Antibiogram and Biofilm Development among *Klebsiella pneumoniae* from Clinical Isolates

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ABSTRACT

Objectives: This study was aimed to evaluate antibiotic resistance pattern and biofilm formation in *K. pneumoniae* strains isolated from different clinical specimens and to study on association of drug resistance pattern with biofilm formation.

Methods: A total of 944 clinical samples from patients attending Sahid Gangalal National Heart Center were processed from September 2019 to March 2020 to identify possible bacterial pathogens following standard microbiological procedures. *K. pneumonaie* isolates were further subjected to antibiotic susceptibility testing using modified Kirby Bauer disc diffusion technique. Biofilm formation was evaluated by tissue culture plate technique.

Results: Of the total 944 samples, 146 (15.47%) samples showed bacterial growth, among which 35 (23.97%) were *K. pneumoniae*. Out of 35 *K. pneumoniae* isolates, 16 (45.71%) were multidrug-resistant and 15 (42.86%) were extensively drug-resistant. Twenty-one (60%) *K. pneumoniae* feebly produced biofilm. Significant association was observed between biofilm production and exhibition of multidrug resistance ($p < 0.05$).

Conclusion: Prevalence of antibiotics resistant *K. pneumoniae*in hospital setting is high and alarming. Significant association between drug resistance pattern and biofilm production implicates need of an immediate response to limit growth and spread of drug resistant microbes in clinical settings.

Keywords: *Kleibsella pneumoniae*, multidrug resistance, biofilm, antibiotic susceptibility test, Nepal

INTRODUCTION

Antimicrobials are substances or drugs such as antibacterial, antivirals, antifungals and antiparasitics used to prevent or treat infections caused by microorganisms. Antimicrobial resistance (AMR) occurs when microorganism undergo the alteration in their genetic constitution and resist the antimicrobial agents making the treatments ineffective (Adegbite et al., 2022). AMR has become a matter of global concern. All the countries across globe have been affected by antimicrobial resistance but the burden is higher in low-income and middle-income countries. Every year 7,00,000 people lose their life because of antimicrobial resistance and it is estimated that if no any prompt actions are taken against it, by 2050, AMR will cause loss of life of 10 million peoples and $UD 100 trillion (Pokharel et al., 2019). WHO has declared AMR as one of the top 10 global public health threats to humanity (WHO 2021).

AMR problem is more frequent in a developing country like Nepal where indiscriminate, inadequate and inappropriate use of antimicrobials and self-medication are quite common. Studies have reported high burden of drug resistant/ bacteria in Nepal (Gurung et al., 2020; Raut et al., 2020).

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Klebsiella pneumonia is a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It is an important nosocomial pathogen involved in urinary tract infections, hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), surgical-wound infection, bacteremia, and septicemia (Li et al., 2014).The most important virulence factors contributing to ***K. pneumoniae*** pathogenesis are capsular polysaccharides, type 1 and type 3 pili, which can contribute to biofilm formation (Lie et al., 2014: Murphy and Clegg 2012 and Podschun and Ullmann 1998).These bacteria can produce a thick layer of extracellular biofilm as a virulence factor that helps the organism in attaching to living or abiotic surfaces, preventing the effects of antimicrobial agents (Vutto et al., 2019). The first biofilm-forming ***K. pneumoniae*** strain was described at the end of the 1988s (Le Chevallier et al., 1998).

WHO has listed *K. pneumoniae* in critical category, which includes multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes, and among patients, whose care requires devices such as ventilators and blood catheters (WHO 2017).

MATERIALS AND METHODS

Study design and sites

This prospective cross sectional study was conducted from September 2019 to March 2020. All the samples were received and processed in Microbiology Department of Sahid Gangalal National Heart Center, (SGNHC), Bansbari, Kathmandu while the biofilm detection was done at Med-Micro Research Laboratory, (MMRL), Babarmahal, Kathmandu. Patient who provided their socio-demographic information and expressed their interest to be a part of study by providing samples were included in the study. Only properly collected and well labeled samples were included in study. A total of 944 samples were collected and processed for the study.

