May-August, 2012/Vol 32/Issue 2
doi: http://dx.doi.org/10.3126/jnps.v32i2.5804

Original Article

Molecular Characterization of *Citrobacter freundii* Isolated from Neonates in Neonatal Intensive Care Unit of Nepal

Thapa B[1], Tribuddharat C[2]

[1]Dr. Badri Thapa, MBBS. Ph D, Lecturer, Kathmandu Medical College, Genesis Laboratory and Research Centre, Communicable Disease Poly Clinic and Research Centre, Kathmandu, Nepal, [2]Dr. Chanwit Tribuddharat, MD, Ph D, Assistant Professor, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Address for correspondence: Dr. Badri Thapa, E-mail: badri_bishal@yahoo.com

Abstract

Introduction: Nosocomial *Citrobacter* spp. is emerging as a successful nosocomial pathogen in neonates in Nepal. The important risk factor being poor infection prevention and control practices. The objective of this study was to investigate the clonal relatedness of *Citrobacter freundii* isolated from clinical and non-clinical sources in Neonatal Intensive Care Unit (NICU) and to determine the presence of Extended Spectrum Beta-Lactamase (ESBL) genes and class 1 integron element. **Materials and Methods:** Polymerase chain Reaction (PCR) and PCR-Randomly Amplified Polymorphic DNA typing of the isolates were performed in three isolates to amplify class 1 integron element integrase gene, ESBL genes, and to study the clonal relatedness, respectively. **Results:** Two isolates harbored class 1 integron element. The bla_{CTX-M} was present in all isolates and bla_{TEM-1} was present in one isolate. An isolate carried bla_{CTX-M} and bla_{TEM-1} genes. All of these isolates were not clonally related. **Conclusion:** The study for the first time documented the emergence and spread of ESBL genes and class 1 integron element in multidrug resistant *C. freundii* in Nepal and urge for monitoring and surveillance of these strains.

Key words: *Citrobacter* spp., ESBL, PCR-RAPD, class 1 integron element

Introduction

Among nosocomial pathogens causing nosocomial infections, *Staphylococcus aureus*, coagulase negative *Staphylococcus* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Escherichia coli, Kleibsella spp., Enterobacter* spp., and *Citrobacter* spp. are most common[1]. *Citrobacter* nosocomial infection in neonates is a cause of substantial rise in morbidity and mortality of neonates in the hospital in Nepal and globally[1,2,3,4]. The poor infection prevention and control practices have been a major determining factor for the spread of this nosocomial pathogen[1]. The infection in neonates is either horizontally transferred as a nosocomial infection or vertically transferred from mother during delivery[5]. The imprudent and less potent use of antibiotics has also led to the emergence of the multidrug resistant nosocomial pathogens[6]. Numerous studies have already shown *Citrobacter* carrying Extended Spectrum Beta-Lactamase genes (bla_{CTX-M}, bla_{TEM-1}, and bla_{SHV}) and a carriage of class 1 integron element[7].

Citrobacter spp. is commonly infecting adult patients admitted to the hospitals in Nepal[8,9]. Infections like, respiratory tract, urinary tract, intra-peritoneal, wound, sepsis, meningitis, and brain abscess are common to this pathogen[5]. Neonates and infants are also at risk of developing *Citrobacter* osteomyelitis, septic arthritis, lung abscess, skin infection and urinary tract infection[10,11,12,13]. Among *Citrobacter* spp., *Citrobacter freundii* is the commonest species that has been described as a nosocomial pathogen[14]. However, this pathogen infecting neonates was never described in Nepal. We have recently described the isolation of *Citrobacter* spp. from various clinical and non-clinical specimens originated from the neonates admitted in Neonatal Intensive Care Unit (NICU)[2]. In light with this fact, here we compared the genotypes of *C. freundii* isolated from the clinical and non-clinical sources and

Manuscript received: 6th February 2012.
Reviewed: 31st March 2012
Author Corrected: 2nd April 2012
Accepted for Publication: 30th April 2012

determined the possession of class 1 integron element and ESBL genes in these isolates.

