ESBL production.

2.4. Statistical analysis

SPSS software (SPSS Inc no. 17) was used for data analysis. Chi-square (χ^2) test was used for analysis of categorical data. A *P*-value of $<$ 0.05 was considered statistically significant.

2.5. Ethical consideration

This study was performed according to the Helsinki Declaration and approved by the Institutional Review Committee (IRC) of CMCTH, Bharatpur, Chitwan, Nepal (Ref No. CMC-IRC-64). Informed written consent was obtained from each of the study subjects.

3. Results

3.1. Biofilm forming abilities of the isolates

Among 60 isolates of *K. pneumoniae*, 44 (73.3%) isolates were biofilm producers. Out of 29 isolates from IPDs and 31 isolates from OPDs, 24 (82.7%) and 20 (64.5%) were biofilm producers, respectively (Table 1).

Table 1

Detection of biofilm formation by TCP method [n (%)].

Type of isolates	No. (%) of isolates		Total
	From IPDs	From OPDs	
BPs	24 (82.7)	20 (64.5)	44 (73.3)
BNPs	5 (17.3)	11 (35.5)	16 (26.7)
Total	29 (48.3)	31 (51.7)	60 (100)

BPs: Biofilm producers; BNPs: Biofilm non-producers.

3.2. Distribution of biofilm forming isolates in various specimens

The highest number of isolates was recovered from urine samples of which 71.7% produced biofilm. In addition, 3/3 isolates (100%) obtained from ET tubes, 5/6 isolates (83.3%) from pus and 8/12 isolates (66.6%) from sputum were also biofilm positive (Table 2).

Table 2

Distribution of biofilm producing isolates in various specimens (n = 44).

Collection sites	No. (%) of the biofilm producing isolates				Total
	Pus	Sputum	Urine	ET tubes	
IPDs	3/3 (100.0)	4/5 (80.0)	14/18 (77.8)	3/3 (100)	24 (54.5)
OPDs	2/3 (66.7)	4/7 (57.1)	14/21 (66.7)	0 (0)	20 (45.5)
Total	5/6 (83.3)	8/12 (66.6)	28/39 (71.7)	3/3 (100)	44 (100.0)

3.3. Antimicrobial susceptibility test

K. pneumoniae showed varying degrees of resistance against all the antimicrobials tested. The biofilm forming isolates exhibited significantly higher rate of resistance compared to biofilm non-forming isolates against 10/21 (47.6%) antimicrobials tested ($P < 0.05$) (Table 3).

Table 3

Antimicrobial resistance pattern of *K. pneumoniae*.

Antimicrobials	Total isolates (n = 60)	Resistance pattern of BPs vs. BNPs		P value
		Resistant BPs No. (%) (n = 44)	Resistant BNPs No. (%) (n = 16)	
		I. Aminoglycosides		
Amikacin (30 µg)	5 (8.3)	4 (9.0)	1 (6.2)	0.725
Gentamicin (10 µg)	8 (13.3)	7 (15.9)	1 (6.2)	0.330
Netillin (30 µg)	5 (8.3)	4 (9.0)	1 (6.2)	0.725
Tobramicin (10 µg)	5 (8.3)	4 (9.0)	1 (6.2)	0.725
II. Anti-pseudomonal penicillins with β lactamase inhibitors				
Piperacillin-tazobactam (100/10 µg)	18 (30.0)	16 (36.3)	2 (12.5)	0.074
Amoxycillin-clavulanic acid (75/10 µg)	39 (65.0)	36 (81.8)	3 (18.7)	0.000*
III. Carbapenems				
Meropenem (10 µg)	9 (15.0)	7 (15.9)	2 (12.5)	0.744
Imipenem (10 µg)	2 (3.3)	2 (4.5)	0 (0.0)	0.386
Ertapenem (10 µg)	1 (1.6)	1 (2.2)	0 (0.0)	0.543
IV. Cepheems				
Cefoxitin (30 µg)	41 (68.3)	35 (79.5)	6 (37.5)	0.002*
Cefazolin (30 µg)	41 (68.3)	34 (77.2)	7 (43.7)	0.014*
V. Extended spectrum cephalosporins				
Cefotaxime (30 µg)	32 (53.3)	29 (65.9)	3 (18.7)	0.001*
Ceftazidime (30 µg)	35 (58.3)	32 (72.7)	3 (18.7)	0.000*
Cefepime (30 µg)	31 (51.6)	28 (63.6)	3 (18.7)	0.002*

(continued on next column)

Table 3 (continued)

