Extended spectrum beta-lactamase production and biofilm formation in *Klebsiella pneumoniae* isolates from urinary tract samples: A tertiary care hospital experience

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Abstract
Multidrug resistant Gram negative bacteria belonging to family *Enterobacteriaceae* are responsible for urinary tract infections (UTIs) that are difficult to treat. Nosocomial and community acquired UTIs are known to be existing with resistance recently. Higher drug resistance among these healthcare associated pathogenic bacteria increases the mortality, morbidity rates and the medical costs. UTIs caused by *Klebsiella pneumoniae* (*K.pneumoniae*) isolates are a major public health problem because of their multidrug-resistance to third generation cephalosporins and for their ability to produce extended spectrum beta- lactamases (ESBLs). To accesses the formation of biofilm formation and ESBL production especially in *K.pneumoniae* isolates from urine samples, this study has been designed in a tertiary care medical college hospital in Mangalore, Dakshina Kannada District, Karnataka, India. According to established standard methods, about 80 urine samples containing *K. pneumoniae* isolates were characterized and subjected for screening to antibiotic susceptibility test using Kirby Bauer disc diffusion and and presumptive ESBL production by double disk synergy test (DDST). In this study, we found that 55 (68.75%) were found to be biofilm producers. 19 (23.75%) isolates were ESBL producers and all them produced biofilm. *K. pneumoniae* isolates producing ESBL had a significantly greater capacity to form strong biofilm (72.4%) than non ESBL producing *K. pneumoniae* isolates (27.58%).

Keywords: Urinary tract infections, *Klebsiella pneumoniae*, Multidrug, ESBL production, Biofilm.

Introduction
Colonization of microbial flora is common in urogenital system which may be opportunistic most of the times.[1] Urethritis, cystitis and acute and or chronic pyelonephritis are terms used commonly to describe Urinary Tract Infections (UTIs).[2]

Common cause for hospital-acquired and community-acquired infections such as UTI, pneumonia, and pyogenic liver abscess is *K. pneumonia*[3] and the most common being UTI due to presence of indwelling urinary catheters.[4] Biofilms appear on any surface as an aggregation of bacteria enclosed in a polysaccharide matrix and favors the bacteria to develop resistance to antibiotics and also against host defense.[5] Pneumonia, UTIs and pyogenic liver absess are nosocomial and community-acquired infections caused predominantly by opportunistic pathogen especially *K. Pneumoniae.*[3]

Biofilm formation can be assessed by methods such as congo red agar method, tissue culture plate (TCP) method and test tube method.[6] *K. pneumonia* belongs to among members of family *Enterobacteriaceae* which is known to produce ESBL. ESBLs are *Beta lactamase* enzymes that cleave beta lactam ring containing antibiotics such as penicillins and broad-spectrum cephalosporins. ESBL and *Carbapenemase* producing strains are cause of concern for Multidrug resistance in *K. pneumoniae* from urinary tract samples. Double Disk Synergy Test (DDST) is a cost effective laboratory diagnostic method to detect ESBL production in clinical isolates of *K.pneumoniae*. Many studies have shown that there is a positive correlation of antibiotic resistance and biofilm formation in *K. pneumoniae* isolates from microbiological clinical samples.[8] The present study was conducted to know the local antibiotic susceptibility pattern, ESBL production and formation of biofilm among *K. pneumoniae* isolated from urine samples in a tertiary care hospital, Mangalore, Dakshina Kannada District, Karnataka.

Materials and Methods
Phenotypic Isolation and identification *K. pneumoniae* from urine samples: A prospective study was designed and conducted in the Department of Microbiology, Yenepoya Medical College and Hospital, Derlakatte, Mangalore, Karnataka, India. Urine samples (midstream clean catch) were collected from suspected UTI patients. Our study collected about 80 *K. pneumoniae* isolates from the urine samples followed by inoculating the same on Mac Conkey's agar and also with 5% Sheep Blood agar and subjected for incubation overnight at 37ºC. The cultured bacterial colonies grown on the agar plates were identified based on morphology and biochemical reactions of the colony using standard microbiological tests.(9) Pure and predominant growth from urine samples containing *K. pneumoniae* isolates were obtained.

