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Original Research Article

Intestinal carriage and molecular characteristics of carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* among patients in intensive care units and outpatients in a tertiary care hospital

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ABSTRACT

Introduction: Intestinal carriage of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* (CREK) plays an important role in the epidemiology of carbapenemase producers. Less is known about the carriage of CREK among the outpatients when compared to carriage among patients in Intensive Care Units. (ICU) Therefore the present study was performed to detect colonization of CREK among ICU patients and outpatients of our tertiary care hospital.

Materials and Methods: Rectal swabs from ICU patients and stool specimens from outpatients were collected. Identification and antimicrobial susceptibility were performed using Vitek 2 compact system. Screening for CREK was done by two methods and confirmed for carbapenemase production by mCIM. Carbapenemase genes were detected by multiplex polymerase chain reaction.

Results: Overall 460 patients were analyzed for the intestinal carriage of CREK, 230 patients each from ICU and outpatients. 10.4% of outpatients and 26% of ICU patients found positive for CREK carriage. The target genes for carbapenemase production found in 78/86 CREK isolates. The majority of isolates 50/78 (64%) harbored beta-lactamase (bla) NDM gene followed by blaOXA-48 like in 18/78 (23%) isolates and 10/78(12%) isolates had both the genes.

Conclusion: Detection of carbapenem-resistant genes in commensal flora of the gut is worrisome. Building proper awareness about the use of antimicrobials in the community and strict surveillance systems to monitor these resistant bacteria in humans, food-producing animals, and the environment could all help to reduce the colonization of CREK among healthy individuals. Implementation of strict infection control measures and prudent use of carbapenems can help to limit the spread of these superbugs in the hospital.

1. Introduction

India is the major contributor to the global burden of antimicrobial resistance (AMR), which is an ancient evolutionary phenomenon in bacteria. According to recent predictions, deaths caused by AMR could reach 10 million by 2050. Overuse and misuse of the antimicrobials in clinical practice, animal and food industry made the human gut as a reservoir for AMR genes. Consequently, drug resistant genes initially detected in clinical isolates are now noticed in intestinal colonizers.

The effortless horizontal interspecies transfer of genes coding for carbapenem hydrolyzing enzymes, through the mobile genetic elements has led to the emergence and speedy dissemination of carbapenem-resistant Enterobacterales (CRE) both in the community and in hospitals.[1–3] Therefore the distinction between hospital-acquired and community-onset bacterial infections has become imprecise over the past few years.[4] The prevalence

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of carbapenem resistance, available from the previously published literature ranges from 5.9% to 59% in India.[5]

The Gastrointestinal tract of patients in the hospital becomes an epicenter for resistant bacteria. Patients become more vulnerable to infections or carriage by CRE due to selection pressure of antimicrobials, long-term hospitalization, and invasive procedures. Colonization by CRE increases the risk of subsequent invasive infection by two-fold in colonized patients. CRE infections following colonization found to be 16.5%. by Mohapatra et al.[6]

Asymptomatic carriers also play an important role in the epidemiology of carbapenemase producers. Hence it is critical to monitor changes in the prevalence and distribution of CRE not only in infected patients but in asymptomatic carriers as well. Several studies have been conducted to investigate the fecal carriage of CRE among ICU patients but there is limited information about the carriage and spread of CRE among the outpatients.[3,7]

Therefore the present study was performed to detect the rate of colonization by Carbapenemase-producing carbapenem-resistant *Escherichia coli* and K*lebsiella pneumoniae* [CREK] among patients admitted to the ICU and attending the outpatient clinics of our tertiary care hospital during the same period, irrespective of the patient's clinical history or exposure to antimicrobials. The study also aimed to detect the genes responsible for carbapenemase production among CREK isolates circulating in the hospital and the community

2. Materials and Methods

The prospective cross-sectional study was carried out at the Department of Microbiology in a tertiary care hospital. The study was approved by the institutional ethics committee. The samples were collected over 5 months between January and May in the calendar years 2019 and 2021. The sample size was calculated by using the OpenEpi statistical tool at a 95% confidence level. Based on the prevalence of 18% as reported by Mohan et al.,[8] the sample size for the present study was calculated to be 230 from ICU patients. The minimum sample size of OPD patients was calculated to be 138 based on the prevalence of 9.9% reported by Roy et al.[9] However, to have adequate CREK for molecular analysis and to have a comparable number of samples, a total of 230 samples were collected in the same period from outpatients.

