Original Paper

Medical Principles and Practice

Med Princ Pract 2018;27:428–435
DOI: 10.1159/000489885

Received: February 27, 2018
Accepted: May 8,2018
Published online: May 8, 2018

Detection of Antibiotic Resistance Determinants and Their Transmissibility among Clinically Isolated Carbapenem-Resistant *Escherichia coli* from South India

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Significance of the Study

- The increasing rates of carbapenem-resistant Enterobacteriaceae in the community and in hospitals led us to determine the prevalence of NDM and OXA in twenty-one *Escherichia coli* isolates and their ability to be transferred to laboratory strains. We hope that this study will emphasize the importance of antibiotic stewardship programs in health care facilities and improvement of social hygiene in the community.

Keywords
Carbapenem resistance · *Escherichia coli* · Transconjugation · New Delhi metallo-β-lactamase

Abstract

Objectives: The aim of this study was to analyze the prevalence of the CTX-M, TEM, SHV, VIM, NDM, and OXA genes in carbapenemase-producing *Escherichia coli* and their transmissibility at a tertiary care hospital in south India. ***Materials and Methods:*** Twenty-one carbapenem-resistant *E. coli* (carbapenem-resistant Enterobacteriaceae; CRE) were collected from the Sri Sathya Sai Institute of Higher Medical Sciences (Puttaparthi India). Resistance to antibiotics was analyzed by Vitek-2, and the identity of the isolates was confirmed by 16S rDNA sequencing. RAPD and enterobacterial repetitive intergenic consensus (ERIC)-PCR were performed for molecular typing. Metallo-β-lactamase production was confirmed by a double disc synergy test. The presence of the extended-spectrum β-lactamases CTX-M, TEM, and SHV and of the carbapenemases NDM, VIM, and OXA was determined by PCR. Carbapenemase variants were further confirmed by sequencing. The transmissibility of the genes was tested by conjugation. ***Results:*** Twelve of the 21 (57%) carbapenem-resistant *E. coli* isolates were community acquired, indicating the spread of CRE in environmental samples. TEM and NDM-

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5 were found to be the major β-lactamases produced by the pathogens. OXA-181 was found in 5 of the isolates. All 21 isolates were found to harbor more than one of the tested β-lactamases, and all of the isolates were found to have the capacity to participate in conjugation; 15 of the transconjugants were found to have acquired the tested β-lactamases, substantiating their ability to be transferred to other strains of bacteria. ***Conclusion:*** Monitoring of community-acquired carbapenem-resistant bacteria is very important as the association of resistance determinants with mobile genetic elements would present a serious clinical challenge.

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Introduction

Escherichia coli is the most common cause of infections in hospitals and in the community [1, 2]. It is also one of the main pathogens causing nosocomial infections, primarily in immunocompromised patients [3]. Carbapenems such as imipenem, meropenem, and ertapenem are the last drugs of choice for treating seriously ill patients who harbor organisms producing extended-spectrum β-lactamases (ESBL) such as CTX-M, TEM, and SHV [4]. More recently, carbapenem-resistant Enterobacteriaceae (CRE) are being increasingly reported [5]. The most prevalent mechanism of resistance among these is the production or acquisition of carbapenemases. Some carbapenemases that are encountered commonly include KPC, NDM, OXA, VIM, and IMP; these genes are generally located on mobile genetic elements which enable their transmission [6–8]. Globally, variants of OXA have been reported among Enterobacteriaceae. However, reports on the prevalence of OXA from India are sparse [9–11].

The rapid dissemination of antibiotic resistance genes globally is exemplified by the NDM carbapenemase. The spread of NDM-1-positive isolates in India and the global spread of NDM metallo-β-lactamase (MBL) was compiled and elucidated in a study conducted by Johnson and Woodford [12]. The prevalence of NDM-1 in Enterobacteriaceae among patients from Pakistan, India, and Bangladesh and among those in the UK who travelled to the Indian subcontinent has been reported. This study explained the epidemiology of NDM-1 and its association with the Indian subcontinent [13].

