Extended Spectrum Beta-Lactamase production, Biofilm Formation and Antibiotic Resistance in Clinical Isolates of *Klebsiella pneumoniae*

Aisha Shitu Sa'id[1*], Mukhkar, M. D.,[2] Bukar, A.,[2] and Yusha'u, M.[2]
[1]*Department of Microbiology, School of Life Sciences, Modibbo Adama University of Technology, Yola
[2]Department of Microbiology, Faculty of Basic Sciences, Bayero University Kano
*Corresponding Author: ayshashitu@gmail.com; Mobile phone No: 07035818250

Abstract*: Klebsiella pneumoniae* is an opportunistic pathogen frequently multidrug-resistant, responsible for both health care and community associated infections. The appearance of extended-spectrum β- lactamase in addition to the biofilm-forming phenotype, is a major problem in the clinical environment. This study aimed to detect ESBL production, biofilm formation and antibiotic resistance profile of clinical isolates of *K. pneumoniae*. Three hundred and twenty five samples of urine and sputum were analyzed by conventional bacteriological techniques. Kirby-Bauer disc diffusion method was used for antimicrobial susceptibility testing. ESBL detection was done by the double‐disc synergy tests using the Clinical and Laboratory Standards Institute guidelines. Biofilm formation was determined by microtiter plate assay. Out of the 325 samples analyzed, only 74 (22.7%) yielded *Klebsiella pneumoniae* isolates. Isolation rate was 25.5% for urine higher than that of sputum (20%). Isolates displayed 100% resistance to cefotaxime and ceftazidime and decreased resistance to imipenem and ciprofloxacin. ESBL production was observed in 31.1% of all the isolates. ESBL-producing isolates formed more biofilm than non ESBL producers. A significant association was observed between ESBL production and biofilm which may be as a result of uptake of ESBL carrying plasmids that activate the virulence factor. However, increased alertness of clinicians and enhanced testing by laboratories are important to reduce treatment failure and prevent the spread of resistance strains.

Key words: Antibiotic resistance; Biofilm formation; Extended Spectrum Beta lactamase; *Klebsiella pneumoniae*

INTRODUCTION

Microorganisms growing in biofilms exhibit phenotypic characteristics that are distinct from those of planktonic organisms, including increased resistance to host immune defenses and to antimicrobial compounds (Stewart, 2002). The resistance could be due to the slowly growing state of the cells in the deeper layers of thick biofilms, which have less access to antibiotics and nutrients, and to the impaired diffusion of antimicrobial molecules within the biofilms (Ito *et al.,* 2009; Hoiby *et al*., 2010). The possession of virulence traits particularly biofilm formation has aggravated the problem of *K. pneumoniae* antibiotic resistance. This pose a serious challenge to infection management worldwide and are likely to be associated with high level of morbidity, increased mortality, longer hospitalization and excessive health care costs compared with infections associated with antibiotic susceptible microorganisms (Hoiby *et al*., 2010; Hou *et al*., 2015). This study aimed to detect ESBL production,biofilm formation and antibiotic resistance profile of clinical isolates of *K. pneumoniae*.

Klebsiella pneumoniae is an opportunistic pathogen correlated with both community and health care associated infections, such as pneumonia, urinary tract infections, septicemia, and wound infections worldwide and the main population at risk is the neonates and immunocompromised hosts (Podschun and Ullmann, 1998). The increasing frequency of multidrug-resistant *K. pneumoniae* has led to it being classified as a major public health concern (Cao *et al*., 2014). However, *K. pneumoniae* has become a clinically important microorganism, particularly in the last two decades due to its tendency to develop antibiotic resistance and cause fatal outcomes (Podschun and Ullmann, 1998; Nordmann *et al*., 2011). Beta-lactamase production by several gram negative and gram positive organisms is the