Antibiotic susceptibility testing: Bacterial susceptibility to antimicrobial agents was determined by conventional Kirby Bauer's disc diffusion method

(in vitro) using Mueller- Hinton agar (MHA) plates as described by Clinical Laboratory Institute (CLSI) guidelines.[10] The MHA plates were inoculated with a suspension of *K. pneumoniae* adjusted to 0.5 McFarland turbidity standards, ($1x10^8$ cfu/ml).The antimicrobial disks tested were ceftazidime (30µg), cefotaxime (30µg), ampicillin ((10µg), amikacin (30µg) cefpodoxime (10µg), ciprofloxacin (5µg), netilmicin (30µg), piperacillin (100 µg), piperacillin-tazobactam (100/10µg), amoxicillin-clavulanic acid (20/10µg) and imipenem (10 µg). The plates were incubated overnight at 37ºC. The zones of inhibition were measured and compared with the standard measurement chart.

Detection of ESBL production by Double disk synergy test: *K. pneumonaie* isolates that showed resistance to third generation cephalosporins were tested for ESBL production by double disk synergy test (DDST) in accordance with CLSI guidelines.[11] The disk containing amoxicillin – clavulanic acid was placed at the centre of the lawn culture made on Muller Hinton Agar (MHA) plate inoculated with each of the test isolates of *K.pneumoniae* found to be resistance towards any one or all the antibiotic disks of ceftazidime, cefotaxime and cefpodoxime. The discs of ceftazidime, cefotaxime and cefpodoxime each having a disc concentration of 30µg were placed around the central amoxicillin – clavulanic acid disc with a centre to centre distance of 30 mm .The plate was incubated at 37°C for 24 hrs. If there was any enhancement of zone of inhibition between any one of the cephalosporin disks with the central disk the isolate was considered to be an ESBL producer.

Detection of biofilm formation: The standand tissue culture plate (TCP) assay (Christensen *et al*.,) was used for assessment of biofilm formation.[12] *Trypticase* Soy Broth (TSB) (10ml) containing glucose (1%) was inoculated with loopful of *K.pneumoniae* on an overnight culture on nutrient agar. The resultant broth was incubated at 37°C (24 hours). The culture was further diluted 1:100 with fresh medium. The wells in the TCPs were filled with 0.2ml of diluted cultures individually and were incubated at 37°C for 24 h. Following incubation, the entire contents of the wells were removed and the plate was tapped gently. The wells were washed with phosphate buffer saline (0.2 ml) to remove excess of free floating bacteria if any. Those biofilms which remained adherent to the walls and the bottoms of the wells, were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with de-ionised water and plates were dried. Optical densities (OD) of stained adherent biofilms were obtained using a micro ELISA reader at wave length 570 nm. Table 1 shows the criteria for interpretation of OD values.

Results and Discussion

In this study, *K. pneumonia* (80 isolates) from hospital urine samples which was associated with UTIs to know their antibiogram profile with ESBL production was used to detect biofilm formation. Kirby Bauer disc diffusion method for antibiotic susceptibility test revealed increased resistance to ciprofloxacin, but the bacterial isolates were very sensitive to amoxicillin-clavulanic acid, piperacillin-tazobactam and imepenem. Antibiogram of *K. pneumoniae* isolates is presented in **Table 2**. Among 80 of *K. pneumoniae* isolates 26 and 31 isolates were resistant to ceftazidime and cefotaxime respectively. DDST confirmed ESBL production among 19 (23.75%) of these isolates. 55 (68.75%) of the isolates of *K. pneumoniae* were biofilm producers. Fig. 1 shows ESBL production in *K. pneumoniae* isolates by using DDST. Among the ESBL producers 14 (73.68%) were strong biofilm producers and 5 (26.31%) were moderate biofilm producers. Among the 61 non-ESBL producers 10 (16.39%), 7 (11.47%) and 44 (72.13%) isolates were strong, moderate and non-biofilm producers respectively. Fig. 2 shows TCP assay for detection of biofilm formation by *K. pneumoniae* isolates from the urine samples.