Antimicrobials	Total isolates (n = 60)	Resistance pattern of BPs vs. BNPs		P value
		Resistant BPs No. (%) (n = 44)	Resistant BNPs No. (%) (n = 16)	
		VI. Fluoroquinolones		
Ciprofloxacin (5 µg)	9 (15.0)	7 (15.9)	2 (12.5)	0.744
VII. Folate pathway inhibitor				
Cotrimoxazole (25 µg)	21 (35.0)	20 (45.4)	1 (6.2)	0.005*
VIII. Monobactam				
Aztreonam (30 µg)	37 (61.6)	33 (75.0)	4 (25)	0.001*
IX. Penicillin with β lactamase inhibitor				
Ampicillin/sulbactam (10/10 µg)	24 (40.0)	22 (50.0)	2 (12.5)	0.009*
X. Polymyxin B (300 units)	2 (3.3)	2 (4.5)	0 (0.0)	0.386
XI. Tetracycline				
Tetracycline (30 µg)	22 (36.6)	20 (45.4)	2 (12.5)	0.019*
XII. Phenol				
Chloramphenicol (30 µg)	8 (13.3)	8 (18.1)	0 (0.0)	0.067

*: P values are significant (< 0.05).

Among the total isolates, 39 (65.0%) were identified as MDR isolates of which 36 (92.3%) were biofilm producers and 3 (7.7%) were biofilm non-producers. Similarly, 26 (43.3%) isolates were found to be ESBL producers, of which 24 (92.3%) were biofilm producers and remaining 2 (7.7%) were non-producers.

4. Discussion

K. pneumoniae with the ability to adhere, multiply and persist on inanimate surfaces in the hospital environment has been reported as a significant cause of severe nosocomial infections. Biofilm is the most vital virulence factor in the pathogenesis of this organism[15]. In the present study, we have demonstrated, for the first time, the prevalence of biofilm formation by *K. pneumoniae* isolated from OPDs and IPDs of tertiary care center of Nepal and their correlation with the antimicrobial resistance pattern.

We found that 73.3% of *K. pneumoniae* strains produced biofilm. Our result was similar to the finding of Mishra *et al.*[16] who elucidated that 75% of *K. pneumoniae* isolated from various indwelling medical devices formed biofilm. Some previous reports have shown variable data of biofilm such as 93.6% by Seifi *et al.*[15]and 60.3% by Kaur *et al.*[17]. This variation may be due to differences in geographical area, types of specimens, sample sizes, techniques used for detection, *etc.*

In our study, the isolates from IPDs showed higher rate of biofilm (82.7%) formation compared to the ones from OPDs (64.5%). This may be attributed by the use of medical devices among hospitalized patients. Medical implants and catheters are particularly susceptible to biofilm formation by *K. pneumoniae* because immune responses are significantly reduced in proximity to foreign bodies[16]. In the present study, all three isolates originated from medical devices (ET) tubes were found to be the biofilm producers. Though, it is difficult to derive conclusion only with these three isolates, our finding is in an agreement with the report of Singhai *et al.*[18] who noticed that a high rate of *K. pneumoniae* strains isolated from ET tubes of patients affected by ventilator-associated pneumonia produced biofilm. However, biofilm formation in our study was also observed in high rate in the isolates obtained from other specimens: 83.3%, 71.7% and 66.6% of isolates from pus, urine and sputum, respectively. Our observations were close to those of Seifi *et al.*[15] who also reported that *K. pneumoniae* isolated from urine, sputum, blood and wound swabs were able to produce biofilm. Detection of biofilm formation even in the isolates originated from OPDs in the present study

indicates that *K. pneumoniae* has the capacity to form biofilm not only on the medical devices used in the hospitalized patients but also directly on the host tissues.

The treatment of *K. pneumoniae* infection has been challenged by the emergence of antimicrobial resistance[19]. The resistance rate may be further increased when the organism forms the biofilm[20]. Biofilm-forming bacteria can combat antibiotic concentration 10–10000 folds higher than the essential concentration needed to hinder the growth of free-floating bacteria and can hamper antimicrobial therapy[6]. *K. pneumoniae* exhibited varying degrees of resistance against all the antimicrobials tested in the current study. Highest resistance was seen for cepheims (68.3%) followed by amoxicillin-clavulanic acid (65.0%) whereas the least resistance was observed for ertapenem (1.6%) followed by imipenem (3.3%) and polymyxin B (3.3%). The findings indicate the effectiveness and clinical utility of latter three antimicrobials agents in *K. pneumoniae* infections. Another interesting finding of this study is that the resistance shown by biofilm positive isolates against all the antimicrobials was higher compared to biofilm negative isolates. The result was significant ($P < 0.05$) for 47.6% of the antimicrobials tested: amoxicillin-clavulanic acid, both cepheims (cefotaxime and cefazolin), all extended spectrum cephalosporins (cefotaxime, ceftazidime and cefepime), cotrimoxazole, aztreonam, ampicillin/sulbactam and tetracycline. All the biofilm negative strains were sensitive to imipenem, ertapenem, polymyxin B, and chloramphenicol whereas biofilm positive strains exhibited resistance of 4.5%, 2.2%, 4.5%, and 18.1%, respectively against them. The higher incidence of antibiotic resistance in biofilm producers than in the biofilm non-producers was also reported by Mishra *et al.*[16] and Naparstek *et al.*[21]. Our observation also concurs with a finding of a prospective analysis that has revealed higher resistance rates against nalidixic acid, ampicillin, cefotaxime and co-trimoxazole among the biofilm positive isolates compared to biofilm negative isolates[22].

