Multidrug-resistant nosocomial *Citrobacter* in a Hospital in Kathmandu

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ABSTRACT

Citrobacter sp. is a commensal flora and an infrequent nosocomial pathogen to cause nuisance in hospital settings. Recently, the isolation of this pathogen in health care setting is rising and the multidrug resistant strains are emerging. This pathogen cause wide array of infections and the mortality rate is unexpectedly high of 30.0-60.0%. Extended spectrum cephalosporins have been used to treat this pathogen and due to the emergence of resistant strains to these drugs newer treatment protocols have to be devised. Epidemiology and antibiotic susceptibility pattern of clinical isolates of *Citrobacter* sp. isolated in a hospital were investigated. Specimens were collected from patients and implicated pathogens were isolated. Disk diffusion test was performed on these isolates and resistant patterns were. Antibiogram typing was used to resolve the clones of the isolated bacteria. The results showed that *Citrobacter* sp. was highly prevalent and commonly isolated from the sputum sample of patients diagnosed as Chronic Obstructive Pulmonary Disease (COPD). The antibiogram pattern suggested the circulation of three clones of *Citrobacter* sp. They were multidrug resistant and were sensitive to only cefoperazone and sulbactam suggesting the production of â-lactamse inhibitors sensitive molecular class A and D extended spectrum â-lactamases (ESBL). In conclusion, although, ESBL producers are always treated with carbapenems, we recommend to use combination therapy of â-lactam and â-lactamse inhibitors to treat this multidrug resistant *Citrobacter* sp. and carbapenems should be kept as a reserve drug and we should aim to prevent the spread of this resistant pathogen between different patients to decrease the morbidity and mortality associated with this pathogen.

Keywords: *Citrobacter* sp., nosocomial pathogen, COPD, treatment option.

INTRODUCTION

Nosocomial infection is a major public health concern these days and a cause of substantial mortality and morbidity for hospitalized patients. Methicillin Resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Kleibsella pneumoniae*, and *Citrobacter* are most common nosocomial pathogens. Although, *Citrobacter* sp. is less frequently isolated, they are emerging as a nosocomial multidrug resistant pathogen across the globe and also in Nepal.[1] They are facultative anaerobe, oxidase negative Gram-negative bacilli within the family of *Enterobacteriaceae* and are ubiquitous in nature (food, soil, and water) and colonizer of human gastrointestinal tract. A study focusing on pathogens in discharging otitis media in the same hospital showed that *Citrobacter* sp. was 1.4%.[2] Another study conducted in Tribhuvan University Teaching Hospital, Kathmandu revealed that *Citrobacter* sp. was one among other pathogens to be isolated from surgical wound infections.[3] *Citrobacter* sp. was also isolated in urinary sample in the pediatric patient in Manipal Medical College Hospital.[4] Among 11 different species under this genus, *C. freundi* and *C. Koseri* (*formerly C. diversus*) are the major species implicated in infections. A retrospective cohort study conducted in 700-beded hospital during ten year period revealed *C. frundii* as a most common species followed by *C. koseri,*[5] while a study conducted in France showed that *C. koseri* as a most common pathogen.[6] SENTRY, antimicrobial surveillance program reported that *C. frundii* rates 11th most common pathogens to cause blood stream infections.[7] Other major infections are respiratory tract infections, urinary tract infections, and meningitis. In infants, meningitis due to *Citrobater* sp. has a mortality rate of approximately 30.0% and on those who survive, more than 80.0% have some degree of mental retardation.[8]

Urinary tract infection by *C. koseri* has been reported to be 12.0% in 1961[9] and the prevalence rate is rising. Invasive procedures like, catheterization helps them to colonize urinary bladder and during intensive chemotherapy this bacterium disseminates to the blood stream to cause severe bacteremia. Intact immunity helps to control the pathogen to certain extent but when the patients are immunocompromised, the situation is grave. The problem is further intensified by the emergence of multidrug resistance *Citrobacter* sp. resulting into treatment failure.

