ISSN 2249-3522

International Journal of Research in Pharmacy and Science

Research Article

Reassuring no blaNDM-1 harboring *K. pneumoniae* in neonatal intensive care unit of Aligarh Hospital, Uttar Pradesh, India

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ABSTRACT

This study was conducted to find the *K. pneumoniae* were screened for ***bla***$_{\text{NDM-1}}$ in clinical isolates from neonatal intensive care unit. The *bla*$_{\text{NDM-1}}$ producing *K. pneumoniae* has been emerged in recent years because highly resistant to different groups of antibiotics including carbapenems. We attempted to screen carbapenemase producers *K. pneumoniae* strain from NICU at Aligarh hospital located in North India, which is a tertiary care hospital. A total of 627 samples, 560 clinical strains were examined from 280 admitted patients and 67 strains from environmental, from Neonatal Intensive Care Unit (NICU) of Aligarh hospital located in North India, which is a tertiary care hospital. Antibiotic susceptibility testing was done by standard disc diffusion method and MIC was determined using two fold agar dilution methods according to CLSI guidelines. PCR amplification and sequencing were performed to detect the presence of various resistant markers. We found that 76.71 % isolates were positive for MBL and 12 % of them were resistant towards imipenem and meropenem. PCR amplification and sequence analysis confirmed the presence of ***bla***$_{\text{CTX-M-3}}$, ***bla***$_{\text{TEM-1}}$, ***bla***$_{\text{SHV-1}}$, ***bla***$_{\text{VIM}}$, ***bla***$_{\text{OXA-1}}$ and arm-A. None of the MBL producers were positive for ***bla***$_{\text{NDM-1}}$ and the resistance towards carbapenem was due to the presence of ***bla***$_{\text{VIM}}$ and ***bla***$_{\text{OXA-1}}$ genes.

Key words: *MBL, NICU, Antibiotic resistance, K. pneumoniae and NDM-1*

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen responsible for large proportion of nosocomial infection in neonatal intensive care unit (NICU). *K. pneumoniae* isolates are increasingly resistant to multiple antimicrobial agents[1]. Metallo-β-lactamases (MBLs) constitute the most clinically important group of carbapenemases since they hydrolyze virtually all β-lactams except the monobactam (aztreonam)[2]. Initially MBLs were detected in *Pseudomonas aeruginosa*, however nowadays they are frequently found in *K. pneumoniae* and other Enterobacteriaceae. MBLs spread easily on plasmids and cause nosocomial infections and outbreaks with excess mortality. Since the outbreak of a new subgroup of metallo-β-lactamase (MBL), designated New Delhi metallo-β-lactamase (NDM-1), originating from New Delhi, India which was first reported from a Swedish patient of Indian origin who travelled to New Delhi, India, and acquired a urinary tract infection caused by a carbapenem resistant *Klebsiella pneumoniae* strain[3]. There are many articles reporting ***bla***$_{\text{NDM-1}}$ possessing isolates from a tertiary care hospital in India[4], isolation of gram negative bacilli during a point prevalence survey carried out on a single day in the sick newborn care unit (SNCU) of a rural hospital in West Bengal, India[5]. Moreover, contrary to these reports, recently, Deshpande *et al*., showed that no ***bla***$_{\text{NDM-1}}$ carriage was observed among the clinical isolates from healthy persons at Hinduja National Hospital, Mumbai, India.

In view of the current situation we attempted to screen carbapenemase producers *K.pneumoniae* strain from Neonatal Intensive Care Unit (NICU), of Aligarh hospital located in North India, which is a tertiary care hospital.

ISSN 2249-3522

MATERIAL AND METHODS

Materials

Sterile cotton swabs, nutrient agar, nutrient broth, mueller hinton agar, muellerh hinton broth, Mac-Conkey agar, and antibiotic discs used were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Reagents of PCR were purchase from Sigma Aldrich USA.

