APPENDICES

Annexure 1: Doctoral Committee

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Annexure 2: Ethical clearance certificate

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ETHICAL CLEARANCE

The PhD thesis Titled “Study of virulence factors and antibiogram patterns among clinical isolates of klebsiella pneumoniae” by Mr. Amar Sunil Lobo, PhD Scholar, Yenepoya Medical College, on scrutiny by the Yenepoya University Ethics Committee has been given Ethical Clearance to conduct the study for the stipulated period. Please inform the ethics committee when the study is complete.

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Biofilm formation and extended spectrum beta-lactamase production in Klebsiella pneumoniae isolates from respiratory samples in a tertiary care hospital

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Abstract
Nosocomial and community acquired infections are being caused by Klebsiella pneumoniae (K. pneumoniae) worldwide. K. pneumoniae among gram negative organisms is an important bacterium responsible for site specific infection when they grow as biofilm. Survival of K. pneumoniae is facilitated by biofilm formation that makes it easier to multiply and spread to various sites. Extended-spectrum beta-lactamase (ESBL) producing K. pneumoniae isolates from respiratory samples were used to know the occurrence of biofilm formation conducted in a tertiary care medical college hospital in Mangalore, Dakshina Kannada District, Karnataka, India. A total of 87 K. pneumoniae isolates from respiratory samples were characterized according to standard microbiological specific procedures. Screening was done for K. pneumoniae Biofilm formation in terms of their antibiotic sensitivity pattern by using standard Kirby Bauer disc diffusion method and presumptive ESBL production by double disk synergy test (DDST). Out of 87 clinical isolates from respiratory samples, 45 (51.7%) were found to be biofilm producers. 29 (33.3%) isolates were ESBL producers and all them produced biofilm. ESBL forming K. pneumoniae isolates had a significantly greater capacity to form strong biofilm (72.4%) than non ESBL producing K. pneumoniae isolates (27.5%).

Keywords: Klebsiella pneumoniae, Nosocomial infections, ESBL production, Kirby-Bauer, Biofilm formation.

Introduction
Hospital and community acquired infections are usually attributed to gram negative organisms mainly K. pneumoniae which is being the predominant bacteria involved in these infections with a increased tendency to form biofilm. Nosocomial pathogens K. pneumoniae is one among the eight known bacteria to cause various infections with ability to form biofilm. With the formation of biofilm, there is association of pathogenicity and chronicity of infections caused by this species. The ability of K. pneumoniae to form biofilm can be assessed by various methods such as tissue culture plate method, test tube method and congo red agar method.

K. pneumoniae showing antibiotic resistance is a cause of concern because of ESBL and Carbapenemase producing bacterial strains are being isolated frequently from various part of the world. ESBLs are beta lactamase enzymes that hydrolyze beta lactam ring containing antibiotics such as penicillins and broad-spectrum cephalosporins, ESBL production could be tested making use of Double Disk Synergy Test (DDST) which is a cost effective laboratory diagnostic method. Studies have shown that there is an association between biofilm formation and production of ESBL in K. pneumoniae isolates from various clinical samples. The present study was conducted to know the local antibiotic resistance profile, ESBL production and biofilm formation among K. pneumoniae isolated from respiratory tract samples such as sputum, Bronchoalveolar Lavage (BAL) and Endotracheal aspirate (ET) in a tertiary care hospital, Mangalore, Dakshina Kannada District, Karnataka.

Materials and Methods
Phenotypic identification K. pneumoniae from respiratory samples: A prospective study was conducted in the Department of Microbiology, Yenepoya Medical College and Hospital, Mangalore, Karnataka, India. A total of 87 isolates of K. pneumoniae obtained from various respiratory samples such as sputum, BAL and ETA were included in this study. Sputum samples quality was graded according to Bartlett’s criterion. Clinical samples were inoculated on Mac Conkey’s agar and 5% sheep blood agar and incubated overnight at 37°C. Agar plates were processed and identified to accesses bacterial colony growth based on criteria of morphology and biochemical reactions using standard microbiological tests. Isolates of K. pneumoniae isolates that were obtained as a pure and predominant growth from the above samples were included in this study.

Antibiotic susceptibility testing: As per Clinical Laboratory Institute (CLSI) guideline, antibiotic susceptibility testing was done by using Kirby Bauer’s disc diffusion method using Mueller- Hinton agar (MHA) plates conventionally. By using a suspension of K. pneumoniae adjusted to 0.5 McFarland turbidity standards, (1x10⁸ cfu/ml) MHA plates were inoculated. The antimicrobial disks tested were ceftriaxone (30µg), cefotaxime (30µg), ampicillin (10µg), nafcillin (30µg)
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 Urthritis, 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. pneumoniae* and the most common being UTI due to presence of indwelling urinary catheters.3 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.4 Pneumonia, UTIs and pyogenic liver abscess 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. pneumoniae* belongs to among members of family *Enterobacteriaceae* which is known to produce ESBL. ESBLs are *Beta lactam* 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, Derikatte, 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 Cokney’s agar and also with 5% Sheep Blood agar and subjected for incubation overnight at 37°C. 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.
Hypermucoviscous uropathogenic strains of *Klebsiella pneumoniae* producing extended spectrum beta-lactamase: An experience in South Indian tertiary care hospital

