ORIGINAL ARTICLE

First report of Molecular Epidemiology of Carbapenem Resistant *Enterobacteriaceae* from a Tertiary Level Hospital in Rajasthan, Western India

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ABSTRACT

Background: Carbapenem Resistant *Enterobacteriaceae* (CRE) are emerging at an alarming rate and pose a significant global threat. **Objective:** To conduct phenotypic and genotypic characterization of CRE strains from Rajasthan, Western India. **Methodology**: This was a prospective observational study conducted in Department of Microbiology, Dr S.N. Medical College, Jodhpur, Rajasthan from October to December 2018. All clinical samples received during the study period were processed and bacterial identification and antimicrobial susceptibility tests were performed according to standard microbiological guidelines. A total of 14 non duplicate carbapenem resistant clinical isolates of *E coli* and *K pneumoniae* were included in the study and subjected to Rapidec Carba NP test. Carbapenemase- encoding genes were amplified by multiplex polymerase chain reaction (PCR). PCR amplified products from three random isolates were subjected to Sanger sequencing. **Results**: Amikacin remained active against 36% isolates. All isolates were found to be susceptible to colistin and tigecycline. Carbapenemase production by Rapidec Carba NP test was noted in all (14/14) study isolates. All isolates were found to harbour ≥ 1 carbapenemase gene. The most common resistance gene observed was blaoxa (86%) followed by bla_{NDM} (79%). None of the CRE isolates included in our study showed production of KPC enzymes. The sequences were analysed using BLAST analysis and were confirmed to be matching to OXA-48/181 and NDM-1. **Conclusions**: Growing carbapenem resistance is an important issue which needs urgent attention and bla_{OXA} is an emerging mechanism of resistance among clinical CRE isolates in our setting

KEY WORDS: Carbapenem resistant Enterobacteriaceae, Phenotypic tests, Carbapenemase gene, Polymerase chain reaction.

Introduction

Enterobacteriaceae are a large family of Gram negative bacteria commonly present as normal commensal flora in the gastrointestinal tract of humans. They are increasingly associated with a plethora of infections, both community and hospital acquired. There is a growing trend of resistance seen to commonly used antibiotics amongst these isolate.[1,2] Carbapenems are last resort agents to treat infections caused by organisms found resistant to other classes of antibiotics. However, Carbapenem Resistant Enterobacteriaceae (CRE) are emerging at an alarming rate and pose a significant global threat.[3,4] Infections caused by these organisms are associated with increased morbidity and mortality.[5]

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They pose a huge financial burden on economies of nations all over.[6] Resistance to carbapenems is mediated by various mechanisms. These include hyperproduction of Amp C type or extended spectrum β-lactamases (ESBLs) coupled with outer membrane porin loss, hyperproduction of efflux pumps, decreased affinity of penicillin binding proteins and production of carbapenemases. Carbapenem resistance due to acquisition of carbapenemases is now being increasingly reported. Three types of carbapenemases are commonly identified in Enterobacteriaceae family. They are Ambler class A (KPC), class B (MBL) and class D (OXA) types.[1,2,7] Emergence of carbapenem resistance among Enterobacteriaceae isolates due to carbapenemases is particularly worrisome because this is plasmid mediated. These bacteria can then easily disseminate not only among inpatients causing hospital associated infections but also spread into the community.[3,4] Early recognition and controlling spread of these isolates is therefore crucial for better patient care, infection control and public health at large.

Currently armamentarium for handling CRE is very limited. Studies claim variable level of success with drugs like colistin, tigecycline, minocycline and amikacin. Novel combinations with various β lactamase inhibitors are available but their spectrum of action needs more evaluation.[8–10] There are very few reports on prevalence of CRE, their mechanisms of resistance and antimicrobial susceptibility pattern from Rajasthan. To the best of our knowledge this is the first study presenting a comprehensive phenotypic and genotypic analysis of CRE strains from a tertiary level hospital in Rajasthan, Western India. This will help in better understanding of existing epidemiology of CRE in this geographical area and help reinforce corrective and preventive measures.

Methodology

This was a prospective observational study conducted in the Department of Microbiology, Dr S.N. Medical College, Jodhpur, Rajasthan from October to December 2018. Ethical clearance for study was obtained by Institutional Ethical committee.