We aimed to investigate the prevalence of antibiotic resistance genes among CRE *E. coli* obtained from infected patients visiting a tertiary care hospital in Andhra Pradesh, India, and to characterize them with regard to their ability to transfer antibiotic resistance to laboratory strains. We believe that the importance of this study stems from the increased occurrence and spread of CRE in the Indian subcontinent [13].

Table 1. List of primers used for PCR amplification

Primer	Sequence	Size, bp
OPA 2	5'-TGCCGAGCTG-3'	
ERIC-F	5'-ATGTAAGCTCCTGGGGATTCA-3'	
ERIC-R	5'-AAGTAAGTGACTGGGGTGAGC-3'	
NDM-F	5'-GGT TTG GCG ATC TGG TTT TC-3'	621
NDM-R	5'-CGG AAT GGC TCA TCA CGA TC-3'	
OXA-F	5'-GCG TGG TTA AGG ATG AAC AC-3'	389
OXA-R	5'-CAT CAA GTT CAA CCC AAC CG-3'	
VIM-F	5'-TTTGGTCGCATATCGCAACG-3'	500
VIM-R	5'-CCATTCAGCCAGATCGGCAT-3'	
CTX-M F	5'-SCSATGTGCAGYACCAGTAA-3'	543
CTX-M R	5'-CCGCRATATGRTTGGTGGTG-3'	
TEM-F	5'-TCGGGGAAATGTGCGCG-3'	971
TEM-R	5'-TGCTTAATCAGTGAGGCACC-3'	
SHV-F	5'-TTATCTCCCTGTTAGCCACC-3'	948
SHV-R	5'-GATTTGCTGATTTCGCTCGG-3'	

Materials and Methods

Setting

This study included 20 patients with urinary tract infections visiting the Department of Urology and 1 patient with a fracture visiting the Department of Orthopedics of the Sri Sathya Sai Institute of Higher Medical Sciences (Puttaparthi, India). Urine samples and pus from these patients were cultured in the Department of Microbiology. Only one isolate from each patient was included in this study.

Bacterial Identification and Antibiotic Susceptibility Testing

During January 2013 to December 2014, a total of 233 carbapenem-resistant clinical isolates were obtained. Among these, 101 were found to be Enterobacteriaceae. The number of *E. coli* resistant to carbapenems was 21. Identification of the isolates was performed by Vitek-2 and confirmed by 16s rDNA sequencing. Antibiotic susceptibility testing (AST) and determination of minimum inhibitory concentrations were done using AST-N280 Vitek cards and interpreted as per Clinical Laboratory Standards Institute (CLSI) guidelines [14].

Phenotypic Detection of Carbapenemase Expression

Imipenem susceptibility testing was performed using the disc diffusion method as per CLSI guidelines [14]. A double disc synergy test using imipenem and imipenem-EDTA discs was performed as described earlier [15] by placing the 2 discs 30 mm apart on Müller-Hinton agar medium inoculated with a 0.5 McFarland suspension of the test organism. An increase in zone size of more than 5 mm for the imipenem-EDTA disc as compared to the imipenem disc was considered positive for the production of an MBL.

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Table 2. PCR amplification conditions

Primer	Initial denaturation	Denaturation 2	Annealing	Extension 1	Final extension	Cycles, *n*
RAPD	95°C for 5 min	95°C for 1 min	36°C for 1 min	72°C for 2 min	72°C for 15 min	40
ERIC-PCR	94°C for 7 min	94°C for 30 s	50°C for 1 min	72°C for 3 min	72°C for 15 min	30
NDM	94°C for 10 min	94°C for 30 s	52°C for 40 s	72°C for 50 s	72°C for 5 min	36
OXA	95°C for 5 min	94°C for 45 s	52°C for 1 min	72°C for 1 min	72°C for 10 min	36
CTX-M	95°C for 5 min	94°C for 45 s	55°C for 1 min	72°C for 1 min	72°C for 5 min	30
TEM	95°C for 5 min	94°C for 45 s	55°C for 1 min	72°C for 1 min	72°C for 5 min	30
SHV	95°C for 5 min	94°C for 30 s	55°C for 2 min	72°C for 1 min	72°C for 10 min	30
VIM (touchdown PCR)	95°C for 10 min	95°C for 30 s	69°C to 61°C (30 s)	72°C for 30 s	72°C for 10 min	2 at each annealing temperature (18 cycles in total)