most important single mechanism of cephalosporins, monobactams and carbapenems (Chaudary and Aggarwal, 2004), which are the commonly used antimicrobials in treatment of bacterial infections in hospitals. Resistance to beta lactams antibiotics has become a growing problem in the treatment of nosocomial and community acquired infections caused by *K. pneumoniae* due to acquisition and expression of Extended Spectrum β-Lactamase. Extended-Spectrum Beta-lactamases (ESBLs) are a group of diverse, complex and plasmid-mediated rapidly evolving enzymes that pose a major therapeutic challenge in the treatment of patients (Bradford, 2001). They are able to inactivate beta-lactam antibiotics containing an oxyimino-group such as oxyimino-cephalosporins (e.g. ceftriaxone, cefotaxime, ceftazidime), oxyimino-monobactam (e.g. aztreonam) as well as the penicillins (Azekhume *et al*., 2015). ESBLs are most commonly produced by *Escherichia coli* and *Klebsiella* species, with *Klebsiella pneumoniae* seemingly the major ESBL producer (Olowe *et al*., 2011; Raji *et al*., 2015). Extended Spectrum β- Lactamase *K. pneumoniae* turned into nosocomial pathogen of utmost significance in recent decades. It has not only disseminated extensively in hospitals but also acquired a variety of resistance mechanisms which turns it into a formidable infectious agent.

MATERIALS AND METHODS

Study Area

This study was carried out at the Federal Medical Centre, Yola. Thishospital is a both referral and tertiary health facility located in Yola, the capital of Adamawa State, North-East region of Nigeria.

Study Design

The study was cross sectional, hospital-based and descriptive in design carried out for a period of six months (February – August, 2018).A non-probability convenient type of sampling was used. Patients attending the out-patient clinics and in-patients at both hospitals were recruited into the study. They include patients from resistance to penicillins, Paediatric wards, General Outpatient Department (GOPD), Medical and Antenatal clinic attendees. The patients were of different ages and sex. Sample size was determined using the formula derived by Cochran, with a 95% confidence interval, error margin of 5% and prevalence rate of 27% (from a study conducted Maiduguri on the prevalence of ESBL *K. pneumoniae*).The formula is as follows:

Sample size (n) = Z x P x (1 – P)/C^2

Where Z = 1.96 (for 95% confidence level), C = Confidence interval (0.05) and P = prevalence rate of 27%

Ethical clearance was received from the Research Ethical Committee of Federal Medical Centre, Yola (Ref. No.: FMCY/SUB/96N/T/36). Samples from the participants were only included in the study after the consent was taken from each one of them. Any refusal to contribute the sample was well respected.

Sample Collection and Identification

A total of 325 clinical samples of urine, sputum and nasal swabs were collected. The samples were collected from 165 and 160 patients with clinical evidence of urinary and respiratory tract infections respectively, as obtained by the physician from both General Out-patient Department (GOPD) and wards. The samples were immediately transported to the Microbiology laboratory for processing.

The organisms were isolated and identified by conventional bacteriological tests (Podschun and Ullaman, 1994; Collee *et al*., 1996). Bacterial colonies with characteristic mucous and pinkish colour on MacConkey agar were presumptively identified as *Klebsiella* spp. Further confirmation was done by microscopy and biochemical tests using the API 20 E kit.

Antibiotic Susceptibility Test

Antimicrobial susceptibility assay was performed according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2016) using the Kirby- Bauer disc diffusion method to evaluate the sensitivity of the test organisms. Antimicrobial agents used include ciprofloxacin (10 µg), tazobactam-

piperacillin (10/100 µg), cefotaxime (30 µg), gentamicin (10 µg), nitrofurantoin (15 µg), aztreonam (30 µg), cefpodoxime (30 µg), ceftazidime (30 µg), imipenem (10 µg) and trimethoprim- sulfamethoxazole (1.25/23.75 µg) (Oxoid, UK). The result of the assay was interpreted according to the CLSI guidelines by measuring the diameter of the zone of inhibition that arise from diffusion of the agent into the medium surrounding the disk.