Fecal specimens of the outpatients, that were collected in clean leak-proof containers and sent to the Microbiology laboratory for routine investigations were used for the detection of CREK. Samples were processed within a maximum of 2 hours after collection. If there was a delay in processing, samples were stored at 4^0 C in the Cary-Blair transport medium.

Even though the stool is an appropriate sample for the detection of intestinal carriage of CREK, it is not easy to collect a fresh stool sample in the ICU owing to its association with medically complicated and nil by mouth patients. Bassis et al. showed that the composition of bacteria in stool specimens can be comparable with rectal swab specimens.[10] Since rectal swabs have been used as an alternative for stool samples by many hospital epidemiologists and clinical microbiologists,[11] we have collected rectal swabs from ICU patients. Informed consent was taken by the patients or patient attendants in the ICU before collecting rectal swabs. Rectal swabs were collected, one each on the day of admission and the seventh day or before discharge from ICU. Patient demographic details and brief clinical history were collected from the medical records.

Rectal swabs were collected using sterile cotton swabs by inserting the swab 1cm into the rectum while rotating the swab, with the help of a trained nurse. Then the swab was placed in the transport tube containing sterile Cary-Blair medium and vortexed for 1 minute upon arrival at the laboratory.

Screening for Carbapenem-resistant *E.coli* and *K. pneumoniae* was carried out by culturing saline fecal suspension or Cary-Blair transport medium (CBT)containing rectal swab specimen (one full planting loop in 3 ml of saline) by CDC recommended broth enrichment protocol(CETP) and direct ertapenem disc protocol (DETP).

CETP-protocol was carried out by adding 100μL of the saline suspension in 5 mL of tryptic soy broth containing a 10μg disk of ertapenem. Following overnight incubation at 37°C, It was streaked onto MacConkey agar and incubated for 18-24 hrs. The presence or absence of bacterial growth was recorded.[12]

DETP- direct ertapenem disc method was a single step, direct process wherein saline suspension was directly inoculated onto MacConkey agar and streaked to 4 quadrants. An ertapenem disk was applied at the junction of quadrants1&2.Isolates growing within a zone diameter of ≤ 21 mm were recorded.[13]

Lactose-fermenting colonies with different morphotypes grown in method CETP and within a zone diameter of ≤ 21mm in DETP protocol were picked up and subjected to Gram stain. Further identification and antimicrobial susceptibility testing of all Gram-negative bacilli were done by Vitek 2 automated system (bioMérieux, Marcy l'Étoile, France).

All CREK isolates detected by anyone or both the screening methods were subjected to the modified carbapenem inactivation method (mCIM) recommended by the Clinical laboratory standards institute (CLSI) as a phenotypic confirmatory test for carbapenemase production.

2.1. Modified carbapenem inactivation method [mCIM]

mCIM was performed according to Pierce et al. In brief, 1 μL saline suspension of the test organism was added into

a tube containing 2 ml of tryptic soy broth (TSB), then a 10μG MEM (meropenem) disk was aseptically added to the bacterial suspension. After 4 hrs of incubation at 37^0c, the meropenem disk was removed from the TSB bacterial suspension and was placed on the lawn culture of *Escherichia coli* (ATCC 25922). Then incubated at 18 to 24 h at 35°C in an inverted position. The absence of an inhibition zone was considered positive, whereas a clear inhibition zone of ≥19mm was considered negative [14]

All CREK isolates were further confirmed by Multiplex PCR for the presence of blaVIM, blaIMP, blaNDM, blaKPC, and blaOXA-48 like genes by using previously published primers and cycling conditions [15]

