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Act Fast as Time Is Less: High Faecal Carriage of Carbapenem-Resistant *Enterobacteriaceae* in Critical Care Patients

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ABSTRACT

Introduction: Carbapenem-resistant *Enterobacteriaceae* (CRE) are drug-resistant Gram-negative bacteria that are present in the community as well as in hospitals. Their infection and colonisation puts critically ill patients at high risk due to the drug-resistant nature of the strains and possible spreading of these organisms, even in a hospital environment.

Aim: To examine the presence and types of *Enterobacteriaceae* species in patients admitted directly from the community.

Materials and Methods: The present study was a one-month pilot conducted in the ICU of a tertiary care hospital in Mumbai, India in 2015. Faecal samples of patients admitted from the community directly to the ICU were analysed using tests like MHT (Modified Hodge) and EDTA for the presence of IMP (action on Imipenem) and KPC (*Klebsiella* Test Pneumoniae Carbapenemase) producing strains of *Enterobacteriaceae*. Polymerase Chain Reaction (PCR) was performed to look for *VIM*, *IMP*, *NDM*1, *OXA*, and KPC genes. Antibiotic Sensitivity Test was carried out as per CLSI guidelines.

Results: The results showed an alarming level of faecal carriage rates in adult ICU patients. *Klebsiella pneumonia* was the most common carbapenem-resistant isolate, closely followed by *Escherichia coli*. PCR results revealed nine strains were positive for *bla*(KPC) gene, from which 7 were *Klebsiella pneumoniae* and one each of *Escherichia coli* and *Klebsiella oxytoca* was observed. Antibiotic Sensitivity Test results showed that the isolates had maximum sensitivity to Colistin (100%) and Tigecycline (95%).

Conclusion: These levels indicate that in the absence of CRE screenings, proper isolation of carrier patients is not possible, leading to possible spreading of these resistant bacteria strains in ICUs. A longer period of study is required to obtain more substantial data to validate the results of this pilot.

Keywords: EDTA, KPC, MHT, PCR

INTRODUCTION

It has been extremely difficult to treat patients with sepsis admitted in the Intensive Care Unit (ICU) due to various reasons. Unfortunately, due to the prevalence of resistant bacteria, even in the community, our in-house antibiotic armamentarium fails in such patients. This has been observed to be even more predominant in patients that are referred from another hospital. In fact, most intensive care units in India encounter the same problem. The advent of the so-called post antibiotic era seems imminent where we will be at the mercy of the so called “superbugs.”

From the time of the discovery of penicillin by Alexander Fleming until today, the beta-lactam group of antibiotics has been the mainstay in treating various infections [1]. However, these bacteria carry plasmids that harbour antimicrobial-resistant coding genes, which are responsible for the emergence of resistant bacteria. Therefore, these plasmids ensure resistance of the bacteria to beta-lactam and its inhibitors. They also encode resistant determinants for other antibiotics. Selective pressure from human and veterinary medicine promotes enhancement of these resistance mechanisms, limiting the choice of antibiotics for treatment. Methicillin was introduced in the year 1960 for treatment of penicillin-resistant *Staphylococcus* and in 1964, the first case of MRSA (methicillin-Resistant *Staphylococcus aureus*) was reported [2], followed by the emergence of similar conditions such as VISA (Vancomycin Intermediate *Staphylococcus aureus*), VRSA (Vancomycin-Resistant *Staphylococcus aureus*), ESBL (Extended Spectrum Beta Lactamase), etc [3-6]. Carbapenems (imipenem, meropenem, ertapenem, and doripenem) are often used as the last resort to inhibit ESBL producing and MDR Gram negative organisms [7]. Nevertheless, an increased resistance pattern has been observed with carbapenems drugs. Among these, the emergence of carbapenem-resistant *Enterobacteriaceae* has been recognized by Thomas Frieden, Director of CDC, as a triple threat [8]. It must be considered before it is too late.