Inclusion criteria

Here, we have collect the samples from nosocomial infected baby, was admitted to the NICU of Department of Pediatrics, Medical college, Aligarh (North India), with the complaints of fever, lethargy, refusal to suck and poor cry and haematuria on fifth day of post natal life. Newborn babies were clinically suspicious for late onset sepsis and the blood samples were sent for septic screen and culture under aseptic precaution. Since septic screening was in favour of sepsis so intravenous antibiotics cephotaxim and amicacin were started and kept under close observation. However, culture report was received during the treatment of sick babies. These newborn babies were found positive for *K. pneumoniae* as identified by using Hi-Crome Kleb selective agar base identification kit (Hi-media, Mumbai, India). Samples were collected from patients by sterile intra-vinous catheter and urine catheter.

Study design and patient population

The study was conducted on the neonates admitted in NICU (sick babies) of one of the hospitals of north India. It is a tertiary care unit of 1300 bed capacity, in which 90 beds were allotted for paediatric patients and 20 beds to NICU. 4000 neonates get admitted to NICU in a calendar year. The study period from January 2011 to February 2012. A total number of 2500 samples were screened from NICU, out of them 627 (25.08%) samples were found to have *K. pneumoniae*. Among these 627 clinical samples, 560 (eyelid, body surface, nose, urine, catheter etc but not rectal) were examined from 280 admitted patients and remaining 67 strains from environmental (instruments like mechanical ventilator, radiant warmer, phototherapy, cot, stethoscope, refrigerator and weighing machine) from Neonatal Intensive Care Unit (NICU), of Aligarh hospital located in North India, which is a tertiary care hospital. All samples were collected through sterile cotton swabs. Each neonates admitted to the ward was repeated. Information regarding study was obtained from the parents and consultants of NICU and clearance was obtained from institutional ethical committee held on 6 July, 2009. Clinical samples were incubated on Mac-Conkey agar at 37^{o}C and were characterized biochemically for *K. pneumoniae.* Moreover, 16S rDNA sequencing confirmed its presence.

Antibiotic susceptibility profile of MBL producing K. pneumoniae isolates

The antimicrobial susceptibility of isolates was performed by the standard disc diffusion method using Mueller Hinton agar as per Clinical and Laboratory Standards Institute, 2011 guidelines[6]. The antibiotic discs used (Table 1) were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India.

Metallo -β-lactamase (MBL) detection

MBL production was detected by combined disk diffusion method employing two disks of imipenem, meropenem and ertapenem (10 µg in each disk), in which one of the disks contained 292 mg (10 ml of 0.1M) anhydrous EDTA and placed 25 mm apart (centre to centre) on Muellar-Hinton agar plate. An increase in the diameter of inhibition zone by ≥4 mm around the imipenem + EDTA, meropenem + EDTA and ertapenem + EDTA disks as compared to that of the imipemen, meropenem and ertapenem disks alone indicated the presence of MBL[7]. All MBLs positive strains were subjected to ***bla***$_{\text{NDM-1}}$ specific colony PCR[8].

Minimum inhibitory concentration (MIC) of MBLs producing K. pneumoniae isolates

The MIC of all MBLs isolates was determined by the CLSI micro-broth dilution methods (CLSI, 2011). Appropriate dilutions of β-lactam antibiotic solutions were prepared according to the report of international collaborative study in which one part of the antimicrobial solution was added to nine parts of liquid Muller-Hinton agar. The MIC values were compared with the break points recommended by CLSI-2011 guidelines. *E. coli* ATCC 25922 strain was used as ESBL negative control and *K. pneumoniae* ATCC 700603 strain was used as ESBL positive control.