Isolation and identification

The collected specimens were cultured simultaneously on Mac Conkey agar (MA), Blood agar (BA) and Michrom UTI agar by quadrant streaking method and incubating them at 37°C for 24 hours as per standard protocol (Cheesebrough 2006). The identification of growth of bacterial colonies from respective media was carried out on the basis of conventional microbiological procedures which includes colony characteristics, gram staining, standard biochemical tests. *K. pneumoniae* isolates obtained from the clinical specimens were considered for further study.

Antibiotic susceptibility testing

Antibiotic susceptibility test (AST) was performed in-vitro using modified Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA) as per CLSI guidelines (2019).

Following antibiotic discs (Hi Media Laboratories, Pvt. Limited, India) were used: Ampicillin (AMP, 10 mcg), Piperacillin/tazobactum (PIT, 100/10 mcg), Ampicillin/sulbactum (AMS, 10/10 mcg), Ciprofloxacin (CIP, 5 mcg) Norfloxacin (NX, 10mcg), Cefalexin (CN, 30 mcg), Gentamycin (GEN, 10 mcg), Amikacin (AK, 30 mcg), Imipenem (IMP, 10mcg), Meropenem (10 mcg), Amoxycillin/clavulanic acid (AMC, 20/10 mcg), Cefotaxim (CTX, 30mcg), Cefepime (CPM, 30mcg), Nalixidic acid (NA, 30mcg), Nitrofurantoin (300 mcg), Cotrimoxazole (COT, 25mcg), Ceftazidime (CTZ, 30 mcg), Ceftriaxone (CTR, 30 mcg), Polymyxin-B (PB, 300 units) and Colistin (CL 10mcg). Results were interpreted on the basis of CLSI guidelines (2019).

MDR and XDR classification

Isolates showing non-susceptibility (either resistant or intermediate) to at least one agent in three or more antimicrobial categories were identified as multi drug resistant (MDR) and those isolates which acquired non-susceptibility to ≥3 classes of antibiotics of antimicrobial class were identified as XDR (Magiorakos et al. 2012). Confirmed *K. pneumoniae* isolates were preserved by using 20% glycerol in TSB for further analysis.

Detection of biofilm formation

Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth (TSB) with 1% glucose. Broths were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh TSB medium. Individual wells of sterile 96 well flat bottom polystyrene cell culture plates were filled with 200 µL of the diluted cultures. The plates were incubated at 37°C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess attain was removed by using deionized water and plates were kept for drying. Optica; density (OD) of stained adherent biofilm was obtained by using ELISA reader (Biorad, UK) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times.

Purity plate

The purity plate was used to make sure that the inoculums used for biochemical tests was pure culture or not. Thus, while performing biochemical tests; the same inoculums were sub-cultured on one half of NA before inoculation (pre purity) and other half ot that NA after inoculation (post purity). The maintenance of aseptic condition is indicated by the growth of same organism in pure form in both pre and post purity halves of the NA medium.

Data analysis

Data analysis was done using computer based software program Statistical Package for the Social Package version 21.0 and the p-value was calculated using Chi-square test.

RESULTS

All-together 944 non-duplicative samples were processed, in which bacterial growth was observed only in 146(16%) samples, while 798 (84%) were culture negative. Out of 146 isolates, *K pneumoniae*were predominant isolates with highest frequency 35 (23.97%) followed by *E coli* 33 (22.60%), *Staphylococcus aureus* 28 (19.17%), *Acinetobacter*spp 19 (13.01%), *CONS* 10 (6.8%), *Serratia*spp 7 (4.7%), *Pseudomonas aeruginosa* 6 (4.1%), *Citrobacterfreundii* 2 (1.36%), *Proteus mirabilis* 2 (1.36%), *Streptococcus pneumonia* 2 (1.36%), *Providencia* spp 1 (0.68%), *Enterobacter*spp 1 (0.68%) (Table 1).

