Carbapenem resistant Enterobacteriaceae neonatal gut colonization: A future concern in healthcare settings

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Abstract

Introduction: Carbapenem-resistant Enterobacteriaceae (CRE) isolates are resistant to carbapenem and other beta-lactam drugs. Infections with these CREs have been reported in different age groups and are difficult to treat because of their resistant pattern and thus, they have become epidemiologically important.

Materials and Methods: Rectal swabs from 150 term/ late preterm neonates (> 35 weeks) and 150 preterm neonates (< 35 weeks) were collected who were hospital delivered and admitted in NICU and likely to stay > 3 days. Three rectal swabs were taken; 1st within 24hrs of birth (day 0), 2nd on day 3 and 3rd before discharge (day 4-10). They were screened for CRE in stool/rectal swab according to CDC criteria. These were further confirmed by following CLSI guidelines (MHT) to observe the carbapenemases production and real-time PCR for bla_{NDM-1}, bla_{IMP}, bla_{VIM}, bla_{KPC}.

Results: A total of 8.7% (26/300) possible CREs were isolated in term/late preterm (15/26, 57.7%) and preterm neonates (11/26, 42.3%). *Klebsiella pneumoniae* was the commonest organism. In total, 22/26 (84.6%) possible CREs were MHT positive and rest 4 were MHT negative. The majority of MHT positive CREs were multidrug-resistant whereas MHT negative CREs was sensitive to tested carbapenem drugs. Carbapenemases genes ($bla_{NDM-1=10}$, $bla_{VIM=8}$, $bla_{IMP=1}$, $bla_{KPC=0}$) were identified in 19/26 of possible CREs, but multiple carbapenemases genes were not found.

Conclusions: In this study, neonates were colonized with CRE within their gut as early as 72 hours after birth. This can cause infection in the postnatal period and may lead to spread of CRE in the community.

Keywords: Carbapenem-Resistant *Enterobacteriaceae*, Neonatal Intensive care Unit, Modified Hodge Test.

Introduction

Carbapenems are potent beta-lactam antibiotics having broad-spectrum activity and thus, have been traditionally rendered as 'last resort' antibiotics. They are active against many aerobic and anaerobic gram-positive and gram-negative organisms including *Enterobacteriaceae*.[1]

Inappropriate empirical antibiotic therapy is independently associated with poorer outcomes in patients with bacteremia due to *Enterobacteriaceae*.[2] The emergence and spread of extended-spectrum beta-lactamases (ESBL) and plasmid-mediated AmpC producing *Enterobacteriaceae* has led to use of carbapenems as frontline empirical antibiotics in many more clinical situations than before. Until recently, carbapenems were mostly used in the treatment of serious bacterial infection eg. post-operative wound infections and in intensive care units (ICU) settings. Now with the emergence of transferable resistance among the carbapenem-resistant *Enterobacteriaceae* (CRE) isolates which render them non-susceptible to all beta-lactam antibiotics, a public health emergency situation has arisen, as very limited available antimicrobials show activity against these isolates.[1]

The first carbapenemases producer in *Enterobacteriaceae* was identified in 1993.[3] Since then, a large variety of carbapenemases has been identified in *Enterobacteriaceae*. In developed countries like the United States, carbapenem-resistant *Klebsiella pneumoniae* strains harbor the *Klebsiella pneumoniae* carbapenemases (KPC's). There are also reports of the presence of carbapenemases in organisms other than *Enterobacteriaceae* like *Acinetobacter baumannii*, *Pseudomonas aeruginosa* around the globe.[3,4]

In developing countries like India, the epidemiological status of carriage of CRE is still unknown. There are reports of CREs in India isolated from blood, pus, stool and other body fluids from adults as well as children.[5-7] But till now, studies have not been conducted among neonates who are institutionally delivered and admitted in neonatal intensive care unit (NICU), to find out their gut colonization, as they can act as a potential source of transmission of such isolates in the community. Thus, keeping this fact as front-runner, the present study was conducted to find out the colonization of CRE in the naive gut of neonates who were institutionally delivered and admitted in NICU of our hospital.

