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Carbapenem resistance mechanisms among blood isolates of *Klebsiella pneumoniae* and *Escherichia coli*

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The purpose of this study was to characterize the different mechanisms of carbapenem resistance among blood isolates of *Klebsiella pneumoniae* and *Escherichia coli*. Meropenem resistant isolates were included. Antimicrobial susceptibility testing, phenotypic and genotypic detection of carbapenemase production were performed. Genetic relatedness of *bla*_{NDM-1} producers was determined by pulsed-field gel electrophoresis (PFGE) typing. The relatedness of *bla*_{NDM-1} carrying plasmids was studied by plasmid restriction fragment length polymorphisms (pRFLP) and polymerase chain reaction (PCR) based replicon typing (PBRT). For the carbapenem gene negative isolates, other possible mechanism of carbapenem resistance such as role of outer membrane porin loss with ESBL or AmpC production, and efflux pumps were analyzed. Among the 162 isolates studied, 1 (0.6%) was found to be *bla*_{KPC-3} producer and 42.5% were *bla*_{NDM-1} producers. All the isolates were multidrug resistant; two isolates carried both *bla*_{NDM-1} and *bla*_{VIM-2}. PFGE determined *bla*_{NDM-1} producers were non-related. The plasmids harbouring *bla*_{NDM-1} belonged to two major incompatibility plasmid types, IncL/M and IncA/C. IncL/M is a novel plasmid group reported firstly from this region. A clonal outbreak of *bla*_{IMP-1} *K. pneumoniae* was identified during this study. This is the first report on the emergence of *K. pneumoniae* producing *bla*_{IMP-1} from South India and *bla*_{KPC-3} from India. The study suggest including ertapenem in the routine susceptibility screening to find the true rate of KPC producers in Indian hospitals. Colistin and tigecycline are two drugs that have activity but both have developed resistance. Selection of an appropriate initial antibiotic regimen for empiric therapy, rotation of different antibiotic classes and judicious use of antibiotics are essential.

Key words: Blood isolates, carbapenemase, multiplex polymerase chain reaction (PCR), pulse field gel electrophoresis, tigecycline.

INTRODUCTION

Antibiotic resistance in gram-negative bacteria is of increasing concern because of the lack of new antibiotics

to treat these infections. Of particular importance from the healthcare epidemiology standpoint is the resistance

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towards carbapenems (Zhanal et al., 2007). Of all the β -lactam antimicrobial drugs, carbapenems (imipenem, meropenem, doripenem and ertapenem) have the most consistent activity against enterobacteriaceae. Activity is retained against isolates that produce AmpC and extended spectrum beta-lactamases (ESBLs) which makes them a drug of choice to treat these resistant infections (Jacoby et al., 1997).

Carbapenems were first introduced in 1980s and are now frequently used as the last choice in treating serious infections caused by multidrug resistant (MDR) strains of gram-negative bacilli (Rodloff et al., 2006). Within a decade, resistance to carbapenems started emerging and has been reported in non-fermenter gram-negative bacilli worldwide over the years with varying frequencies (Spencer et al., 2002). Over the past two decades, while carbapenem resistance has become a serious problem among the non-lactose fermenting bacteria, it has remained uncommon in enterobacteriaceae. Recently however, identification of carbapenem-resistant enterobacteriaceae is increasing (Spencer et al., 2002; Zhanal et al., 2007).

Carbapenem-resistant gram-negative bacteria pose a serious problem due to the genes encoding most of these carbapenemases reside on plasmids or transposons which carry additional genes encoding resistance to other classes of antimicrobial agents (Rasmussen et al., 1996). These transferable structures can readily be acquired by gram-negative pathogens, facilitating the dissemination of these potent resistance mechanisms and, in many cases, conferring on the isolate a MDR profile (Walsh et al., 2005).

The incidence of MDR ESBL producing pathogens is an increasingly difficult problem in hospitals. Carbapenems have been the last line of drug treatment for serious infections caused by these pathogens due to the stability of these agents against the majority of β -lactamases and their high rate of permeation through bacterial outer membranes. This is of great concern as presently carbapenems are considered the last resort especially in ICUs. With *Klebsiella pneumoniae* and *Escherichia coli* remaining the leading pathogen causing infection in our hospital, close monitoring for the possibility of developing antimicrobial resistance is imperative.

With this background, this study was undertaken to identify the different mechanisms of carbapenem resistance, the antimicrobial profile, and the clonal relatedness of the isolates which could provide important information on the future impact of antibiotic resistance.

MATERIALS AND METHODS

Bacterial isolates

In this study, meropenem resistant blood isolates of *K. pneumoniae* and *E. coli* were obtained from patients attending a major tertiary care teaching hospital, Puducherry. Duplicate isolates from the same patients were excluded.