All clinical samples received from inpatients and outpatients during the study period were processed. These included pus, urine, stool, broncho alveolar fluid (BAL), wound swab, blood, sputum, endotracheal aspirate (ET) etc. Bacterial identification and antimicrobial susceptibility tests were performed according to standard microbiological guidelines.[11,12] The antibiotics tested included Amikacin (30μg), Cefoperazone-sulbactam (75/30μg), Ceftazidime (30μg), Ceftriaxone (30μg), Ciprofloxacin (5μg), Cotrimoxazole (25μg), Cefepime (30μg), Meropenem (10μg) by disc diffusion method. Colistin and Tigecycline susceptibility were determined by E test (AB Biodisk, Solna, Sweden). E. coli ATCC 25922 and *Klebsiella pneumoniae* ATCC BAA-1705 strains were used for quality control.

A total of 14 non duplicate carbapenem resistant (meropenem zone size <23 mm) clinical isolates of *E. coli* and *K. pneumoniae* were included in the study and subjected to following further tests:

Rapidec CARBA NP test

This test is based on direct detection of carbapenem hydrolysis by carbapenemase producing bacteria and was done as per manufacturer's instructions.[13] After incubation, readings were done by visually comparing with control well, starting at 30 min and after no more than 2 hours. A positive test corresponded to red to yellow or red to orange colour change. This visual evaluation was performed blindly by two doctors who did not know about the carbapenemase status of the strains.

Detection of beta lactamases using multiplex PCR

Genomic DNA isolation was done using method described by Maniatis et al.[14] Multiplex PCR was performed for detection of beta lactamase genes in carbapenemase positive isolates using three PCR reactions and conditions mentioned by Poirel et al.[15] Primers were used to amplify the following 11 genes: bla$_{IMP}$, bla$_{VIM}$, bla$_{NDM}$, bla$_{SPM}$, bla$_{AIM}$, bla$_{DIM}$, bla$_{GIM}$, bla$_{SIM}$, bla$_{KPC}$, bla$_{BIC}$ and bla$_{OXA-48}$. Table 1 Amplified products were visualized using the DNA agarose gel electrophoresis. PCR amplified products from three random isolates were subjected to Sanger sequencing.

Statistical analysis

All data was evaluated using SPSS Windows version 16. Descriptive statistics, frequencies and percentages were given.

Results

A total of 1240 samples received in Department of Microbiology during the study period October to December 2018 were processed and 58 *Enterobac-*

Table 1: Primer sets for multiplex PCR

Primer set	Primers	T_m	Primers		Amplicon Size
AIM set	bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}	52°C	blaAIM-F	CTGAAGGTGTACGGAAACAC	322bp
			blaAIM-R	GTTCGGCCACCTCGAATTG	
			blaDIM-F	GCTTGTCTTCGCTTGCTAACG	699bp
			blaDIM-R	CGTTCGGCTGGATTGATTTG	
			blaGIM-F	TCGACACACCTTGGTCTGAA	477bp
			blaGIM-R	AACTTCCAACTTTGCCATGC	
			blaSIM-F	TACAAGGGATTCGGCATCG	570bp
			blaSIM-R	TAATGGCCTGTTCCCATGTG	
KPC set	bla_{KPC}, bla_{NDM-1}, bla_{OXA}, bla_{BIC}	56.5°C	blaKPC-F	CGTCTAGTTCTGCTGTCTTG	232bp
			blaKPC-R	CTTGTCATCCTTGTTAGGCG	
			blaNDM-F	GGTTTGGCGATCTGGTTTTC	621bp
			blaNDM-R	CGGAATGGCTCATCACGATC	
			blaOXA-F	GCGTGGTTAAGGATGAACAC	438bp
			blaOXA-R	CATCAAGTTCAACCCAACCG	
			blaBIC-F	TATGCAGCTCCTTTAAGGGC	537bp
			blaBIC-R	TCATTGGCGGTGCCGTACAC	
IMP set	bla_{IMP}, bla_{SPM}, bla_{VIM}	57°C	blaIMP-F	GGAATAGAGTGGCTTAAYTCTC	232bp
			blaIMP-R	GGTTTAAYAAAACAACCACC	
			blaSPM-F	AAAATCTGGGTACGCAAACG	271bp
			blaSPM-R	ACATTATCCGCTGGAACAGG	
			blaVIM-F	GATGGTGTTTGGTCGCATA	390bp
			blaVIM-R	CGAATGCGCAGCACCAG	

teriaceae isolates were obtained. When subjected to antibiotic susceptibility testing, 14 (24%) were found to be meropenem resistant by disc diffusion method. These 14 consecutive non duplicate clinical isolates of *E. coli* and *K. pneumoniae* (7 each) were included in the study and subjected to further tests.

These included 10 (71%) and 4 (29%) isolates from inpatients and outpatients respectively. The breakdown of various samples from which CRE isolates were recovered is as shown in Figure 1. Maximum number of CRE isolates were obtained from urine (5/14, 35%) followed by wound swab and blood (3/14, 21% isolates each). One isolate each was obtained from pus, BAL fluid and ET aspirate.