The emergence of such dangerous bacteria has created a major public health problem, which is compounded by the shortage of new antibiotics for inhibiting CRE [9]. Members of the *Enterobacteriaceae* family are the most common clinical isolates found, and can be acquired in the community or at the hospital. *Enterobacteriaceae* are gut flora. They serve as reservoirs for contaminating the environment and fomites and therefore can spread more easily in the ICU. The resistance mechanisms in these organisms are easily transferred as they are located in jumping genes or transposons. The selection pressure of antibiotic abuse in veterinary and human medicine contributes to the emergence of resistance patterns like ESBL [10]. Carbapenems are one of the drugs we use as a last resort in such cases of resistance. However, there seems to be a disturbing trend of carbapenem-resistant *Enterobactericeae* among clinical isolates. Since we are aware of the colonising capabilities and the emergence of various resistance patterns in the community, we embarked upon this pilot study to understand the baseline epidemiology of CRE carriage rates among critically ill adult patients admitted directly from the community to the hospital ICU.

MATERIALS AND METHODS

This pilot study was performed in January 2015 among patients admitted to the 16-bedded ICU of a tertiary care hospital in western Mumbai, Maharashtra, India. The study was approved by the hospital ethics committee.

Inclusion criteria: All adult patients directly admitted to the ICU of the hospital over a period of one month, i.e., in January 2015,

from whom we could collect first stool samples at the time of admission were included in the study.

Exclusion criteria: Patients shifted from other units of our hospital/other hospitals/nursing homes to the ICU, paediatric patients and postoperative patients kept for observation were excluded. The target population was selected for two reasons. First, defined patient population and second patients had many reported risk factors for CRE colonization. A stool sample was collected on the day of admission and thereafter on the same day each week over the entire study period.

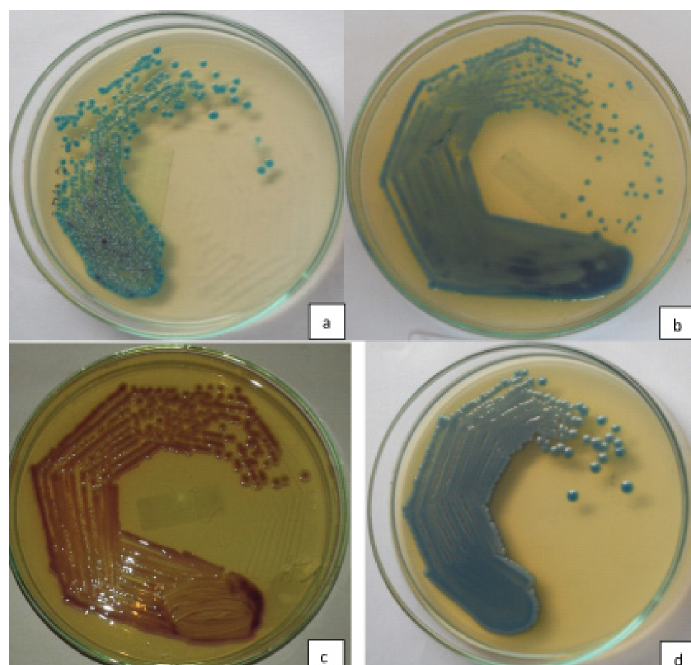
Microbial Culture and Identification

Sample Processing: CRE screening: On day one samples were inoculated on *KPC* agar (Hi-Media, Mumbai), and on day 2 any positive growth was identified according to the colony morphology on the *KPC* agar [Table/Fig-1]. Further identification and antibiotic sensitivity was confirmed by standard laboratory technique.

Antibiotic Sensitivity Test: The *Enterobacteriaceae* isolates were tested for antibiotic sensitivity based on standard laboratory technique as per Clinical and Laboratory Standards Institute (CLSI) guidelines with commercially available discs (Hi-Media, India [11]. *Escherichia coli* ATCC 25922 was used as control.

Phenotypic Confirmation of CRE was done by Modified Hodge test and Imipenem + Imipenem-EDTA disc synergy Test [12] (I+IE DST).

Modified Hodge test [11] was carried out in MHA (Hi-Media, Mumbai, India) as per CLSI 2014 guidelines. Overnight culture suspension of *Escherichia coli* ATCC 25922 and 10µg Ertapenem disc (BD-BBL) were used. After overnight incubation, the plates were observed for the presence of a "cloverleaf shaped" zone of inhibition. The plates with such zones were interpreted as modified Hodge test positive.