Material and Methods

The study had isolated 12 multidrug resistant *Citrobacter* spp. from various clinical and non-clinical sources. They were grouped into five antibiotypes (I to V). The isolate C109, isolated from the nasal prong and C316709 were grouped with four other isolates in antibiotype I. These isolates along with C3409 belonging to antibiotype V were selected for the present study. The identification of *C. freundii* was carried out as described previously[15]. Hydrogen sulfide production on Triple Sugar Iron Agar slant along with other biochemical tests was used to confirm as *C. freundii*. Overnight grown bacterial colonies were emulsified in 1 ml sterile distilled water and boiled for 1 minute. The emulsified colonies were centrifuged at 13,000 g for 5 minutes and supernatant was used for colony Polymerase Chain Reaction (PCR). PCR-Randomly Amplified Polymorphic DNA (PCR-RAPD) was performed as described earlier using 1 µl of supernatant as a DNA template[16]. The class 1 integron element integrase gene (*intI1*) and ESBL genes (bla_{CTX-M}, $bla_{TEM-1,}$ and bla_{SHV}) were amplified using the protocol published[17]. Briefly, the reaction mixture contained 0.5 M each primer, 250 M each deoxynucleoside triphosphate, 1U Taq DNA polymerase (Finzymes), 2.5 µl of 10X PCR buffer, and 100-500 ng of total genomic DNA in a final volume of 25 µl. Amplification was performed in a Perkin-Elmer thermocycler 2400 model for 5 min at 95°C, followed by 30 consecutive cycles of 1 min at 95°C, 1 min at 50°C for bla_{TEM} gene, 55°C for bla_{SHV}, 58°C for bla_{CTX-M} gene, and 1 min at 72°C, with a single final extension step of 10 min at 72°C. PCR products were separated by electrophoresis in a 1.5 percent agarose gel and stained with ethidium bromide.

Results

The isolate C316709 and C109 were sensitive to ofloxacin, ciprofloxacin and norfloxacin while isolate C3409 was resistant to all eleven antibiotics tested (Table 1). The *IntI1* was amplified from two isolates (C3409 and C109). All isolates were positive for bla_{CTX-M} and an isolate C3409 also harbored bla_{TEM-1}. bla_{SHV} was not amplified from all isolates. The PCR-RAPD genotyping of these isolates showed three different genotypes (a, b and c) and they were not clonally related (Table 1 and Fig. 1).

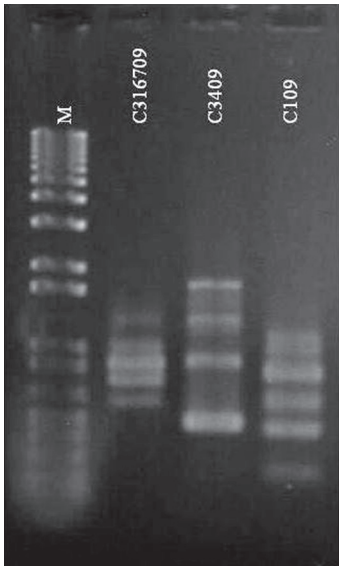

Fig1: PCR-RAPD patterns of *C. freundii* isolates. The lanes have been marked with the respective isolates. M, Molecular weight marker (1 Kb+ Invitrogen, USA). Lane 1, pattern a; lane 2, pattern b and lane 3, pattern c. The PCR-RAPD was performed in Department of Microbiology, Siriraj Hospital, Bangkok, Thailand.

Discussion

In this study, the *C. frundii* isolated from nate and innate sources from Neonatal intensive Care Unit (NICU) were characterized. Isolates C316709 and C109 had similar antibiotype, but were not clonally related by PCR-RAPD typing. The typing also revealed that the *C. freuidii* isolated from clinical and non-clinical sources were not related highlighting for other sources that could not be traced. PCR-RAPD is an arbitrarily primed PCR and is more discriminatory. Less discriminatory and more robust molecular typing tools like, ribotyping and multi-locus sequence typing (MLST) are necessary for clonal analysis[18]. We could not include all isolates for genotyping and the relatedness of those isolates to these clones (a, b, and c) could not be ascertained.