2.2. Polymerase chain reaction (PCR)

DNA was isolated from bacterial colonies using the boiling lyses method. PCR was performed in 20 μL of the reaction mixture containing 10 μL Quick-load Taq 2X PCR Master Mix, 2 μL primer mixture, and 2 μL of DNA template with the following cycling conditions: 95°C for10 min; 95°C for 1min, 59°C for 30 sec, and 72°C for 1 min for 30 cycles, with a final extension at 72°C for 10 min. PCR products were visualized on a 1.8% agarose gel stained with ethidium bromide. [15]

2.3. Sequencing

Amplified DNA (25uL) of the randomly selected two *E.coli* and two *K.pneumoniae* isolates were sequenced at Eurofin genomics India Pvt. Ltd. The data was subjected to a nucleotide sequence similarity search using the BLAST available at the National Centre for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Statistical analysis

The data were analyzed in EPI INFO 2005 software of the World Health Organization. Data were expressed in terms of percentages for qualitative variables, mean and standard deviation for quantitative variables. Statistical differences between groups were tested using Fisher's exact test.

3. Results

Overall 460 patients were analyzed for the intestinal carriage of CREK, 230 patients each from ICU and outpatient clinics. In the ICU, 145(63.6%) were male and 85 (36.4%) were female patients of different age groups. A total of 87 (37.8%) patients were transferred from other hospitals or shifted to ICU after a period of hospitalization in the ward. The mean age was 48.5 years and the mean duration of ICU stay was 5.1 days. Among the outpatients, 137(59.6%) were male and 93(40.4%) were female patients of different age groups. The mean age was 35.1 years.

In general colonization rate of CREK was 18.6% (86/460). A total of 24/230(10.4%) outpatients and 62/230 (26%) ICU patients of different age groups were found positive for the intestinal carriage of CREK. A single carbapenem-resistant *E.coli* or *K.pneumoniae* was isolated from each patient. The proportion of outpatients positive for CREK colonization was highest in the age group of 71-80 years (25%), and the least was in 1-10 years(8%). (Table 1)

In the ICU highest proportion of patients colonized with CREK was found in the age group of 51-60 years (63%) and none were colonized in the age group 18-20 years. (Table 2) However, the gender and age of the patients did not show a statically significant association with intestinal carriage of CREK ($p > 0.05$).

The mean duration of ICU stay among colonizers was 8.6 days and among noncolonizers, it was 3.9 days. A statistically significant association was found ($p < 0.05$) between the duration of ICU stay and colonization by CREK. But the number of patients decreased with the increase in the duration of stay in ICU. More colonizers were found during 3-10 days of ICU stay. (Figure 1)

Overall 143 patients were admitted directly to the ICU and 87 were transferred from wards or other hospitals. In the colonizer group, 20(32.3%) were admitted directly to the ICU and 42 (67.7%) were shifted from wards or another hospital. Hospitalization just before shifting to ICU was found statistically significant, ($p<0.05$) provided directly admitted patients should not stay for a longer duration (>6 days) in the ICU.

A total of 46 carbapenem-resistant *E.coli* and 40 *K.pneumoniae* were isolated. (Figure 2) In ICU 37 (59.6%) carbapenem-resistant *K.pneumoniae* and 25(40%) *E.coli* were isolated from the 62 colonized patients. From outpatients, 21 carbapenem-resistant *E.coli* (87.5%) and 03(12%) *K. pneumoniae* were isolated. *E.coli* was the predominant species isolated from outpatients while *K.pneumoniae* was found to be significantly higher among ICU patients. ($p < 0.05$)

All these isolates were detected by one of the screening protocols performed. All 86 carbapenem-resistant isolates were detected by DETP protocol. CETP detected 44/86(51.6%) CREK isolates. Except for 03 isolates, all were resistant to carbapenems tested.(MIC ≥8μg/ml-≥16μg/ml) All 86 isolates were tested for carbapenemase production by the mCIM test. Among them, 07/86 isolates tested negative for carbapenemase production.