Materials and Methods

The study was conducted in the Department of Microbiology and the samples were collected from Department of Paediatrics of the same tertiary care hospital, East Delhi for over a period of one year and approved by the institutional Ethical committee.

A total of 150 term/late preterm (> 35weeks) and 150 preterm (< 35 weeks) hospital delivered neonates who were admitted in NICU and likely to stay for > 3 days were included in the study after taking written consent from the parents. Since no studies have been conducted to best of our knowledge, there is no prevalence value available to calculate the sample size in our study. However, this was a pilot study and thus, it was decided to take 10% of the total admission in NICU. The data collected from NICU shows that a total number of neonates staying for more than three days is approximately 1500 in each group over the year. Hence, 150 neonates in each study group were considered as sample size.

Random samples were collected twice weekly to achieve the sample size. The rectal swabs (sterile cotton swab) from neonates were taken on day 0 (within 24hrs of birth), day 3 (on 3rd day birth) and before discharge (day 4 – day 10) and were processed within 15 - 20 minutes of collection of the samples according to centre for disease control (CDC) criteria of detection of carbapenem-resistant or carbapenemase-producing, *Klebsiella sp.* and *E. coli* from rectal swabs.[8] Briefly, rectal swabs were put in trypticase soy broth containing ertapenem disc (10 µg) and incubated overnight at 37 °C and on the next day, subcultured on Mac-Conkey agar plate and looked for lactose fermenting colonies, which were identified to species level.[9] Modified Hodge Test (MHT) was performed on these possible CRE isolates.[10]

Antibiotic Susceptibility Testing: The antibiotic susceptibility of all the *Enterobacteriaceae* isolates was determined by disc diffusion (Kirby-Bauer) method for the following antibiotics: amikacin (30 µg), ampicillin-sulbactam (10/10 µg), ciprofloxacin (5 µg), colistin (10 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), cefotaxime (30 µg), gentamicin (10 µg), netilmicin (30 µg), piperacillin/tazobactam (100/10) polymyxin B (300 units), tigecycline (15 µg).[10] For epidemiologic purposes, microorganisms predominantly bacteria, that are resistant to one or more classes of antimicrobial agents are labeled as a multidrug-resistant organism (MDR).[11]

Modified Hodge Test: CLSI guidelines for MHT were followed.[10] Briefly, *E. coli* ATCC 25922 suspension corresponding to 0.5 McFarland dilutions was further diluted 1:10 using normal saline. This was used to make the lawn culture on Muller Hinton agar plate and allowed to dry for 3-5 minutes. An ertapenem disc (10 µg) was placed in the center of the plate and the test organisms were streaked at the right angle from the edge of the disc to edge of the plate along with positive control (wild strains of the previous study) and negative control (*E.*

coli ATCC 25922). On one plate, four organisms were tested and plates were incubated at 35°C ± 2°C in ambient air for 16 - 24 hours and clover leaf-type indentation within the zone of inhibition of *E. coli* at the intersection of the test organism was observed.

Real-Time Polymerase Chain Reaction (RT-PCR): DNA was extracted from CRE isolates using 'HiPura^{TM} Bacterial Genomic DNA Extraction Kit' (Himedia, India) The DNA was subjected to Real-time PCR for bla_{NDM-1}, bla_{VIM}, bla_{IMP}, bla_{KPC} using SYBER green chemistry. Briefly, 20 µl of the reaction mixture which consisted of 5 µl extracted DNA, 1 µl of forward and reverse primer (Genex Life Science, India) each (conc. 10 pmol/µl), 10µl Lightcycler DNA master mix SYBER green-I (Roche) and 3 µl nuclease free water in one well of 96 well reaction plate was used. The reaction plate was loaded and real-time PCR was performed along with respective positive and negative controls on Roche light cycler 480 (Roche Applied Sciences, India) and the results were studied by T$_m$ calling. In the case of SYBER green I DNA binding dye, the melting temperature for or T$_m$ is defined as the point at which half of the DNA is double-stranded and half is single-stranded. The resultant amplicons were electrophoresed on 2% agarose gel.