Double Disk Synergy Test for ESBL Production

Isolates with zone diameters suspicious of ESBL production as predetermined by the susceptibility test results (Cefpodoxime: ≤17 mm; Ceftazidime: ≤22 mm; Cefotaxime: ≤27 mm) were subjected to the Double Disk Synergy Test (CLSI, 2016) to check for the presence of ESBL producing enzymes. Briefly, test organisms (suspected of ESBL production) were cultured overnight on nutrient agar, and a suspension prepared to match a 0.5 McFarland turbidity standard was inoculated onto the surface of each of the molten Mueller Hinton agar plates using a sterile cotton swab. Amoxicillin (20 µg)/ clavulanic acid (10 µg) combination disc was placed at the center of each inoculated Mueller Hinton agar plate. Cefotaxime (30 µg) and ceftazidime (30 µg) single discs were then placed 20 mm (center to center) from the amoxycillin/clavulanic acid disc and incubated at 37ºC overnight (18-24 hrs). Enhancement of the zones of inhibition of any of the cephalosporin beta-lactam antibiotic discs towards the amoxycillin/clavulanic acid disc caused by the synergy with clavulanate was taken as an evidence of ESBL production. *K. pneumoniae* ATCC 700603(ESBL-producer) was used as reference strain for quality control.

Detection of Biofilm by Microtitre Method

Determination of biofilm production was carried out according to the procedure described by O'Toole and Kolter (1998). *K. pneumoniae* isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth containing 1% glucose and incubated at 37^{o}C for 24 h. The overnight cultures were then diluted in a ratio of 1:100 with fresh medium. Individual wells of sterile 96 well- flat bottom polystyrene tissue culture treated plates (Sigma- Aldrich, Costar,USA) were filled with 200 µL of the diluted cultures. The control organism was also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37^{o}C for 24 h. After incubation, contents of each well were removed by gentle tapping and the wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was removed by rinsing with deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength 570 nm.

Statistical Analysis

Data from the work were entered in the worksheet of Statistical Package for Social Science (SPSS) software version 25.0. Frequency and percentages were analyzed as descriptive findings. Inferential statistics were analyzed using Chi square test to find the association between ESBL producing *K. pneumoniae* and biofilm formation.

RESULTS

The morphological characteristics and the biochemical test result of the *K. pneumoniae* isolates is summarized in Table 1a and 1b. Out of the overall 325 samples screened for the presence of *K. pneumoniae*, only 74 (22.7%) were positive. The rate of occurrence of the isolates was found to higher among those with urinary tract infections with frequency of 25.5% when compared to those with respiratory tract infections (20%) as shown in Table 2.

Table 1a: Colony characteristics of the *K. pneumoniae* isolates on MacConkey agar medium

Colony Characteristics				
Size	Form	Colour	Margin	Elevation
Small	Circular (mucoid encapsulated)	Pink	Entire	Raised

Table 1b: Analytical Profile Index test results of the isolates

Test	**Result**
ONPG	+
ADH	-
LDC	-
ODC	-
CIT	+
H_2S	-
URE	+
TDA	-
IND	-
VP	+
GEL	-
GLU	+
MAN	+
SOR	+
RHA	+
SAC	+
MEL	+
AMY	+
ARA	+
INO	+

Key: ONPG-O-Nitrophenyl-b-d-Galactopyranoside; ADH- Arginine dyhydrolase; LDC- Lysine decarboxylase; ODC- Ornithine decarboxylase; CIT- Citrate; H_2S- Hydrogen sulfide; URE- Urease; TDA- Tryptophan deaminase; IND- Indole; VP- Voges Proskauer; GEL- Gelatinase; GLU- Glucose; MAN- Mannose; INO- Inositol; SOR- Sorbitol; RHA- Rhamnose; SAC- Sucrose; MEL- Melibiose; AMY- Amygdalin; ARA- Arabinose