Phylogenetic Grouping

Random amplified polymorphic DNA (RAPD) [16–18] and enterobacterial repetitive intergenic consensus (ERIC)-PCR [19–21] were performed to understand the clonal relatedness of the study isolates. Genomic DNA was recovered using NucleoSpin® tissue kits (Macherey-Nagel). RAPD and ERIC-PCR were performed using the primers mentioned in Table 1. The PCR conditions for the phylogenetic analyses involving RAPD and ERIC-PCR are described in Table 2.

PCR Amplification of β-Lactamase Genes

PCR amplification of the CTX-M, TEM, SHV, NDM, VIM, and OXA families of genes among the 21 carbapenem-resistant *E. coli* isolates was performed in a final volume of 50 µl containing PCR Master Mix – K0171 (25 µL; Thermo Scientific, USA), 10 pmol of each primer, and 50 ng of the template DNA. PCR amplifications were performed using primers (Table 1) and conditions as mentioned in Table 2. NDM- and OXA-positive amplicons were purified using a GeneJET PCR Purification Kit (Thermo Scientific). Sequencing was performed to identify the variants of the NDM and OXA amplicons only, while only PCR amplification was performed to confirm the presence of CTX-M, TEM, and SHV. Template DNA extracted from *E. coli* ATCC 25922, sensitive to carbapenem and cephalosporins, was used as a negative control.

Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported in the current study has been assigned by the GenBank database under the accession numbers KY817193.1-KY817205.1 (NDM), MG865741-MG865744 (NDM), KY777229.1, KY777228.1, KY801334.1, KY817191.1, and KY817192.1 (OXA).

Conjugal Transfer

The DNA transfer capacity of the study isolates was tested by selecting dual resistant transconjugants after broth mating experiments with a recipient plasmid-free, azide-resistant, conjugation-compatible *E. coli* J53Azi^R strain [22]. Exponentially growing cells (0.8 at OD_{600}) were mixed at a ratio of 1:1 (donor:recipient) and transconjugants were selected on LB agar containing ampicillin (100 µg/mL) and sodium azide (100 µg/mL). The presence of *bla* genes in the transconjugants was confirmed by PCR amplification of the respective genes.

Results

Clinical Characteristics

Twenty of the isolates were obtained from patients with UTI, while 1 sample was obtained from surgical site pus of a 78-year-old patient who presented to the Department of Orthopedics with an intertrochanteric fracture. The age of the patients ranged from 2 to 78 years. Five of the patients were female while the remaining 16 were male. Urine from a catheter was collected from 4 of the patients, while it was collected from right kidney in 1 patient. In all of the other patients, mid-stream urine was collected for culture. Clinical abnormalities in these patients included intertrochanteric fracture, calculus, voiding dysfunction, hyperplasia of the prostate, and urethral stricture among other structural and functional abnormalities of the urinary tract and kidney (Table 3).

Twelve of the patients were found to have community-acquired infections, while 9 patients had nosocomial infections. The demographic details of the patients include the following data: West Bengal (n = 6), Karnataka (n = 1), Orissa (n = 3), Telangana (n = 1), and Andhra Pradesh (n = 10). Eight of the patients from Andhra Pradesh belong to the Anantapur district.