Multiplex PCR detected the target genes for carbapenemase production in 78/86 CREK isolates. None of the target genes were detected in the remaining 08 isolates. Among these 08 isolates, 02 *E.coli*, and 01 *K.pneumoniae* were sensitive to carbapenems and tested negative by mCIM. 03 *K.pneumoniae*, 01 *E.coli* isolates were resistant to carbapenems but tested negative by mCIM. The remaining 01 *E.coli* isolate was resistant to

carbapenems and tested positive by mCIM.

The majority of isolates 50/78 (64%) harbored the blaNDM gene followed by blaOXA-48 like in 18/78 (23%) isolates and 10/78(12%) isolates had both the genes. Other target genes blaVIM, bla IMP and bla KPC were not detected. blaNDM was found in 37/46(80.4%), bla OXA in 3/46 (6%) and both genes in 2/46 (4%) of *E.coli* isolates. None of the target genes were detected in 4/46(8.6%) *E.coli* isolates. Among *K.pneumoniae* 13/40(32.5%) harbored the blaNDM gene, 15/40(37.5%) harbored bla OXA-48 like gene and 8/40(20%) had both the genes. None of the target genes were detected in 04/40(10%) *K.pneumoniae* isolates. (Figures 3 and 4)

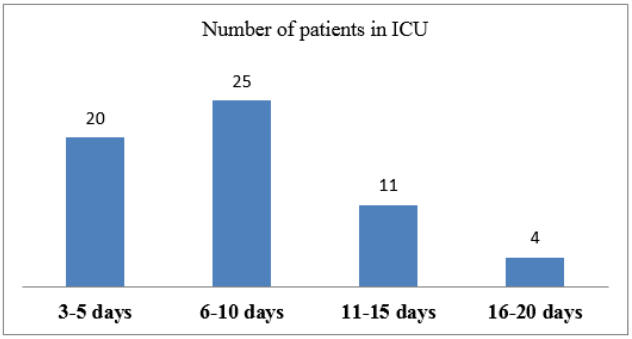

Figure 1: Distribution of patients according to number of days in ICU

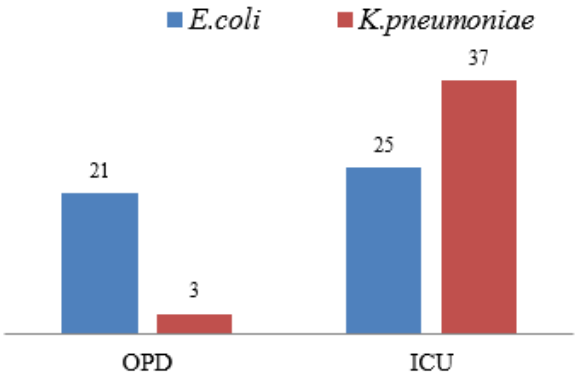

Figure 2: Distribution of CREK among colonizers

Among *E.coli* isolates, blaNDM was a more frequent gene than blaOXA-48-like gene and statistically significant.(p <0.05) whereas blaOXA -48 like and blaNDM genes were distributed almost equally among *K. peneumoniae* isolated from ICU patients. Only bla NDM gene was detected among *K. peneumoniae* isolated from outpatients.