Table 2: Percentage occurrence of *K. pneumoniae* isolates among the clinical samples

Samples collected	Site of infection	No. of isolates	% occurrence
Sputum (n = 160)	RTI	32	20
Urine (n = 165)	UTI	42	25.5
Total (n = 325)		74	

Legends: RTI- Respiratory Tract Infections; UTI- Urinary Tract Infections; %- Percentage; n= number of samples

ESBL production was detected in 28 (37.7%) out of the 74 isolates recovered from the clinical samples. The results indicated that the isolates were able to enhance the inhibition zones of cefotaxime and ceftazidime on the site facing the amoxicillin-clavulanate disk. This enhancement toward towards the amoxicillin-clavulanate disk was interpreted as a positive test (Plate 1).

The activities of the antibiotics tested against the ESBL producers are presented in table (3). All ESBL producing isolates showed resistance to cefotaxime and ceftazidime (100%), while lowest resistance was seen in imipenem (16.7%) and ciprofloxacin (20.5%).

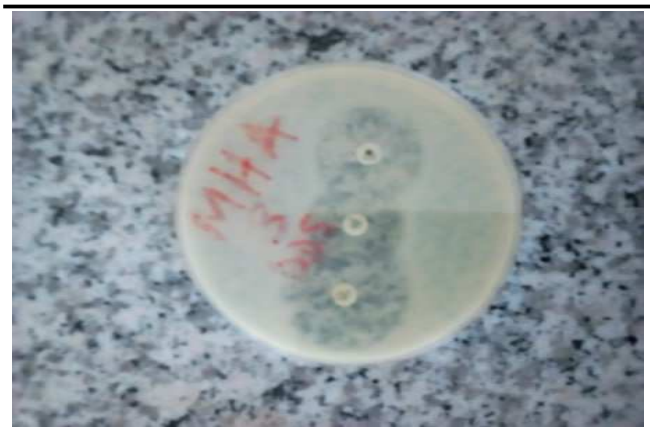

Plate 1: A positive double disk synergy test for *K. pneumoniae* using cefotaxime disk, ceftazidime disk and amoxicillin clavulanate at the centre.

Table 3: Antibiotic Resistance Profile of ESBL Producing *K. pneumoniae*

Antibiotics (µg)	R(%	S (%)
Ceftazidime	100	0
Cefotaxime	100	0
Cefpodoxime	89.6	10.4
Aztreonam	78.4	21.6
Imipenem	16.7	83.3
Ciprofloxacin	20.5	79.5
Gentamicin	86.8	13.2
Nitrofurantoin	35.3	64.7
Piperacillin/Tazobactam	35.3	64.7
Trimethoprim/Sulfamethoxazole	74.6	25.4

Key: R: Resistance, S: Sensitive

Biofilm intensity was categorized as strong, moderate and weak. Optical densities (OD) of the isolates which were higher than biofilm negative control were considered as strong biofilm producers. Our findings indicated that out of the 28 ESBL *K. pneumoniae* isolates, 25 (93.9%) of ESBL producers were detected as strong biofilms, 2 (4.1%) as moderate biofilms and only 1 (2.0%) was found to be a weak or nonbiofilm (Figure 1a). Moreover, strong biofilm production among the non ESBL producers was found to be 3 (9%), while 2(24%) of the non ESBL producers were showed to appear as moderate biofilm producers. Non biofilm production among the non ESBL producers 23(89%) was found to be higher compared to ESBL producers (Figure 1b).