Strain Characterization and Profiling of Antibiotic Resistance

All study isolates were confirmed as *E. coli* by Vitek-2 and 16S rDNA sequencing. Antibiotic sensitivity analyses revealed that the isolates were resistant to penicillins (ampicillin and ampicillin-clavulanic acid), cephalosporins (cefuroxime, ceftriaxone, cefoperazone-sulbactam, and cefepime), carbapenems (ertapenem, imipenem, and meropenem), and quinolones (ciprofloxacin and nalidixic acid). More than 52% (11 isolates against amikacin and

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Table 3. Clinical details of the study patients

Isolate	Age, years	Gender	Specimen	Diagnosis
VIR 1	57	F	fresh catheter urine	urinary tract infection
VIR 2	53	M	midstream urine	calculus in the urethra
VIR 3	62	M	midstream urine	calculus of the kidney
VIR 4	18	M	midstream urine	voiding dysfunction
VIR 5	61	M	midstream urine	diverticulum of the bladder
VIR 6	51	M	suprapubic catheter urine	hyperplasia of the prostate, obstructive and reflux uropathy
VIR 7	4	F	midstream urine	obstructive and reflux uropathy
VIR 8	66	M	midstream urine	hyperplasia of the prostate
VIR 9	49	M	right kidney urine	calculus of the kidney with calculus of the ureter
VIR 11	2	M	midstream urine	urethral stricture
VIR 12	27	M	midstream urine	redundant prepuce, phimosis, and bladder-neck obstruction
VIR 13	26	F	midstream urine	multiple left secondary calculus
VIR 14	12	M	fresh catheter urine	calculus of the ureter
VIR 16	55	M	midstream urine	congenital posterior urethral valves
VIR 17	51	M	suprapubic catheter urine	vesicoureteral-reflux-associated uropathy
VIR 18	52	F	midstream urine	trauma with complete urethral stricture
VIR 19	66	M	midstream urine	urethral stricture
VIR 20	4	F	midstream urine	urethral stricture
VIR 21	65	M	midstream urine	grade 1 prostatomegaly with significant postvoid residue
VIR 22	74	M	fresh catheter urine	obstructive and reflux uropathy
VIR 25	78	M	surgical site pus	intertrochanteric fracture

15 isolates against gentamycin) of the isolates were resistant to aminoglycosides. Seventeen (81%) were found to be resistant to co-trimoxazole, while only 4 isolates were resistant against nitrofurantoin. All of the isolates except Vir-4 (intermediate resistance) were found to be sensitive to tigecycline. All of the isolates were sensitive to colistin (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000489885). The MBL-producing capacity of the isolates was confirmed by double disc synergy testing involving imipenem and imipenem-EDTA discs.

All of the isolates were found to carry NDM and/or OXA carbapenemase genes. NDM was found in 17 (81%) of the isolates, while OXA-181 was present in 5 (24%). Among the isolates positive for the NDM gene, NDM-5 was prevalent in 12 isolates, while NDM-7 was found in 3 isolates; NDM-4 and NDM-6 variants were present in 1 isolate each. One isolate, i.e., Vir14, was found to possess both NDM-4 and OXA-181. VIM was not identified in any of the 21 isolates. Of the 21 *E. coli* isolates, 13 (62%) were found to possess the CTX-M type of ESBL, while TEM β-lactamases were found in 20 (95%). Twelve of the 21 (57%) isolates were found to concomitantly encode CTX-M and TEM genes. None of the isolates were found to possess SHV ESBL's.

Transmissibility of the Resistance Determinants and Genetic Relatedness

After mating the study isolates with the recipient EJ53 *E. coli*, the resultant transconjugants resistant to sodium azide (100 μg/mL) and ampicillin (100 μg/mL) were screened for the presence of CTX-M, TEM, NDM, and OXA β-lactamases. PCR amplification of genomic DNA from the transconjugants revealed the presence of the NDM gene in 9 isolates, OXA in 1 isolate, CTX-M in 2 isolates, and TEM in 13 isolates. Analyses of the transconjugants of the 6 isolates (Vir-5, Vir-12, Vir-13, Vir-17, Vir-19, and Vir-20) did not reveal the presence of any of the four β-lactamases, indicating that they may not be on mobilizable elements or, because the selection of the transformants was performed in the presence of ampicillin, they may not have been transferred (Table 4). Further analyses are required to characterize the mobilizable elements of the study isolates.