Antimicrobial susceptibility testing

Susceptibility of the isolates to carbapenems (ertapenem, imipenem and meropenem), tigecycline, colistin and polymyxin-B were determined by the standard Kirby Bauer disk diffusion method. Antimicrobial susceptibility of the isolates to other β -lactam and non β -lactam broad spectrum antimicrobials such as amikacin, trimethoprim/sulfamethoxazole, tetracycline, ciprofloxacin, aztreonam, cefotaxime, ceftriaxone, ceftazidime, cefepime, ceftazidime/sulbactam, and piperacillin/tazobactam (Hi Media, India) were also determined by disk diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2010). *E. coli* ATCC 25922 was used as the control strain in each series. Determination of MIC was performed by E-test (AB Biodisk, Solna, Sweden) method for carbapenems, polymyxin-B, tigecycline, colistin and by agar dilution method for other β -lactam and non β -lactam antibiotics. The results were interpreted according to the CLSI (CLSI, 2010). Isolates concomitantly resistant to ≥ 3 different antimicrobial classes were defined as multi drug resistant (MDR).

Phenotypic detection of carbapenemase production

All isolates were screened for metallo- β -lactamase (MBL) and *Klebsiella pneumoniae* carbapenemase (KPC) production by modified Hodge's test (MHT) (Lee et al., 2001). Further, all imipenem resistant isolates were tested by imipenem-EDTA (I-EDTA) synergy test for MBL production (Lee et al., 2001). The MBL positive isolates were confirmed by the MBL E-test using E-strips (bioMérieux). A class A carbapenemase producer was defined as any isolate which displayed reduced susceptibility to carbapenems and tested positive in MHT, and negative in I-EDTA synergy test. A MBL producer was defined as, any isolate which displayed reduced susceptibility to carbapenems and tested positive in MHT test and positive in I-EDTA synergy test.

Molecular detection of carbapenemase and ESBL genes

Polymerase chain reaction (PCR) was used to screen and sequence the following carbapenemase genes: *bla*_{KPC}, *bla*_{NDM-1}, *bla*_{OXA-48}, *bla*_{IMP} and *bla*_{VIM} genes as described previously by Mulvey et al. (2011). The ESBL genes screened were *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M} and *bla*_{OXA-1} (Nuesch et al., 1996; Boyd et al., 2004). The plasmid mediated AmpC β -lactamase (PMABL) genes *bla*_{MOX}, *bla*_{FOX}, *bla*_{EBC}, *bla*_{ACC}, *bla*_{DHA} and *bla*_{CIT} were also detected as described earlier (Perez and Hanson, 2002).

Determination of genetic relatedness

Molecular typing of *bla*_{VIM} and *bla*_{IMP} positive isolates were done by RAPD-PCR technique using the HLWL74 (5'-ACG TAT CTG C-3') primer (Tribudharat et al., 2008). Clonal relatedness of *bla*_{NDM-1} positive isolates was determined by macrorestriction analysis using *Xba*I according to the PulseNet program developed for *E. coli* (Gautom, 1997). Analysis of images was carried out using BioNumerics software (Applied Maths) using the dice coefficient and UPGMA to generate dendrograms with 1.0% tolerance values. Salmonella ser. Braenderup standard strain H9812 was used as the ladder.

Plasmid analysis

Plasmids harbouring *bla*_{NDM-1} were extracted using commercial kits (Qiagen Inc., Mississauga, ON, Canada). *bla*_{NDM-1} containing plasmids were transformed in electrocompetent *E. coli* DH10BTM

Table 1. Distribution of carbapenemase genes detected by multiplex PCR from blood isolates of *K. pneumoniae* and *E. coli*.

Carbapenemase gene(s)	<i>K. pneumoniae</i> (n=108) (%)	<i>E. coli</i> (n=54) (%)	Total isolates (n=162) (%)
<i>bla</i> _{NDM-1}	48 (44.4)	19 (35.1)	67 (41.3)
<i>bla</i> _{VIM-2}	7 (6.4)	2 (3.7)	9 (5.5)
<i>bla</i> _{VIM-24}	2 (1.8)	0 (0)	2 (1.2)
<i>bla</i> _{VIM-1}	3 (2.7)	0 (0)	3 (1.8)
<i>bla</i> _{IMP}	5 (4.6)	1 (1.8)	6 (3.7)
<i>bla</i> _{KPC}	1 (0.9)	0 (0)	1 (0.6)
<i>bla</i> _{VIM} and <i>bla</i> _{NDM-1}	2 (1.8)	0 (0)	2 (1.2)
Total	68 (62.9)	22 (40.7)	90 (55.5)

(Invitrogen, Carlsbad, CA) (Boyd et al., 2004) and restricted using *Bgl*II (Roche Diagnostics, QC) by standard procedures (Mulvey et al., 2009). PCR based replicon typing (PBRT) was also conducted as per the standard method (Caratolli et al., 2005). Conjugation was carried out by both broth-mating and filter-mating assays at 30°C using NDM-1-producing isolates as donor and azide-resistant *E. coli* J53 as recipient. Transconjugant selection was performed on MacConkey agar containing meropenem (0.5 mg⁻¹) and sodium azide (100 mg⁻¹). Transconjugants were tested for *bla*_{NDM-1} and other beta-lactamase genes by PCR as described earlier. Susceptibilities of transformants were performed by disk diffusion method and MIC was determined by agar dilution and/ or E-test as described earlier.