Out of 35 *K. pneumoniae* isolates maximum number of isolates were isolated from urine samples 18 (51.43%), followed by sputum 7 (20%), pus/wound 4 (11.43%), CVP tips 3 (8.57%), blood 2 (5.71%), pleural fluid 1 (2.86%) (Figure 1).

Antibiotic susceptibility testing revealed that all the isolates i.e. n=35 were found to be sensitive to colistin (100%). Other antibiotics to which majority of bacterial isolates were sensitive to polymyxin B 63 (89%) followed by meropenem 21 (60%).All these isolates were resistant to ampicillin (100%). Besides this, only 9% of isolates were found to be sensitive towards ciprofloxacin, cephalexin (Table 2).

Out of 35(100%) isolates, 15 (43%) were found to be XDR, 16(46%) were found to be MDR, and 4 (11%) were found to be susceptible isolates. Detection of biofilm was carried out by Tissue culture method, in which 60% (n=21) *K. pneumoniae* were found to be weak biofilm producers and 40% (n=14) were found to be non-biofilm producers (Figure 2).

Analyzing antibiotic resistance pattern of all 35 isolates of *K. pneumoniae* 15(43%) of them were found to be XDR, 16(46) of them were MDR and 4(11%) were susceptible isolates against antibiotics. Statistical association (chi square) calculated between MDR and Biofilm producers was also found significant ($p<0.01$) (Table 3).

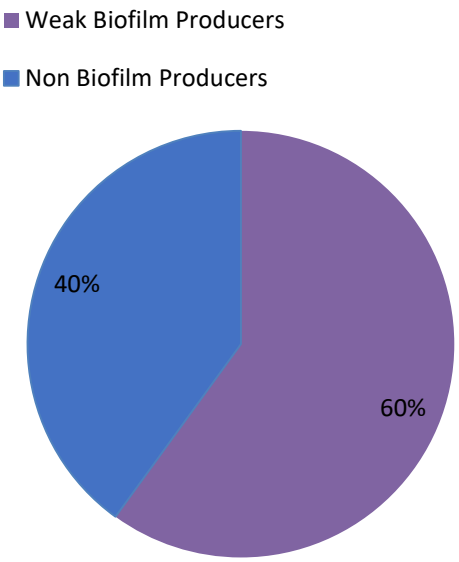
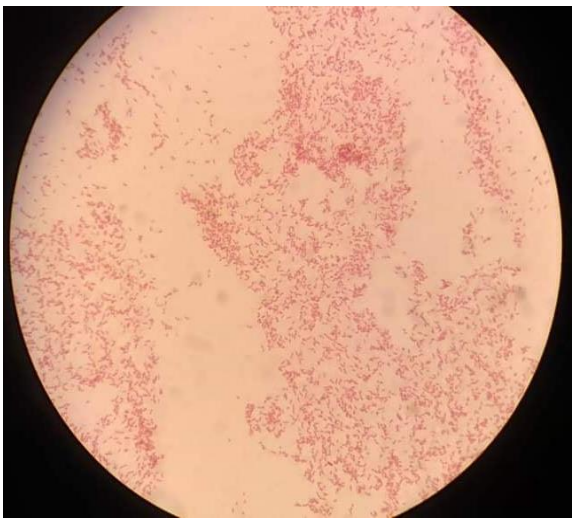


Figure 2: Biofilm Prodution among *K. pneumoniae* isolates



Photograph 1: Gram staining for identification of Gram negative bacilli under 100x magnification