RT-PCR Conditions: NDM-1: The primers and PCR cycles for NDM-1 were performed as per the protocol by Manchanda et al.[12]

VIM: The primers were used from Mandes et al[13] and PCR cycles used in the present study for VIM were standardized as follows: Initial denaturation at 95 °C for 10 mins Σ 1 cycle, amplification 45 cycles of 95 °C for 10 second(s), 60 °C for the 30s and 72 °C for 20s, melting step 1 cycle of 95 °C for 5s, and 65 °C for 1 min, cooling at 40 °C for 10s.

IMP: The primers were used according to Mandes et al[13] and PCR cycles used in the present study for IMP were standardized as follows: initial denaturation at 95 °C for 10 mins Σ 1 cycle, amplification 45 cycles of 95 °C for 10s, 58 °C for 45s and 72 °C for the 30s, melting step 1 cycle at 95 °C for 5s and, 65°C 1 min, cooling at 40 °C for 10s.

KPC: The primers were used according to Wang L et al[14] and PCR cycles used in our study for were standardized as follows initial denaturation at 95 °C for 10 mins Σ 1 cycle, amplification at 45 cycles of 95 °C for 10s, 60 °C for 30s and 72 °C 10s, melting 1 cycle at 95 °C for 5s and 65 °C for 1min, cooling at 40 °C for 10s.

Results

Out of 300 neonates studied, 26 (8.7%) had possible CRE according to CDC criteria for detection of CRE in stool/rectal swab. No possible CRE were isolated on day 0 of admission. Six out of 26 (23.08%) of possible CRE isolates were obtained on day 3 of admission and 20 (76.92%) at the time of discharge (day 4 - 10) the antibiogram of the 6 neonates colonized on day 3

remained the same at the time of their discharge. (Table 2).

Klebsiella pneumoniae (18/26, 69.2%) was the commonest organism among the possible CREs followed by *Citrobacter* spp. (4/26, 15.3%), *Enterobacter* spp. (2/26, 7.6%) and *Escherichia coli* (2/26, 7.6%). Eleven (11) and seven (7) *Klebsiella pneumoniae* were isolated in term/late preterm and preterm neonates, respectively and 2 *Citrobacter* spp. each in both the groups. Two (2) *Enterobacter* spp. were isolated in term/late preterm neonates and 2 *Escherichia coli* in preterm neonates only (Fig. 1).

Twenty-two of the 26 possible CREs were MHT positive, 18 being resistant and 4 sensitive to all three tested carbapenem drugs. Out of 26 possible CREs, 25 were MDR showing five different drug resistance patterns, while 1 strain was sensitive to all the tested antimicrobials (Table 3).

Real Time-PCR: Carbapenemases genes were identified in 19 possible CREs by Real-Time PCR ($bla_{NDM-1=10}$, $bla_{VIM=8}$, $bla_{IMP=1}$, $bla_{KPC=0}$). (Fig. 2-5)

More than one gene was not found in any isolates. 18 of these were resistant to carbapenem drugs and MHT positive. One isolate which harbored the carbapenemases gene was sensitive to the tested carbapenem drugs, but MHT positive. (Table 4)

Table 1: Primers

Primer	Sequence	Amplicon size (bp)
NDM-1F	5`-GCTGGCGGTGGTGACTC-3`	106
NDM-1R	5`-GGCAAGCTGGTTCGACAAC-3`	
VIM-F	5`- GTTTGGTCGCATATCGCAAC-3`	382
VIM-R	5`-AATGCGCAGCACCAGGATAG-3`	
IMP-F	5`-GAATAGGGTGGCTTAATTCTC-3`	188
IMP-R	5`-CCAAACCACTACGTTATC-3`	
KPC-F	5`TTGTTGATTGGCTAAAGGG-3`	106
KPC-R	5`-CCATACACTCCGCAGGTT-3`	