Table 1: Characteristics of *Citrobacter freundii* isolates

			ESBL genes				
ID	Source	AST (sensitive to)	bla_{TEM-1}	bla_{SHV}	bla_{CTX-M}	intI1	PCR-RAPD
C316709	US	CF, OF, NX	-	-	+	-	A
C3409	US	None	+	-	+	+	B
C109	NP	OF, CF, NX	-	-	+	+	C
US, Umbilicab Swab; NP, Nasal Prong; Antibiotics tested (µg/disc): Ampicillin (10), Piperacillin (100), Amoxy-Clav (20/10), Ceftriaxone (CI) (30), Cephalexin (30), Amikacin (30), Netilmicin (30), Ciprofloxacin (CF) (5), Ofloxxacin (OF) (5), Norfloxacin (NX) (10), Sulphamethoxazole+Trimithorprim (23.75+1.25).							

Despite the fact that *Citrobacter* spp. infection is prevalent in Nepal, it is unfortunate that no systematic molecular studies have been conducted, however the isolation of *Citrobacte*r spp. have been described[2,8,9]. In this study, we have shown the emergence of *C. freundii* carrying class 1 integron element and bla_{CTX-M} and bla_{TEM-1} ESBL genes for the first time in Nepal. The present study has also identified *C. freundii* strain simultaneously harboring bla_{CTX-M} and bla_{TEM-1} which has also been described in India[18]. These Indian isolates also carried bla_{SHV} and bla_{ampC} along with bla_{CTX-M} and bla_{TEM-1}. *C. freundii* carrying bla_{CTX-M} has also been described elsewhere[7]. *Enterobacteriaceae* carrying class 1 integron element have been described, however the reports describing the presence of this mobile genetic element in *Citrobacter* is scarce. We have also identified *C. freundii* carrying this mobile genetic element. Most of the ESBLs are carried in this mobile genetic element[19] and the carriage of ESBL genes in this element was not investigated. C*itrobacter* spp. infection has frequently been increasing as a cause of serious concern, especially for neonates and immunocompromised adults. The increasing antibiotic resistance is also noteworthy for *C. freundii*.

The neonatal mortality of Nepal was 33/1000 live births in 2006 and the preliminary findings of National Demographic and Health Survey 2011 has shown no difference in NMR[20]. To address maternal health, Ministry of Health and Population, Government of Nepal has started "AMMA" program, which aims to motivate institutional delivery to decrease maternal mortality. In light of emergence of class 1 integron element and ESBL harboring *C. freundii*, government's effort of advocating institutional delivery and saving maternal lives will position neonates at increased risk of neonatal morbidity and mortality if infection prevention and control policies and practices are not in place. Furthermore the spread of mobile genetic element and resistance genes will help in emergence of multidrug resistant pathogens and might pose challenge for saving newborns.

In conclusion, this study has highlighted the spread of oligoclonal population of *C. freundii* neonatal nosocomial infection in NICU, Nepal and has also documented the spread of bla_{CTX-M} and bla_{TEM-1} and class 1 integron element carrying *C. freundii* in Nepal.

Acknowledgements: Authors would like to acknowledge Asst. Prof. Dr. Chanwit Tribuddharat for allowing the authors to conduct the experiment at his laboratory located at Department of Microbiology, Siriraj Hospital, Bangkok, Thailand.

