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Co-existence of bla_{OXA-23} and bla_{NDM-1} genes of *Acinetobacter baumannii* isolated from Nepal: antimicrobial resistance and clinical significance

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Abstract

Background: Molecular analysis of carbapenem-resistant genes in *Acinetobacter baumannii*, an emerging pathogen, is less commonly reported from Nepal. In this study we determined the antibiotic susceptibility profile and genetic mechanism of carbapenem resistance in clinical isolates of *A. baumannii*.

Methods: *A. baumannii* were isolated from various clinical specimens and identified based on Gram staining, biochemical tests, and PCR amplification of organism specific 16S rRNA and bla_{OXA-51} genes. The antibiotic susceptibility testing was performed using disc diffusion and E-test method. Multiplex PCR assays were used to detect the following β-lactamase genes: four class D carbapenem hydrolyzing oxacillinases (bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-24} and bla_{OXA-58}). Uniplex PCRs were used to detect three class B metallo-β-lactamases genes (bla_{IMP}, bla_{VIM} and bla_{NDM-1}), class C cephalosporin resistance genes (bla_{ADC}), aminoglycoside resistance gene (*aphA6*), and IS*Aba1* of all isolates. Insertion sequence IS*Aba125* among NDM-1 positive strains was detected. Clonal relatedness of all isolates were analyzed using repetitive sequence-based PCR (rep-PCR).

Results: Of total 44 analyzed isolates, 97.7% ($n = 43$) were carbapenem-resistant *A. baumannii* (CR-AB) and 97.7% ($n = 43$) were multidrug resistant *A. baumannii* (MDR-AB). One isolate was detected to be extremely drug resistant *A. baumannii* (XDR-AB). All the isolates were fully susceptible to colistin (MICs < 2 μg/ml). The bla_{OXA-23} gene was detected in all isolates, while bla_{NDM-1} was detected in 6 isolates (13.6%). Insertion sequence, IS*Aba1* was detected in all of bla_{OXA-23} positive isolates. IS*Aba125* was detected in all bla_{NDM-1} positive strains. The bla_{ADC} and *aphA6* genes were detected in 90.1 and 40. 1%, respectively. The rep-PCR of all isolates represented 7 different genotypes.

Conclusion: We found high prevalence of CR-AB and MDR-AB with bla_{OXA-23} gene in a tertiary care hospital in Nepal. Systemic network surveillance should be established for monitoring and controlling the spread of these resistant strains.

Keywords: *Acinetobacter baumannii*, Carbapenem resistance, bla_{OXA-23} and bla_{NDM-1} carbapenemase genes

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Background

Acinetobacter baumannii, an emerging pathogen of healthcare centers, shows intrinsic as well as acquired drug-resistance mechanisms [1]. Multidrug-resistant *A. baumannii* can be resistant to all of the currently available antibiotics, and in its deadliest form these are only susceptible to potentially toxic polymyxins and colistins, leaving limited options for treatment [2]. Infections with carbapenem- and colistin-resistant *A. baumannii* are emerging globally [3].

Carbapenem resistance in *A. baumannii* encompasses production of class B, C and class D carbapenemase, decreased membrane permeability, altered penicillin-binding proteins, and overexpression of efflux pumps [4, 5]. Most commonly, *Acinetobacter* spp. develop carbapenem resistance by production of OXA-type carbapenemase and metallo-β-lactamases (MBLs) [6, 7]; $bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-40}}$-like, $bla_{\text{OXA-58}}$-like and $bla_{\text{OXA-51}}$-like carbapenemases are broadly reported, where $bla_{\text{OXA-51}}$-like β-lactamases, intrinsic to *A. baumannii*, is used for species identification [8–10]. Among multiple MBL genes, bla_{IMP} and bla_{VIM} types (chromosomal or plasmid encoded) encode carbapenemase in *A. baumannii* [9]. *A. baumannii* harboring plasmid encoded New Delhi metallo-β-lactamase-1 (NDM-1), a novel carbapenemase gene, is reported from many countries [11, 12]. In addition, detection of class C β-lactamase genes (bla_{ADC}) which mediated cephalosporin resistances and aminoglycoside resistant genes (*aphA6*) has increased in recent years in *A. baumannii* clinical isolates [13, 14].

A. baumannii remains a critical problem in many healthcare settings throughout the world despite the implementation of infection control practices. There are limited data on carbapenem-resistant *A. baumannii* in Nepal. The objective of this study was to determine antibiotic susceptibility profile, antibiotic resistance genes and genetic mechanism of carbapenem resistance of *A. baumannii* in clinical isolates at a tertiary care hospital, Nepal.