Table 1: Distribution of organisms among different clinical sample

Organism	Number of Samples						
	Blood	**Urine**	**Sputum**	**Pus**	***Body fluids**	****Others**	**Total**
Klebsiella pneumoniae	2	18	7	4	1	3	35 (23.97%)
Escherichia coli	-	31	1	-	-	1	33 (22.60%)
Staphylococcus aureus	7	10	1	6	1	3	28 (19.17%)
*Acinetobacter*spp	5	2	3	3	-	6	19 (13.01%)
*CONS***	3	1	2	3	-	1	10 (6.8%)
*Serratia*spp	-	-	-	6	-	1	7 (4.7%)
Pseudomonas aeruginosa	2	1	2	1	-	-	6 (4.1%)
Proteus mirabilis	-	2	-	-	-	-	2 (1.36%)
Citrobacter freundii	-	1	-	1	-	-	2 (1.36%)
Streptococcus pneumoniae	1	-	1	-	-	-	2 (1.36%)
*Enterobacter*spp	-	-	-	1	-	-	1 (0.68%)
*Providencia*spp	-	1	-	-	-	-	1 (0.68%)
Total	**20**	**67**	**17**	**25**	**2**	**15**	**146**

*Body fluids: Pericardial fluid, Pleural fluid

**Others: Endotracheal tube tips Mitral valve tissue, Aortic valve tissue, Foleys tip, CVP tip

***CONS: Coagulase negative *Staphylococci*

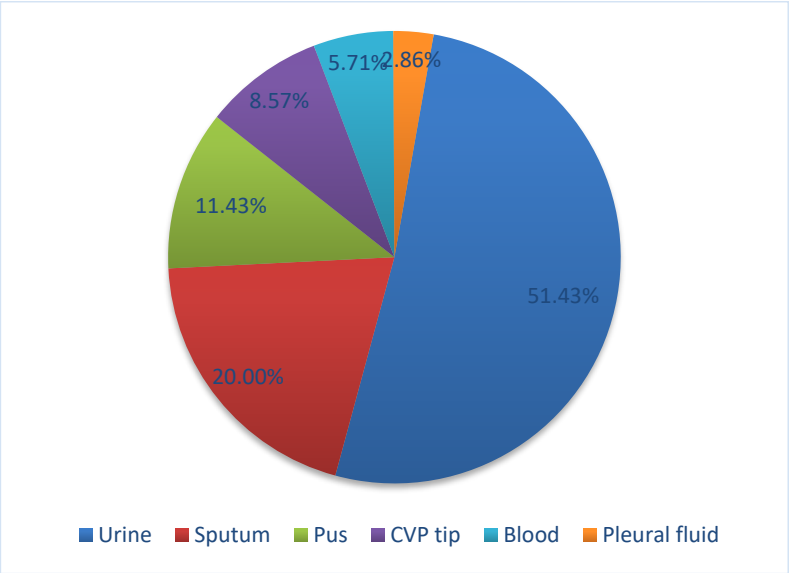


Figure 1: Frequency of *K. pneumoniae* among clinical sample

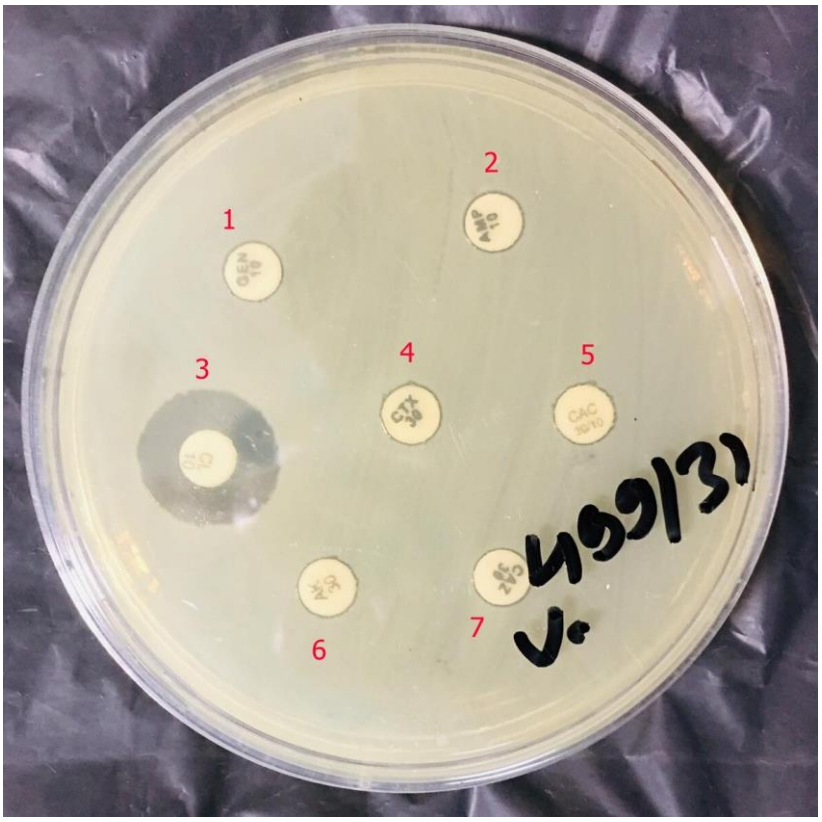
Table 2: Antibiotic Susceptibility Pattern of *K. pneumoniae*

