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Extended-spectrum beta-lactamase and carbapenemase producing *Klebsiella Pneumoniae* isolated from patient with suspected urinary tract infection

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Abstract

Urinary tract infection (UTI) is the most common bacterial infections in humans and serious health problem in many parts of the world. Carbapenem resistant Enterobacteriaceae (CRE) are increasingly reported from healthcare facilities in Nepal and around the world. This study is carried out with an objective to assess the Prevalence of ESBL and carbapenemase producing *Klebsiella pneumoniae* isolate from patient with suspected of UTI. Total 880 urine sample was collected during 6-month period and processed and identified by using conventional microbiological procedure and biochemical test. ESBL screening isolates were done by using Ceftazidime and Ceftazidime with clavulanic acid. Production of ESBL was determine by combination Disc Test. For the confirmation of carbapenemase producing *K. pneumoniae* Modified Hodge Method, Carbapenem Inactivation Method and EDTA Impregnated Disc test (MBL) was performed. Out of total sample, 9.30% (82) showed significant growth. Prevalence of UTI seen more in male as compare to female patients. Out of positive isolates, 37.8% were *K. pneumoniae*. *K. pneumoniae* were resistance with Ceftriaxone (54.8%) followed by 51.61% Ceftazidime, Cotrimoxazole. Multi drug resistance (MDR) was found to be 54.83%. Out of 31 isolates, 10(32%) were confirmed as ESBL positive by Combination Disc Assay. 14 isolates were screened as carbapenemase producer but 2(14.28%) showed positive through Modified Hodge Method, 4(28.57%) Modified Carbapenem Inactivation Method and 7(50%) EDTA impregnated Disc Test (MBL). Analytically, the sensitivity of MHT, CIM, and MBL tests was 42.81%, 58.10%, and 76.96%, respectively, all with 100% specificity. Colistin was the drug of choice, with 93.54% of isolates showing sensitivity to it. Early detection of ESBL and MBL-producing isolates is crucial for reducing mortality rates and preventing the intra-hospital spread of these strains.

Key words: Carbapenemase, *K. pneumoniae*, Modified Hodge Test, CIM,

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Introduction

Normally, the urinary tract is sterile, but bacteria may rise from the perianal region, possibly leading to urinary tract infection (UTI). Pathogens in the bladder may stay silent or can cause irritative symptoms like urinary frequency and urgency. The pathogenesis of UTIs involves complex interaction between an organism, the environment and the potential host. The symptoms of a person with urinary tract infections depend on the age and the location. Several authors around the world have been reported the Gram-negative bacteria of *Escherichia coli and Klebsiella* spp. being the most frequent organisms causing UTIs[1]. UTIs are one of the most common bacterial infections in childhood, accounting for 5% to 14% of pediatric emergency department visits[2]. Recurrent UTIs in children may be indicative of malformation or malfunction of the urinary tract. Common symptoms and signs of UTIs in children include pain and urgency with urination, blood in the urine, abdominal/pelvic pain, fever, flank pain, and vomiting.

Antibiotic resistance occurs when an antibiotic has lost its ability to effectively control or kill bacterial growth. In recent years, the number of multidrug-resistant bacteria has increased rapidly and several epidemics were identified in different regions of the world. Faced with this situation that presents a major global public health concern, the development and the use of new and rapid technologies is critical [3]. The microorganisms eventually become Multi-Drug Resistant (MDR) forms on continuous use of various antibiotics and become "superbugs". The modes of resistance in such bacterial species include altered target sites, specialized efflux pumps, DNA mutations that develop alternate metabolic

pathways and production of enzymes that degrade antibiotics[4]

Extended-spectrum β-lactamases (ESBLs) are plasmid encoded enzymes that hydrolyze β-lactam ring and cause resistance to β-lactam antibiotics which include third-generation Cephalosporins such as Ceftriaxone, Ceftazidime, Cefotaxime and the Monobactam such as Aztreonam[5]. Resistance increased by the production of beta lactamase enzymes (ESBLs and Carbapenemases) has now become a threat around the world and WHO lists such bacteria in a critical priority category for the discovery of new drugs[6]. In the Gram-positive bacteria, beta-lactamases are secreted to the outside membrane environment as exo-enzymes. In the Gram-negative bacteria, they remain in the periplasmic space, where they attack the antibiotic before it can reach its receptor site [7]