All of the 21 *E. coli* isolates were analyzed for their genetic relatedness using both ERIC-PCR and RAPD with 3 primers. Our analyses revealed that RAPD using the primer OPA-2 was able to provide a higher discrimination compared to the ERIC-PCR method. Although the ERIC-PCR method showed the presence of 11 clusters (data not provided), we found that the majority of

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Table 4. Presence of *bla* genes in the *E. coli* isolates and their transconjugants

Isolate	CTX-M	TEM	OXA	NDM	Transconjugants			
					CTX-M	TEM	NDM	OXA
vir 1	negative	positive	negative	NDM 6		positive	positive	
vir 2	positive	positive	negative	NDM 7		positive		
vir 3	positive	positive	negative	NDM 5		positive		
vir 4	negative	positive	negative	NDM 5			positive	
vir 5	positive	positive	positive	negative				
vir 6	negative	positive	negative	NDM 5		positive	positive	
vir 7	negative	positive	negative	NDM 5		positive	positive	
vir 8	positive	positive	positive	negative		positive		
vir 9	positive	positive	positive	negative				positive
vir 11	positive	positive	negative	NDM 7	positive	positive	positive	
vir 12	positive	positive	negative	NDM 5				
vir 13	positive	positive	negative	NDM 5				
vir 14	positive	positive	positive	NDM 4			positive	
vir 16	negative	positive	negative	NDM 7		positive		
vir 17	negative	positive	negative	NDM 5				
vir 18	positive	positive	negative	NDM 5	positive	positive	positive	
vir 19	positive	negative	negative	NDM 5				
vir 20	negative	positive	negative	NDM 5				
vir 21	positive	positive	positive	negative		positive		
vir 22	positive	positive	negative	NDM 5		positive	positive	
vir 25	negative	positive	negative	NDM 5		positive	positive	

the isolates (Vir-9, Vir-7, Vir-16, Vir-12, Vir-22, Vir-21, Vir-20, and Vir-18) were grouped together into a single cluster as ERIC-PCR could not produce good banding patterns for these isolates. On the other hand, RAPD using the OPA-2 primer was able to produce satisfactory banding patterns in the majority of the isolates (Fig. 1). A total of 6 clusters were found by RAPD analysis. In the first cluster, 4 isolates, i.e., Vir-5, Vir-8, Vir-16, and Vir-14, were found. Of these, Vir-14 branched out, indicating its divergence from the remaining 3 isolates. Incidentally, Vir-14 was the only isolate to possess CTX-M, TEM, NDM-4, and OXA-181 β-lactamases. The second cluster contained Vir-6 and Vir-7, both isolated from patients hailing from West Bengal. The third cluster had Vir-11 and Vir-21. The fourth cluster had Vir-4, Vir-3, Vir-18, and Vir-22 branching together. In the fifth cluster, Vir-2, Vir-13, and Vir-1 were found together. Vir-1 was found to further branch out from the remaining 2 isolates. The last cluster had Vir-9 and Vir-20. Vir-19 was found to be different from the remaining isolates based on its unique banding pattern. The RAPD banding pattern was not satisfactory among 3 isolates (Vir-17, Vir-25, and Vir-12) and so they could not be clustered with the other isolates.

Discussion

The emergence of multidrug-resistant *E. coli* and their rapid spread and ability to continuously evolve pose a serious threat to global health [5, 23, 24]. Carbapenemase-producing Enterobacteriaceae due to the presence of NDM and OXA β-lactamases have been reported in various medical centers globally as well as in India [25–29]. The spread of NDM-producing Enterobacteriaceae from the Indian subcontinent through visiting tourists has been documented in 2010 [13]. Ninety-five percent of the isolates at our center were uropathogenic *E. coli* (data not included) with high levels of antibiotic resistance. Similar trends were reported from 2 large hospitals located in Kuwait [30]. In this paper, 21 carbapenem-resistant *E. coli* were analyzed for the presence of ESBL (CTX-M, TEM, and SHV) and carbapenemase (NDM, OXA, and VIM) genes. The demographics of the patients indicate the widespread presence of CRE *E. coli* in community settings. Further, 57% of the study isolates were community acquired, indicating the spread of CRE in the general community, and they may not be solely hospital acquired. The AST pattern obtained from the VITEK 2 compact system of these isolates revealed that they are resistant to