Table-1: Patient diagnosis, specimens collected, and pathogens isolated

Patients	Diagnosis	Ward	Specimen for culture	Pathogen isolated
1	RTAa, multiple Injury	Surgery	Wound swab	Citrobacter spp.
2	HTNb with COPDc	Medicine	Sputum	Citrobacter spp.
3	Laparotomy for intestinal obstruction	Surgery	Pus	Enterococcus spp.
4	COPD with RHFd	Medicine	Urine	Enterobacter spp.
5	Subacute Intestinal obstruction	Surgery	Wound swab	K. pneumoniae
6	Pyrexia of unknown origin	Medicine	Blood	Salmonella typhi
7	Suction tip	Surgery	Swab	K. pneumoniae
8	Diabetes, HTN, COPD, ALDe	Medicine	Sputum	Citrobacter spp.
9	COPD	Medicine	Sputum	Citrobacter spp.
10	Ovarian Cacinoma, Febrile neutropenia	Gynaecology	Urine	E. coli
11	Subacute intestinal obstruction	Surgery	Peritoneal fluid	Citrobacter spp.

[a]Road Traffice Accident, [b]Hypertension, [c]Chronic Obstructive Pulmonary Disease, [d]Right Heart Failure, and [e]Alcohoilic liver Disease.

Recently, we have been frequently isolating *Citrobacter* sp. from various specimens from different patients in this hospital. â-lactam antibiotics like, penicillins; 3rd, and 4th generation cephalosporins either alone or in combination with â-lactamase inhibitors are commonly used to combat these infections but the resistance to most antibiotics in this class has already been noticed. The mechanisms underlying the resistance to â-lactam antibiotics in *Citrobacter* sp. is conferred by the presence of narrow and extended spectrum â-lactamases (ESBL), plasmid and chromosomal AmpC cephalosporinase, metallo â-lactamases, and loss of outer membrane porins. Here, we investigated the epidemiology of *Citrobacter* sp. in patients admitted in a hospital and tracked down antibiotic susceptibility pattern and recommend a guideline for the treatment of multidrug-resistant *Citrobacter* sp.

MATERIALS AND METHODS

Patients, samples, and bacterial identification: This study was conducted in 150 bedded Hospital which is located in Kathmandu, Nepal. Patients included in this study were admitted patients in the hospital during 21-28th February, 2009. Inclusion criteria were, i) patients with culture negative during the time of admission, ii) any form of infections developed after 48 hours of admission (nosocomial), iii) patients with specimen culture positive later during the course of stay in a hospital, and iv) antibiotic uses. Specimens like, blood, sputum, urine, wound swabs, pus, peritoneal fluid, and swab from suction tube were collected and bacterial identification was carried out by performing biochemical tests following the standard procedures.[10]

Antibiotic susceptibility test: Antibiotic susceptibility test was performed on Muller Hinton agar using the Kirby-Bauer disk diffusion test following manufacturer's guidelines (HiMedia Pvt. Ltd, Mumbai, India). The antibiotic disks and the concentration used were as follows; cefoperazone sulbactam (75/10µg), ceftriaxone (30 µg), ampicillin (10 µg), cefixime (5 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cephalexin (30 µg), ceftazidime (30 µg), co-trimoxazole (trimethoprim, 1.25/sulphomethoxazole, 22.75 µg), naledexic acid (30 µg), nitrofurantoin (300 µg), and norfloxacin (10 µg). Inhibition zone size was interpreted as resistant, intermediate, and susceptible following the manufacturer's guidelines.

Bacterial clones

Antibiogram typing was used to resolve the isolated *Citrobacter* to the clonal level.

RESULTS

Sample source and bacteria identified

Ten patients met our criteria and further investigation

Table-2: Antibiotic susceptibility test of pathogen isolated.

