



BIOFILM PRODUCTION AND ANTIBIOTIC SUSCEPTIBILITY OF UROPATHOGENIC *Klebsiella pneumoniae* ISOLATED FROM AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NIGERIA



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Abstract: Uropathogenic *K. pneumoniae* is one of the prominent uropathogens that plays a major role in biofilm formation. Microbial biofilms pose a public health problem for the persons who require indwelling medical devices, as the microorganisms in the biofilms are difficult to treat with antimicrobial agents. This study determined the biofilm production and antibiotic susceptibility pattern of *K. pneumoniae* isolated from Ahmadu Bello University Teaching Hospital, Zaria. Isolates from urine samples submitted to the Medical Microbiology Laboratory of the hospital were collected and identified using standard microbiological techniques. The isolates were evaluated for their biofilm production potentials and their susceptibility to antibiotics was determined. From the 180 uropathogens isolated, 55 (37.93%) were *K. pneumoniae*. The result of biofilm production showed most of the *K. pneumoniae* to be biofilm producers (63.64%). The antibiotic susceptibility testing showed that, the isolates were generally resistant to amoxicillin (78.18%), chloramphenicol (69.09%), tetracycline (63.64), cotrimoxazole (60.00%), ceftriaxone (49.09), ceftazidime (47.30%), ciprofloxacin (47.27%) and gentamicin (40.00%) while being uniformly susceptible to meropenem (90.90%), amikacin (85.45%) and piperacillin-tazobactam (83.64). A total of 76% of the isolates had MAR indices ≥ 0.3 indicating the prior exposure of these uropathogens to these antibiotics and up to 74.55% of the *K. pneumoniae* were multidrug resistant. However, the difference between the multidrug resistance of the biofilm-positive and biofilm-negative *K. pneumoniae* isolates was not statistically significant ($P < 0.05$) in this study.

Keywords: Antibiotics, biofilm, *Klebsiella pneumoniae*, resistance, susceptibility, uropathogens

Introduction

The emergence of antibiotic resistance in the management of urinary tract infections is a serious public health challenge, especially in the developing world where there is high level of poverty, ignorance, poor hygienic practices, fake drugs of questionable quality in circulation and poor prescription pattern (El-Astal, 2005). Apart from the above mentioned predisposing factors of antibiotic resistance, biofilm production seems to have greater impact in facilitating pathogens to develop resistance. The implication of *K. pneumoniae* in biofilm production has resulted in the evolution of resistant strains (Patel, 2005) which cause persistent and recurrent infections (Shirliff and Leid, 2009). Biofilm does not only serve as a training ground that transforms susceptible organisms to resistant strains but also act as a reservoir that disseminates them recurrently (Shigemura *et al.*, 2005). This biofilm production as commonly seen among the uropathogens has been said to worsen the situation of drug resistance by making *K. pneumoniae* multidrug resistant than their planktonic counterpart (Gilbert *et al.*, 1997). The intrinsic and acquired resistance of *K. pneumoniae* is due to several mechanisms which include; active efflux systems, reduced cell wall permeability, plasmid acquisition, expression of various enzymes and biofilm formation which on its own housed the above factors and other mechanisms of resistance. Biofilms can cause significant problems in the medical settings such as persistent, recurrent and device-related infections. Biofilms have major medical significance as they decrease susceptibility to antimicrobial agents and the proximity of cells within biofilms can facilitate plasmid exchange, enhancing the spread of antimicrobial resistance (Watnick and Kotler, 2000). Hence, there is need to determine the biofilm production and antibiotic susceptibility of uropathogenic *K. pneumoniae*.

Materials and Methods

Isolation and Identification of *K. pneumoniae*

This study was carried out in Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. The ethical approval for the work was obtained from the Ethical Committee of Ahmadu Bello University Teaching Hospital Zaria. A total of 180 isolates of urine samples submitted to the Medical Microbiology Laboratory of Ahmadu Bello University Teaching Hospital, Zaria was collected for this work. The isolates were identified by growing on selective media such as Cystein Lactose Electrolyte Deficient agar (CLED) and MacConkey agar for lactose fermentation, Gram staining for morphological identification and other biochemical tests were carried out which include: Triple Sugar Iron Agar (TSI), Citrate utilization test, Urease test, indole test, catalase test, oxidase tests and others as stated by Cheesbrough (2006) and Chakraborty and Nishith (2008).