Methods

Bacterial isolation and identification

A. baumannii isolates were collected from inpatient units of a tertiary hospital, Nepal. Forty-four non-duplicate isolates were collected (24 male and 20 female; age range between 24 to 80 years) over 9 months periods (October 2014 to June 2015). All isolates were identified by classical biochemical methods and confirmed by PCR method for detecting 16S rRNA gene and $bla_{\text{OXA-51}}$ gene [15, 16]. Isolates were identified as *A. baumannii* by PCR result of positive for both PCRs.

Antibiotic susceptibility testing

The antibiotic susceptibility of amikacin (30), cefotaxime (30), ceftazidime (30), ceftriaxone (30), cefepime (30), ciprofloxacin (5), gentamicin (10), imipenem (10), meropenem (10), trimethoprim/sulfamethoxazole (1.25/23.75), tetracycline (30), and piperacillin/tazobactam (100/10) (Oxoid) was determined on Mueller Hinton Agar (High Media, India) according to the antibiotic disk diffusion method [17]. The plates were incubated at 37 °C for 24 h. The zones of inhibition were determined whether the microorganism was susceptible, intermediately resistant, or resistant to each antibiotic according to Clinical and Laboratory Standards Institute (CLSI) guidelines. E-test was performed to determine the Minimum inhibitory concentration (MIC) of ceftazidime, imipenem, tigecycline and colistin (High Media, India) according to manufacturer instructions and interpreted as per CLSI guidelines except for tigecycline. Multidrung-resistant *A. baumannii* (MDR-AB) was defined when *A. baumannii* resistant to multiple antibiotics, often defined as three or more antibiotic classes. Extensively drug resistant *A. baumannii* (XDR-AB) was defined when *A. baumannii* was resistant to all antimicrobial agents except polymyxins (colistin) [18].

PCR amplification of antibiotic resistance genes

PCR assays to detect $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$, $bla_{\text{OXA-58}}$, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{ADC} and *ahpA6* genes were performed using primers as describe previously (Table 1). The amplification reaction was performed using *A. baumannii* cell lysate as DNA template. Each PCR was performed in triplicate in a thermocycler with a PCR condition as described previously [14, 16, 19–21]. All PCR assays used 16S rRNA or $bla_{\text{OXA-51}}$ genes as the internal control. The IS*Aba1* of $bla_{\text{OXA-23}}$ gene was detected using combination of primers IS*Aba1*-F/IS*Aba1*-R and IS*Aba1*-F/$bla_{\text{OXA-23}}$-R (Table 1) [22]. The IS*Aba125* of $bla_{\text{NDM-1}}$ gene were determined in all $bla_{\text{NDM-1}}$ positive strains using combination of primers ISA125-F/ISA125-R and ISA125-F/bla_{NDM}-R (Table 1). PCR products of the $bla_{\text{NDM-1}}$ genes were purified and sequenced. BLAST was used to compare the sequences of $bla_{\text{NDM-1}}$ genes against the GenBank Database. PCR products were analyzed by electrophoresis in 1% agarose gel containing 0.5 μg/ml ethidium bromides.

IPM-EDTA combined disk test

All $bla_{\text{NDM-1}}$ positive strains were tested for MBL production by IPM-EDTA combined disk test. The test was performed as previously described [23]. After 24 h incubation, the difference of inhibition zone diameter between IPM-EDTA disk and IPM disk alone (≥7 mm) was considered the positive criteria for the presence of MBL.