Table 2: Prevalence of possible CRE using CDC criteria

Neonates	Rectal Swabs			Total no. of neonates with possible CRE
	Day 0 n(%)	Day 3 n(%)	Day 4-10 n(%)	
Term/late preterm (150)	0 (0%)	3 (2%)	12 (8%)	15 (10%)
Preterm (150)	0 (0%)	3 (2%)	8(5.3%)	11 (7.3%)
Total (300)	0 (0%)	6 (2%)	20 (6.6%)	26 (8.7%)

Table 3: Drug resistance patterns in CRE

Drug Resistance	No. of organisms (n=26)	Resistant to drugs #
Pattern 1	15	AK, A/S, CIP, CTX, PIT, GEN, NET, MEM ETP, IPM
Pattern 2	4	A/S, CIP, CTX, PIT, GEN
Pattern 3	3	AK, A/S, CIP, CTX, PIT GEN, NET
Pattern 4	2	AK, A/S, CIP, CTX, PIT, NET, MEM, ETP, IPM
Pattern 5	1	A/S, CTX, PIT, GEN, MEM, ETP, IPM
Pattern 6	1	Sensitive to all drugs

AK=Amikacin, A/S=Ampicillin-Salbactam CIP=Ciprofloxacin, CTX=Cefotaxime, PIT=Pipercillin-Tazobactam, GEN=Gentamicin, NET=Netilmicin, MEM=Meropenem, ETP=Ertapenem, IMP=Imipenem

Table 4: Carbapenemases genes in possible CREs

(n=26)	bla_{NDM-1}		bla_{VIM}		bla_{IMP}		bla_{KPC}		No. of isolates harbouring carbapenemases genes
	+	-	+	-	+	-	+	-	
MHT positive C Sensitive (n=4)	1	3	0	4	0	4	0	4	1
MHT positive C Resistant (n=18)	9	9	8	10	1	17	0	18	18
MHT negative C Sensitive (n=4)	0	4	0	4	0	4	0	4	0
MHT negative C Resistant (n=0)	0	0	0	0	0	0	0	0	0
Total	10	16	8	18	1	25	0	26	19

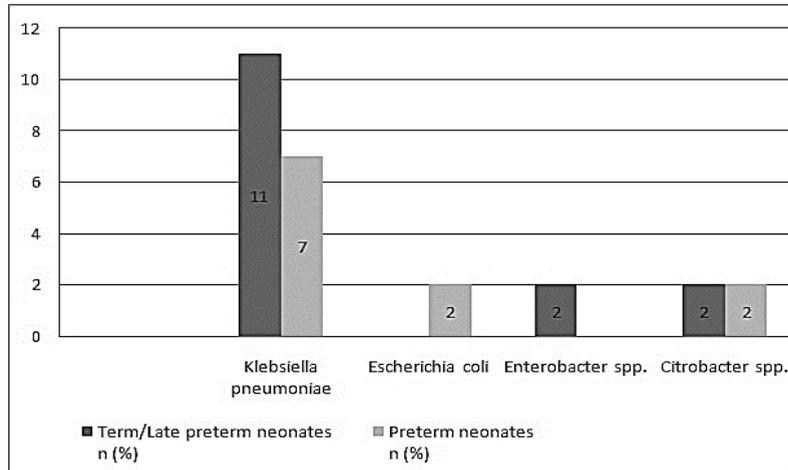MHT = Modified Hodge Test, C= 3 carbapenem drugs tested in the study
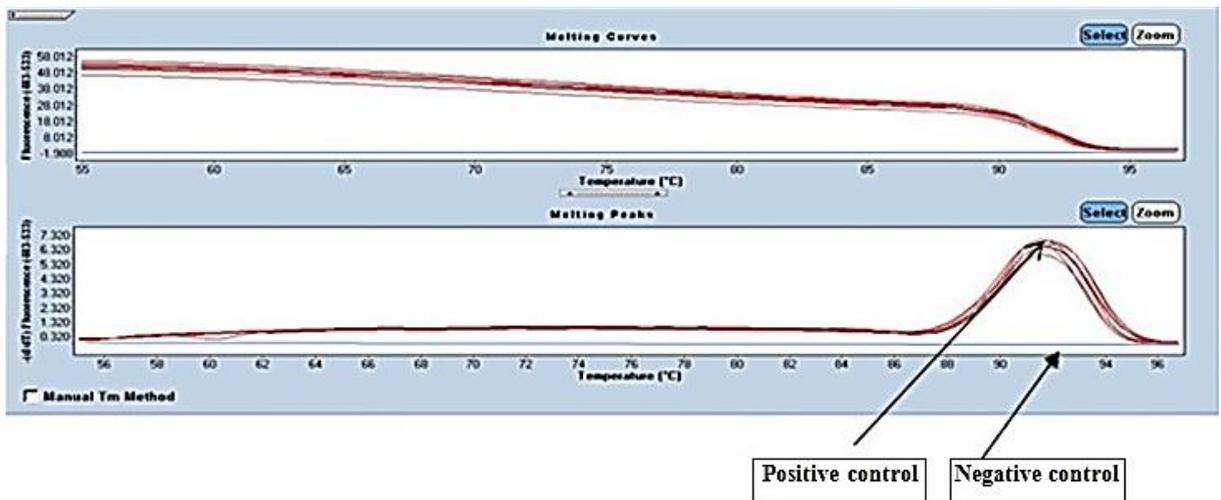
Fig. 1: Possible CRE isolated