K. pneumoniae is a common pathogen associated with both community and hospital-acquired infections including UTIs [13] Biofilm formation is a predominent virulence factor of *K. pneumoniae* and it has a prominent role in several infections which is well documented. Those bacteria which are isolated from biofilms are known to exhibit increased resistance antibiotic when compared to free growing bacteria and this could be attributed to many factors like inability of antibiotics to penetrate to biofilms, expression of drug resistance genes by bacteria and delayed bacterial growth rate.[14] we found that ESBL producing *K. pneumoniae* isolates produced strong biofilms compared to other studies **(Table 1 & 2).**[15-17]

Table 1: Interpretation of OD values for assessment of biofilm formation

Mean OD values	Adherence	Biofilm formation
<0.120	None	None/Weak
0.120-0.240	Moderate	Moderate
>0.240	Strong	High

Table 2: The antibiogram pattern of *K. pneumoniae* isolates from urine samples (N=80)

Antibiotics	Sensitive n (%)	Interme-diate n (%)	Resistant n (%)
Ampicillin	0	0	80 (100)
Amikacin	56 (70)	2 (2.5)	22 (27.5)
Ceftazidime	53 (66.25)	1 (1.25)	26 (32.5)
Cefotaxime	47 (58.75)	2 (2.5)	31 (38.75)
Cefpodoxime	52 (65)	1 (1.25)	27 (33.75)
Ciprofloxacin	23 (28.75)	1 (1.25)	56 (70)
Netilmicin	56 (70)	2 (2.5)	22 (27.5)
Piperacillin	60 (75)	1 (1.25)	19 23.76)

Pip-tazobactum	67 (83.75)	1 (1.25)	12 (15)
Amoxi-clav	68 (85)	0	12 (15)
Imipenem	71 (88.76)	0	9 (11.25)

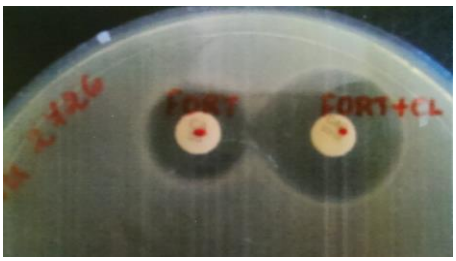

Figure 1: Double disk synergy test showing ESBL production among *K. pneumoniae*

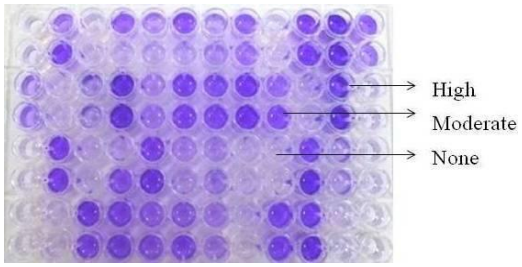

Figure 2: Tissue culture plate assay showing biofilm formation among *K. pneumoniae*

K. pneumoniae has been recognized as predominant pathogen in UTIs due to the emergence of ESBL producing and biofilm forming strains worldwide including India. Our study showed that many of the *K. pneumoniae* strains isolated from urine samples were highly resistant to third generation cephalosporins. There was a positive correlation between antibiotic resistance profile and biofilm forming ability by ESBL producing *K. pneumoniae* isolates. Detection of ESBL-producing microorganisms is required to be performed by every diagnostic laboratory using standard detection methods, so as to detect and control the spread of community and hospital-acquired UTIs. For the detection of ESBL production in a diagnostic laboratory, the DDST is a simple, sensitive, and inexpensive test. However there is a need to emphasize on rational use of antimicrobials especially carbapenems. It is critical to have protocols in place for antimicrobial stewardship and enhanced surveillance control efforts to limit the spread of ESBL producing and biofilm-forming *K. pneumoniae* strains from UTIs. This along with antimicrobial susceptibility surveillance and stringent infection control polices will help in containing the spread of UTIs caused by multidrug resistant *K.pneumoniae* in both the community and hospital-acquired infections.

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