Biofilm production by microtitre plates

Biofilm production of the uropathogenic *K. pneumoniae* was determined by a modification of the method described by Merrit *et al.* (2005) and Christensen *et al.* (1985). The isolates were grown overnight for 24 h at 37°C in Brain Heart Infusion Broth supplemented with 2% glucose and 2% sucrose. The cultures were diluted 1 μ l in 100 ml medium and 150 μ l of the cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtitre plate for 48 h at 37°C. After 48 h, the suspension was poured off and the wells were gently washed 3 times in 3 different trays of water and dried in an inverted position. The dried wells were stained with 250 μ l of 0.1% crystal violet solution in water for 20 min. The excess stain was poured off and the wells washed 3 times in three different trays of water and allowed to dry. A strong biofilm production can be seen as the presence of a layer of stained materials adhered to the inner wall of the wells.

Quantitative assay of biofilm by microtitre plate reader (ELISA)

This was carried out according to the method described by Merrit *et al.* (2005) and Christensen *et al.* (1985). The quantitative assay of the biofilm produced was performed by

adding 250 µl of ethanol-acetic acid (95:5 vol/vol) to destain the wells obtained from the preceding test and 100 µl from each wells were transferred to a new microtiter plate and the Optical Density (OD) of the solution was measured at 545 nm. Each assay was performed in triplicate. The control was uninoculated media, to determine background OD. The mean OD₅₄₅ value from the control wells was subtracted from the mean OD₅₄₅ value of the test wells which gives the amount of biofilm produced. The calibration for the measurement of biofilm was adapted from the method derived by Ando *et al.* (2004) as ≥ 0.5 (strong biofilm formers = S), $< 0.5 \geq 0.2$ (moderate formers = M), $< 0.2 > 0.0$ (weak biofilm formers = W), 0 (non-biofilm formers = N).

Antibiotic susceptibility testing

The antibiotic susceptibility of the uropathogenic *K. pneumoniae* was determined according to EUCAST (2011). The antibiotics selection was based on the standard antibiotics recommended for the definition of multidrug resistance according to Magiorakos *et al.* (2011). A total of 12 antibiotics were used against all the isolates comprising of tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), ceftriaxone (CRO, 30 µg), amoxicillin (AML, 10 µg), gentamicin (CN, 10 µg), meropenem (MEM, 10 µg), tigecycline (TGC, 10 µg), amikacin (AK, 30 µg), ceftazidime (CAZ, 30 µg), piperacilline-tazobactam (TZP, 100 µg) and cotrimoxazole (COT, 25 µg) (Chesbrough, 2006; Magiorakos *et al.*, 2011). The standardized suspension was inoculated on sterile Mueller Hinton agar plate using a sterile swab to ensure even distribution and confluent growth. The sensitivity discs of the various antibiotics were aseptically placed using a sterile forceps on the dried inoculated agar surface. The plates were then incubated at 37°C for 18 h. After incubation, the plates were examined for the zones of inhibition and measured. The result was interpreted using the interpretation criteria published by EUCAST (2011). The isolates were reported as sensitive, intermediate and resistant to the various antibiotics depending on the size of the zone of inhibition.

Statistical analysis

Statistical analysis was carried out to compare the relationship between the drug resistance (MDR and non-MDR) and Biofilm production (BP and BN) using chi square. Values of $p < 0.05$ were considered significant.

Results and Discussion

Out of the 180 isolates that were collected and identified from urine sample submitted to Medical Microbiology Laboratory of Ahmadu Bello University Teaching Hospital, Zaria, 55 (37.93%) of the isolates were *K. pneumoniae*. This high prevalence could be because *Klebsiella* species are ubiquitous in nature. In humans, they may colonize the skin, pharynx or gastrointestinal tract and are regarded as normal flora in many parts of the colon, intestinal tract and in the biliary tract which by chance find their ways to the urinary system through faecal contamination (Osazuwa, 2010). This is in agreement with Osazuwa (2010) who reported *Klebsiella* species as the most prevalent uropathogens isolated from urinary tract infected patients attending University of Benin Teaching Hospital Benin, Nigeria. Bolaji *et al.* (2013) also reported *K. pneumoniae* as the second most prevalent uropathogen after *S. aureus* among pregnant women in Zaria, Kaduna State, Nigeria.

Table 1 shows the percentage values of the biofilms produced by *K. pneumoniae*. The distribution of the isolates into biofilm positive and biofilm negative was considered as: strong and moderate biofilm producers = biofilm positive (PB) while the weak and non-biofilm producers = biofilm negative (NB). The biofilm positive were 63.64% while the biofilm negative were 36.37% as shown in Fig. 1. There is high prevalence of

biofilm production by *K. pneumoniae* as seen in this work and this could likely be the reason for resistance observed frequently with uropathogens especially when associated with catheterization as stated by Nicolle (2005). *K. pneumoniae* has a mucoidal nature of growth or colonies which might have facilitated their biofilm producing ability. Maldonado *et al.* (2007) reported *Klebsella* species to be one of the pathogens that are able to cause biofilm forming catheter associated infections. It was also demonstrated that type 1 and type 3 fimbriae expressed by *K. pneumoniae* enhance biofilm formation on urinary catheters in a catheterized bladder model that mirrors the physico-chemical conditions present in catheterized patients (Stahlhut *et al.*, 2012). The *in vitro* identification of *K. pneumoniae* as biofilm formers in this work also agrees with Donlan (2001) who demonstrated the biofilm forming ability of *K. pneumoniae in vivo*.