Antibiotic susceptibility profile to various antibiotics tested is as shown in Table 2, Figure 2 shows the representative results obtained using the Rapidec Carba NP test. All 14 CRE isolates tested positive implying carbapenemase production and were further subjected to Multiplex PCR for eleven target genes. All isolates were found to harbour one or more than one resistance genes as shown in Figure 3

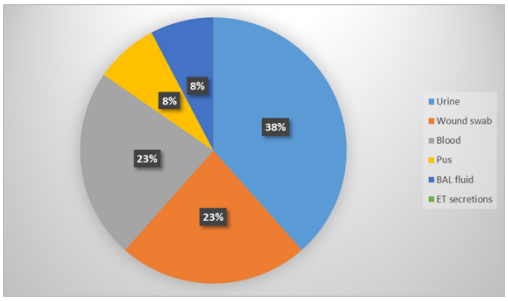

Figure 1: Pie diagram showing distribution of samples from which CRE isolates were obtained

shows a gel electrophoresis image on PCR amplified products.

Discussion

Global spread of various resistance mechanisms have facilitating the emergence and spread of CRE.[2–4] Excessive use of antimicrobials in humans, animals and agriculture, combined with poor public health infrastructure has made antimicrobial resistance a burning issue especially for developing

Table 2: Antimicrobial susceptibility profile of Carbapenem Resistant Enterobacteriaceae isolates

S. No.	Name of Antimicrobial agent	Number of Carbapenem resistant isolates (Total no=14)	Percentage of Carbapenem resistant isolates (Total no=14)
1	Amikacin	9	64%
2	Cefoperazone-sulbactam	13	93%
3	Ceftazidime	14	100%
4	Ceftriaxone	14	100%
5	Ciprofloxacin	14	100%
6	Colistin	0	0
7	Cotrimoxazole	12	86%
8	Cefepime	10	71%
9	Meropenem	14	100%
10	Tigecycline	0	0

Figure 2: Shows representative results obtained using the Rapidec Carba NP test. The test reaction is read in well e while well d serves as control which is required to validate the test. (A) Negative result (red), (B) Strong result (yellow)

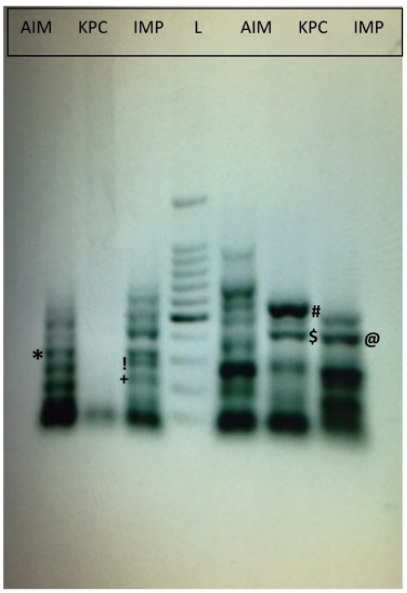

Figure 3: 1% agarose gel electrophoresis showing PCR amplified products of multiplex PCR for AIM (bla_{AIM}, bla_{DIM}, bla_{SIM}, bla_{GIM}), KPC (bla_{KPC},bla_{NDM-1}, bla_{OXA-48}, bla_{BIC}) and IMP (bla_{IMP}, bla_{VIM}, bla_{SPM}) set of primers. L corresponds to the ladder and band sizes matching the respective *bla* genes are marked as :- $^{\#}$ *NDM-1(621bp),* $^{@}$*VIM(390bp),* $^{\$}$*Oxa-48 (438bp), !SPM(271bp),* $^{+}$*IMP(232bp), *AIM(322bp)*

economies.[8,9,16–20]

Novel drugs and drug combinations for countering the menace of CRE are being explored. These newer drugs are developed with unique spectrum of activity against Enterobacteriaceae producing a specific carbapenemase. Ceftazidime/avibactam is active against KPC and OXA-48 producers while meropenem/vaborbactam shows activity against mainly KPC producers. Some isolates particularly OXA-48 producers have also been reported to be susceptible to carbapenems themselves.[21,22]

The aim of this study was to characterize the mechanisms of carbapenem resistance in clinical *E. coli* and *K. pneumoniae* strains and to study their susceptibility profile to other classes of drugs.