Outer membrane protein profiling

The role of bacterial outer membrane proteins (OMPs) in the mechanism of carbapenem resistance was determined. Bacterial cells in logarithmic phase were lysed by sonication. The outer membrane proteins were extracted and analyzed by SDS-PAGE (Filip et al., 1973). The molecular weight of the OMPs was compared with the carbapenem susceptible strain of *K. pneumoniae* KpCS1. The involvement of potential efflux pumps in carbapenem resistance was analyzed by an MIC based assay. Agar dilution method for meropenem was performed on Muller-Hinton agar plates with 25 µg/ml of reserpine and without reserpine. An MIC ratio of 1:≤3 between reserpine to non-reserpine group suggests inhibition test positive, indicating existence of active efflux mechanism (Ribera et al., 2002).

RESULTS

A total of 3,361 patient blood cultures were tested which yielded 162 (4.8%) meropenem resistant (MEM-R) isolates (108 *K. pneumoniae* and 54 *E. coli*). Among the 162 MEM-R isolates, 95 (58.6%) were positive for MHT. Eighty seven (53.7%) isolates were positive by I-I EDTA synergy test and 67 (41.3%) isolates were negative by both the tests.

Molecular detection of the carbapenemases

Of the 162 isolates tested, 90 (55.5%) were PCR positive for the carbapenemase genes tested and 72 (44.4%)

negative. A total of 62.9% *K.pneumoniae* and 40.7% *E. coli* carried carbapenemase genes (Table 1). Two isolates (1.2%) carried both *bla*_{VIM} and *bla*_{NDM-1} genes.

Characterization of *bla*_{VIM} producing isolates

Fourteen (8.6%) isolates were positive for *bla*_{VIM}: *K. pneumoniae*, n=12 (11.1%) and *E. coli*, n=2 (3.7%). All the isolates were positive for I-EDTA synergy test and MHT. Among the MHT done with different substrates, 10 isolates were positive with meropenem, 12 with ertapenem and all the 14 were positive with imipenem. Antimicrobial susceptibility testing revealed that all were resistant to carbapenems, third generation cephalosporins (3GCs), cephamycins, penicillin inhibitor combinations, fluoroquinolones, aminoglycosides and tetracycline (Table 2). Of the 14 isolates, 9 (64.2%) were resistant to polymyxin-B, 6 (42.8%) to colistin followed by 1 (7.1%) to tigecycline, which was pan resistant. Clonal relatedness of the *bla*_{VIM} positive isolates was determined by RAPD-PCR using HLWL74 primer. Among the 14 isolates, 7 *K.pneumoniae* (KpV4 to KpV10) showed clonal cluster type I, 2 *K.pneumoniae* (KpV11, KpV12) showed cluster type II, 2 *E. coli* (EcV1, EcV2) were of cluster type III, and 3 *K.pneumoniae* isolates (KpV1, KpV2, KpV3) were of dissimilar types. Nucleotide sequencing of the VIM was done for representative isolates from each cluster. Cluster I and cluster III showed *bla*_{VIM-2} type, cluster II showed *bla*_{VIM-24} type and all the three dissimilar clones were of *bla*_{VIM-1} type variants. In addition, *bla*_{CTX-M-15} was carried along with the *bla*_{VIM-24} producing isolate. *bla*_{CTX-M-15} and *bla*_{TEM-1} was carried along with *bla*_{VIM-2} isolates and *bla*_{SHV-12} was carried in all the three *bla*_{VIM-1} *K.pneumoniae*.

Characterization of *bla*_{IMP} producing isolates

Among the 162 isolates tested, 6 (3.7%) were positive for *bla*_{IMP-1}: *E. coli* (n=1) and *K. pneumoniae*(n=5) (Table 1). All 6 isolates were positive for MHT and I-I EDTA synergy test. All *bla*_{IMP} containing isolates were MDR. Additionally,

Table 2. Antimicrobial resistance patterns of *bla*_{VIM}, *bla*_{IMP} and *bla*_{NDM-1} producing isolates of *K. pneumoniae* and *E. coli*.