K. pneumoniae carbapenemases (KPC) have the greatest potential for spread due to its location on plasmids. KPCs are capable of hydrolyzing all β-lactams, and strains harboring *bla*KPC often have acquired resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole, creating multi drug resistance organisms (MDRO) [8]

The clinical use of carbapenem is in danger with the emergence of carbapenemases, particularly Metallo-β-lactamase (MBL). MBL has the ability to hydrolyze a wide variety of β-lactam agents, such as Penicillins, Cephalosporins, and Carbapenems. MBLs are inhibited by metal chelators, such as an ethylene diamine tetra acetic acid (EDTA) and thiol-based compounds[9]. MBLs spread easily via plasmids and cause nosocomial infections and outbreaks. Such infections mainly concern patients admitted to the intensive care units with several comorbidities[10]. Therefore, early detection and identification of MBL-producing organisms are of crucial importance for the prevention of nosocomial infection through appropriate treatment [11].

In Nepal, various cases of multidrug resistance are repeatedly being encourage [12,13] ,however, very limited data are available for the resistance pattern of carbapenem resistance Enterobacteriaceae (CRE) though they are frequently being isolated from various clinical samples. The consequences of the treatment of infections caused by these bacteria are very important since, there is practically no therapeutic arsenal for the infections caused by the pathogens producing carbapenemases [14].

Rate of bacterial resistance are markedly higher in many developing countries, probably because of a lack of supervision, poor infection prevention practices, inappropriate use of limited resources, and overcrowding of hospitals[15,16].It seems that the overuse of effective antibiotics is also a potent cause of bacterial resistance especially in these counties. This study aims to determine the occurrence of Extended Spectrum Beta-Lactamase (ESBL) and carbapenemase-producing *Klebsiella pneumoniae* in patients with suspected urinary tract infections (UTIs). The specific objectives are to: isolate and identify bacterial pathogens in urine samples, determine the antibiotic susceptibility patterns of these isolates, evaluate ESBL production using the combination disc test, and assess carbapenem resistance in *K. pneumoniae* using the Modified Hodge Test, Carbapenemase Inactivation Method, and EDTA impregnated disc test. These tests will identify resistance mechanisms and confirm the presence of carbapenemase enzymes.

Materials and methods

The cross-sectional study was carried out in Pathology Department of International Friendship Children's Hospital, Maharajgunj, and Kathmandu from July 2017 –January 2018. During the period, a total of 880 urine samples were collected and processed according to the standard laboratory methods. Patients under the age of 12 years or their guardians visiting the Pathology Department were directly interviewed for his/her clinical history during the sample collection.

Clinical sample (Midstream urine) was collected and transported to the laboratory by using standard laboratory procedures. The quality of the clinical samples was evaluated before processing and the improper specimens were rejected and patients were requested to submit another sample properly.

Bacterial isolation and identification

The urine sample was macroscopically examined by observing its color and appearance, and the findings were reported accordingly [17,18].

The urine culture was conducted by inoculating samples on McConkey agar and Blood agar plates using a semi-quantitative culture technique with a standard calibrated loop. The plates were then incubated at 37°C overnight (18-24 hours). Colony counting was performed to determine the number of colony-forming units (CFU) per milliliter of urine, and the bacterial count was reported.

The bacterial cultures from the selective and differential media were further cultured on Nutrient Agar (NA) and incubated overnight at 37^0 C for isolation of pure

cultures. The isolated bacteria were identified using standard microbiological tools and techniques as described in the Bergey's Manual of systemic bacteriology[19]which involves examining the morphological, staining and biochemical properties.

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing of Enterobacteriaceae members towards various antibiotics was performed using the modified Kirby-Bauer disk diffusion method, as recommended by Clinical and Laboratory Standards Institute [12]on Muller Hilton Agar. Antibiotics such as Amoxicillin clavunate (20+10 µg), Amikacin (30 µg), Cotrimoxazole (25 µg), Ciprofloxacin (5 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), Gentamicin (10 µg), Imipenem (10 µg) and Meropenem (10 µg) were used for antimicrobial susceptibility testing and their zone size interpretive chart. Zone of Inhibition of Meropenem and Imipenem were observed for the screening of organisms for the production of carbapenemase. Finally, isolates producing β-lactamase were selected as described by Livermore and Brown [20]and multi drug resistant (MDR) isolates were selected as described by Magiorakos [21]

After conducting antimicrobial susceptibility testing, multidrug-resistant (MDR) isolates in pure culture were preserved in 20% glycerol-containing Tryptic Soy Broth and stored at -70°C until subsequent tests for the detection of carbapenemase production could be performed.