Patient	Pathogen isolated	Antibiotics tested													Clone
		CF	C	CO	G	A	CH	CA	CK	CFX	CS/S	NX	NF	NA	I
1	Citrobacter spp.	S	R	R	R	R	R	R	R	R	S	NT	NT	NT	I
2	Citrobacter spp.	S	R	R	R	R	R	R	R	R	S	NT	NT	NT	
3	Enterococcus spp.	R	R	R	R	S	R	R	R	R	S	NT	NT	NT	
4	Enterobacter spp.	R	S	R	R	R	R	R	R	R	S	NT	NT	NT	
5	K. pneumoniae	R	R	R	R	R	R	R	R	R	S	NT	NT	NT	
6	Salmonella typhi	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	
7	K. pneumoniae	R	R	R	R	R	R	R	R	R	S	NT	NT	NT	
8	Citrobacter spp.	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	III
9	Citrobacter spp.	R	R	R	R	R	R	R	R	R	S	NT	NT	NT	III
10	E. coli	S	S	S	S	S	R	R	NT	NT	S	S	S	S	
11	Citrobacter spp.	R	R	R	R	R	R	R	R	R	S	NT	NT	NT	II

Abbreviations: CF, Ciprofloxacin; C, Chloramphenicol; CO, Co-Trimoxazole; G, gentamicin; A, Ampicillin; CH, Cephalothin; CA, Ceftazidime; CK, Ceftriaxone; CFX, Cefixime; CS/S, Cefoperazone/ Sulbactam; NX, Norfloxacin; NF, Nitrofurantoin; NA, Naledexic Acid . Cephalothin disk is used for testing susceptibility for Cephalexin and Cephadroxil and Ampicillin disk is used to interpret susceptibility to Amoxicillin.

was carried out on these patients (Table-1). These patients were admitted with different diseases and various specimens were collected from them. Most of the specimens represented sputum (3/11). Wound swab and urine represented two each, and each of them was pus, blood, and peritoneal fluid. A swab from the suction tip from the surgery ward was also included.

Citrobacter, K. pneumoniae, E. coli, S. typhi, Enterbacter , and *Enterococcus* were isolated from various specimen sources (Table-1). All bacteria isolated from sputum were *Citrobacter* (patients 2, 8, and 9) and was also isolated from wound swab (patient 1) and peritoneal fluid (patient 11). All three sputum samples were collected from the patients diagnosed as Chronic Obstructive Pulmonary Disease (COPD). Blood sample yield *S. typhi*. From urine samples *Enterobacter* and *E. coli* were isolated. *K. pneumoniae* was isolated from wound and swab from suction tip. *Enterococcus* was isolated from pus.

Antibiotic susceptibility test

Antibiotic susceptibility test is given in Table-2. Four isolates of *Citrobacter* were resistant to two or more than two classes of antibiotics as suggested by no zone or small zone of inhibition (patients 1, 2, 9, and 11). All of them were multi-drug resistant. One Citrobacter isolated from sputum (patient 8) was sensitive to all antibitotics. All of the five *Citrobacter* sp. were sensitive to cefoperazone/sulbactam combination and two of them were sensitive to ciprofloxacin. Other species isolated from the patients were also sensitive to this combination. *K. pneumoniae* was sensitive to only this combination. *Enterobacter* and *Enterococcus* were further sensitive to amoxyxillin and chloramphenicol respectively. *E. coli* was sensitive to most of the antibitotics except for the cephalexin and ceftazidime. *S. typhi* was sensitive to all antibiotics tested.

BACTERIAL CLONES

Bacteria with the same antibiogram can be considered as a same clone. Antibiogram for *Citrobacter* isolated from patients 1 and 2 were same and designated clone I, *Citrobacter* isolated from patient 9 and 11 also had same antibiogram but different from cone I, and this represented clone II, and *Citrobacter* , isolated from patient 8 had antibiogram different from clone I and II and represented clone III (Table-2). *K. pneumoniae* isolated from the patient and the suction tips had the same antibiogram and represented the same clone. Other species of bacteria represented individual clone.