[Table/Fig-1]: CRE Isolates on KPC Agar (a) *Klebsiella oxytoca, (b) Enterobacter aerogenes, (c) Escherichia coli* and (d) *Klebsiella pneumonia*.

EDTA –Disc Diffusion Synergy Test: A 10µg of imipenem disc (Hi-Media, India) was placed on the agar and a 10µg imipenem-EDTA disc was placed 10mm apart from edge-to-edge. After overnight incubation, the presence of an enlarged zone of inhibition was interpreted as EDTA synergy positive.

Molecular Confirmation: CRE grown in *KPC* agar were further studied for molecular characterization [13]. We looked for *VIM*, *IMP*, NDM1, *OXA*, and *KPC* genes by PCR using the set of primers and PCR conditions mentioned in [Table/Fig-2].

Risk Factors for CRE Carriage: A case-control study was performed to identify factors associated with CRE faecal carriage. Cases were patients with a positive CRE screening culture and controls were patients with negative screening cultures.

STATISTICAL ANALYSIS

Statistical analysis was carried out using Yates correction method for pooling the data under Chi-Square test among clinical conditions and CRE positive and CRE negative.

RESULTS

Twenty eight (51.85%) Carbapenem-Resistant *Enterobacteriaceae* (CRE) isolates were obtained from 54 stool samples on *KPC* agar. The species distribution of the observed CRE isolates is listed in [Table/Fig-3]. *Klebsiella pneumonia* was the most common carbapenem-resistant isolate, closely followed by *Escherichia coli*. Phenotypic confirmation of the CRE isolates revealed 24 out of 28 screen positive isolates were MHT positive [Table/Fig-4]. All the *Escherichia coli*, 9 *Klebsiella pneumoniae*, 3 *Klebsiella oxytoca*, and 1 *Enterobacter aerogenes* strains were MHT positive [Table/Fig-5]. Four isolates that were MHT negative were subjected to MBL detection by I+IE DST. Two *K.pneumoniae* strains and one *Klebsiella oxytoca* strain were found to be positive and one K pneumoniae strain was MHT and I+IE DST negative.

For genotypic characterization PCR was performed to look for *VIM*, *IMP*, NDM1, *OXA*, and *KPC* genes [Table/Fig-6]. Nine strains were positive for *bla*(*KPC*) gene, from which 7 were *K.pneumoniae* and one each of *E.coli* and *K.oxytoca* was observed. Fourteen strains were positive for *VIM* and 15 were positive for the *IMP* gene [Table/Fig-7,8].

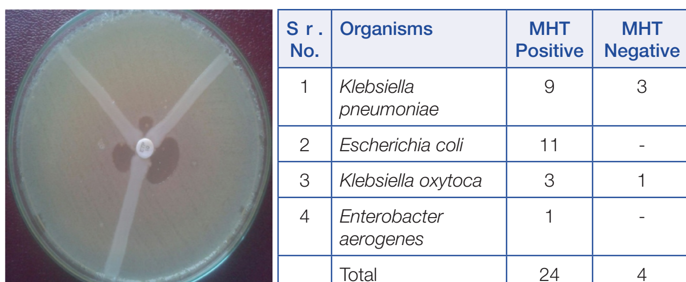
Six isolates were harbouring all the three genes and among them four were *K.pneumoniae*, one was *E.coli*, and one was *K.oxytoca*.

Organisms	Total
Klebsiella pneumonia	12 (42.8%)
Escherichia coli	11(39.2%)
Klebsiella oxytoca	4(14.2%)
Enterobacter aerogenes	1(3.5%)
Total	28

[Table/Fig-3]: Species distribution of CRE Isolates in Stool Samples.