ESBLs test

The Clinical and Laboratory Standards Institute (CLSI) recommends a phenotypic confirmatory combined-disk test for ESBL production in *K. pneumoniae*. This test involves measuring the growth-inhibitory zones around Ceftazidime (CAZ) disks with and without clavulanate (CA) (CLSI 2012). An increase in zone size of ≥5 mm from combination disk i.e. clavulanate containing disk compared to Ceftazidime alone indicate the presence of ESBL in the test organism.

MBL test

The strains resistant to Ceftazidime and carbapenems were confirmed for MBL by CDDT using IMP (10mg) + 10µl-0.5 M EDTA (750µg). An increase of 7mm or more in zone diameter in the presence of EDTA compared to those with IMP tested alone was considered to be a positive test for the presence of MBL [22].

Phenotypic confirmation of Carbapenemase production

The confirmation of carbapenemase production was determined by Modified Hodge Test (CDC, 2009). According to the guidelines, a cloverleaf-type indentation appears after 16-24 hours of incubation of test organism streaked on an MHA plate with a lawn culture of *E. coli ATCC* 25922 within the zone of inhibition of carbapenem susceptibility disc, confirming the production of carbapenemase.

Carbapenem Inactivation Method [23],

According to guidelines, the test is based on the principle that when a 10-µg Meropenem (MEM) disk is incubated for 2 hours in an aqueous suspension of a carbapenemase-producing microorganism, the carbapenem in the disk is degraded by the carbapenemase. In contrast, if the test microorganism does not produce carbapenemase, MEM retains its antimicrobial activity after incubation in the bacterial suspension. The disk is removed from the suspension and placed onto a Mueller-Hinton agar (MHA) plate seeded with a suspension of a carbapenem-susceptible *E. coli ATCC* 25922. Following overnight incubation, the zone of inhibition is measured to determine whether the MEM had been hydrolyzed (growth of the indicator organism close to the disk), or is still active (a large zone of inhibition around the disk).

Result

A total of 880 urine samples were processed for bacterial culture, revealing that 82 (9.30%) exhibited significant growth. The highest incidence of significant growth was observed in the 9-12 age group (10.5%), followed by <4 (9.8%) and 5-8 (7%). Overall, 51.22% of males and 48.78% of females showed significant growth. Among the 82 positive urine samples, the predominant isolates were 41 (50%) *E. coli*, 31 (37.8%) *K. pneumoniae*, 6 (7.30%) *Pseudomonas* spp, and 4 (4.9%) *Citrobacter* spp. The sensitivity and specificity of conventional microbiological procedures and biochemical tests used in identifying bacterial species can vary based on the specific tests employed and the conditions under which they are conducted. They are designed to distinguish *Klebsiella pneumoniae* from other bacteria based on specific biochemical characteristics. The Voges–Proskauer test, Simmon's citrate test, and catalase tests were positive for *Klebsiella* spp. On Triple Sugar Iron (TSI) test, the slant was yellowish with no changes in butt and no H2S produced, but gas bubble appeared. Notably, 93.54% of *K. pneumoniae* isolates were sensitive to colistin, followed by Amikacin (71%), Nalidixic acid

(71%), Ciprofloxacin (64.5%), Gentamicin (54.8%), Imipenem (54.8%), and Meropenem (51.6%). Out of the 31 *K. pneumoniae* isolates, 17 (54.8%) were classified as multidrug-resistant (MDR), with 16 (94%) displaying resistance to Ceftazidime, and high resistance rates to Meropenem (82%), Imipenem (82%), Cotrimoxazole (82%), Ceftriaxone (71%), Nitrofurantoin (71%), and Ciprofloxacin (65%).