DISCUSSION

Citrobacter, K. pneumoniae, E. coli, S. typhi, Enterbacter sp, and *Enterococcus* were implicated in several infections in this hospital. Most common nosocomial pathogen was *Citrobacter* and the prevalence rate was 50.0%. Similar high prevalence (62.0%) has been reported elsewhere.[6] Most of this bacterium (3/5) were isolated from the respiratory tract infection (COPD) and might suggest as a common pathogen to infect this group of patients. It has also been demonstrated that this pathogen is frequently isolated from sputum. This pathogen was also isolated form surgical wounds and

peritonitis.[11] All of these infections with this pathogen have already been reported from different parts of world and also from Nepal.[2,3,5] Urinary tract infection, bacteremia, and meningitis were also common[9] but we did not isolate this pathogen in these infections in our setting. This might be because of less number of urinary and blood samples analyzed, no case of meningitis, and short duration of period studied. To our surprise, *P. aeruginosa* and *A. baumannii* which are frequently isolated in Intensive care units and various units were not isolated from any samples.

We investigated the clonality using antibiogram. Same clones of bacteria have same antibiotic patterns and most likely same resistance genes and same clones can be presumed. Antibiogram resolved *Citrobacter* into three clones. Clone I and Clone II were multidrug resistant and clone III was a susceptible clone. This reflects the oligoclonal situation. This is true for a leading private hospital which is equipped with acute care facilities, intensive care units, and infectious disease wards and an inter- and intra-hospital transfer of infected patients are common and polyclonal nature of this pathogen is a true phenomenon. Horizontal gene transfer is common among members of *Enterobacteriaceae* and this may distort the clonal framework. Endemicity of the individual clones couldn't be confirmed as the time of study was too short. Bacterial isolates representing different time points should be included to confirm this data. *K. pneumoniae* isolated from patient and suction tips from surgery ward had same antibiogram pattern means that the source of this pathogen was the suction tip. This highlights that the pathogen is spreading from patient to patient through the use of suction tip.

Antibiogram is cost effective, easy, rapid tool and can be used as typing tool in a low resource country like ours where molecular typing tools are not accessible but it is not a reliable marker for bacterial typing. This is because nosocomial pathogens like *Citrobacter* and other gram negative bacteria like, *E. coli, K. pneumonia, P. aeruginosa, and A. baumannii* share same ecological niche and are very promiscuous. Resistance genes can transfer via horizontal gene transfer (conjugation, transduction, and transformation) among these pathogens. This transfer is perpetuated by antibiotic and environmental selection pressure which favors acquisition of resistance genes or mutation in drug target sites. These resistance genes can be lost, and back mutation can occur at any time when selection pressure is withdrawn. This suggests that resistant genes can be lost and gained at any time and can't be used as a stable marker for typing bacterial isolates. More robust polymerase chain reaction (PCR) based molecular typing tools like randomly amplified polymorphic DNA (RAPD) and multi locus sequence typing (MLST) and other tools like restriction fragment length polymorphism (RFLP); pulse field gel electrophoresis (PFGE), and ribotyping are highly recommended for clonal analysis.[12] However, these techniques were not used in this study.