Table 1 List of primer for detection of genes used in this study

Target genes	Primer name	Sequence 5′-3′	Size/ Annealing temp.	References
16S rRNA	16S rRNA-F	AGAGTTTGATCCTGGCTCAG	1500/58	[15]
	16S rRNA-R	ACGGCTACCTTGTTACGACTT		
bla_{OXA-23}	bla_{OXA-23}-F	GATCGGATTGGAGAACCAGA	501/52	[16]
	bla_{OXA-23}-R	ATTTCTGACCGCATTTCCAT		
bla_{OXA-51}	bla_{OXA-51}-F	TAATGCTTTGATCGGCCTTG	353/52	[16]
	bla_{OXA-51}-R	TGGATTGCACTTCATCTTGG		
bla_{OXA-24}	bla_{OXA-24}-F	GGTTAGTTGGCCCCCTTAAA	246/52	[16]
	bla_{OXA-24}-R	AGTTGAGCGAAAAGGGGATT		
bla_{OXA-58}	bla_{OXA-58}-F	AAGTATTGGGGCTTGTGCTG	599/52	[16]
	bla_{OXA-58}-R	CCCCTCTGCGCTCTACATAC		
bla_{IMP}	bla_{IMP} –F	GGAATAGAGTGGCTTAAYTCTC	232/52	[20]
	bla_{IMP} –R	GGTTTAAYAAAACAACCACC		
bla_{VIM}	bla_{VIM} –F	GATGGTGTTTGGTCGCATA	390/52	[20]
	bla_{VIM} –R	CGAATGCGCAGCACCAG		
bla_{NDM}	bla_{NDM}–F	GGTTTGGCGATCTGGTTTTC	621/52	[21]
	bla_{NDM}–R	CGGAATGGCTCATCACGATC		
bla_{ADC}	bla_{ADC}-F	TAAACACCACATATGTTCCG	663/56	[19]
	bla_{ADC}-F	ACTTACTTCAACTCGCGACG		
aphA6	*aphA6*-F	ATGGAATTGCCCAATATTATTC	736/55	[14]
	aphA6-R	TCAATTCAATTCATCAAGTTTTA		
IS*Aba1*	IS*Aba1*-F	CATTGGCATTAAACTGAGGAGAAA	451/52	[22]
	IS*Aba1*-R	TTGGAAATGGGGAAAACGAA		
IS*Aba125*	ISA125-F	TGTTGAAGCGATCCGTTGTT	755/57	This study
	ISA125-R	GTGCGACAGTTTCAAAAGCCA		
Rep-PCR	ERIC-2	AAGTAAGTGACTGGGGTGAGCG	variable length/45	[24]

Repetitive element PCR-mediated DNA fingerprinting (rep-PCR)

Genomic DNA of each isolates was extracted from the overnight cultures using GF-1 bacterial DNA extraction kit (Vivantis, Malaysia). Rep-PCR was performed by using genomic DNA as a template for PCR amplification with the ERIC-2 primer (Table 1) using condition as describe previously [24, 25]. PCR-banding patterns and rep-PCR types were analyzed and interpreted as previously described [25].

Results

Demographic characteristic of patients

Demographic characteristics of the inpatients with *A. baumannii* infection were analyzed; 24 (54.5%) were male and 20 (45.5%) were female. Most of the specimens were from ICU wards ($n = 27$, 61.4%) (Fig. 1). Isolates were collected from sputum ($n = 26$, 59.1%), tracheal aspirates ($n = 9$, 20.4%), catheter tip, ($n = 4$, 9.1%), pus ($n = 4$, 9.1%) and urine ($n = 1$, 2.3%) (Fig. 1).

Antibiotic susceptibility

Of the 44 isolates, resistance was found against ciprofloxacin ($n = 43$, 97.7%), cefotaxime ($n = 43$, 97.7%), ceftazidime ($n = 42$, 95.4%), ceftriaxone ($n = 41$, 93.2%), cefepime ($n = 39$, 88.6%), amikacin ($n = 19$, 43.2%), gentamicin ($n = 23$, 52.3%), trimethoprim/sulfamethoxazole ($n = 41$, 93.2%), tetracycline ($n = 21$, 47.7%) and piperacillin/tazobactam ($n = 43$, 97.7%). Only one isolate of *A.baumannii* was susceptible to all tested antibiotics. Most isolates (97.7%, $n = 43$) were carbapenem resistant *A. baumannii* (CR-AB); all CR-AB were MDR-AB. One isolate was detected to be XDR-AB. All the isolates were fully susceptible to colistin (MICs < 2 μg/ml) and MIC of tigecycline was determined to be <2.5 μg/ml (Table 2).

Antibiotic resistance genes and IS element in *A. baumannii*

Aminoglycoside resistance gene, *aphA6* and cephalosporin resistance genes, bla_{ADC} were detected in 40.1% (18/44) and 90.1% (40/44), respectively. The bla_{OXA-23} was present in all isolates. Other class D β-lactamase