Table: 1 Antibiotic susceptibility pattern

Antibiotic	Sensitivity	Intermediate	Resistance
Gentamicin	17 (54.8%)	2 (6.5%)	12(38.7%)
Ciprofloxacin	20 (64.5%)	0	11 (35.5%)
Cotrimoxazole	15 (48.4%)	0	16 (51.6%)
Ceftazidime	14 (45.2%)	1 (3.22%)	16 (51.6%)
Meropenem	16 (51.6%)	1 (3.22%)	14 (45.2%)
Ceftriaxone	14 (45.2%)	0	17 (54.8%)
Imipenem	17 (54.8%)	0	14 (45.2%)
Nitrofurantion	15 (48.4%)	2	14 (45.2%)
Amikacin	22 (71%)	0	9 (29%)
Polymixin B	19 (61.3%)	5 (16.13%)	7(22.6%)
Colistin	29 (93.5%)	0	2 (6.5%)
Nalidixic acid	22 (71%)	3(9.7%)	6(19.4%)

Seventeen *K. pneumoniae* isolates were suspected of extended-spectrum beta-lactamase (ESBL) production, and 10 (32.25%) were confirmed as ESBL producers. Among the ESBL producers, the highest percentage was observed in the ≤4 age group (60%), followed by 5-8 (30%) and 9-12 (10%). Out of the 31 *K. pneumoniae* isolates, 7 (23%) males and 3 (10%) females were ESBL positive.

Out of the 31 *K. pneumoniae* isolates screening positive for carbapenemase, 14 were confirmed as metallo-beta-lactamase (MBL) producers, with 7 (50%) confirmed by the EDTA impregnated disc test. Among the carbapenemase screening-positive isolates, 2 (14.3%) were positive for the modified Hodge test (MHT), 4 (28.60%) for the Carbapenem Inactivation Method (CIM), and 7 (50%) for MBL. The MHT, CIM, and MBL tests demonstrated sensitivities of 42.81%, 58.10%, and 76.96%, respectively.

Table 2: Phenotypic conformation of carbapenemase confirmation

S.N	Confirmation tests	No. of suspected carbapenemase producer	No. of confirmed cases
1.	Modified Hodge test	14	2(14.3%)
2.	Modified Carbapenem inactivation test	14	4 (28.6%)
3.	EDTA impregnated disc test (MBL)	14	7 (50%)

Discussion

Urinary tract infection (UTI) stands out as one of the most prevalent bacterial infections in humans, posing a significant health concern globally. In this study, the prevalence rate of urinary pathogen isolation was 9.30%, with 82 cases identified out of 882 patients. This rate contrasts with findings from Pradhan[23]at Kathmandu Medical College Teaching Hospital, Duwakot (13.8%), and Saffar et al. 2008 from Iran (12.6%)[24]. The prevalence among male patients (51.22%) was higher compared to female patients (48.78%), consistent with Gautam[25]report of 57% in males and 43% in females. UTI incidence varied by age and sex in children [2]with male children exhibiting a significantly higher UTI rate than females, possibly attributed to the longer urethra in females [1]. The study identified four gram-negative bacterial species, with *E. coli* accounting for 50% of UTI cases, followed by *K. pneumoniae* (37.8%), *Proteus* (7.30%), and *Citrobacter* (4.87%). Similar patterns were observed in studies by [26,27].

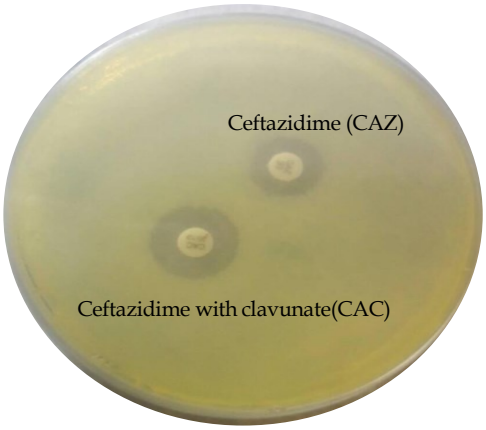
Antibiotic susceptibility testing revealed that 93.54% of *K. pneumoniae* were sensitive to Colistin, with varying sensitivities to other antibiotics Amikacin and Nalidixic acid (70%), Ciprofloxacin (64.52%), Gentamicin (54.84%), Nitrofurantoin (48.39%) and Ceftazidime, Ceftriazone (45.16%)). Similarly, Gentamicin susceptibility was 57.45%, followed by Ceftriaxone (51.06%) and Nitrofurantoin (45.75%) in a study at Seti Zonal Hospital[28].

The emergence of multi-drug resistance (MDR) in uropathogens, potentially due to plasmid transfer of resistance genes [29],poses a major challenge. This study identified 54.83% MDR isolates, consistent with Poudyal [30], though Thakur [31]reported a higher occurrence (64%). Urgent measures such as improved infection control, prudent antibiotic use, and revisiting empirical regimes are needed to curb further resistance[32]. In Nepal, studies have documented diverse rates of multidrug resistance in *E. coli* (38.2-95.52%) and *K. pneumoniae* (25-100%)[15,33] Hospitalization and prior antibiotic use were identified as common risk factors [34].