S.N.	Categories	Antibiotics	Frequency of *K. peumoniae* isolates	
			Sensitive No. (%)	Resistance No. (%)
A	Aminoglycosides	Amikacin	14 (40.0)	21 (60.0)
		Gentamicin	13 (37.0)	22 (63.0)
B	Antipseudomonal penicillin+ ß-lactamase inhibitors	Piperacillin/tazobactam	12 (34.0)	23 (66.0)
C	Carbapenems	Meropenem	21 (60.0)	14 (40.0)
		Imipenem	18 (51.0)	17 (49.0)
D	Extended spectrum cephalosporins 3rd and 4th generation cephalosporins	Ceftriaxone	10 (29.0)	25 (71.0)
		Ceftazidime	8 (23.0)	27 (77.0)
		Cefepime	7(20.0)	28 (80.0)
		Cefotaxime	6 (17.0)	29 (83.0)
E	Fluroquinolones	Ciprofloxacin	3 (9.0)	32 (91.0)
F	Folate pathway inhibitor	Cotrimoxazole	17 (49.0)	18 (51.0)
G	Penicillin	Ampicillin	0 (0.0)	35 (100.0)
H	Penicillin +ß-lactamase inhibitors	Amoxycillin/clavulanic acid	11 (31.0)	24 (69.0)
		Ampicillin/sulbactam	10 (29.0)	25 (71.0)
I	Polymyxin	Polymyxin B	31(89.0)	4 (11.0)
		Colistin	100 (100.0)	0 (0.0)

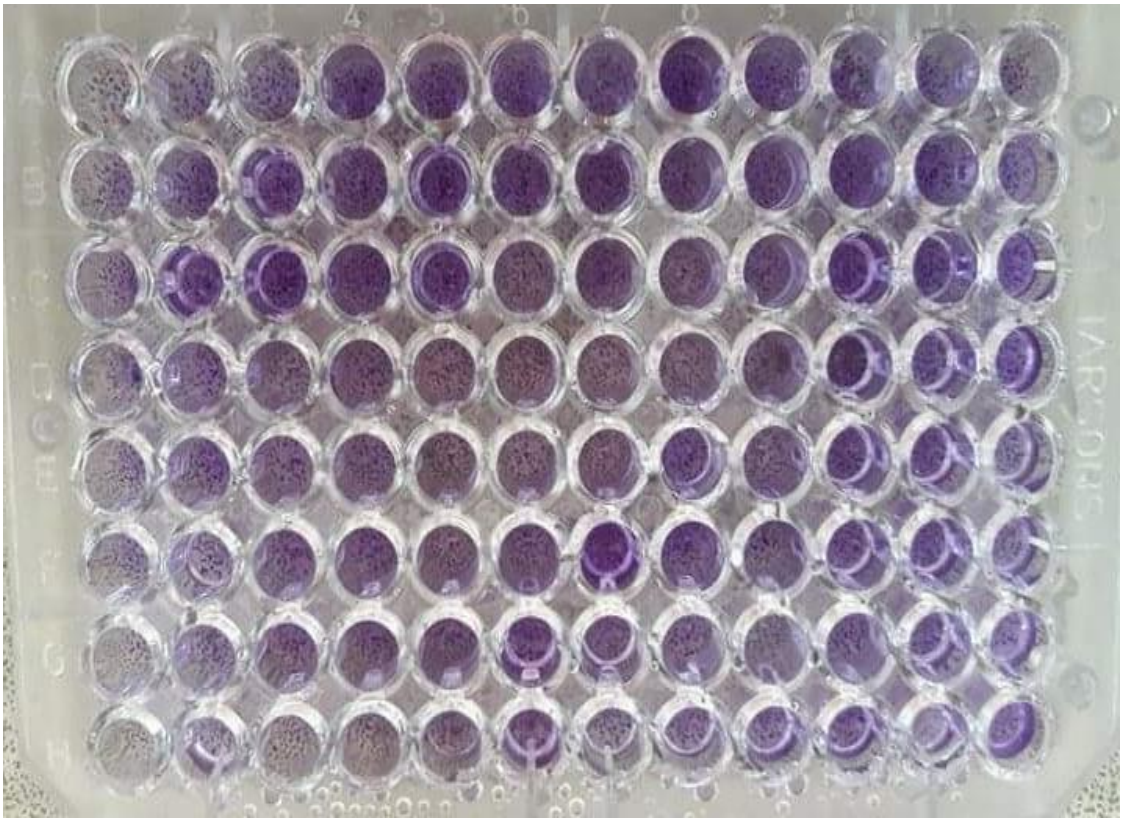
Table 3: Distribution of biofilm among multi drug resistance *K. pneumoniae*