PCR amplification and sequence analysis of bla genes

Plasmids from clinical isolates were screened by PCR for the following β-lactamase genes ***bla***$_{\text{CTX-M}}$, ***bla***$_{\text{TEM}}$, ***bla***$_{\text{SHV,}}$ ***bla***$_{\text{OXA-1,}}$ ***bla***$_{\text{OXA-9,}}$ ***bla***$_{\text{OXA-10,}}$ ***bla***$_{\text{OXA-48}}$, ***bla***$_{\text{NDM-1,}}$ ***bla***$_{\text{VIM,}}$ AmpC, arm-A, rmt-A, rmt-B and ***bla***$_{\text{KPC}}$ using the oligonucleotide specific primer as given in table 2 [9,10]. The amplified products were sequenced using an ABI 3130 genetic analyzer (Applied Bio-systems). The obtained nucleotide sequences were searched for similar sequences in National Centre for Biotechnology Information (NCBI) database by using its BLAST program.

RESULTS

The present study was carried out on samples isolated from NICU of one of the hospitals of north India over a period of one year. A total of 2500 neonates that were screened for the nosocomial infection in the NICU, 627 (25.08%) were found to have *K. pneumoniae* strains.

Antibiotic susceptibility profile of MBLs producing K. pneumoniae isolates

Antibiotic susceptibility testing was performed for all the isolates and the result is given in table 1. Our study clearly indicated that 45-86% resistant against different groups of antibiotics (β-lactam, aminoglycosides, fluoroquinolones, quinolone and other groups of antibiotics) whereas 45-56% resistance was observed against 1st, 2nd and 3rd generation cephalosporins. However, all the isolates were found to be

susceptible to carbapenems (imipenem, meropenem and ertapenem) whereas, 7.48% of them showed resistance against imepenem and 4.57% resistance against meropenem. It is clear that MBL producing *K. pneumoniae* isolates showed significant resistance against broad spectrum of antibiotics.

Out of 627 clinical strains, 76.71% (481/627) were found to be positive for ESBLs and MBLs, Among the MBLs producers 12.05% (58/627) isolates showed carbapenem resistance (36 imipenem, 22 meropenem). The remaining 146 strains showed moderate resistant pattern to different antibiotics.

MIC of ESBL producing K. pneumoniae isolates

All 481 MBLs producing clinical isolates were used to determine MIC against cephalosporins and carbapenem groups of antibiotics the results are presented in supplementary table 1. All the isolates showed high level of resistance against the antibiotics of different generations of cephalosporins except ceftazidime. The individual activity by the antibiotic showed a resistant pattern against all the tested strains whereas, in carbapenem groups their MIC dramatically reduced by many folds.

PCR amplification and sequence analysis of bla genes

Among 58 carbapenemes resistant isolates, PCR amplification and sequence analysis revealed the presence of $\boldsymbol{bla}_{\text{CTX-M-3}}$ in 53 isolates, $\boldsymbol{bla}_{\text{TEM-1}}$ in 32 isolates, $\boldsymbol{bla}_{\text{SHV-1}}$ in 28 isolates, $\boldsymbol{bla}_{\text{VIM}}$ in 15 isolates, $\boldsymbol{bla}_{\text{OXA-1}}$ in 15 isolates and arm-A in 4 isolates. It is interesting to note that none of the MBL producers showed the presence of $\boldsymbol{bla}_{\text{NDM-1}}$, $\boldsymbol{bla}_{\text{KPC}}$, $\boldsymbol{bla}_{\text{OXA-9}}$, $\boldsymbol{bla}_{\text{OXA-10}}$ and $\boldsymbol{bla}_{\text{OXA-48}}$.

Table 1: Resistance pattern of *Klebsiella pneumoniae* strains from NICU, Aligarh hospital