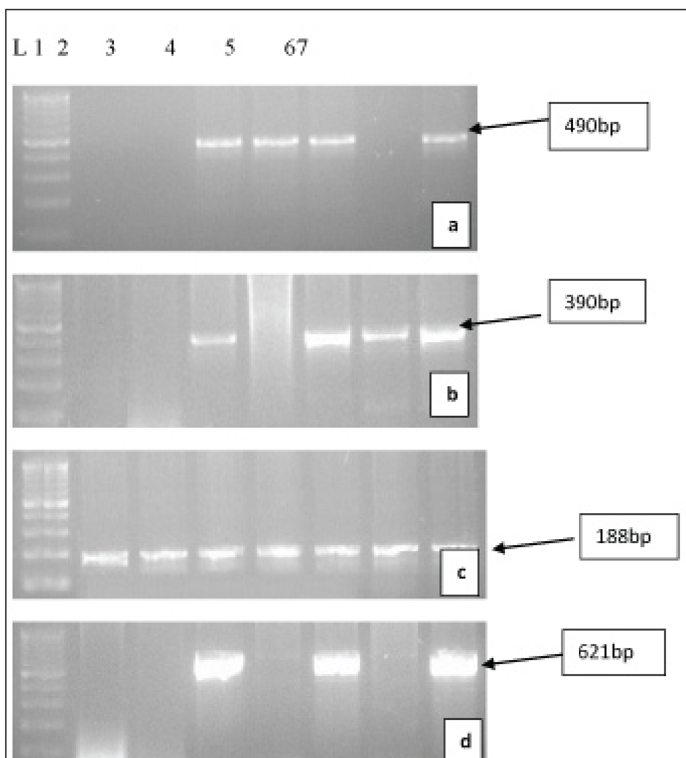
Sr. No.	Gene	PCR conditions	Primers	Amplicon size
1	*bla*-VIM	10 minutes at 94°C and 36 cycles of amplification consisting of 30 s at 94°C, 40 s at 52 °C and 50 s at 72 °C, with 5min at 72 °C for the final extension	VIM-F-5-GATGGTGTTTGGTCGCATA-3 VIM-F-5-CGAATGCGCAGCACCAG-3	390bp
2	*bla*IMP	94°C for 5 minutes followed by 36 cycles of 94°C for 30 s, 52 °C for 40 s and 72 °C for 50 s and 5min at 72 °C	Imp-F-5-GGAATAGAGTGGCTTAAYTCTC-3 Imp-R-5-CCAAACYACTASGTTATCT-3	188bp
3	*bla*KPC1	94°C for 5 minutes followed by 36 cycles of 94°C for 30 s, 52 °C for 40 s and 72 °C for 50 s and 5min at 72 °C	Kpc-F-5-CTTGCTGCCGCTGTGCTG-3 Kpc-R-5-GCAGGTTCCGGTTTTGTCTC-3	490bp
4	*bla*NDM1	940 C for 10 min, followed by 36cycles of 940 C for 30 s, 520 C for 40 s and 720 C for 30 s and 720C for 5 min	NDM-F-5 GGTTTGGCGATCTGGTTTTC-3 NDM-F-5-CGGAATGGCTCATCACGATC-3	621 bp
5	*bla*OXA	950 C for 5 min, followed by 32cycles of 950 C for 30 s, 560 C for40 s and 720 C for 50 s and 720 C for 10 min	OXA-F 5-ATGGAAGGGCGAGAAAAGG-3 OXA-R 5-TTGCATGAGATCAAGACCGATA-3	127 bp

[Table/Fig-2]: Polymerase Chain Reaction Conditions, Primers and Amplicon Size for Detection of Carbapenemase Genes among CRE Isolates.



S r . No.	Organisms	MHT Positive	MHT Negative
1	*Klebsiella pneumoniae*	9	3
2	*Escherichia coli*	11	-
3	*Klebsiella oxytoca*	3	1
4	*Enterobacter aerogenes*	1	-
	Total	24	4

[Table/Fig-4]: Positive modified hodge test. **[Table/Fig-5]:** Modified hodge test results.



[Table/Fig-6]: Gel Electrophoresis Pictures of Resistant Genes ["L" is DNA ladder of 100 base pairs] a) KPC gene: 490 bps; b) VIM gene: 390 bps; c) IMP gene: 188 bps and d) NDM gene: 621 bps.

Seven isolates had both *VIM* and *IMP* genes: four *E.coli*, 2 *K.pneumoniae*, and 1 *K.oxytoca*. Among *K.pneumoniae*, one had *VIM* and *KPC* genes and one had *IMP* and *KPC* genes together. One of the *K.oxytoca* isolate, which was DST positive, did not contain any of the genes [Table/Fig-8]. Antibiotic Sensitivity Test of the isolates showed maximum sensitivity to Colistin (100%) and Tigecycline (95%), followed by Cotrimoxazole (23%), Tobramycin, and Gentamicin 7.69%. Levofloxacin and Amikacin showed 3.85% sensitivity pattern.