Fig. 2: Demonstrates real-time PCR (Syber green based) amplification and melting curves for *bla*NDM-1
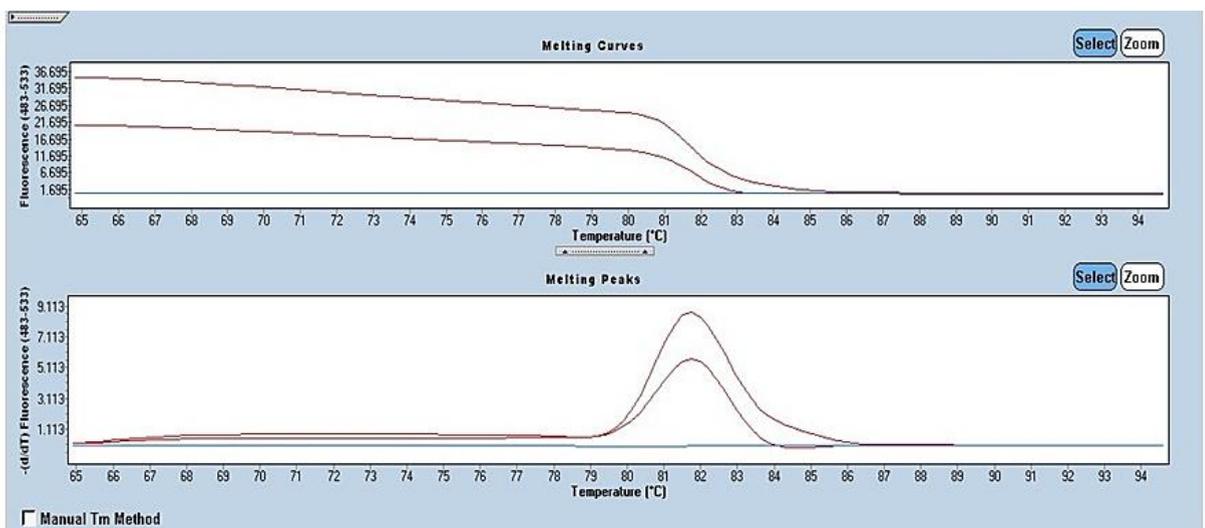