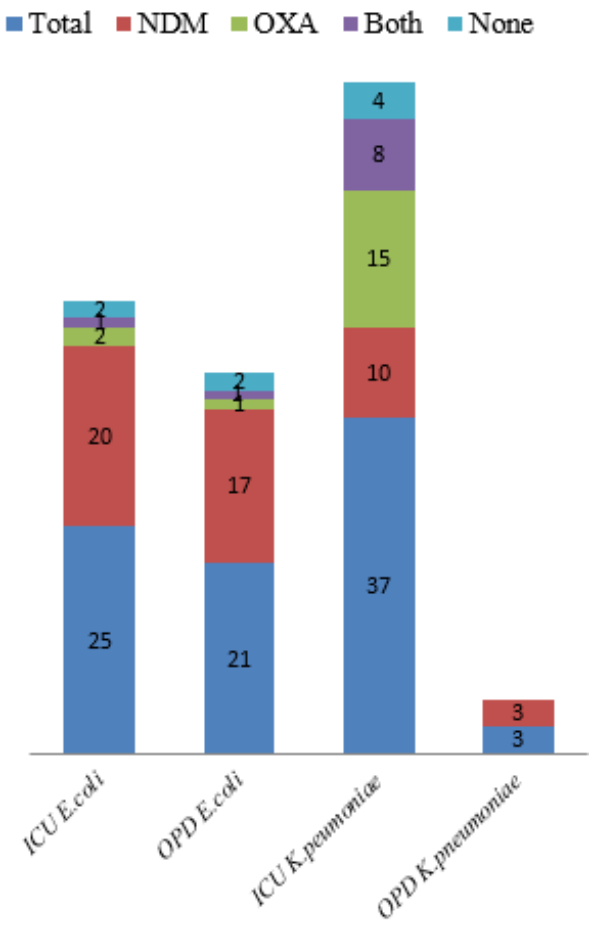

Figure 3: Distribution of carbapenemase genes among CREK isolates.

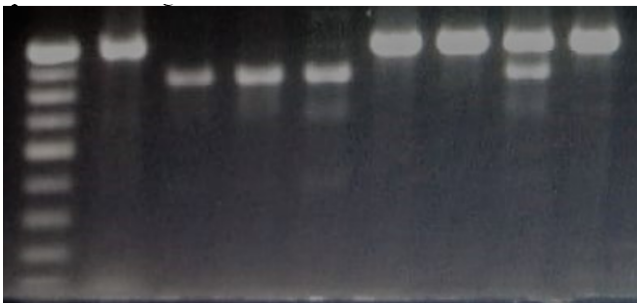

Figure 4: Agarose gel image of multiplex PCR: 100 bp ladder, well 1,5,6 & 8 - blaNDM -980bp, well 2,3&4 OXA-48 like gene–800bp, well 7- both genes

Table 1: Age and sex distribution of CREK Colonizer and non-colonizers among OPD patients

Age in years	No of male patients		No of female patients	
	CREK Colonizers	CREK Non Colonizers	CREK Colonizers	CREK Non Colonizers
1-10	01(3.8%)	25(96.2%)	02(18%)	09(82%)
11-20	02(11.7%)	15(88.3%)	Nil(0%)	08(100%)
21-30	01(5.5%)	17(94.5%)	03(18%)	13(82%)
31-40	05(16%)	25(84%)	Nil(0%)	14(100%)
41-50	02(10.5%)	17(89.5%)	02(12.5%)	14(87.5%)
51-60	03(17%)	14(83%)	Nil(0%)	16(100%)
61-70	02(20%)	08(80%)	Nil(0%)	08(100%)
71-80	Nil	Nil	01(25%)	03(75%)
81-90	Nil	Nil	Nil	Nil
Total	16	121	08	85

Table 2: Age and sex distribution of CREK colonizer and non-colonizers in the ICU

Age in years	No of male patients		No of female patients	
	CREK Colonizers	CREK Non Colonizers	CREK Colonizers	CREK Non Colonizers
18-20	Nil(0%)	05(100%)	Nil(0%)	08(100%)
21-30	06(26%)	17(74%)	01(14%)	06(86%)
31-40	08(25%)	24(75%)	09(34%)	15(66%)
41-50	05(22%)	17(78%)	02(22%)	07(78%)
51-60	13(52%)	12(48%)	01(9%)	10(91%)
61-70	06(23%)	20(77%)	02(15%)	11(85%)
71-80	03(30%)	07(70%)	04(44%)	05(66%)
81-90	01(50%)	01(50%)	01(25%)	03(75%)
Total	42	103	20	65

Table 3: DNA sequencing results of carbapenamase genes

Amplicon	Source	Gene detected	BLAST result
Amlicon 1	*E.coli*(from ICU patient)	bla NDM	bla NDM-5
Amlicon 2	*E.coli*(from outpatient)	bla NDM	bla NDM-5
Amlicon 3	*K.pneumoniae*(from ICU patient)	bla NDM	bla NDM-1
Amlicon 4	*K.pneumoniae*(from ICU patient)	bla OXA-48 like	bla OXA-232

The nucleotide BLAST results at 97% query cover and 99% identity showed that Amplicon 1and Amplicon 2 are producing significant alignment with bla NDM-5 and Amplicon 3 with NDM-1. Amplicon 4 was producing significant alignment with bla OXA-232. The sequences obtained by sequencing amplicon-1, amplicon-3, and amplicon-4 have been submitted to GenBank under accession numbers OM276847, OM276848, and OM460174 respectively.