Ciprofloxacin, aminoglycosides, and â-lactam (extended spectrum cephalosporins, carbapenems) antibiotics are widely used in the treatment of *Citrobacter* sp. infection and resistance to these drugs have already been emerged.[9,12,13] Mechanism of resistance to â-lactam antibiotics are; mutation in the drug target sites (penicillin binding protein), acquisition of resistance genes like â-lactamase (plasmid encoded AmpC cephalosporinase, integron elements carrying narrow and extended spectrum cephalosporinases (bla_{TEM}, bla_{SHV}), efflux pumps and alteration in the porins which acts as a channel for a drug entry.[14] â-lactamase are classified by Ambler into molecular class A, B, C, and D.[15] Important mechanism of resistance to â-lactams is the possession of molecular class C chromosomal AmpC cephalosporinase which are well described in this species. The mechanism of resistance due to AmpC is unlikely in these multiresistant *Citrobacter* isolated here, as this enzyme is not inhibited by â-lactamase inhibitors. TEM, SHV, and OXA are narrow spectrum, molecular class A and D â-lactamase but when mutation occurs in these enzymes they can hydrolyze extended spectrum cephalosporins and are called ESBL. â-lactamse inhibitor sensitive ESBL production is likely mechanism of resistance to â-lactam antibiotics as they can hydrolyze penicillin, narrow and extended cephalosporins and are inhibited by â-lactamase inhibitors, which was seen in multi-resistant *Citrobacter* sp. studied here. ESBL are plasmid borne and are widely disseminated in this species and other Gram-negative bacteria.[16]

Multidrug resistance *Enterobacteriaceae* are characterized by the presence of mobile genetic element like, class 1 integron element which has been described as "natural cloning and expression vector for resistance genes".[17] Aminoglycosides resistance gene (ant (3")-I-b), narrow spectrum â-lactamses (bla_{TEM}, bla_{SHV}), carbapenemases (bla_{IMP}, bla_{VIM}), sulfonamide resistance gene (*sul*1), and trimithoprim resistance gene (*dfr*1) are carried as an array of resistant cassettes in this mobile genetic element. When one antibiotic is challenged to the bacteria carrying the corresponding resistance gene cassette, all of the resistance gene cassettes in this element are expressed and simultaneously confer resistance to all antibiotics. Gentamicin resistance and co-trimoxazole resistance might correlate with the possession of resistance genes in class 1 integron

element. Other mechanisms like, mutation in drug target sites and efflux pumps, might act synergistically to confer resistance. Genetic analysis is mandatory to conclude the molecular mechanisms of resistance to these antibiotics and to choose the appropriate therapy.

Multidrug resistant *Citobacter* sp. that was isolated in this study was sensitive to only cefoperazone and sulbactam. At the present moment we highly recommend the use of combination therapy for the treatment of *Citrobacter* sp. infections, mostly respiratory tract infections in COPD patients. Although ESBL producing organisms show susceptibility in vitro to this combination they are resistant in vivo and carbapenem should only be choice for ESBL producing organisms. All patients treated with this combination showed clinical improvement and suggest this combination can still be used. Although, carbapenems are widely used and resistance has already been demonstrated[14], it is not widely used for the treatment of this pathogen in our setting and we assume that these drugs are still active against this pathogen. Hence, carbapenems like, imipenem, meropenem, and doripenem could be kept as a reserve drug if in case they become resistant to this combination. Isolates bearing AmpC cephalosporinase can hydrolyze all â-lactams except carbapenems and are not inhibited by â-lactamase inhibitors. This cephalosporinase is inducible on exposure to cephalosporins and hence can confer resistance to these combinations.[18] Minimizing the use of extended spectrum cephalosporins for the treatment of this pathogen and other Gram-negative pathogens like, multidrug resistant *E. coli, K. pneumoniae, P. aeruginosa,* and *A. baumannii* is must to prevent the further development of resistance and to prevent the resistance gene sharing among these pathogens.

In conclusion, we report the high prevalence of multidrug-resistant *Citrobacter* sp. as a nosocomial pathogen in this hospital and thus recommend revising the treatment protocol. At present scenario where ESBL genes are predominant among Citrobacters, cefoperazone and sulbactam combination and carbapenems are ideal choices. This bacterium can become resistant to these drugs at any time, hence indiscriminate, inadequate, and prophylactic use of antibiotics should be avoided. Infection control strategies like, disinfection of wards, barrier precaution against infectious and colonized patients, using disposable gloves and drapes, compulsory hand washing with 70% alcohol before and after nursing care of the patient, washing of stands for IV drips, door knobs, taps, and bed rails should be strictly done to prevent the spread of this pathogen and to prevent the spread of their resistance genes.

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