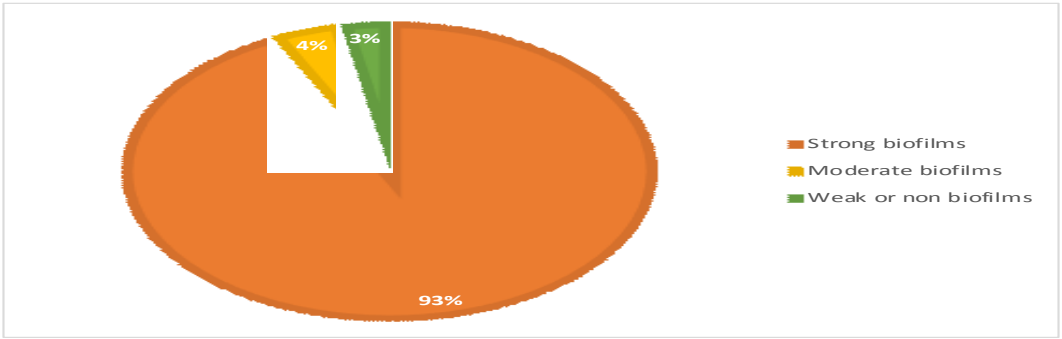

Figure 1a: Biofilms formed with ESBL producing *K. pneumoniae*

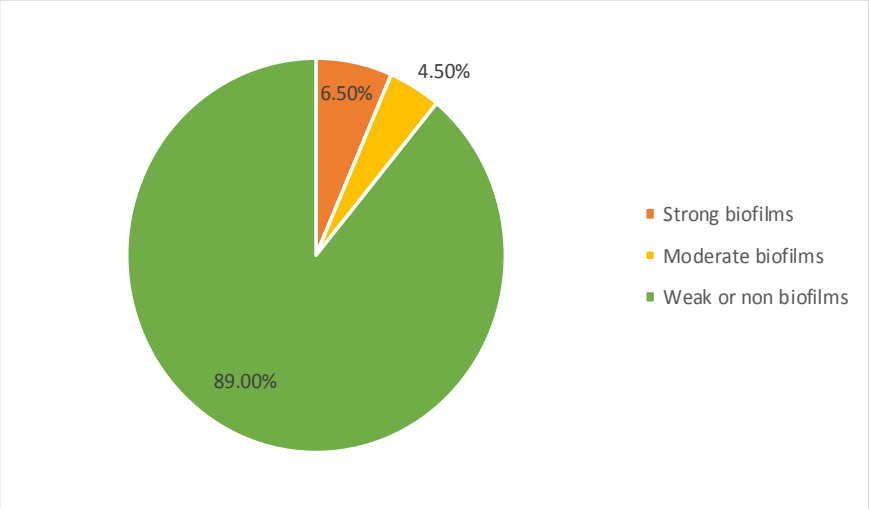

Figure 1b: Biofilms formed with Non-ESBL producing *K. pneumoniae*

DISCUSSION

K. pneumoniae have become major well recognized causative pathogens in both healthcare and community associated infections including respiratory and urinary tract infections and wound infections globally. They are among the major causes of preventable morbidity and mortality in developing countries where infection rates are fairly higher as a result of poor infection control practices, inadequate surveillances, congestion in hospitals and improper use of limited resourcesIn the present study, *K. pneumoniae* was mostly isolated from urine samples with highest prevalence rate of 25.5% in comparison with respiratory samples with 20%. Similar findings were reported by Giwa *et al.* (2018) from Northwestern Nigeria and Al-Yousef *et al.* (2016) from Saudi Arabia. In contrast, higher prevalence rate of 54.4% was reported by Akujobi (2007) in Ebonyi, 71.1% by Chibueze (2014) in Enugu while Ali and Ismael (2017) in Erbil city found a higher rate of 75.9% and a very low rate of 10% for urine and sputum respectively.