4. Discussion

The human intestinal tract plays a prominent role in the development, selection, multiplication, and dissemination of CREK in the community and hospital in an unnoticed manner. Colonization with CREK often goes undetected unless carriage leads to infection or is identified through surveillance cultures. Roughly 16.5% of CREK colonized patients in the hospital progressed to clinical infection but less is known about the association of CREK in the community.[16,17] The overall fecal carriage rate of carbapenemase-producing CREK isolates was 18.6%(86/620). The carriage rate was significantly higher in ICU (26%) than in the outpatients (10.4%). These results are in concurrence with previous studies[8,9] and lend support to the existence of selective antibiotic pressure and bacterial transmission in ICU. The predominance of *E.coli* among outpatients and *K. pneumoniae* in ICU patients was also found in other studies.[3,8,18,19] Detection of CREK in fecal samples from outpatients raises the possibility of community-acquired CREK infections, which is concerning.

In the study conducted by Konar et al.[6] prior hospitalization just before being shifted to ICU was

a significant risk factor for the colonization, which is in parallel with the findings of our study, wherein CREK colonization among patients shifted from wards or transferred from other hospitals was significantly higher ($P \leq 0.05$) when compared to direct admission to ICU. The association between the length of ICU stay and colonization by CREK was found to be statistically significant. This finding was further emphasized by the findings of Mohan et al.[8] where the duration of ICU stay was a significant risk factor for CREK colonization. A maximum number of colonizers were found between 5-10 days of ICU stay. Similarly, Konar et al.[6] found that the colonization of MBL-producing organisms increased with an increase in the duration of stay in the ICU. Mittal et al.[18] found more number of patients colonized with CRE on day four than on day one of ICU admission. However, other factors like gender and age did not show statistical significance of association in ICU and outpatients. The results are comparable to Mohan et al.[8] and Antony et al.[3] respectively.

Screening of CREK isolates was carried out by using DETP and CETP protocols. DETP protocol allowed to detect 42 more CREK isolates than CETP. This was substantiated by Loans et al.[20] where the direct MacConkey plate method was found more sensitive than broth enrichment methods. Segarra et al.[21] stated that the CETP is highly sensitive for the detection of KPC carbapenemases. The lack of KPC producers in the present study could be the reason for the low detection rate of the CETP protocol.

All presumptively identified CREK isolates were further confirmed by mCIM and multiplex PCR for carbapenemase production. Among CREK isolates 78/86 showed the presence of target genes. DETP detected 02 carbapenem-sensitive isolates. Carbapenem resistance in remaining PCR negative isolates could be due to other mechanisms or the existence of variants of target genes tested for carbapenemase production.

In India NDM Metallo beta-lactamase is widely disseminated among the enteric pathogens and has been reported in isolates from public tap water and sewage. Oral-fecal transmission plays an important role in the spread of these bacteria to the community in India with poor sanitation facilities.[22]

The PCR results showed that blaNDM (64%) was the predominant gene followed by OXA-48(23%) gene. The association of bla NDM and *E.coli* ($P > 0.05$)was found statistically significant. Gupta et al.[18] found that *E.coli* was the most resistant isolate from the stool samples of healthy individuals in a semi-urban community. They found blaVIM in one isolate which reflected the low-level circulation of carbapenemases in the community. Antony et al.[3] revealed the high carriage rate(31%)of multidrug-resistant *E.coli* followed by *K.pneumoniae* in the rural adult population. Among them, 0.65% were NDM producers. The low prevalence of NDM producing CREK may be due to exclusion of individuals from the study with risk factors that could potentially affect the normal flora of the gut. Patients from an urban or semi-urban community with high access to hospitals and collection of samples regardless of the patient's clinical history and antibiotic exposure could explain the high carriage rate of NDM producers in our study compared to other studies. The findings of our study are in concurrence with that of Shen et al.[23] who reported 47 % NDM producing CRE from a healthy population in China. This devastating predominance and dissemination of NDM producing CREK in the community indicate the percolation of NDM producing CREK through the weakened border between the hospital and community habitation.