Table 1: The biofilm production by uropathogenic *K. pneumoniae* isolated from ABUTH, Zaria

Level of biofilm produced	Number of isolates (%)
Strong	18 (32.73)
Medium	17 (30.91)
Weak	18 (32.73)
Non	2 (3.64)

Table 2: The percentage susceptibility of uropathogenic *K. pneumoniae*

Antibiotics	Resistant	Intermediate	Sensitive
Ciprofloxacin	47.27	5.45	47.27
Gentamicin	40.00	14.55	45.45
Chloramphenicol	69.09	0.00	30.91
Tigecycline	16.36	25.45	58.18
Ceftazidime	47.30	9.09	43.60
Cotrimoxazole	60.00	3.60	36.36
Tetracycline	63.64	0.00	36.36
Amoxicillin	78.18	0.00	21.81
Meropenem	0.00	9.09	90.9
Amikacin	10.91	3.63	85.45
P-tazobactam	10.91	5.45	83.64
Ceftriaxone	49.09	0.00	50.91

Disc diffusion method was used to test the susceptibility of the fifty five (55) *K. pneumoniae* isolates and the result was interpreted as sensitive, intermediate and resistant as shown in Table 2 according to EUCAST (2011). Meropenem, amikacin and piperacillin-tazobactam were the most effective antibiotics against all the isolates. This is in agreement with various studies which identified meropenem, amikacin and imipenem as the most effective antibiotics against Gram-negative uropathogens (Savas *et al.*, 2006; Varsha, 2007; Zahar, 2009 and Abdallah, 2011). The high activity observed on these drugs indicates that they have not been abused and probably their high cost has limited their indiscriminate usage. Hence, these antibiotics could be used as effective therapeutic agents in the treatment of urinary tract infections.

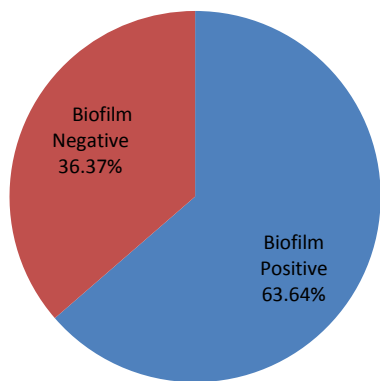


Fig. 1: The summary of biofilm production by uropathogenic *K. pneumoniae*

Figure 2 showed the percentage resistance of the antibiotics indicating clearly those that were not effective against the *K. pneumoniae*. Amoxicillin, tetracycline and cotrimoxazole were extremely poor against *K. pneumoniae*. This work agrees with Daniyan and Ojo (2013) who recorded the antibiotics with the highest resistance by the uropathogens to be tetracycline, amoxicillin and cotrimoxazole. It was similarly reported also that, 100% of *K. pneumoniae* isolates were resistant to amoxicillin (Getnet *et al.*, 2011). Olufunmiso (2011) has reported resistance of uropathogens to cotrimoxazole and ceftriaxone. Resistance of uropathogens to ciprofloxacin, gentamicin and ceftriaxone has also been reported by Laupland *et al.* (2007). These antibiotics have been abused by their indiscriminate usage, as they are cheap and easily available. Some of these antibiotics can be found even with the street vendors in the environment of study. The ease in their accessibility and route of administration have gained them this resistance development.

The resistance of the uropathogenic *K. pneumoniae* to aminoglycosides (gentamicin), fluoroquinolones (ciprofloxacin) and cephalosporins (ceftazidime and ceftriaxone) as seen in this work (Fig. 2) calls for concern since these antibiotics have been known to be effective against Gram negatives bacteria. This is in line with the finding of Ruiz (2003) who stated that, the extensive use of quinolones in the treatment of urinary tract infections have resulted in bacteria rapidly developing resistance to these agents. This resistance observed among the uropathogenic *K. pneumoniae* is supported by the work of Hryniewicz *et al.* (2001) who also stated that, the worldwide data is showing an increasing resistance among uropathogens to the conventional drugs. The high activity of piperacillin – tazobactam seen in Table 2 compared to the less activity of amoxicillin against *K. pneumoniae*, as shown in Fig. 2 calls for concern as both are penicillins. This could indicate that, some of the isolates are β -lactamase producers because, tazobactam, a β -lactamase inhibitor, might have played it role, thereby increasing the activity of piperacillin - tazobactam.