Among MDR isolates, 94% were resistant to Ceftazidime, followed by 82% for Cotrimoxazole, Meropenem, Imipenem and 71% for Ceftriaxone. In contrast, 10(32.25%) *K. pneumoniae* isolates were indicated as ESBL producers, exhibiting higher antibiotic resistance to carbapenem and cephalosporins, which are essential drugs for the treatment of UTIs. According to Metri [35], ESBL production was reported

44.9 % and Taneja et al [36] reported 51.2%. In this study, the high prevalence of ESBL presents a threat to the effective treatment of serious infections caused by these isolates. Ampicillin, Cotrimoxazole, Ciprofloxacin, and Ceftazidime were found to be ineffective against the majority of ESBL-producing isolates. Limited options, such as expensive carbapenems and Tigecycline, exist for treatment. Notably, ESBL-producing organisms, associated with poor outcomes and increased hospital expenses, were prevalent [37]

From clinical specimens of patients visiting a tertiary care hospital, Ghimire et al. [38]reported that 25.8% of *K. pneumoniae* were identified as ESBL producers. In the capital hospital and research center, Kathmandu, Chaudhary [39] identified 18.4% of *K. pneumoniae* isolates as ESBL producers. Meanwhile, from a tertiary care hospital in Kathmandu, Nepal, Chander [40] reported that 16.55% of *K. pneumoniae* isolates were confirmed ESBL producers. In this study, ESBL producer was found in male as compare to female mostly in ≤4 age group (60%). This data is insignificant with gender.



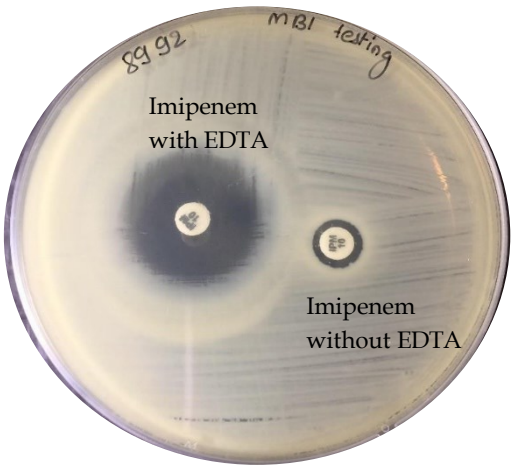
Photograph 1: Confirmation of ESBL production using Combine Disc Assay

Carbapenem resistance in *Klebsiella* spp. is primarily attributed to the production of carbapenemases, with the most prevalent types belonging to Ambler class A, notably KPC, or Ambler class B, (MBLs) such as IMP and VIM types, representing the most common mechanisms[41]. Unfortunately, in the present study, we were unable to perform the genotypic tests due to limited resources. Traditional phenotypic methods like CIM, MBL and MHT remain essential due to their accessibility and reliability, newer methods such as Carba NP test, WGS, and PCR-based assays offer higher accuracy, rapid turnaround, and broader detection capabilities for ESBLs and carbapenemases. The choice of method often depends on the specific clinical or epidemiological context, resource availability, and desired level of detail in resistance profiling.

Early detecting MBL-producing isolates is crucial for reducing mortality rates and preventing the intra-hospital spread of these strains. In a nutshell, this test can identify and differentiate resistant pathogens, leading to improved patient outcomes, enabling effective infection control, and mitigating the escalation of resistance.

This study demonstrated 50% *K. pneumoniae* isolate were MBL producer. In comparison to Humera [41]where MBL producer *K. pneumoniae* was found 67.16%, was higher than this study finding.

From the pool of 31 *K. pneumoniae* isolated, 14 *K. pneumoniae* isolates were resistant to at least one cephalosporin and producing carbapenem zone diameter of < 21mm (Imipenem or Meropenem) were screened as indicative of carbapenemase production or as a possible carbapenem resistant bacterium in this study. However, only 7 were found to be carbapenemase producer through EDTA impregnated test. In a similar study done by Datta et al from India, the prevalence of carbapenem resistant bacteria belonging to Enterobacteriaceae family was 7.87% and the rate of MBL type carbapenemase production was 5.75% [33] have reported that the combined disk test (IPM-EDTA) is the most sensitive method, suitable as a convenient screening approach for detecting MBL production in Gram-negative bacilli in routine microbiological practice.