Statistical analysis showed significant difference between the clinical conditions of patients and colonization/carriage of CRE at 5% and 1% level of significance.

DISCUSSION

Members of the family *Enterobacteriaceae* are among the most important bacterial human pathogens, accounting for the majority of the bacteria isolated from clinical samples. A major concern is that these Gram-negative bacilli rapidly acquire resistance to one or more antimicrobial agents traditionally used for treatment. One of the most concerning emerging resistance traits among Gram-negative bacteria is the ability of the organisms to produce carbapenem-hydrolyzing β-lactamases, which confer resistance to almost all β-lactams [14]. The occurrence of multidrug resistant carbapenem hydrolysing Gram-negative bacteria is increasing worldwide.

The most clinically significant carbapenemases are KPC, MBLs (namely *VIM*, *IMP*, NDM types) and *OXA*. These genes are located on mobile genetic elements, allowing them to spread easily. Recognition of the presence of carbapenemase producers is of paramount importance for effective treatment and control. Currently, MBLs and *KPC* are considered a major threat in *Enterobacteriaceae* [Table/Fig-9], representing a potential source of clinical failure in patients treated with almost all beta-lactam agents [15].

Sr. No	Organism	MHT	I/IE Test	Genotypic Characterization				
				OXA	*VIM*	*IMP*	*NDM1*	*KPC*
1	Kpn	+	-	-	+	+	-	-
2	Kpn	+	-	-	+	+	+	+
3	Kpn	-	+	-	+	+	+	-
4	Koxy	-	+	-	-	-	-	-
5	Kpn	+	-	-	-	-	-	-
6	Kpn	-	+	-	+	+	+	+
7	Kpn	+	-	-	+	+	+	+
8	Kpn	+	-	-	+	+	+	+
9	Eco	+	-	-	-	-	-	-
10	Kpn	+	-	-	-	-	+	+
11	Eco	+	-	-	-	-	-	-
12	Kpn	+	-	-	-	-	-	-
13	Eco	+	-	-	+	+	-	-
14	Eco	+	-	-	-	-	-	-
15	Koxy	+	-	-	-	+	-	-
16	Eco	+	-	-	-	-	-	-
17	Koxy	+	-	-	+	+	+	+
18	Eaer	+	-	-	+	+	+	-
19	Eco	+	-	-	-	-	-	-
20	Kpn	-	-	-	-	-	-	-
21	Eco	+	-	-	+	+	-	-
22	Eco	+	-	-	+	+	-	+
23	Koxy	+	-	-	-	-	-	-
24	Kpn	+	-	-	+	-	-	+
25	Eco	+	-	-	-	+	-	-
26	Eco	+	-	-	+	+	-	-
27	Kpn	+	-	-	-	+	-	+
28	Eco	+	-	-	+	+	+	-
				0	14	16	9	9

[Table/Fig-7]: Phenotypic and genotypic characteristic of CRE isolates.

Sr. No.	Organisms	KPC Producers	KPC Non-Producers
1	*K. pneumonia*	7	5
2	*E. coli*	1	10
3	*K. oxytoca*	1	3
4	*E aerogenes*	Nil	1
	Total	9	19

[Table/Fig-8]: Distribution of *KPC* and *OXA* Genes among Different Isolates.

Sr. No.	Organisms	VIM	IMP	NDM1
1	*Klebsiella pneumoniae*	7	7	6
2	*Escherichia coli*	5	5	1
3	*Klebsiella oxytoca.*	1	2	1
4	*Enterobacter aerogenes*	1	1	1
	Total	14	15	9

[Table/Fig-9]: Distribution of Metallobetalactamase Genes (VIM, IMP and NDM1).

Characteristics	Carbapenemase Producers (n=26)	Non-Carbapenemase producers (n=26)	p-value*
Presensence of comorbidities	19	07	0.0095
Prior antibiotic use (3months before)	20	05	0.0013

[Table/Fig-10]: Significant risk factors associated with CRE.