Antibiotic groups	Antibiotics		Resistance to antibiotics		MIC range of MBL producer	
			MBL N=481 (76.71%)	Remaining N=146(23.28%)		
Aminoglycosides		G	302 (62.78)	78 (53.42)	-	-
		Tb	365 (75.88)	61 (41.78)	-	-
		Ak	245 (50.93)	43 (29.45)	-	-
Fluoroquinolones		Na	403 (83.78)	89 (60.95)	-	-
		Cf	318 (66.11)	87 (59.58)	-	-
		Gf	221 (45.94)	31 (21.23)	-	-
β-Lactams		A	414 (86.07)	71 (48.63)	-	-
		P	421 (87.52)	81 (55.47)	-	-
		Pc	329 (68.39)	76 (52.05)	-	-
Others		T	366 (76.09)	61 (41.78)	-	-
		Co	415 (86.27)	52 (35.61)	-	-
Cephalosporins	1st g	Ch	273 (56.75)	49 (33.56)	256-512	
		Cz	259 (53.84)	49 (33.56)	256-512	
	2n g	Cu	280 (58.21)	50 (34.24)	64-256	
		Cn	238 (49.48)	42 (28.76)	32-128	
	3rd g	Ca	226 (46.98)	32 (21.91)	02-04	
		Ce	220 (45.73)	29 (19.86)	128-256	
		Ci	217 (45.11)	18 (12.32)	32-128	
	4th g	Cpm	159 (33.05)	14 (9.58)	04-32	
Carbapenems		Imp	36 (07.48)	00 (00.00)	02-04	
		Mr	22 (04.57)	00 (00.00)	02-04	
		Ert	00 (00.00)	00 (00.00)	0.5-02	

Generation (g), Gentamicin (G), Tobramycin (Tb), Amikacin (Ak), Nalidixicacid (Na), Ciprofloxacin (Cf), Gatifloxacin (Gf), Ampicillin (A), Penicillin (P), Pipracillin (Pc), Tetracycline (T), CoTrimoxazole (Co), Cephalothin (Ch), Cefazolin (Cz), Cefuroxime (Cu), Cephoxitine (Cn), Ceftazidime (Ca), Cephotaxime (Ce), Ceftriaxone (Ci), Cefepime (Cpm), Imipenem (Imp), Ertapenem (Ert), Meropenem (Mr)

Table 2: Primers used in this study

S. N.	Primer name	Oligonucleotide Primer sequence (5'-3')		References
		Forward	Reverse	
1.	**CTX-M**	SCS ATG TCG AGY ACC AGT AAT	CCG CRA TAT GRT TGG TGG TG	Poirel *et al.*, 2011
2.	**TEM**	GTA TCC GCT CAT GAG ACA ATA	TCT AAA GTA TAT ATG AGT AAA CTT GGT CTG	--- do ---
3.	**SHV**	ATG CGT TAT WTT CGC CTG TGT	TTA GCG TTG CCA GTG CTC G	--- do ---
4.	**OXA-1**	TCA ACT	GTG TGT	--- do ---

ISSN 2249-3522

		TTC AAG ATC GCA	TTA GAA TGG TGA	
5.	**OXA-9**	TTC GTT TCC GCC ACT CTC CC	ACG AGA ATA TCC TCT CGT GC	Poirel *et al.*, 2011
6.	**OXA-10**	GTC TTT CGA GTA CGG CAT TA	ATT TTC TTA GCG GCA ACT TAC	--- do ---
7.	**OXA-48**	TTG GTG GCA TCG ATT ATC GG	TTG GTG GCA TCG ATT ATC GG	--- do ---
8.	**NDM-1**	GGT TTG GCG ATC TGG TTT TC	CGG AAT GGC TCA TCA CGA TC	--- do ---
9.	**VIM**	GTT TGG TCG CAT ATC GCA AC	AAT GCG CAG CAC CAG GAT AG	--- do ---
10.	**AmpC**	AACAGCC TCAGCAG CCGGTTA	TTCGCCG CAATCAT CCCTAGC	--- do ---
11.	**ArmA**	ATT TTA GAT TTT GGT TGT GGC	ATC TCA GCT CTA TCA ATA TCG	--- do ---
12.	**RmtA**	AAA CTA TTC CGC ATG GTT C	TCA TGT ACA CAA GCT CTT TCC	--- do ---
13.	**RmtB**	ACT TTT ACA ATC CCT CAA TAC	AAG TAT ATA AGT TCT GTT CCG	--- do ---
14.	**KPC**	CAGCTCA TTCAAGG GCTTTC	AGTCATT TGCCGTG CCATAC	Sabine *et al*., 2009.