Variable	Total	Weak biofilm	Non biofilm	P-value
Susceptible strain	4(11%)	1(4.8%)	3(21.4%)	<0.05
MDR	16(46%)	5(23.8%)	11(78.6%)	
XDR	15(43%)	15(71.4%)	0	
Total	**35 (100%)**	**21 (100%)**	**14 (100%)**	

Photograph 2: Biochemical test for confirmation of *K. pneumoniae* (From left to right) (Fermentative, Motility –ve, Indole –ve, MR –ve, VP +ve, Citrate +ve, TSI A/A $^{gas+/H2S\text{-}ve}$, Urease +ve)



Photograph 3: Antibiotic susceptibility test for *K. pneumoniae* (1: GEN=R, **2:** AMP=R, **3:** CL=S, **4:** CTX=R, **5:** CAC=R, **6:** AK=R, **7:** CAZ=R) *S= Sensitive and **R= Resistant



Photograph 4: **Biofilm detection by Microtitre plate method.**

DISCUSSION

Even though *K. pneumonia* can be frequently found in the mouth, on the skin, and in the intestine of an individual, it can be detrimental on immunocompromised patients causing urinary tract infections, respiratory tract infections, and septicemia. Multidrug Resistant *K. pneumoniae* leaves the therapeutic options limited than the susceptible one. The prevalence of multidrug resistance has risen remarkably since the introduction and unrestricted use of new generation antibiotics. High pathogenic capacity, ability to produce extended spectrum-lactamase (ESBLs), carbapenemase and biofilms enables this organism to develop antibiotic resistance faster than other bacteria (Munita JM and Arias CA 2016). This study was perfomed to detect the biofilm formation *K. pneumoniae* isolates and their relation to the antibiotic resistance.

Of the total 944 clinical samples analyzed, 16% (146) showed growth on different culture media. This finding is in harmony with the finding of Parajuli et al., (2017), Adhikari et al., (2018) and Shrestha et al., (2019) who reported similar bacterial growth of 19.68%, 17.79%, 16% respectively during their study. Out of 146 isolated organisms, *K. pneumoniae* were predominant isolates with the number of 35 (23.97%) followed by the *E. coli* 33 (23.60%) which is in concordance with the study conducted in the Department of Microbiology, B.P. Koirala Institute of Health Sciences (BPKIHS), Dharan, Nepal by Shrestha et al., (2019). High percentage of *K. pneumoniae* was isolated from urine 51%, followed by sputum (20%), pus (11%), CVP tip (8%), blood (6), pleural fluid (3%). These finding identify *K. pneumoniae*as an important cause of hospital acquired urinary tract infection. Beirao et al., (2011) and Singh et al., (2015) also reported that during his study, highest number of *K pneumoniae* were obtained from urine. Central venous catheter is frequently used in ICU settings however, their colonization with different types of organism increases the hospital stay and mortality in these patients. It is usually done in an