In India, there is large variance in the reported carbapenem resistance rate among GNB with the lowest documented occurrence of carbapenem resistance among *Enterobacteriaceae* being 1.8% and the highest being approximately 51% [16]. Taneja et al., found it to be 36.4%, whereas Datta et al., reported the presence of 7.87% carbapenem resistant strains in their study [17,18].

Either stools or rectal swabs (with or without enrichment in the presence of a carbapenem) can be plated on selective media for CRE screening; however, rectal/ peri-rectal swabs are less sensitive than stool specimens [19]. Hence we choose stools as the samples for our study.

No consensus on the optimal method for CRE screening is available yet. The CDC method (CDC-TS) of CRE screening comprises inoculation of the sample onto a Tryptic soy broth with a 10µg meropenem disc then swabbing it the next day onto MacConkey agar. Different studies have used different culture-based techniques like MacConkey agar plates supplemented with 1µg/ml imipenem, CHROM agar *KPC*, MacConkey Agar with imipenem, meropenem and ertapenem disc (10µg), and two-step selective broth enrichment method using a 10µg carbapenem disc to evaluate gut colonization with CRE with good performance [20]. We have used CHROM agar *KPC* for CRE screening of our patients. Evaluation of CHROM agar *KPC* for Rapid Detection of Carbapenem-Resistant *Enterobacteriaceae* by Samra et al., showed a sensitivity and specificity of 100% and 98.4%, respectively, relative to PCR [21]. Gilad et al., reported that CHRO Magar *KPC* can be employed for processing of specimens used for monitoring carbapenem-resistant *Enterobacteriaceae*; however, in the presence of *KPC*-negative ertapenem-resistant strains or in the detection of *KPC*-producing *E.coli*, its application may be limited due to its limited performance [22]. In our study, the overall CRE carriage rate was 51%. There is a paucity of data on carriage rates of CRE in stool samples. In one study by Swaminathan et al., the overall prevalence of CRE carriage was 5.4% [23]. CRE carriage rate was 7.1% in another study [24]. In a prospective study by Kothari et al., 75 healthy, vaginally delivered, antibiotic naive, breast fed neonates were studied for gut colonization and it was found that colonization with CRE was rare with only one detected isolate of *Enterobacter* [25]. Twenty four (9.9%) isolates demonstrated carbapenemase activity among 242 screened *Enterobacteriaceae* isolates in a study from Delhi [26]. Out of 62 isolates, 12 (29.26%) *E.coli* and 8 (38.09%) *Klebsiella* spp. were found to be positive for MBL production in a study by Aggarwal et al., [27]. Our results demonstrate a disturbingly high carriage rate, which could just be the tip of the iceberg and more patients might be carrying such resistant bugs, which could have been successfully screened if a supercarba medium was used. In one study, various screening methods were compared and it was found that the supercarba medium was highly sensitive in the detection of CRE carriage in stool, including the detection of *OXA* type genes [28].

Accurate detection of CRE by phenotypic and genotypic assays has an important clinical and epidemiological value [29]. The MHT based on in-vivo production of a carbapenemase by a carbapenemase-producing strain has been suggested for phenotypic confirmation of carbapenem resistance by CLSI, and can be used as the first step in detecting the carbapenemase activity of candidate isolates [29]. The sensitivity and specificity of MHT in our study, when compared with the genotypic method, were 85.71% and 28.57%, respectively. MHT often lacks specificity (e.g., false-positive results for high-level AmpC producers or CTX-M-type ESBL producers, *Enterobacter* species) and sensitivity (e.g., weak detection of NDM producers), but works well for the detection of *KPC* and *OXA*-48 producers [29].