The correlation between biofilm formation and multi-drug resistance has not been clearly defined. However, the presence of large number of extra cellular DNA and also the large number of immobile distinct bacterial cells in close contact is believed to favor exchange of some anti-microbial resistance genes resulting in the development of MDR phenotypes[7,23]. The present study revealed that 65.0% of the total isolates of *K. pneumoniae* were MDR phenotypes of which 92.3% were biofilm producers. Our results are close to the observation of Sahal *et al.*[24] who found that 80% MDR *K. pneumoniae* were strong biofilm producers. Sanchez *et al.*[25] also elucidated that MDR *K. pneumoniae* strains formed very rich biofilm.

Beta-lactam group of drugs have been the choice for the treatment of *Klebsiella* infections because of the presence of aminoglycoside-modifying enzymes, macrolide esterases, and efflux systems that render many other drug classes ineffective. However, the use of beta-lactams has become difficult in the recent years as various classes of beta-lactamases have been detected in clinical isolates of *Klebsiella*[26]. One of these enzymes is the ESBL which hydrolyzes oxyimino beta-lactam agents such as third-generation cephalosporins and aztreonam[9]. The plasmids coding ESBLs also contain resistance genes to other antibiotics including aminoglycosides, chloramphenicol, sulfonamides, trimethoprim, and tetracycline. Thus, Gram-negative bacilli which carry these plasmids are also found to be MDR[9].

The data of ESBL producing *K. pneumoniae* in Nepal ranges from 18.4%–30.0%[27,28]. Our study showed higher percentage of ESBL (43.3%) than the previous studies from Nepal, however, was similar to data of another study from South East Asia (46.7%)[29]. Our

result was lower than that found in India which showed 67.04% of ESBL producing *Klebsiella* spp.[30]. Epidemiological studies have highlighted that the increasingly widespread use of third-generation cephalosporins is a major risk factor contributing to the emergence of ESBL producing *K. pneumoniae*[31-33]. Several additional risk factors for an infection with ESBL-producing organisms include arterial and central venous catheterization, mechanical ventilation, prolonged length of stay in an intensive-care unit and prior antibiotic use[9].

Management of ESBL producing *K. pneumoniae* infection becomes difficult due to limitation of treatment options. However, carbapenems (imipenem, meropenem, ertapenem, doripenem) are stable drugs even in the presence of ESBL enzymes and also easily passes through porins into the Gram-negative bacilli. Therefore, this feature allows carbapenem to be the first choice of the treatment for serious infections with ESBL producing *K. pneumoniae*[34].

It has been reported that > 98% of the ESBL producing *K. pneumoniae* are still susceptible to these drugs[18]. Tigecycline is another drug that can be considered for the treatment of infections caused by these organisms[35].

ESBL producing *K. pneumoniae* isolates have also a greater ability to form biofilm[18]. The present study revealed that 92.3% of ESBL producing strains were biofilm positive whereas only 58.8% of ESBL non-producing strains were identified as biofilm positive. Our findings correlate with the results of Subramanian *et al.*[36] who also observed that 94.4% of ESBL strains of *K. pneumoniae* produced biofilm. This shows that ESBL producing *K. pneumoniae* are associated with the biofilm formation, indicating that biofilm provides protective environment for the survival of the organisms and spread of the resistance genes. Therefore, proper management of biofilm is essential to control the infection related to ESBL production.

In conclusion, the clinical isolates of *K. pneumoniae* have a high degree of biofilm forming abilities and exhibit varying degrees of resistance to all commonly used antimicrobials. The biofilm positive strains are more resistant to the antimicrobials than the biofilm negative strains. Therefore, it is recommended to identify the biofilm producers in regular clinical practice for the effective use of antimicrobial agents to control *K. pneumoniae* related infections.

Conflict of interest statement

We declare that we have no conflict of interest.

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