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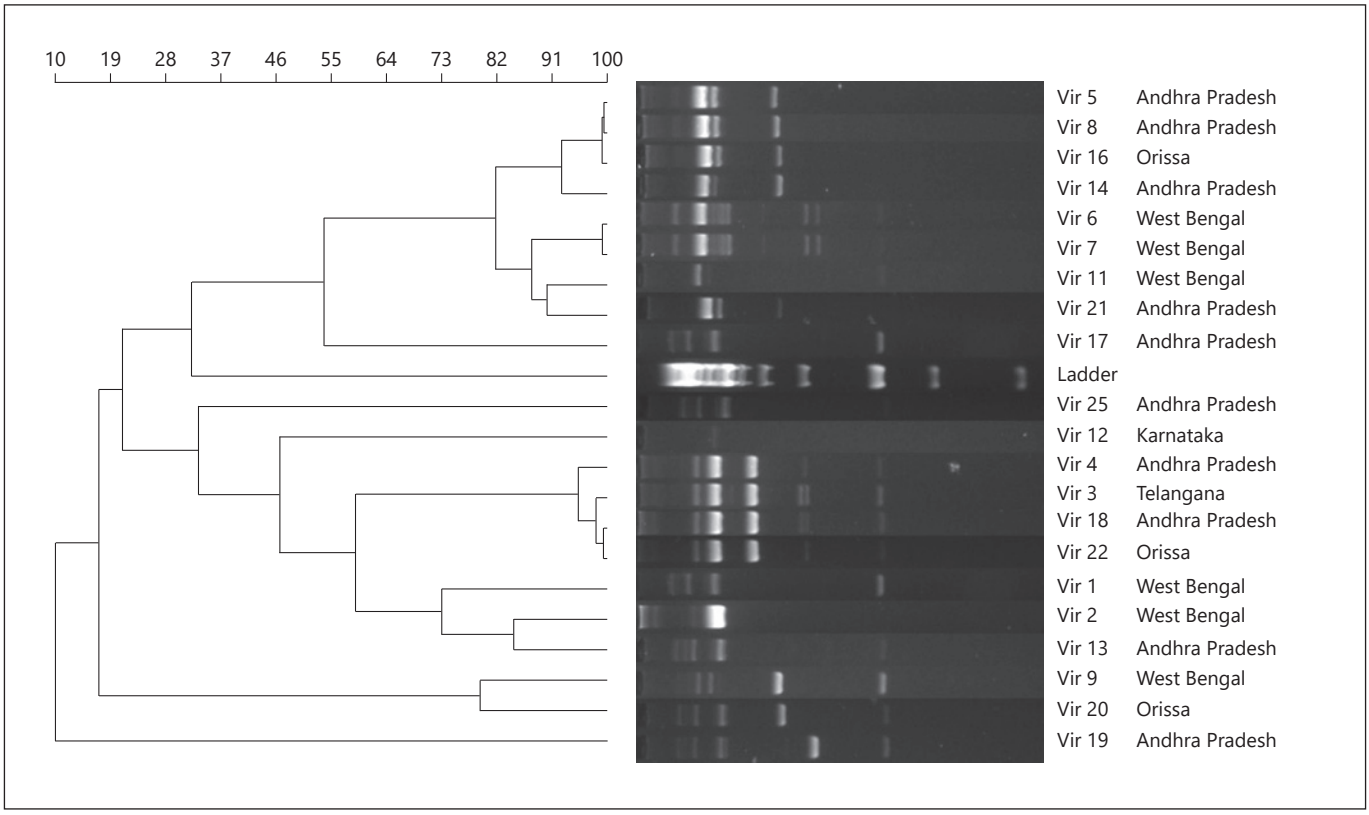

Fig. 1. RAPD profile of 21 carbapenem-resistant *E. coli* using the OPA-2 primer. The dendrogram was generated using UPGMA the method from the RAPD band patterns with the Opa-2 primer for the 21 CRE *E. coli* isolates.

a majority of the antibiotics, except colistin, leaving clinicians with limited therapeutic options to treat the CRE infections. RAPD and ERIC-PCR revealed that the CRE *E. coli* involved in the current study belong to multiple distinct clades.