Table 3 showed the multiple antibiotic resistance (MAR) indices of the *K. pneumoniae*. A total of 76% of the isolates had MAR indices ≥ 0.3 as shown in Fig. 3. Multiple antibiotics resistance (MAR) index is a tool that reveals the spread of bacteria resistance in a given population (Krumpermann, 1983). The high percentage of MAR index \geq

0.3 seen in this work is a possible indication that a very large proportion of the bacteria isolates have been exposed to several antibiotics which have induced this resistance.

Table 3: Multiple antibiotic resistance indices of uropathogenic *K. pneumoniae*

MAR index	No of isolates	Percentage
0	4	7.27
0.1	5	9.09
0.2	4	7.27
0.3	13	23.64
0.4	4	7.27
0.5	5	9.09
0.6	7	12.73
0.7	6	10.91
0.8	6	10.91
0.9	1	1.82

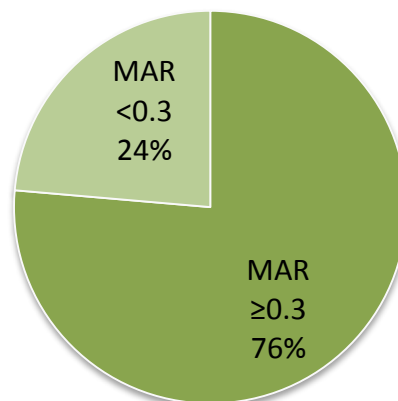


Fig. 3: The summary of MAR indices of the uropathogenic *K. pneumoniae*

Table 4: The distribution of the uropathogenic *K. pneumoniae* into multidrug resistance and non-multidrug resistance

Resistance	No of isolates	Percentage
SA	4	7.27
NON-MDR	10	18.18
MDR	43	74.55

An International Expert Proposal for Interim Standard defines Multidrug Resistance (MDR) as non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories (Classes) (Magiorakos *et al.*, 2012) Table 4 showed the distribution of the isolates into MDR, non-MDR (non-susceptible to ≥ 1 agent in <3 antimicrobial categories) and SA (susceptible to all the antibiotics). A total 74.55% of the isolates were Multidrug Resistant. Olayinka (2004) stated that, the multiple antibiotic resistances in bacteria populations is currently one of the greatest challenges to the effective management of infections and this is becoming increasingly true just as seen in this work. This high prevalence of MDR obtained in this work indicates a great threat for persistent and recurrent infections in the study area particularly with the *K. pneumoniae*. This result of MDR agrees with the study of Babinchak *et al.* (2005) who stated that, the multiple drug resistance to β -lactams, aminoglycosides and quinolones among the Gram negative bacteria has become a major nosocomial problem worldwide.

The statistical analysis also compared the relationship between the drug resistance (MDR and non-MDR) and Biofilm production (BP and BN). The P value of 0.297 was obtained as shown in Table 5. This is greater than 0.05 which indicates, no significant difference in the MDR level among the biofilm producers and the non- biofilm producers of the *K. pneumoniae* isolates.

Table 5: The relationship between multidrug resistance and biofilm formation among the uropathogenic *K. pneumoniae*

Drug Resistance	Biofilm		Total	Chi-square	Df	p value	odd ratio
	Positive	Negative					
MDR	26 (63.4)	15 (36.6)	41 (100.0)	1.089	1	0.297	0.473
NMDR	11 (78.6)	3 (21.4)	14 (100.0)				
Total	37 (67.3)	18 (32.7)	55 (100.0)				

It can therefore be deduced that, biofilm formation potentials and resistance development do not necessarily have a singular factor for their both expression in an organism; rather, biofilm formation predisposes pathogens to resistance development, as a result of the mechanisms of resistance and other complexity involved in biofilm community. This shows that, a susceptible pathogen that has the ability to form biofilm is a potential resistant strain. This finding is in agreement with Eyoh *et al.* (2014) who found no significant relationship between multidrug resistance and biofilm production but in contrast to the work of Fitzpatrick *et al.* (2005) who stated that the multidrug resistance strains were found more among biofilm producers than non- biofilm producers.

Conclusion

This study has revealed uropathogenic *K. pneumoniae* as a high biofilm producer. High prevalence of multidrug resistant was also seen among *K. pneumoniae*. Amoxycillin, tetracycline, ceftazidime, cotrimoxazole and ceftriaxone showed less activity while meropenem, amikacin and piperacillin-tazobactam showed high activity. This work also showed that, there is no singular factor responsible for the expression of both biofilm and resistance in an organism rather, biofilm formation induces resistance development.

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