DISCUSSION

Emerging carbapenem resistance in *K. pneumoniae* has become a major problem in community acquired and nosocomial infections worldwide [11,12,13], most typically attributed to production of *K. pneumoniae carbapenemase* (KPC) and is a cause of concern as many nosocomial *Klebsiella spp*. are detected to be resistant to carbapenem groups of antibiotics. However Carbapenems are used as last-resort drugs because increasing resistance against b-lactam groups of antibiotics has developed due to their excessive use in treating a wide range of infections[14].

One of the latest resistance enzymes, NDM-1 (New Delhi metallob-lactamase) was first identified in isolates from a Swedish patient of Indian origin in 2008. There is a limited literature available regarding the prevalence of resistance to carbapenems in *Klebsiella spp* from clinical isolates in our country. The emergence of these drug resistant strains has necessitated the requirement of a rapid and accurate identification and characterization of resistant markers in *K. pneumoniae*. Moreover, the analysis of antibiotic susceptibility, MIC of MBL, PCR amplification and sequence analysis have been reported as key players in emergence of NDM-1 harboring *K. Pneumonia* in NICU.

One of the most striking findings in the present study was 33-58 % resistance to first, second, third and fourth generation of cephalosporins among *K. pneumoniae* isolates. The SENTRY surveillance program reported the frequency of ESBLs producing *K. pneumoniae* to be approximately 37% in Latin America and 7% in the United States[15]. Within the Asian Pacific region, the prevalence of ESBLs producing *K. pneumoniae* isolate was reported to be 5%, 21.7%, 31% and 38% in Japan, Taiwan, Philippines and Malaysia/Singapore, respectively[16] .The present data show resistance against multiple group of antibiotic (β-lactam 68-87%, aminoglycosides 50-75%, fluoroquinolone 45-83% and others (tetracycline) 76). This is consistent with the previous findings[17]. In the present study, *K. pneumoniae* strains were also found to be highly resistant to tetracycline and cotrimoxazole. This is probably due to the fact that this antibiotic has been widely used over the past decade in this region because of the low cost and easy availability to the poor people residing in various under developed pockets of the otherwise developing nation. Similar studies have also been performed in other parts of India. Our data share harmony to previous reports[18]. Our study revealed the presence of ***bla***$_{\text{CTX-M-3}}$, ***bla***$_{\text{TEM-1}}$ and ***bla***$_{\text{SHV-1}}$ and ***bla***$_{\text{VIM}}$ genes on the plasmids which has also been reported earlier in Europe [19].

In our earlier work (2010), the presence of NDM-1 in two samples collected from patients admitted in ICU was reported. These two patients had history of taking advance generation of antibiotics for infectious disease treatment. One of the patients, a 69 year-old male (patient A), was admitted to the ICU of Aligarh Hospital, North India, with a diabetic foot and severe sepsis. To treat severe sepsis he was given intravenous antibiotics (including imipenem for a week) but the patient showed no response. Eventually, amputation at the knee had to be performed. The other patient, a 60-year-old male (patient B), was admitted to the endocrinology ward of the same hospital during the same period also with a diabetic foot ulcer. He underwent the same treatment with no recovery, finally developing severe sepsis which led to foot amputation. Fortunately, our present study shows that NDM-1 has not spread to the same extent as reported in the past[20]. The findings of our study are also contrary to those reported by Perry et al [21] .Therefore, our study reassures that ***bla***$_{\text{NDM-1}}$ harboring *K. pneumoniae* strains are not present in NICU of Aligarh Hospital, Our study revealed the no NDM-1 harboring *k. pneumoniae* in neonatal intensive care unit which has also been reported earlier in India one of the hospitals of North India[22]. In spite of the fact that no NDM-1 producers was observed during the study period

but still infection control and surveillance programme for dissemination measurements should be taken into consideration.

ACKNOWLEDGEMENTS

Author acknowledges BSR, government of India for the support and internal facilities of the department. This work was supported by internal funds of Biotechnology Unit, AMU, Aligarh.

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