Eighty-one percent of the isolates were NDM positive, and nearly a quarter of the isolates were positive for OXA carbapenemases. Interestingly, 1 isolate (Vir-14) was positive for all of the 4 tested ESBL and carbapenemases. Since the first description of NDM, several variants of the gene have been reported [31]. More recently, 7 NDM (1–7) variants were described from an Indian health care center [29]. In the current study, the majority of the isolates (i.e., 17) were found to possess the NDM gene. Of these, NDM-5 was found in 12 isolates, NDM-7 was detected in 3 isolates, and NDM-4 and NDM-6 variants were present in 1 isolate each. OXA-181 was first reported in 2011 [10]. All of the OXA genes in our population were found to be OXA-181.These findings confirm that NDM and OXA-181 are endemic to the Indian subcontinent and are highly prevalent in this region.

In the current study, all of the isolates were found to be compatible for conjugal transfer when mated with sodium-azide-resistant, plasmid-deficient *E. coli* J53 [22, 29]. ESBL and/or carbapenemases were detected in 15 (71%) of the transconjugants, indicating that the majority of these resistance determinants are transferable and capable of bestowing enhanced resistance features to the bacterial host. These findings suggest that NDM, TEM, and CTX-M genes are major causes of ESBL and carbapenem resistance in the Indian subcontinent and the predominant β-lactamase among our isolates is TEM (95%) [9].

These pathogens are a reservoir of antibiotic resistance genes and, together with their capacity to disseminate via horizontal gene transfer, they pose a grave threat to infection control strategies, with very few treatment options. Further, the spread of these resistance determinants in environmental samples should be a cause for alarm. A wide range of bacteria including *E. coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Shigella boydii*, *Vibrio cholerae,* and *Aeromonas caviae* harboring the *bla*NDM-1

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gene were isolated from 2 drinking water and 12 seepage samples [32]. Consistent with previous reports, our analysis revealed the presence of non NDM-1 variants (NDM-4, NDM-5, NDM-6, and NDM-7) among patients with serious urological disorders [29]. If this increase in antibiotic resistance continues and spreads to bacteria which were previously sensitive and easily treatable, it may lead us to dire straits without any available treatment options for simple infections.

Conclusion

Molecular epidemiology of carbapenem-resistant *E. coli* harboring multiple resistance-encoding genes is rapidly evolving. In this study, CRE *E. coli* were predominantly isolates from community-acquired clinical samples and the pathogens were found to express resistance against a variety of antibiotics except colistin. CTX-M, TEM, NDM, and OXA were identified among the 21 study isolates. Isolates positive for OXA and/or NDM were found to harbor genes encoding other resistance determinants. NDM-5 and OXA-181 were found to be prevalent in the study isolates. The widespread coexistence of ESBL and carbapenemases on transferrable elements is a major clinical challenge for control of infections.

Acknowledgement

We thank the Department of Microbiology of Sri Sathya Sai Institute of Higher Medical Sciences for providing the specimen and clinical information. We greatly acknowledge Dr. Kashinath Prasad (Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India) for providing the *E. coli* J53AzR strain. We acknowledge UGC-SAP (DRS), DST-FIST, and DBT-BIF, and the Government of India for infrastructural support to the Department of Biosciences of the Sri Sathya Sai Institute of Higher Medical Sciences.

Disclosure Statement

None.

Funding Sources

This research project received no specific grant from any funding agency in the public, commercial, or not-for-profit sector.

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