Antimicrobial agents tested	MIC (µg/mL)		
	<i>bla</i> _{VIM} producers (n=14)	<i>bla</i> _{IMP} producers (n=6)	<i>bla</i> _{NDM-1} producers (n=69)
Piperacillin/tazobactam	≥128 (n=9); 64 (n=5)	≥128 (n=6)	≥256 (n=10); 128 (n=35); ≥64 (n=24)
Cefotaxime	≥256 (n=14)	≥256 (n=6)	≥256 (n=69)
Ceftriaxone	≥256 (n=14)	≥256 (n=6)	≥256 (n=69)
Ceftazidime	≥256 (n=14)	≥256 (n=6)	≥256 (n=69)
Cefoxitin	Not done	≥128 (n=6)	32 (n=37); 64 (n=17); ≥128(n=15)
Cefepime	≥256 (n=14)	≥256 (n=6)	16 (n=9); 32 (n=12); ≥64 (n=48)
Aztreonam	≥128 (n=12)	64 (n=6)	Not done
Meropenem	≥64 (n=14)	≥128 (n=6)	≤32 (n=12); 64 (n=18); ≥128 (n=39)
Ertapenem	32 (n=5); 64 (n=9)	≥128 (n=6)	≤16 (n=8); 32 (n=14); 64 (n=17); >128 (n=30)
Imipenem	32 (n=2); 64 (n=3); ≥128 (n=9)	≥128 (n=6)	≤16 (n=19); 32 (n=7); 64 (n=21) ≥128 (n=22)
Ciprofloxacin	32 (n=3); 64 (n=9); 128 (n=2)	128 (n=6)	32 (n=48); ≥64 (n=21)
Colistin	64 (n=4); ≥128 (n=2)	32 (n=6)	32 (n=12); 64 (n=4); ≥128 (n=10)
Polymyxin-B	16 (n=6); ≥32 (n=3)	≥32 (n=6)	32 (n=9); 64 (n=12); ≥128 (n=3)
Tetracycline	64 (n=9); 128(n=2); ≥128 (n=3)	Not done	Not done
Amikacin	64 (n=3); ≥128 (n=11)	≥128 (n=6)	128 (n=11); ≥256 (n=58)
Gentamicin	≥128 (n=8); ≥256 (n=6)	≥128 (n=6)	128 (n=21); ≥256 (n=48)
Tigecycline	8 (n=1)	≤1 (n=6)	8 (n=6); 16 (n=1)

all were susceptible to tigecycline, polymyxin-B, and 83.3% were susceptible to colistin (Table 2). Genetic relatedness was determined by RAPD-PCR using HLWL74 primer. Among the 5 *K. pneumoniae*, all belonged to a single clone, cluster I. Additionally, all *K. pneumoniae* carried *bla*_{SHV-12} and *bla*_{TEM-1} and *E. coli* carried *bla*_{TEM-1} and *bla*_{OXA-1} genes.

Characterization of *bla*_{NDM-1} producing isolates

Among the 162 isolates tested, 67 (41.3%) were *bla*_{NDM-1} producers: *K. pneumoniae* (n=48, 44.4%) and *E. coli* (n=19, 35.1%) (Table 1). Two *K. pneumoniae* isolates were positive for both *bla*_{NDM-1} and *bla*_{VIM} genes. All isolates were positive by MHT and I-I EDTA synergy test. All of these isolates were resistant to all antimicrobial groups (Table 2). Seven (7.2%) isolates were resistant to tigecycline, 26 (37.6%) were resistant to colistin and 24 (34.7%) to polymyxin-B. PFGE was performed on a convenience sample of 20 *bla*_{NDM-1} *K. pneumoniae* and 15 *bla*_{NDM-1} *E. coli*. Among *K. pneumoniae*, 2 clusters with >80% similarity were observed cluster 1 (n=3) and cluster 2 (n=9), (Figure 1). Among *E. coli*, 10/12 isolates clustered into one of three groups sharing >80% (Figure 2). Two isolates were non-typeable.

Plasmid analysis was conducted on twenty *bla*_{NDM-1} *K.*

pneumoniae. Seventeen of the *bla*_{NDM-1} bearing plasmids were successfully transferred to *E. coli* DH10B by electroporation. *bla*_{NDM-1} genes were located on plasmids that ranged in size from 89 to 131 Kb (Figure 3). Replicon typing revealed that the *bla*_{NDM-1} bearing plasmids were distributed among two incompatibility groups: 12 (70.5%) IncA/C and 5 (29.4%) IncL/M. The RFLP patterns of plasmid transformant DNA consisted of 2 clusters corresponding to incompatibility groups (Figure 3). Cluster I consisted of 8 IncL/M plasmids that were genetically similar (>85% homology), varying in size from 89 to 122 Kb. The cluster II consisted of IncL/M plasmids consisted of highly similar RFLP patterns of ~131 Kb in size. Susceptibility testing among the transformants revealed that in addition to carbapenems resistance determinants of other antimicrobial groups was also co-transferred in all the isolates.

Characteristics of both *bla*_{NDM-1} and *bla*_{VIM} positive isolates

The two isolates positive for both carbapenemase genes showed by sequencing that they were of *bla*_{NDM-1} and *bla*_{VIM-2} types. PFGE revealed a single clonal type. The isolates were pan resistant including resistance to tigecycline and colistin. Replicon typing revealed they both were on IncA/C plasmids.