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Abstract
*Klebsiella pneumoniae* (K. pneumoniae), belongs to Enterobacteriaceae family and is the most pathogenic species that is prominently responsible for infections such as septicemia, pneumonia and also urinary tract infections (UTIs). The UTIs are due to Gram negative bacteria and could be both nosocomial and also community acquired which are very difficult to treat as a consequence of antibiotics resistance. The increased incidence of antibiotic resistance among these healthcare associated uropathogenic strains of *K. pneumoniae* leads to increase in mortality, morbidity rates makes the treatment of UTIs less cost effective and predominant emergence and production of extended spectrum Beta-lactamases (ESBLs) by uropathogenic *K. pneumoniae* strains which are of a greater public health concern. This study was conducted to know the occurrence of hypermucoviscosity and ESBL production in *K. pneumoniae* isolates from urine samples in a tertiary care medical college hospital in Dakshina Kannada, Mangalore, Karnataka, India. *K. pneumoniae* isolates were characterized from urine tract samples by using standard microbiological procedures showing hypermucoviscosity which were screened to test antibiotic sensitivity by using Kirby Bauer disc diffusion and also by double disc synergy test (DDST) for presumptive ESBL production. In our study we found, among 80 samples of uropathogenic *K. pneumoniae* isolates, 78 (97.5%) were hypermucoviscous or hypervirulent, 1 (12.5%) isolates were found to be ESBL producers. This shows that ESBL producing *K. pneumoniae* isolates had a greater capacity to produce hypermucoviscosity (100%) than non-ESBL producing *K. pneumoniae* isolates (96.7%).

Keywords: *Klebsiella pneumoniae*, Hypermucoviscous, Uropathogenic, Hypervirulent, ESBL, production.

Introduction
Genitourinary system is often colonized by normal microbial flora including bacteria many of which may act as opportunistic infectious agents. Key opportunistic pathogen for both hospital-acquired and community-acquired infections such as pneumonia, UTI and pyogenic liver abscess is found to be *K. pneumoniae* and group of disorders named urethritis, cystitis and acute and chronic pyelonephritis comes under Urinary Tract Infections (UTIs).1,2

The presence of indwelling urinary catheters is closely associated with UTIs caused by uropathogenic *K. pneumoniae* isolates.3 *K. pneumoniae* UTI symptoms include increased frequency, dysuria, urgency of voiding, and hematuria. *K. pneumoniae* pathogenicity is attributed to several virulence factors like fimbrial adhesins, lipo polysaccharides, capsule and siderophores. The exopolysaccharide capsule is associated with muco viscosity and an increase in its production confers the hypermucoviscous or hypervirulent phenotype of *K. pneumoniae*.1 The extracapsular polysaccharide or hypercapsule is responsible for the expression of hypermucoviscous *K. pneumoniae* strains and aids bacteria to develop resistance to both antibiotics and host defense mechanisms. Hypermucoviscous of *K. pneumoniae* strains can be assessed by a positive string test based on their ability to form mucoviscous strings using colonies grown on 5% sheep blood agar culture plates.6

UTIs are often associated Hypermucoviscous strains of *K. pneumoniae* and are known to produce ESBL among members of family Enterobacteriaceae.7 Beta lactam ring containing antibiotics such as penicillins and broad-spectrum cephalosporins can be made ineffective by ESBLs that shows resistance to antibiotics including carbapenemase producing strains isolated from urinary tract samples. A reliable, simple and economic test is Double Disk Synergy Test (DDST) to detect production of ESBL strains *K. pneumoniae* which is cost effective too. Many studies have shown link between antibiotic drug resistance and hypermucoviscosity from clinical isolates of *K. pneumoniae*.8

The present study was conducted to know the local antibiogram pattern, ESBL production and hypermucoviscosity among uropathogenic strains of *K. pneumoniae* in a tertiary care hospital, Mangalore, Dakshina Kannada District, Karnataka.

Materials and Methods
Phenotypic Isolation and identification of uropathogenic *K. pneumoniae* strains:
A prospective study was conducted in the Department of Microbiology, Yenepoya Medical College and Hospital, Mangalore, Karnataka. Urine samples were collected from suspected UTI patients using standard specimen collection guidelines. 80 *K. pneumoniae* uropathogenic strains were included in this study. Clinical samples were inoculated on Mac Conkey’s agar and 5% Sheep Blood agar and incubated overnight at 37°C. Colonies of bacteria grown on the agar plates were identified by its morphology and biochemical reactions utilizing standard microbiological tests including pure and predominant growth from urine samples containing *K. pneumoniae* isolates.9