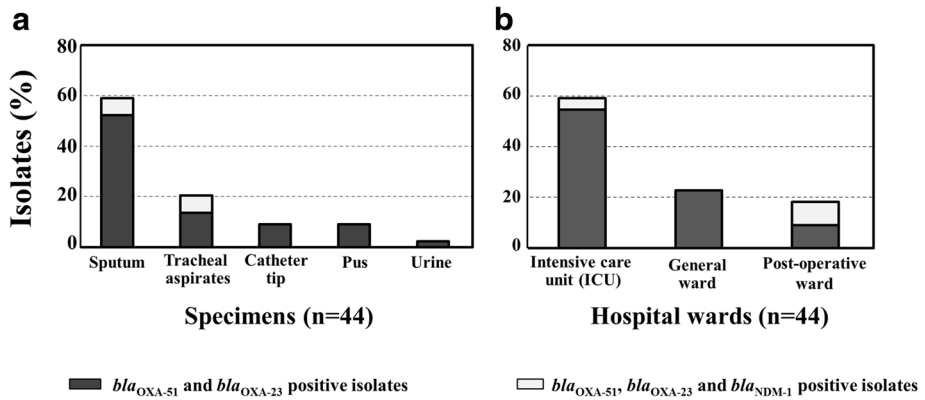

Fig. 1 Distribution of *A. baumannii* carrying carbapenemase genes in different specimen types **a** and wards **b**

genes, including $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$, markers of carbapenem resistance in *A. baumannii*, were not detected in analyzed isolates. IS*Aba1* was detected in all of $bla_{\text{OXA-23}}$ positive isolates (100%). Of total analyzed isolates, 6 (13.6%) also harbored $bla_{\text{NDM-1}}$ gene in addition to $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$. All NDM-1 positive strains exhibited insertion sequence IS*Aba125* detecting with primers ISA125-F/ISA125-R. All isolates also detected a band of 1.6 kb in a PCR using ISA125-F/bla_{NDM}-R primers. Metallo-β-lactamase (MBL) genes, including bla_{VIM} and bla_{IMP}, were not detected in all isolates. The sequences of the $bla_{\text{NDM-1}}$ gene yielded 99-100% sequence identity to the $bla_{\text{NDM-1}}$ gene from *Acinetobacter lwoffii* strain WJ10621 plasmid pNDM-BJ01 (Accession: JQ001791) obtained from the GenBank Database.

MBL production

Six *A. baumannii* isolates harbored $bla_{\text{NDM-1}}$ gene were detected for MBL production. All of $bla_{\text{NDM-1}}$ positive strains were positive for MBL production. MBL positive strains showed resistance to fluoroquinolones and β-lactam.

Epidemiological typing

Clonal relationship among isolates were studied using rep-PCR typing. The fingerprinting represented 7 different DNA patterns consisting of 2 to 5 DNA fragment sizes. The amplicons size for ERIC-2 PCR was 500–4000 bp. The genotype was named A-G as shown in Fig. 2. The high prevalence genotype was type C ($n = 14$; 31.8%) and D ($n = 12$; 27.3%). Genotype A, B, C and D were disseminated in all isolated ward (ICU, general ward and post-operative ward). Among 44 isolates, one isolate of type F (2.3%) and G (2.3%) was found. Type F was obtained from a catheter tip specimen from the ICU ward. Type G was obtained from sputum of a patient from a general ward. All NDM-1 positive strains exhibited genotype A ($n = 1$), B ($n = 1$), C ($n = 3$) and D ($n = 1$).

Discussions

A. baumannii harboring $bla_{\text{OXA-51}}$-like gene has been identified as a marker for species identification. An intrinsic $bla_{\text{OXA-51}}$-like gene detected in all isolates in this study supports the use of this gene as a surrogate marker of *A. baumannii* identification [8–10]. High prevalence of cephalosporin resistance genes, bla_{ADC} (90.1%) was found in this study. In addition, we found a high rate of cepharosporin resistant antibiotics (cefotaxime, ceftazidime, ceftriaxone) using the disk diffusion method. These data indicated that cephalosporins no longer work to treat *A. baumannii* isolated from Nepal. Carbapenem resistance in *A. baumannii* is a major concern and is most often associated with class D

Table 2 The carbapenemases gene patterns, rep-PCR types and MIC determination of *A. baumannii* isolated from difference wards

Sites	β-lactamase gene patterns	No. of isolates	Rep-PCR Types (*n*)	MIC (μg/ml) range			
				CAZ	IMP	TG	CL
Intensive care unit	$bla_{\text{OXA-51/ OXA-23}}$	25	A (2), B (6), C (8), D (6), E (2), F (1)	4– > 256	1– > 32	1.6–3.9	0.13–2
	$bla_{\text{OXA-51/ OXA-23/ NDM-1}}$	2	B (1), C (1)	>256	>32	1.7–3.2	0.61–0.79
General ward	$bla_{\text{OXA-51/ OXA-23}}$	9	A (1), B (1), C (1), D (5), G (1)	>256	>24–>32	2–3.4	0.32–0.88
Post-operative ward	$bla_{\text{OXA-51/ OXA-23}}$	4	A (1), B (1), C (2)	>256	>32	2.3–3.3	0.54–0.78
	$bla_{\text{OXA-51/ OXA-23/ NDM-1}}$	4	A (1), C (2) D (1)	>256	>32	2.1–3.2	0.23–0.51