The most classified carbapenemase gene in India is NDM. Two hundred and thirty-five ertapenem-non-susceptible (MIC≥0.5 mg/L) isolates of *Enterobacteriaceae* from the worldwide Study for Monitoring Antimicrobial Resistance Trends (SMART) 2009 programme were screened using a multiplex PCR for the presence of bla (*KPC*), bla (*OXA*-48), bla (*VIM*), and bla (NDM-1) genes. Bla (NDM-1) was identified in 33 isolates and all of them were from India [30]. Recent studies on the isolation of CRE (NDM-1) from environmental samples and community acquired infections indicate that the NDM-1 enzyme, which produces CRE, may be widely distributed in India [31]. However, there is paucity of data regarding faecal carriage of CRE. Regarding the faecal carriage of *VIM/IMP* genes, perhaps this is the first study from India. As per our knowledge There are very few reports of *VIM/IMP* type MBLs in *Enterobacteriaceae* from clinical isolates in India [19,25]. The most common MBL subtype in one study was the bla(*IMP*), followed by bla(*VIM*) and bla (SIM), again from clinical isolates. The study also demonstrated concurrent occurrence of multiple MBL genes in a single isolate [32].

Stool samples were screened for the presence of CRE in a Chinese university hospital and out of the eight CREs detected two were *KPC* (*IMP*-4 and NDM-1) [33]. In another study, from Greece with a total of 226 patients, 164 (72.6%) were colonized with *KPC*-Kp within an average of 9.1 days of ICU stay [34]. In our study, 9 out of 28 patients (32.1%) were found to be harbouring the *bla*(*KPC*) gene. Colonization with CRE was found to be rare in one of the Indian studies where only one baby harboured *Enterobacter* species and was found to be positive for *bla*(*KPC*-2) [25]. We did not find any carriage of *OXA* gene in our study. Shanti M et al., observed that *OXA*-48/*OXA*-181 is not a major mediator of carbapenem resistance among *Enterobacteriaceae* [35].

Yet again, there is limited data related to the risk factors for acquiring CRE and its clinical outcomes. Considering these facts, this study attempted to analyze the factors influencing the acquisition of carbapenemase producing *Enterobacteriaceae* and its clinical outcomes. Analysis revealed that past usage of more than 1 class of antibiotics emerged as a significant risk factor that influenced the acquisition of CRE [Table/Fig-10].

Use of several classes of antimicrobials, namely cephalosporins, fluoroquinolones, metronidazole, and carbapenems, contributes significantly to the development of resistance to carbapenems [36]. Mechanical ventilation {odds ratio (OR), 11.5}, pulmonary disease (OR, 5.2), days of antibiotic therapy (OR, 1.04), and CRE colonization pressure (OR, 1.15) were independently associated with CRE acquisition [23]. In another study, the independent predictors for CRE colonization included Charlson's score greater than 3 {OR, 4.85 (95% confidence interval (CI), 1.64-14.41)}, immunosuppression {OR, 3.92 (95% CI, 1.08-1.28)}, presence of indwelling devices {OR, 5.21 (95% CI, 1.09-2.96)}, and prior antimicrobial exposures {OR, 3.89 (95% CI, 0.71-21.47)} [24].

This is the first study where we have looked for the carriage rate of CRE among critically ill patients along with molecular characterization. There are not many, hardly any reports from India that have studied the presence of genes like *VIM/ IMP* or *KPC*.

LIMITATION

A major drawback of our current study is that it was performed as a pilot study over a very short duration. Due to time and resource constrains, characterization of other non-enzymatic mechanisms mediating carbapenem resistance, such as upregulated efflux pumps, porin defects, and hyper production of AmpC beta-lactamase, were not performed in this study.

CONCLUSION

The results of this study indicate a high prevalence of faecal carriage among patients hospitalized in our ICU, which is a cause of concern. Microbial surveillance of rectal flora in stools of patients in ICUs can guide clinicians about gut colonization of CRE as these groups of patients are at risk of possible endogenous infection,

underlining the necessity for proper management of antibiotic therapy within healthcare units as well as stringent infection control and prevention practices like strict contact precautions for all CRE positive carriers. In order to prevent the spread of CRE prospective cases, a controlled study with adequate sample size and a primary objective to identify the risk factors for infection with carbapenemase-resistant *Enterobacteriaceae* and the outcome of such infections is essential to have a clear understanding of this problem. Based on this study, it seems that the prevalence of CRE colonisation in patients admitted to our hospital is very high. This should be confirmed with a larger study to assess causality. From this study, it seems prudent to screen patients for CRE as contact isolation precautions for these patients would go a long way in restricting the spread of these organisms and contamination of the environment.

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