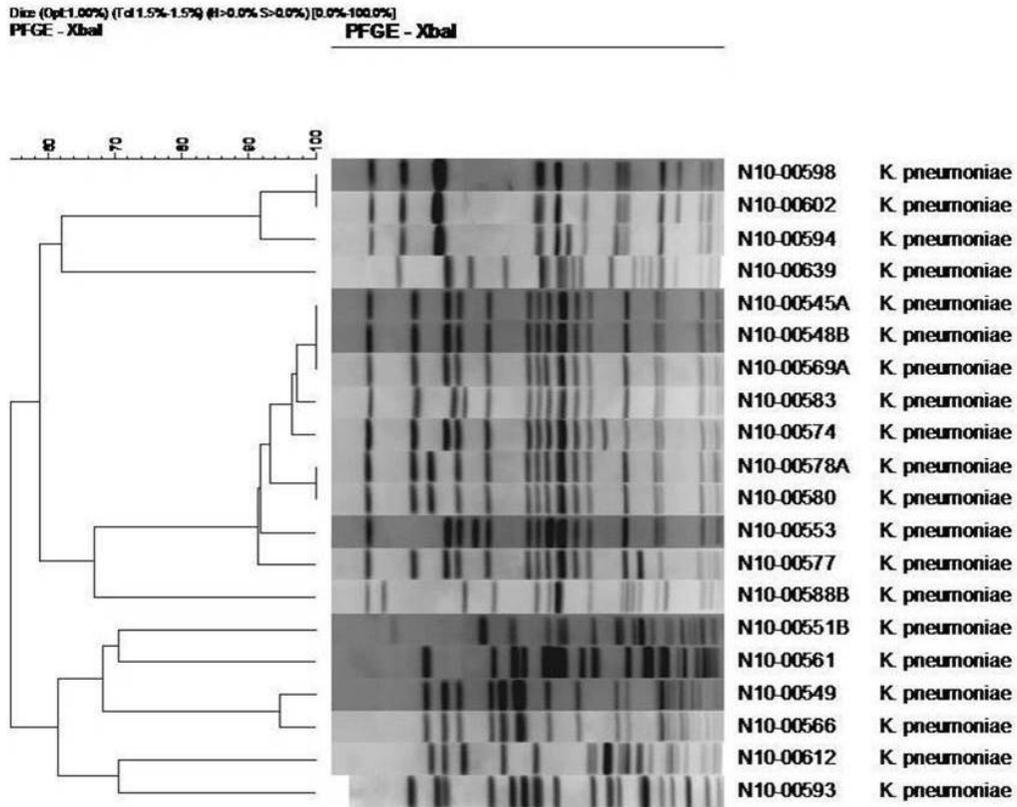


Figure 1. PFGE dendrogram depicting the genetic relatedness of *bla*_{NDM-1} producing blood isolates of *K. pneumoniae*.

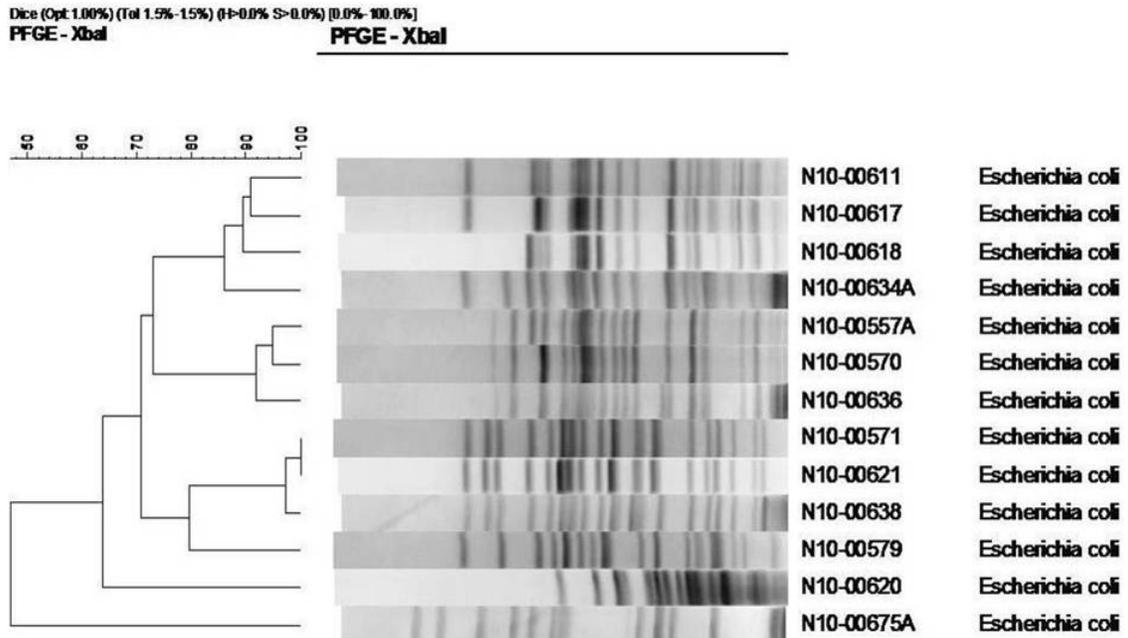


Figure 2. PFGE dendrogram depicting the genetic relatedness of *bla*_{NDM-1} producing blood isolates of *E. coli*.