Since the discovery of bla NDM twenty-four variants have been identified in more than 60 species of 11 bacterial families. *K. pneumoniae* and *E.coli* are the predominant carriers of the blaNDM gene. In India, carbapenem resistance in *E.coli* is commonly mediated through the blaNDM-5gene with enhanced carbapenemase activity than other blaNDM gene variants.[24,25]

Jaggy et al.[26] stated that NDM-5 is the most common variant in *E.coli* isolated from stool samples which suggest the existence of bla NDM-5 harboring *E.coli* in the gut microflora in the community. Shen et al.[23] found NDM-5 as the major mediator of carbapenem resistance in *E.coli* isolated from healthy volunteers in China. The amplicon sequencing results of our study showed that *E.coli* isolated from ICU and outpatients harbored the balNDM-5 gene indicating the circulation of NDM-5 producers in the community and hospital. *K.pneumoniae* from ICU patient harbored blaNDM-1 gene. Raghupathi et al.[25] stated that, the NDM-1 is still a common mechanism of carbapenem resistance in other species of MDR bacteria in India.

In our study bla OXA-48 like genes were predominant among *K.peumoniae* isolated from ICU patients but absent among outpatients. Among *E.coli*, blaOXA-48-like genes were detected in only 02 isolates from ICU and one isolate from outpatients.

India is considered to be endemic for OXA48-like carbapenemases and frequently reported in clinically important nosocomial pathogens *E. coli* and *Klebsiella pneumoniae*.[27,28] Bakthavatchalam et al.[27] stated that most of the OXA-48-like carbapenemases are reported in *K. pneumoniae*. Even though oxa-48-like carbapenemase producers are less among OPD patients, rapid dissemination of plasmid carrying bla OXA 48-like gene associated with mobile genetic elements is worrisome. The enhanced transfer of plasmid carrying bla OXA-48 like gene could be due to insertional inactivation of tir gene by Tn1999 transposon which codes for the transfer inhibition protein[28,29]

K.pnemoniae isolated from ICU showed the presence of bla OXA-232 gene, a predominant variant of OXA-

48 like carbapenemase among carbapenem-resistant *K.pneumoniae*.[28] More isolates need to be sequenced to recognize the varients of bla NDM and bla OXA-48 like genes circulating among the carriers in the hospital and community.

Additionally, co-existence of bla NDM and blaOXA-48-like genes were found among *K.pneumoniae* and *E.coli* isolates. Defray et al.,[30] Mittal et al.[18] and Azour et al.[31] found co-occurrence of genes coding for carbapenemases in the same isolate from carriers. This highlights the horizontal transfer of plasmids carrying these resistance genes between clinical isolates and the intestinal flora.

5. Conclusion

Detection of carbapenem-resistant genes in commensal flora of the gut is worrisome. Colonization by resistant bacteria can increase the risk of subsequent infection in colonized individuals. The plasmids carrying the resistance genes have the potential for rapid dissemination and expression under antibiotic pressure. Overuse and misuse of antibiotics in the community accelerate the spread of resistant bacteria in the gut of healthy individuals which in turn adversely affects the treatment strategy for the community-acquired infections. Moreover hidden influx of resistant strains into the ICU could challenge the limited treatment options and affect the infection prevention and control measures. Building proper awareness about the use of antibiotics in the community, supply of safe drinking water, efficient sanitization system, and strict surveillaince systems to monitor these resistant bacteria in humans, food-producing animals, and the environment could all help to reduce the colonization of CREK among healthy individuals. Implementation of strict infection prevention and control measures and prudent use of carbapenems can help to limit the spread of these superbugs in the hospital.

6. Conflict of Interest

None.

7. Source of Funding

None.

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