Based on the result of this study, the overall prevalence of ESBL *K. Pneumoniae* was 37.7%.This study is similar to findings of Bajfal *et al.* (2014); Aggrawal *et al.* (2008); Akanbi *et al.* (2013) and Giwa *et al.* (2018) where ESBL production was found to be 34.3%, 36.8%,33.6% and 34.4% respectively.Several reports regarding the prevalence of ESBL producing *K. pneumoniae* have been documented all over the world (Al Jasser, 2006; Siraj *et al.*, 2014). The profile of the ESBL producing species may vary geographically particularly in isolates which are rapidly changing with time due to complex epidemiology of ESBLs and methods used for ESBL detection among other factors (Al-jasser *et al.*, 2006; Azekhueme *et al.*, 2015). However, in some studies from other part of Nigeria, Chikwendu *et al.* (2010), Ogefere *et al.* (2013), Yusha'u *et al.* (2010), Iroha *et al.* (2010), Azekhueme *et al.* (2015) in Calabar, Benin, Kano, Ibadan and Uyo respectively, recorded prevalence of 48.3%, 44.3%, 66.7%, 76.9% and 47.1%. The increased prevalence of ESBL *K. pneumoniae* in different regions of the world especially the developing countries of the world is disturbing. This problem may be as a result of multiple factors such as inappropriate use of antibiotics for febrile infections as well as limited infection control measures to reduce the spread of multidrug resistant strains. This reported prevalence differs with findings of some continental studies carried out in South America (18.1%), Asia-Pacific (14.2%), Europe (11%) and North America (7.5%) region (Hawser *et al.*, 2011; Siraj *et al.* 2014).

Perhaps, this high prevalence from our study as compared to other developed countries might be due to the fact that developed nations have strict infection management policies and practices, brief or average hospital stay, proper nursing barriers that can significantly reduce the chances of transmission and spread of ESBL producing strains (Azekhueme *et al*. 2015; Giwa *et al*. 2018).

All ESBL producing isolates showed maximum resistance to cefotaxime and ceftazidime (100%), while lowest resistance was seen in imipenem (16.7%).This agrees with studies reported by Raylane *et al*. (2018) and Yangzan *et al*. (2018) with resistance rate of 95.5% and 100% respectively for third generation cephalosporins. Commonly prescribed antibiotics in our community settings such as third generation cephalosporins, gentamicin, aztreonam and trimethoprim/sulfamethoxazole were among the least effective as seen in this study. The reason for such could be linked with increased misused and abuse of these cheap antibiotics which are easily accessible as over-the-counter (OTC) drugs and can even be taken without physician's prescription (Anikpo *et al*., 2009).

Infections caused by ESBL producing *K. pneumoniae* are connected to harsh conditions due to expression of virulence features by these strains and have been reported to have major role in their pathogenicity (Sahly *et al*., 2008). Furthermore, biofilms have the ability to protect bacteria from the effect of antimicrobials when compared with other nonbiofilm forming bacteria (Bellifa *et al*., 2013). This current study revealed that strong biofilm formation was noticed among ESBL producers (93%) when compared to for non ESBL producers (6.5%) with highly significant difference (P<0.05) (figure 1a and b). The results are in consistent with that of Gharrah *et al*. (2017) who reported that development of biofilms is found to be higher with ESBL producing *K. pneumoniae* strains than non ESBL producing strains.This high rate of biofilm formation amongESBL producing *K. pneumoniae* may be as a result of uptake of ESBL carrying plasmids which activate the virulence factor by upregulation of some genes or adding new virulence genes such as type 1 or type 3 fimbriae that are greatly responsible for invasion and biofilm production in *K. pneumoniae* (Vuotto *et al*., 2014; Yazgan *et al*., 2018).

CONCLUSION

The present study clearly highlightsthat there is a strong relationship between ESBLproduction and biofilm formation which led to immense antibiotic resistance in *K. pneumoniae* isolates. All the isolated ESBL producers were resistant to third generation cephalosporins but are still sensitive to carbapenems like Imipenem. There is an alarming increase in resistance rateto a number of commonly used antibiotics. However, increased alertness of clinicians and enhanced testing by laboratories are important to reduce treatment failure and prevent the spread of ESBL producing *K. pneumoniae.* Furthermore, formation of a strict antibiotic policy in hospitals is also necessary as it will help in reducing the resistance level.

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