Abbreviations: *CAZ* ceftazidime, *IPM* imipenem, *TG* tigecycline, *CL* colistin

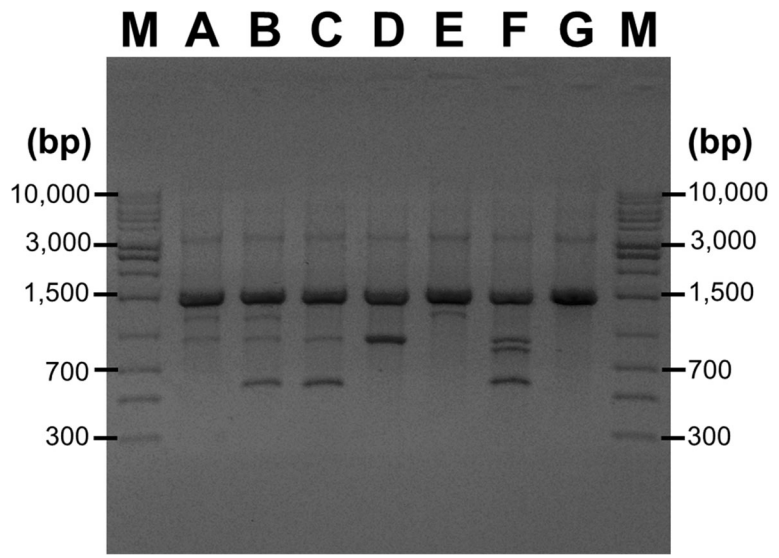

Fig. 2 Rep-PCR-based DNA fingerprint patterns of *A. baumannii* isolates. The *lanes* marked M contain molecular markers. Each lane represents genotype patterns of *A–G*

β-lactamases and MBLs. The full susceptibility of all CR-AB to colistin in this study indicates that colistin is still an option of drug for the treatment of infections caused by *A. baumannii* in Nepal hospital.

OXA-type carbapenemases are predominant in *A. baumannii* [6, 7]. In agreement with this finding, high prevalence of $bla_{\text{OXA-23}}$ carrying *A. baumannii* strains has been reported in Nepalese patients [26]. The acquired $bla_{\text{OXA-23}}$ is the dominant genetic determinant in Asia. The $bla_{\text{OXA-23}}$ gene located on plasmid can be transferred between *A. baumannii* through conjugation. Thus, antibiotic resistant bacteria have been rapidly increasing worldwide [27]. The $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$ were not detected in any isolates from this study. The $bla_{\text{OXA-24/40}}$ and $bla_{\text{OXA-58}}$ genes were common in *A. baumannii* isolated from Europe [2, 28]. Recently, $bla_{\text{OXA-143}}$ and $bla_{\text{OXA-235}}$, which are novel class D β-lactamase genes in *A. baumannii* have been identified. To date, these determinants were detected only in Brazil, Mexico and the USA [29, 30]. IS*Aba1* was detected in widespread clones of *A. baumannii* worldwide. Our study found IS*Aba1* upstream of $bla_{\text{OXA-23}}$ in all *A. baumannii* isolates. A correlation between *A. baumannii* clusters carrying the IS*Aba1*/$bla_{\text{OXA-23}}$ gene and increased minimal inhibitory concentrations for carbapenems was reported [31]. One isolate (AB-13) that was recovered from catheter tips of long-stay hospital patients showed an extreme drug resistance pattern (Additional file 1: Table S1). This isolate represented $bla_{\text{OXA-23}}$, bla_{ADC} and *aphA6* genes. Further molecular study to detect other antibiotic resistance genes is needed to explain what factors correlated with extreme drug resistance. We also found one isolate (AB-25) harboring $bla_{\text{OXA-23}}$, bla_{ADC} and *aphA6* genes was sensitive to all tested drugs (Additional file 1: Table S1). This may be due to the lack of promoter or mutation of IS*Aba1* or $bla_{\text{OXA-23}}$ gene. Further study is needed to warrant the conclusion.