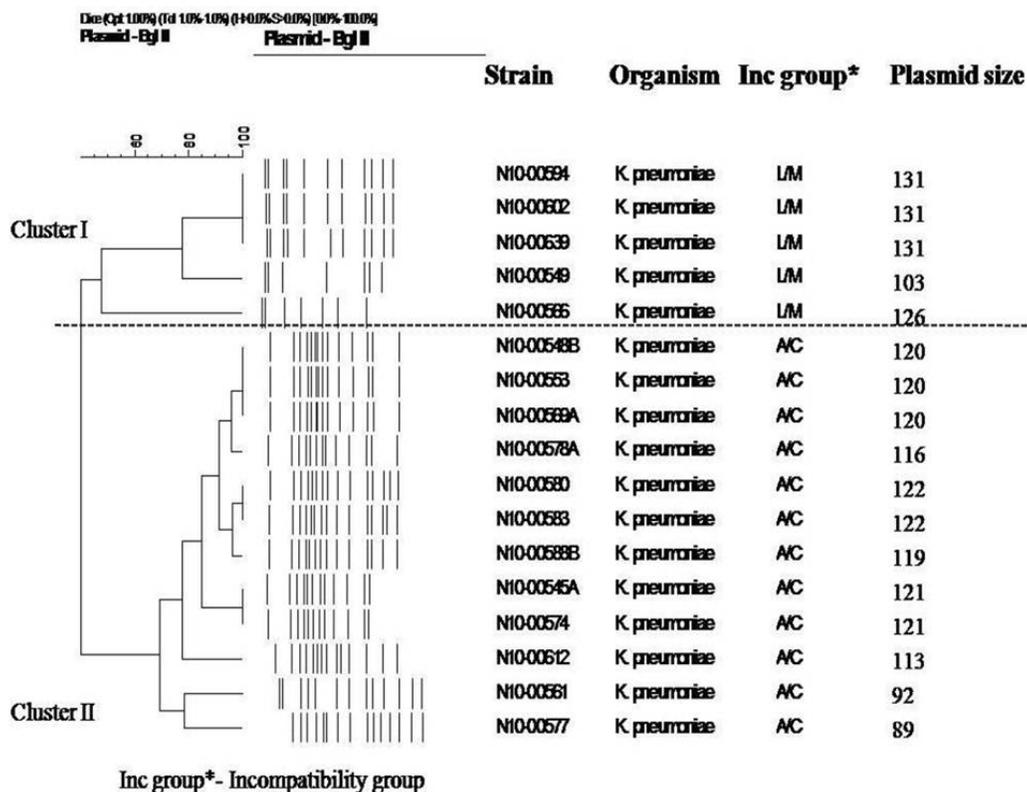


Figure 3. RFLP dendrogram depicting genetic relationships of the *bla*_{NDM-1} bearing plasmids from blood isolates of *K. pneumoniae*.

Non-carbapenemase resistance mechanisms

A total of 44.4% (n=72) of MEM-R *K. pneumoniae* and *E. coli* blood isolates were negative for all carbapenemase genes tested using PCR. A convenience sample of 41 isolates was selected for an efflux assay. Fifteen (36.5%) isolates showed a 1 to 2 dilution decrease in the MICs of meropenem with reserpine, suggesting that an efflux may be responsible, at least in part, for the carbapenem resistance in these isolates. Additional beta-lactamase PCR was conducted on 57 isolates that were negative by both efflux testing and carbapenemase gene PCR. Forty three (75.4%) possessed ESBL genes and 9 (15.7%) carried PMABL genes. The OMP profile was studied for randomly selected 22 carbapenem resistant, carbapenemase gene negative, efflux negative, ESBL (n=19) and or AmpC positive (n=3) *K. pneumoniae* isolates. OMP profile showed loss of 36kDa porins in 13 ESBL positive and 1 AmpC positive isolate. Thus, 14 (63.6%) showed OMP porin loss.

DISCUSSION

Carbapenems are antibacterial agents with broad spectrum of activity against gram positive, gram negative

and anaerobic bacteria. In the last two decades, carbapenems (example, imipenem and meropenem) have become the drugs of choice against ESBL and AmpC producing MDR isolates causing infections in a host of medical centers, but are being compromised by the emergence of carbapenem- hydrolysing β -lactamase (carbapenemase) of molecular classes B and D (Livermore et al., 2006; Lee et al., 2006). This newer antimicrobial resistant species such as carbapenem-resistant enterobacteriaceae (CRE), including *E. coli* and *K. pneumoniae* are emerging which is an increasing therapeutic problem worldwide. Therefore, investigating the mechanisms underlying the resistance has an impact in treatment measures.

MBL production was screened by using the MHT and I - I EDTA synergy test which gave 100% specificity and sensitivity with that of PCR results. MHT detects carbapenemase production and EDTA synergy test confirms MBL production and thus, in the study experience it is suggested to test by both the phenotypic tests which will not miss out any carbapenemase producing isolate. However, specific gene target PCR has to be performed for confirmation.

KPC enzymes are class A carbapenemases that mediate resistance to extended-spectrum cephalosporins in addition to carbapenems and are usually plasmid encoded.

The KPC β -lactamase occurs most commonly in *K. pneumoniae*, but has also been reported in other species of Enterobacteriaceae and in *P. aeruginosa* (Anderson et al., 2007). In this study, a KPC-3 producing *K. pneumoniae* was identified from a blood isolate.