14 isolates of CRE obtained from various clinical samples were included in the current study, CRE prevalence being 24.1 %. Two thirds (71%) of these isolates were obtained from inpatients. *E. coli* and *K. pneumoniae* each constituted 50%. i.e. seven isolates each. In India, studies have recorded CRE prevalence to be varying from 3-65%.[23–25] Higher

rates from some centres may be explained by the fact that these studies were done in high risk areas like intensive care units.[26] The variable data reflects the distinct distribution of resistance determinants which might be related to the different antibiotic-prescribing habits of local doctors. All the 14 isolates that were carbapenem resistant by disc diffusion method were further evaluated by phenotypic and genotypic tests.

Higher preponderance of CRE among hospital acquired strains is reported by various researchers.[21,26]The presence of CRE among community isolates (29%) in our study is quite worrisome and can be explained by injudicious use of antibiotics leading to multiple drug resistance. Growing carbapenem resistance in *E. coli* particularly may lead to difficult to treat community-acquired infections.[3,20]Over the counter availability of drugs without a valid medical prescription is an important factor which needs to be addressed.

High level of resistance was noted to various commonly used antimicrobials like ceftazidime, ceftriaxone, cefepime, cefoperazone-sulbactam, cotrimoxazole and ciprofloxacin in this study. Table 2 Amikacin remained active against 36% isolates. Aminoglycosides have shown optimistic results in other studies as well both on national and global level.[16,24,27–29]All isolates were found to be susceptible to colistin and tigecycline. Similar findings have been reported by other authors too.[16,25] However, resistance in CRE to both the wonder drugs has been reported recently.[30,31] This warrants extreme caution against any inappropriate use of these drugs.[30]

Carbapenemase production by Rapidec Carba NP test was noted in all (14/14) study isolates, all isolates showing colour change from red to orange. Figure 2. This test provides rapid results thereby saving up to a day in making a clinical decision of whether patient needs isolation. It is easy to use, easy to implement and interpret. It is not only cost effective in comparison to PCR but may also be successful in picking up newer potential resistance mechanisms. Further corroborative evidence is provided by the fact that all isolates that tested positive by this test also showed the presence of $\geq$1 carbapenemase gene by PCR, thereby implying 100% agreement between the two. Similar encouraging results have been reported by other studies too.[32,33] Thus, in the current scenario of rising carbapenem resistance, this test may offer a high medical value as it is highly sensitive and specific (97.8% each) and provides an all-in-one solution.

The type of carbapenemase present was characterized by multiplex PCR using previously described primers and conditions for all 14 CRE isolates. All isolates were found to harbour $\geq$ 1 carbapenemase gene. Carbapenemase production was found as the mode of carbapenem resistance in all isolates thus dwarfing the role of other mechanisms. This in turn implies easy transmissibility of resistance trait because of their presence on plasmids. The most common resistance gene observed was bla$_{oxa}$ (86%) followed by bla$_{NDM}$ (79%) while the bla$_{VIM}$ and bla$_{GIM}$ gene were least frequent (7%). The most frequent combination seen in isolates with multiplicity of genes was bla$_{NDM}$ and bla$_{OXA}$ as observed in 79% of study isolates. No bla$_{DIM}$ and bla$_{kpc}$ genes were found in any of the study isolates. Table 3

Table 3: Occurrence of beta lactamase genes

Primer set	Gene	Presence	Presence of more than one bla	N (%)
AIM set	AIM	8/14	NDM+OXA	11/14 (79%)
	DIM	0/14		
	GIM	1/14		
	SIM	3/14	NDM+OXA+BIC	8/14 (57%)
KPC set	KPC	0/14	NDM+OXA+AIM	6/14 (43%)
	NDM-1	11/14		
	OXA	12/14	NDM+OXA+SPM	5/14 (36%)
	BIC	8/14		
IMP set	IMP	2/14	NDM+OXA+SIM	3/14 (21%)
	SPM	6/14		
	VIM	1/14		

In early 2000's when CRE emerged and were recognized as potential threats, they were largely restricted to specific geographical locations. Regions and countries considered as having the highest prevalence of the various carbapenemase-producing CRE are the Indian subcontinent (NDM CRE), United States, Israel, Greece and Italy (KPC CRE), Turkey, the Middle East and North Africa (OXA-48 CRE). However increasing global travel and trade has resulted in blurring of these geographical boundaries.[1,2,4,18–20]

In India, considerable geographical variation was reported by a study done on clinical isolates collected from hospitals across North and South India. Carbapenem resistance was found to be higher in north Indian strains. bla$_{OXA-1}$ was the most common carbapenemase gene among the South Indian strains, while NDM-1 followed by OXA-1 were the two most prevalent genes in North Indian isolates.[34] In a wide based study from UP involving 8973 clinical samples, bla$_{NDM}$ (63%) was found to be most prevalent and bla$_{NDM}$ and bla$_{OXA-48}$ were co-observed in 20 per cent isolates.[24] NDM as the predominant carbapenemase amongst clinical Enterobacteriaceae strains has been reported by various other studies as well.[26,35,36]