Funding: None
Conflict of interest: None to declare.
Permission from IRB: Yes

References

1. Kamath S, Mallaya S, Shenoy S. Nosocomial infections in neonatal intensive care units: profile, risk factor assessment and antibiogram. *Indian J Peadiatr* 2010;77:37-9.
2. Khadka SB, Thapa B, Mahat K. Nosocomial *Citrobacter* Infection in Neonatal Intensive Care Unit in a Hospital of *Nepal. J Nepal Paediatr Soc* 2011;31:105-09.
3. Saraswathi KDA, Gogate A, Fernande AB. *Citrobacter* sepsis in infants. *Indian Peadiatr* 1995;31:359-62.
4. Shrestha P, Das BK, Bhatta NK, *Jha DK, Das B, Setia A et al.* Clinical and Bacteriological profiles of blood culture positive sepsis in newborns. *J Nepal Paediatr Soc* 2008;27:64-7.
5. Mohanty S, Singhal R, Sood S, Dhawan B, Kapil A, Das BK. *Citrobacter* infections in a tertiary care hospital in Northern India. *J Infect* 2007;54:58-64.
6. Thapa B, Mahat K. In-vitro activity of antibiotics and brands of ceftriaxone against clinical isolates. *J Nepal Med Assoc* 2010;49:225-7.
7. Kim J, Lim YM. Prevalence of derepressed AmpC mutants and extended-spectrum beta-lactamase producers among clinical isolates of *Citrobacter freundii*, *Enterobacter* spp., and *Serratia marcescens* in Korea: dissemination of CTX-M-3, TEM-52, and SHV-12. J. *Clin Microbiol* 2005;43:2452–5.
8. Banjara MR, Sharma AP, Hoshi AB, Tuladhar NR, Ghimire P, Bhatta DR. Surgical wound infections in patients of Tribhuvan University Teaching Hospital. *Nepal Health Res Counc* 2003;3:41-5.
9. Thapa B, Adhikari P, Mahat K, Chettri M, Joshi LN. Multidrug-resistant nosocomial *Citrobacter* in a hospital in Kathmandu. *J Nepal Med Coll* 2009;11:195-9.
10. Jansen RD, Meadow WL, Schwartz IK, Ogata ES. "Bacteriological bit", *Citrobacter diversus* osteomyelitis in a neonate. Clin Pediatr (Phila) Bacteriol 1981;20:791.
11. Bruehl CL, Listernick R. *Citrobacter freundii* septic arthritis. *J Paediatr Child Healt* 1992;28:402-3.
12. Shamir R, Horev G, Merlob P, Nutman J. *Citrobacter diversus* lung abscess in a preterm infant. *Pediatr Infect Dis J* 1990;9:221–2.
13. Barton LL, Walentik C. *Citrobacter diversus* urinary tract infection. *Am J Dis Child* 1982;136:467-8.

14. Samonis G, Karageorgopoulos DE, Kofteridis DP, Matthaiou DK, Sidiropoulou V, Maraki S et al. *Citrobacter* infections in a general hospital: characteristics and outcomes. *Eur J Infect Dis* 2009;28:61-8.

15. Ng SY, Kwang L, Tan TY. Identification of Gram-negative bacilli directly from positive blood culture vials. *J Med Microbiol* 2007;56:475-9.

16. Thapa B, Tribuddharat C, Srifuengfung S, Dhiraputra C. High prevalence of bla_{OXA-23} in oligoclonal carbapenem-resistant *Acinetobacter baumannii* in Siriraj Hospital, Mahidol University, Bangkok, Thailand. *Southeast Asian J Trop Med Public Health* 2010;41:625-35.

17. Tribuddharat C, Srifuengfung S, Chiangjong W. A Correlation between phenotypes and genotypes of extended-Spectrum beta-Lactamase (ESBL)-producing *Klebsiella pneumoniae* in a University Hospital, Thailand. *Dis Antimicrob Agents* 2007;24:117-23.

18. Norskov-Lauritsen N, Sandvang D, Hedegaard J, Fussing V, Mortensens KK, Sperling-petersen HU, et al. Clonal origin of aminoglycoside-resistant *Citrobacter freundii* isolates in Danish county. *J Med Microbiol* 2001;50:636-41.

19. Shahid, M. *Citrobacter* spp. simultaneously harboring bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{ampC}, and insertion sequences IS*26* and *orf513*: an evolutionary phenomenon of recent concern for antibiotic resistance. *J Clin Microbiol* 2010;48:1833–38.

20. Ministry of Health and Population. Government of Nepal. Nepal Demographic and Health Survey, 2011. Nepal.

How to cite this article ?

Thapa B, Tribuddharat C. Molecular Characterization of Citrobacter freundii Isolated from Neonates in Neonatal Intensive Care Unit of Nepal. *J Nepal Paediatr Soc* 2012;32(2):132-135.