In a recent study from this centre, of the 103 meropenem resistant *K. pneumoniae* from blood, urine and exudates samples tested 6 were MHT positive, I-I EDTA synergy test negative and were found to KPC producers phenotypically (Parveen et al., 2010). Among the 9 blood isolates tested, one was KPC positive phenotypically and it was included in this study for further molecular characterization. By PCR and sequencing analysis, the isolate was confirmed to be KPC-3 producer. The phenotypically positive isolates from other sources were not subjected to molecular studies, however by Randomly Amplified Polymorphic DNA (RAPD) typing all the 6 KPC producers showed dissimilar patterns (Parveen et al., 2010). No discernible epidemiological linkage could be seen between these isolates. This finding reveals the diversity of these strains and its non-clonal dissemination in this hospital.

This isolate was found to be resistant to all the antimicrobials except tigecycline and colistin. However, before this information could be used, the patient had died of septic shock, presumably caused by KPC producing *K. pneumoniae*. The source of the isolate could not be found out. Although the study could not determine whether this case was sporadic in nature or attribute to the existence of an outbreak, this enzyme has not been previously identified in this geographic region.

The present study also reveals the presence of VIM MBL producing isolates of *K. pneumoniae* and *E. coli* in the study hospital. Fourteen (8.6%) of carbapenem-resistant *K. pneumoniae* and *E. coli* harboured bla_{VIM} . Phenotypic tests indicated that imipenem was inactivated by all the 14 imipenem-resistant isolates by MHT, they were also positive for EDTA synergy test indicating presence of MBL. Sequence identification of bla_{VIM} revealed 9 (64.2%) bla_{VIM-2} , 3 (21.4%) bla_{VIM-1} and 2 (14.2%) bla_{VIM-24} variants. To this study knowledge, this is the first report of VIM variants in *K. pneumoniae* and *E. coli* from India.

RAPD analysis of the 12 bla_{VIM} *K. pneumoniae* revealed seven cases of infection by *K. pneumoniae* producing bla_{VIM-2} was found to have been caused by a single clone (data not shown). These findings suggest clonal *K. pneumoniae* harbouring bla_{VIM-2} are contributing to the dissemination of bla_{VIM} in this hospital. The present study reports, 3.7% (n=6) of carbapenem resistant isolates as harbouring bla_{IMP} . All were multidrug resistant and susceptible to tigecycline.

In this study, among the 12 VIM positive *K. pneumoniae*, seven cases of infection by *K. pneumoniae* producing bla_{VIM-2} was found to have been caused by a single clone. During the study period, the other outbreak involved two cases of infection caused by a single clone

of bla_{VIM-2} producing *E. coli*. These findings suggest an increasing prevalence rate of bla_{VIM-2} positive isolates in this hospital. Further, three cases of bla_{VIM-1} *K. pneumoniae* of dissimilar clones was also found. This could be in part due to the nosocomial outbreak due to clonal and non-clonal isolates occurring in this hospital over this study period, however, it was not recognized due to the lack of awareness in detecting the MBLs routinely. These results show that bla_{VIM} positive isolates are still confined in different wards and may spread at a low rate and cause sporadic outbreaks in this hospital.

Analysis of other β -lactamases combinations among bla_{VIM} producers showed, $bla_{VIM-2} + bla_{CTX-M-15} + bla_{TEM-1}$; $bla_{VIM-1} + bla_{SHV-12}$ and $bla_{VIM-24} + bla_{CTX-M-15}$ combinations. The coexistence of two enzymes, an MBL and a non-MBL extended-spectrum β -lactamase, in the same strain has been noteworthy. Kassis et al. (2006) reported $bla_{VIM-1} + bla_{SHV-5}$ in *K. pneumoniae* from France and $bla_{VIM-4} + bla_{SHV-12}$ was reported by Luzzaro et al. (2004). From a Tunisian university hospital, a maximum of four enzymes, bla_{VIM-4} , $bla_{CTX-M-15}$, bla_{CMY-4} , and bla_{TEM-1} in a MDR *K. pneumoniae* (Ktari et al., 2006). Since *K. pneumoniae* and *E. coli* are notorious as a hosts' for resistance plasmids and are the major causes of nosocomial infections, they may acquire the resistance genes from non-fermenters. The presence of more than one ESBL or MBL enzymes in the same plasmid may facilitate its potent dissemination to other species or genera.

In this study, five isolates of *K. pneumoniae* possessed the bla_{IMP-1} gene. RAPD-PCR typing showed that all *K. pneumoniae* were from a single clone. The source of the infections was unknown. These isolates were collected over a period of three years and from different wards, thus providing evidence that bla_{IMP-1} producing *K. pneumoniae* though less in percentage exists close to a state of endemicity in this hospital. A more worrying thing is that bla_{IMP-1} has spread in this region, but has remained undetected to date. To date there are no published data on the identification of IMP harboring *K. pneumoniae* and *E. coli* isolates in India.