Photograph 2: Confirmation of carbapenemase production by EDTA Impregnated test (MBL)

According to the World Health Organization (WHO) in 2014, carbapenem resistance rates among *K. pneumoniae* were reported as follows: 68% in Europe, 55% in South-East Asia, 54% in the Eastern Mediterranean region, 11% in the Western Pacific, 9% in the Americas, and 3.5% in Africa. [42]

In this study, Modified Hodge Test was positive in 2

(14.3%) out of 14 screened isolates. Other report a higher occurrence in comparisons to this result, Amjad et al [43]reported 17%, [10]Ramana et al reported 30% and G C[44] reported 16.6%. While carbapenemase resistance poses a significant global challenge, the prevalence of carbapenemase resistance genes varies epidemiologically across different regions. The most common ones include Extended-Spectrum Beta-Lactamases (ESBLs) Genes: bla_TEM, bla_SHV, bla_CTX-M. These genes are widely distributed globally, with bla_CTX-M being the most prevalent ESBL gene in many regions. Carbapenemases Genes: bla_KPC (Predominantly found in the Americas), bla_NDM (First identified in India and now found worldwide), bla_VIM and bla_IMP (Common in Southern Europe and parts of Asia.), bla_OXA-48 (Frequently detected in the Middle East, North Africa, and increasingly in Europe) Plasmid-Mediated AmpC Beta-Lactamases Genes: bla_CMY, bla_DHA, bla_ACC, bla_ACT, bla_MIR, bla_FOX. These genes are also found worldwide, with varying prevalence depending on the region[45]. According to Song studies, he reported the total of sensitivity of MHT were 76.8%. MHT had 97% sensitivity and 100% specificity [46]. The MHT, recommended by CLSI, serves as a phenotypic confirmatory test to detect carbapenemase production in Enterobacteriaceae isolates with elevated MICs for carbapenems or reduced inhibition zones in disc diffusion susceptibility testing. Its sensitivity and specificity for carbapenemase detection are considered acceptable. However, its sensitivity and specificity for detecting low-level MBL production remain unknown. Various studies have also reported instances of weakly positive results for the MHT in the detection of MBL-producing Enterobacteriaceae [10]

In this study, 4 (28.6%) were found to produce enzyme that hydrolyses carbapenem through Carbapenemase Inactivation Method. Lamiaa [47]reported CIM identified 45.8% of isolates as carbapenemase-producers with a sensitivity of 95.7% and specificity of 95.5%. In this study, MHT and CIM were found to be sensitivity with 42.81% and 58.10% respectively. These bacteria exhibit high carbapenem resistance attributed to the production of acquired carbapenemases, encompassing metallo-β-lactamases like IMP, NDM, and VIM types, as well as KPC-type β-lactamases.[48] While the core resistance genes in *Klebsiella pneumoniae* found in Nepal are likely to be similar to those found globally (such as bla_NDM, bla_CTX-M, and bla_SHV), the specific prevalence and combinations of these genes can vary. Nepal may have a higher prevalence of certain genes like bla_NDM due to regional factors such as antibiotic usage patterns and healthcare practices.

Conclusion

The study highlights the alarming prevalence of UTIs and the escalating challenge of antibiotic resistance, particularly multi-drug and carbapenem resistance. Nepal's unique geography, with sparsely populated hilly and mountainous regions, limited healthcare resources, and a low-income economy, centralizes healthcare facilities in Kathmandu, leading people from remote areas to travel there for treatment. These individuals may acquire drug-resistant pathogens and act as carriers when they return to their villages. Multi-center studies in major healthcare facilities across Nepal are necessary to understand the prevalence of ESBL and carbapenemase-producing uropathogens better. Molecular epidemiological studies of resistance genes among these uropathogens are also essential. This report highlights the emergence and prevalence of ESBL-producing *K. pneumoniae* in urinary isolates in Nepal, stressing the need for strict hospital infection control policies, prudent antimicrobial use, and regular monitoring of ESBL-producing clinical isolates in laboratories.

Author's contribution

RB is the principal investigator, also drafted and edited the manuscript. PN and PR helped in laboratory. ST guide during the lab work and RM conceptualized the research.

Competing Interests

This study does not involve any competing interests

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