Castanheira M et al had first demonstrated the presence of bla$_{OXA-48}$ like gene in 26% CRE strains collected from 14 hospitals across India as part of the SENTRY Antimicrobial Surveillance Program, 2006-2007.[37] A recent shift in India from NDM to OXA-48 like carbapenemases has been noted by Shankar C et al wherein OXA-232 was present in around 71% isolates. [30]Sharma A et al reported presence of bla$_{OXA-48}$ and bla$_{NDM}$ in an equal percentage (32%) of tested isolates, co-production of bla$_{OXA-48}$ and bla$_{NDM}$ like was seen in 13% isolates.[16] Endemicity of OXA-48 and NDM-1 carbapenemase producing Enterobacteriaceae has also been reported recently from a tertiary care level hospital in Varanasi, India.[38]

It is noteworthy that OXA-48 like enzyme producers were originally localized mainly in Middle East countries. Now they are found worldwide in Europe, North America and Asia.[17–19]Countries like Belgium and France have also reported a recent rise.[18,22]High prevalence of OXA-48 like carbapenemases in our study is a source of concern since this gene is mostly identified on plasmid and is implicated as a cause for silent spread and outbreaks in hospitalized patients.[17–19] These enzymes impart weak hydrolytic activity against broad spectrum cephalosporins and carbapenems.[18] Due to this they may go undetected in routine screening tests. Thus laboratories handling clinical samples need to develop their own algorithms for detecting these sinister looking isolates. There is an urgent need to develop more reliable phenotypic tests for Class D enzyme detection. Temocillin has been shown to be a good indicator of OXA 48, but this needs to be evaluated further.[39] None of the CRE isolates included in our study showed production of KPC enzymes. Similar findings regarding absence of KPC enzyme have also been highlighted by other studies from India.[6,16,24,40]

Variable data regarding carbapenemases has been presented by contemporary studies done globally too. Considerable geographical variation in epidemiology of carbapenemases within a country has been reported from China. While Liang WJ et al reported bla$_{NDM}$ to be the principal resistance mechanism, Yu X et al claim bla$_{kpc}$ to be the dominant carbapenemase gene.[27,28] KPC was the predominant resistance mechanism reported from a multicentric study done in Colombia amongst the paediatric population.[29] Baran et al have reported from Turkey that while OXA-48 is still the most common source of carbapenem resistance in Enterobacteriaceae in their country, NDM-1 is also increasingly being isolated.[41] All these factors highlight the evolving epidemiology and need for continuous surveillance of CRE in order to develop a sound antimicrobial policy.

The PCR amplified products from three random isolates that were NDM and OXA coproducers were purified and sent for Sanger sequencing (Eurofins ltd). The sequences were analysed using BLAST analysis and were confirmed to be matching to OXA-48/181 and NDM-1. Similar results have been presented by Sharma A et al from a study done in CMC Vellore.[16]OXA-181 which is a variant of OXA-48 was initially reported from India. [37] NDM-1, OXA-48 and OXA-181 have been reported from 65%, 24% and 23% of CRE isolates causing blood stream infections respectively in a recent study from New Delhi.[42] NDM Variants like NDM-5, NDM-6 and NDM-7 have been identified in a study from Lucknow.[23]

This pilot study highlights the importance of conducting a prospective analysis of CRE isolates and their complete characterization. This will help in not only tailoring individual therapy but also formulating standard treatment guidelines. Based on above facts, we recommend that therapy for CRE infections for all patients need to be customized. Clinical decisions should be made based on patient characteristics, disease severity, molecular phenotypes of resistance seen and susceptibility profile of isolates involved.

Strength

To the best of our knowledge, this is the first study presenting molecular epidemiology of CRE isolates in

Rajasthan, Western India.

Limitations

This was a pilot study planned to conduct both phenotypic and genotypic characterization of CRE isolates and hence included a small number of isolates. Also sequencing could not be done for all isolates due to scarcity of funds. Larger multicentric studies need to be carried out to enhance our understanding of CRE burden and epidemiology in Western India.

Conclusion

Growing carbapenem resistance is an important issue which needs urgent attention and bla_{OXA} is an emerging mechanism of resistance among clinical CRE isolates in our setting.

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Nil.

Conflicts of interest

There are no conflicts of interest

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