The most common carbapenamase identified in this study was bla_{NDM-1} (42.5%) which was approximately 45% of *K. pneumoniae* and 35% of *E. coli* carried bla_{NDM-1} . The first report of bla_{NDM-1} producers from India was by Deshpande et al. (2010) from Mumbai, who found 22 out of 24 isolates to be bla_{NDM-1} producers. The study findings were not surprising given the numerous reports of bla_{NDM-1} from South India.

Antimicrobial susceptibility has shown resistance to all classes with tigecycline and colistin as the only treatment options. However, 10 and 37.6% were resistant to tigecycline and colistin respectively, which narrows the therapeutic choices. This is also evident from a recent study from India that, tigecycline still has effective antimicrobial effects against carbapenamase and ESBL producing *K. pneumoniae* and *E. coli* from neonatal septicaemia, but with increased MIC levels (Roy et al.,

2013). Molecular typing by PFGE shows dissimilar patterns in both *K. pneumoniae* and *E. coli*. This diverse clonality indicates that parallel and horizontal evolution of resistance which due to carbapenem antibiotic pressure.

Analysis of *bla*_{NDM-1} plasmids indicated large plasmids of 90 to 1130 kbs which also carried co-resistance to aminoglycosides and fluoroquinolones. The plasmids harbouring the *bla*_{NDM-1} gene were found to belong to two major incompatibility group IncA/C (73.9%) and IncL/M (26%). This is similar to the report of Kumarasamy et al. (2010) where, *bla*_{NDM-1} isolates from Chennai carried IncA/C type plasmids thus showing that this is the major plasmid type circulating in South India.

Of interest, 2 isolates with indistinguishable PFGE patterns were identified which carried both *bla*_{NDM-1} and *bla*_{VIM-2}. They were extensively drug resistant (XDR) including tigecycline. To this study knowledge, this is the first report on the presence of both *bla*_{NDM-1} and *bla*_{VIM} carbapenemase genes from India. Three strains of *K. pneumoniae* producing *bla*_{VIM-1} and *bla*_{KPC-2} from clinical specimens in Greece were reported (Giakoupi et al., 2009). These findings indicate the continued spread of resistance genes among these pathogens.

In the present study, a significant proportion (44.4%) of carbapenem resistance was found to be due to non-carbapenemase producing *K. pneumoniae* and *E. coli*. A total of 63.6% of these isolates were found to be associated with ESBL production along with porin loss OmpK36.

Earlier studies reported the role of both OmpK35 and OmpK36 in increasing the MICs of carbapenems in *K. pneumoniae*, but in this study, lack of OmpK36 seemed to be the only cause of carbapenem resistance (Nikaido, 1989; Nikaido, 1998). It has been previously reported that clinical isolates of *K. pneumoniae* lacking expression of ESBLs express the two porins OmpK35 and OmpK36, whereas most isolates producing these β -lactamases express only the porin OmpK36, while there is either very low expression of the OmpK35 porin or it is not expressed (Doménech-Sánchez et al., 1999). Thus, the study report confirms the specific role of OmpK36 in *K. pneumoniae* clinical isolates with reduced carbapenem susceptibility which produce OmpK35 but with no OmpK36 expression.

False detection of carbapenemase production was observed by the MHT possibly as a result of ESBL production coupled with porin loss (Carvalhoes et al., 2010). However, the study has not experienced this which may be due to the use of ertapenem which is the most sensitive indicator. Hence, keeping this fact in mind when performing MHT for carbapenem resistant strains, especially in high ESBL evidence settings like this study, the study suggest to use ertapenem as the substrate for MHT which gave 100% specific and sensitive results to that of PCR. In analyzing the role of efflux in carbapenem resistance, 36.5% isolates showed that a putative efflux mechanism may be involved in the resistance.

In the study hospital, meropenem use was initiated in 2006. Amikacin and meropenem were the frequently used antibiotics to treat infections by multidrug resistant bacteria in ICUs and soon within a short period resistance to carbapenems developed, thus rendering the treatment options narrow. Similarly, studies have shown that a shift in empirical therapy to the carbapenems, due to the presence of ESBL producers, is associated with emerging resistance and in the ESBL-producing organisms themselves (Kliebe et al., 1985).

Nearly 18% colistin-resistant strains have emerged in this sample collection. The reason for colistin resistance among the isolates in this tertiary care centre, wherein colistin methane sulfonate (CMS) is not in extensive use is inexplicable. Interestingly, none of the patients studied had received colistin prior to the isolation of the resistant strains.

Conclusions

This study records the emergence and rise of *bla*_{KPC-1}, *bla*_{NDM-1}, *bla*_{VIM}, *bla*_{IMP} genes circulating in Puducherry, South India and also the non-carbapenemase resistance mechanisms. These resistance mechanisms create a major public health problem, compounded by a shortage of newer antibiotic options. Physicians must select antibiotics with the specific needs of an individual patient in mind but also in a manner that does not breed further drug resistance. Selection of an appropriate initial antibiotic regimen for empiric therapy, rotation of different antibiotic classes and judicious use of antibiotics are needed.

Conflict of Interests

The authors have not declared any conflict of interests.

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