emergency procedure patient with prolonged hospital stays, critical conditions, especially those on ventilators and patients with multiple invasive devices are most likely to have a greater risk of infections. In a study conducted by Sapkota et al., (2017), they found prevalence of isolation rate of *K. pneumoniae*in CVC tip was 3 %. Colonization of *K. pneumoniae* on CVP tip occurs at the time of catheter insertion at this time skin commensal as well as iatrogenic organisms have chances to colonize in the insertion-site later these organisms reached to the deep portion of skin and colonized in catheter which results in blood stream infection. It has been stated that central venous catheterization longer than five to seven days was associated with a higher risk of catheter-related infection (Moro et al., 1994).

Antibiotic susceptibility test of all the *K. pneumoniae* isolates were performed against 16 different antibiotics of the different chemical classes. In terms of sensitivity most of the bacterial strains were found to be sensitive against colistin (100.0%), followed by polymyxin B (89%). The bacterial isolates exhibited 100% resistant towards ampicillin which is in harmony to the finding of Adhikari et al (2018); Beyene et al (2019): Livermore and Woodford (2006). Aminoglycosides usually have better activity against clinically important Gram-negative bacteria, but in this study, we observed that the isolates exhibited higher resistance rates of 63% to gentamycin and 60% to amikacin respectively. This result resembles to the finding of Khanal et al., (2013) who reported similar resistance rate of 69% and 54% exhibited by *K. pneumoniae* against gentamycin and amikacin respectively. Carbapenems are an important class of β- lactams antibiotic, this study showed higher percentage of resistance towards imipenem 49% followed by meropenem 40%. Meropenem was reported more effective than imipenem in this study which differ with earlier studies conducted by Piller et al., (2008); Mishra et al (2012); Shrestha et al., (2014). This might be due to the reason that meropenem are more active against Gram negative bacilli, while imipenem are more active against Gram positive cocci. Hence, the activity of carbapenem depends on types and species of organism as well as meropenem is a new drug in comparison to imipenem (Zhanel et al., 1998). The increasing trend of carbapenem resistance recorded in different studies of Nepal might be due to overuses/inappropriate uses of carbapenem antibiotics or might be due to prevalence of ESBL producers, which causes extensive use of carbapenem antibiotics as an empirical option (Hawkey and Jones 2009).

Biofilm production test in this study was conducted by Tissue culture plate assay. Since, tissue culture method is the quantitative and termed as gold standard method (Christensen et al., 1985). Using the method 60% of total isolates were found to be biofilm producers. It has been reported that more than 50% of total human infection are associated with biofilm production (Costerton et al., 1987). Biofilm producing strains showed relatively high drug resistance against all antibiotics tested as compared to non-biofilm producing strains. In the present study, strong correlation was noted between biofilm production and resistance to multiple antibiotics at significance level 0.05, where almost biofilm producing strains were MDR phenotypes. A higher proportion of antibiotic resistance in biofilm producers in comparison to non-producers has been documented in many studies (Dumaru et al. 2019; Shrestha et al. 2017). This was in accordance with study carried out by Stewart (2002).

CONCLUSION

Irrational use of antibiotics has resulted in dramatic increase of multi drug resistant K. pneumonia. It has become a matter of challenges to today's world. Periodic study on status of antimicrobial resistance is crucial to assess its spread. Different innate factor or mutational changes in organism can make it adaptive against antimicrobial agents. In this study, we aim to explore whether biofilm produced by organism plays crucial role in impairing resistance against antimicrobials.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of

interest.

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