The $bla_{\text{NDM-1}}$ carrying *A. baumannii* has recently been emerged in many countries, including Germany, Spain, Israel, Egypt, Switzerland, Libya, India, Pakistan and Nepal [11, 26, 32, 33]. The $bla_{\text{NDM-1}}$ gene has been identified as a chimeric gene constructed by the fusion of the aminoglycoside-resistance gene *aphA6* with a mannose-binding lectin gene. This event most likely occurs in *Acinetobacter* spp., indicating that these bacteria are likely the origin of this gene [34]. In this study, we identified 13.6% of *A. baumannii* carrying $bla_{\text{NDM-1}}$ gene. Previous study has identified high prevalence (24.6%) of the *A. baumannii* harbored the $bla_{\text{NDM-1}}$ gene in Nepal in 2013–2014 [26]. Taking into consideration the relationship between India, China and Nepal, the spread of $bla_{\text{NDM-1}}$ is likely to occur rapidly, mostly through *A. baumannii* rather than Enterobacteriaceae. *A. baumannii* able to transfer the $bla_{\text{NDM-1}}$ gene via conjugation to the recipients and Tn*125* appears to be the main vehicle for dissemination of the $bla_{\text{NDM-1}}$ genes in *A. baumannii* [35]. Poirel et al. reported that the $bla_{\text{NDM-1}}$ gene was located within the composite transposon Tn*125* bracketed by two copies of a strong promoter of $bla_{\text{NDM-1}}$ gene called IS*Aba125* [11]. This report was correlated with our finding that found IS*Aba125* in 100% of NDM-1 producing *A. baumannii*.

The previous study reported that the most of *A. baumannii* isolates harboring $bla_{\text{NDM-1}}$ belonged to ST85 and ST25 [35–37]. In Libyan hospital, Libya, the main clone of imipenem-resistant NDM-1-producing *A. baumannii* belonged to ST2 [33]. We used rep-PCR typing

to determine the clonal relationship in NDM-1 producing *A. baumannii*. Our study highlighted that most of NDM-1-producing *A. baumannii* isolates belonged to 4 genotypes using rep-PCR. Rep-PCR is a method that generates DNA fingerprints to discriminate between bacterial strains, and has been used to characterize *A. baumannii* isolates from hospitalized patients [38]. Our rep-PCR typing represented a high genetic diversity (A-G) among *A. baumannii* isolates from Nepal. Some clonally related groups (A, B, C and D) were observed in the all wards represented the disseminated of these clones in the hospital. Four genotypes (A, B, C, and D) of co-existence of $bla_{\text{OXA-23}}$ and $bla_{\text{NDM-1}}$ *A. baumannii* isolates were found. In addition, dissemination of these four genotypes into different wards also confirms as a major epidemic. Since rep-PCR is less discriminatory for molecular typing of bacterial strains, further study using multi-locus sequence typing could be useful for epidemiological investigations.

Conclusion

Antibiotic resistance in *A. baumannii* is considered to be a major future challenge in Nepal. Beyond OXA-type carbapenemase, there is no doubt the emergence and spreads of NDM-1 encoding *A. baumannii*–a superbug–will further limit chemotherapeutic options and threaten the public health of Nepal. The mechanism of hospital adaptiveness beyond antibiotic resistance will be more demanded in order to fully understand and combat MDR and XDR *A. baumannii*.

Additional file

Additional file 1: Table S1. Type of clinical specimen, ward, antibiotic susceptibility patterns, rep-PCR types, resistance genes and MIC of 44 *A. baumannii* isolates. (DOCX 23 kb)

Abbreviations

CR-AB: Carbapenem-resistant *Acinetobacter baumannii*; ICU: Intensive care unit; IPM-EDTA: Imipenem-ethylenediaminetetraacetic acid; MBL: Metallo-beta-lactamase; MDR-AB: Multidrug-resistant *Acinetobacter baumannii*; MIC: Minimum inhibitory concentration; NDM: New Delhi metallo-beta-lactamase; PCR: Polymerase chain reaction; XDR-AB: Extremely drug resistant *Acinetobacter baumannii*

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

PRJ and MA designed the study, collected data, analyzed the data and prepared the manuscript, TK supervised the study, UL and RT collected data, SS, analyzed the data, supervised the study and prepared the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethical Review Board of Nepal Health Research Council (NHRC) (Reg. 27/2015). Informed consent was taken from all the patients or patients' guardians. The research was in compliance with the Helsinki Declaration.

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