Fig. 3: Demonstrates real-time PCR (Syber green based) amplification and melting curves for *bla*IMP
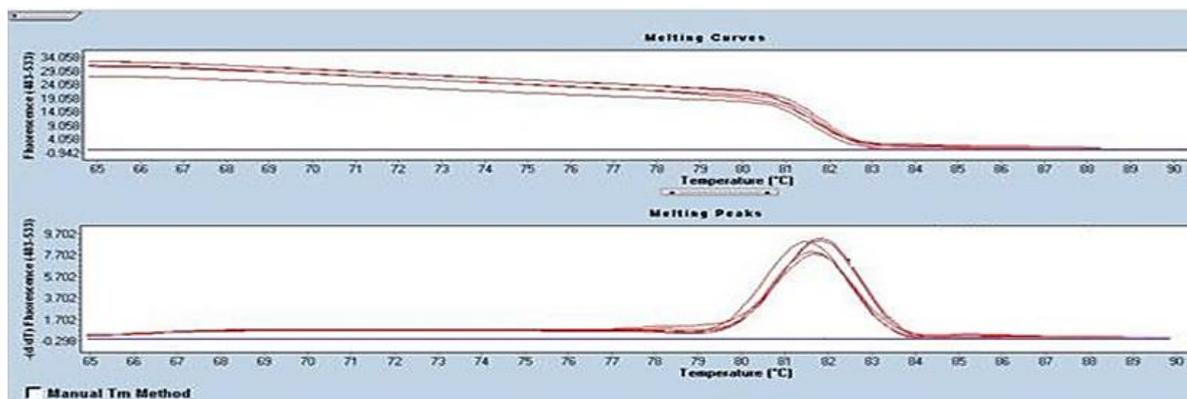
Fig. 4: Demonstrates real-time PCR (Syber green based) amplification and melting curves for *bla*VIM
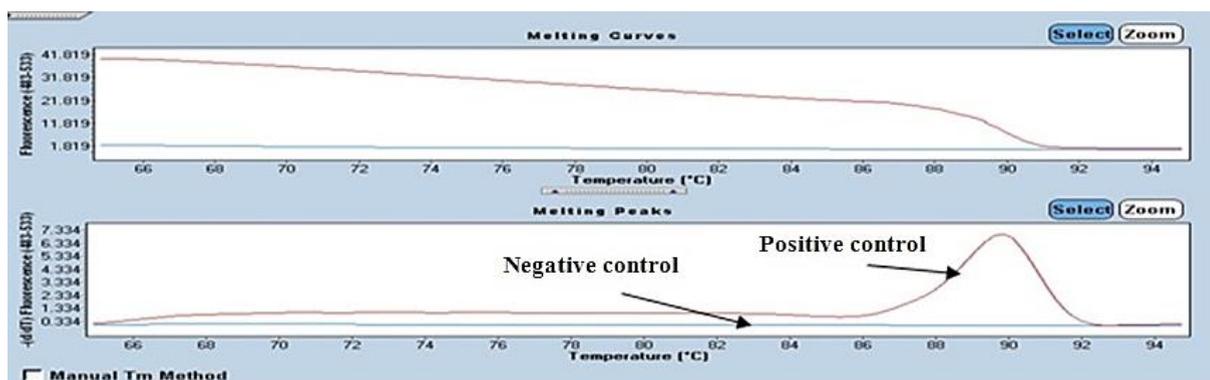

Fig. 5: Demonstrates real-time PCR (Syber green based) amplification and melting curves for *bla*KPC

Discussion

Detection of CRE gut colonization *among* neonates delivered and admitted in NICU being the primary objective of the study was observed in 8.7% (26/300) of the studied population. 10% (15/150) of the term/late preterm neonates and 7.3% (11/150) of the preterm neonates were colonized. Possible CREs were isolated as early as on day 3. Majority of the possible CREs could be isolated by the time the neonates were being discharged (day 4-10). In both the term/late preterm and preterm neonates, *Klebsiella pneumoniae* (69.2%) was the commonest colonizer.

An Indian study showed that the gut colonization with GNB in neonates increased with a longer stay in NICU.[15] This finding was similar to the present study in terms of increased colonization with duration of hospitalization. Another study conducted in a NICU from Chandigarh showed that 45% had sterile guts on day 1 and all infants were colonized by day 3.[16] The focus of the Chandigarh study was only on neonatal gut colonization without reporting AST. While the present study was aimed at CRE gut colonization and no CRE was isolated on day 0. Neonatal gut colonization with multidrug-resistant *Enterobacteriaceae* (MRE) in NICU has been documented from UK, Sweden, USA, and India.[14] However, there is a paucity of data on prevalence of CRE gut colonization in term/late preterm and preterm neonates in these studies.

A study from North India documented *E.coli* as major gut colonizer followed by *Klebsiella pneumoniae* and *Enterococcus fecalis* which is different from the findings of an Indian study from Kolkata that showed the equal prevalence of *E.coli* and *Klebsiella pneumoniae*.[15,16] In a point prevalence survey on CRE transmission among sick children by Roy S et al. found all 20 CREs were *E.coli* from 16 body sites including gut of the neonates.[6] So, it is evident that there might be geographical variation in the gut colonization by the species of *Enterobacteriaceae* family.

Similar to our study, MDR CRE isolates have been also reported around the world from gut of the adults and neonates.[5,6,15,18]

In a study from Italy by Mosca et al., *bla*KPC was reported in 100% of the CREs, where carbapenemases production detected by MHT was seen in 84%[15] An American study by Raghunathan A et al. showed that PCR assay for the *bla*KPC gene was positive in 82% of the MHT positive test isolates and was negative in 87% of MHT negative isolates.[19]

In our study, *bla*KPC gene was not detected in any of the 26 CRE isolates and carbapenemases genes were found in 86.36% (19/22) MHT positive isolates which include 18 carbapenem-resistant and 1 carbapenem sensitive isolates. The MHT false positivity could be the result of the production of a high level of AmpC and due to minor carbapenem hydrolysis by CTX-M and AmpC

enzymes and or presence of other carbapenemases genes with the weak hydrolyzing activity of carbapenemases enzymes.[18]

There are also reports of a difference in carbapenemases genes in different studies around the globe. Logan LK study revealed the presence of NDM, KPC, VIM-1, IMP-4, OXA-181, and SME genes in 64 clinical CRE isolates from the five different countries (India, Israel, Greece, UK, and Spain). Indian CRE isolates of Logan KL study showed the only presence of the bla_{NDM} gene.[21] An Indian study by Roy S et al. from Kolkata on isolates from NICU with CRE infections focused only on the presence of bla_{NDM-1} gene.[6] Deshpande et al. from Mumbai and Seema et al. from Banaras in their studies also focused on bla_{NDM-1} in *Enterobacteriaceae* from various clinical specimens irrespective of age.[22,23] This difference of result from our study may be explained by the differences in focus of other studies, the geographical variation in the presence of different carbapenemases genes.

According to CDC criteria, the prevalence of possible CREs was 8.7% (26/300) while after final confirmation by PCR, the prevalence of CREs was 6.6% (19/300) for studied four carbapenemases genes. The difference can be explained by the fact that we have looked for only four types of carbapenemases genes and other mechanisms of carbapenem resistance were also not studied.

In our study, CREs were isolated as early as on day 3. Though most of the CREs were isolated from the sample taken on day 4-10 i.e. before discharge from NICU and no other studies have been conducted to best of our knowledge to find out the CRE gut colonization of hospital delivered and admitted term/late preterm and preterm neonates in NICU. The limitations of the study could be the small number of studied population, no follow up of the persistence of CRE gut colonization in those neonates after discharge from NICU and MICs were not observed in these multidrug-resistant CRE isolates. So, for a better understanding of resistance pattern and mechanisms of gut colonization in neonates, a study should include large sample size with their follow up.

Conclusion

Neonates in a NICU environment are colonized with potentially pathogenic organisms within the first week of life itself. This colonization may be associated with diseases in the neonatal period (such as sepsis, NEC) as well as post-neonatal diseases (such as atopy, inflammatory bowel disease, irritable bowel syndrome etc). The nosocomial acquisition is the most common cause of CRE colonization. These neonates may carry CREs from the hospital to the community after being discharged. This may increase the likelihood of appearance of CRE infections, new drug resistance patterns and evolution of carbapenemases genes in the community. These CREs have a potential to spread rapidly within the hospital environment, in the community and also across the continents which have become a major public health concern.

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