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Molecular Mechanisms of Carbapenem Resistance Among Enterobacteriaceae Isolated at Teaching Hospital, Batticaloa

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Abstract

The family Enterobacteriaceae includes a group of Gram negative facultative anaerobes. Members of this family are known to develop resistance to β- lactam antibiotics including carbapenems. Present study was carried out to identify presence of selected genetic determinants of carbapenem resistance in carbapenem non-susceptible Enterobacteriaceae at Teaching Hospital, Batticaloa. Isolates which were resistant or intermediate sensitive to any of carbapenems using routine sensitivity testing (CLSI) were collected from October 2015 to March 2016, sub cultured on blood agar and MacConkey agar and incubated in air at 37 ^{0}C for 18 – 24 hours. PCR amplification of bla_{TEM}, bla_{SHV} and bla_{CTX-M} as potential genetic determinants of *Extended Spectrum Beta Lactamases* (ESBLs) and bla_{OXA-48}, bla_{KPC} and bla_{NDM} as genetic determinants for carbapenemases was performed. Total of 768 Enterobacteriaceae were isolated during study period and of these 5 were confirmed as resistant to carbapenem group i.e. carbapenem-resistant Enterobacteriaceae (CRE). All five isolates were intermediate resistant or resistant to imipenem, meropenem, ertapenem, ceftazidime, cefotaxime, ciprofloxacin, levofloxacin and aztreonam. Four isolates carried at least one potential genetic determinant of bla_{CTX-M} in 3, bla_{TEM} in 3 and bla_{SHV} in 2 isolates. Four isolates were found to carry at least one of genetic determinants of bla_{OXA-48} in 3 and bla_{NDM} in 1.

Keywords: Carbapenemases, Enterobacteriaceae, Carbapenem Resistance, $bla_{OXA-48,}$ PCR Amplification

1. INTRODUCTION

Prevalence of *Extended Spectrum β- Lactamases* (ESBLs) producing enterobacteriaceae and CRE are increasing globally and treatment options for these MDR isolates are very limited. A study done at National hospital of Sri Lanka revealed that prevalence of CRE was 7.9 % and showed a statistically significant relationship (P<0.001) with hospitalization for more than 5 days (Gunaseakara, 2015). Presence of CRE where mechanism of resistance is acquired *Metallo β- Lactamases* (MBL) have been reported from several countries (Iseri, 2008). However MBL producing isolates vary in Asian countries (Walsh, 2002). Further, a little information is available on prevalence of MBL producing enterobacteriaceae isolates in Sri Lanka.

MBLs have broad activity profiles that encompass most β- lactam antibiotics, including carbapenems. Genetic determinants of these enzymes are usually associated with mobile genetic structures which have potential of horizontal transfer (Bebrone, 2013). They spread quickly and cause sudden outbreaks in hospitalized patients due to their gene

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transferring ability within and between species. Therefore, identification of these strains is important to prevent their spread.

2. LITERATURE REVIEW

β- Lactam antibiotics

Discovery of β- Lactam antibiotic penicillin by Alexander Fleming in 1928, and its subsequent large scale use from 1944, started era of antibiotics. β- lactam antibiotics are natural or synthetic derivatives which possess diverse anti-bacterial spectrum including both Gram positive and Gram negative bacteria. β- lactam antibiotics include penicillins, cephalosporins, carbapenems and monobactems (Livermore, 1996). Carbapenems are group of potent and highly effective antibiotics among β- lactams. These are used as a last choice to treat infections that are resistant to cephalosporin. Imipenem, Meropenem, Ertapenem and Doripenem are four members of carbapenem (Bassetti *et al.,* 2009).

Antibiotic resistance

Bacteria exhibited resistance to antibiotics long before humans started to utilize antibiotics for medicinal purposes. Some antibiotics are naturally produced by microorganisms in environment. When bacteria would encounter them, they subsequently develop resistance mechanisms for survival. *Penicillium chrysogenum* is a fungus commonly found in soil and it produces penicillin. This fungus shares habitat with clinically related species including *P. aeruginosa* and *Acinetobacter* sp. These organisms are intrinsically resistant to penicillin by production of β- lactamases encoded by their chromosomes (Jacoby, 2009).

Screening for carbapenemases

Detection of carbapenemases is based on screening by carbapenem susceptibility testing in a clinical isolates by increasing *Minimum Inhibitory Concentration* (MIC) or a decreased in inhibition zone diameter. *Clinical Laboratory Standard Institute* (CLSI) has revised and increased carbapenemases screening zone diameter break points against enterobacteriaceae in 2010. Susceptible breakpoints for imipenem and meropenem were both increased from >16 mm to >23 mm; for ertapenem it was increased from >19 mm to >23 mm. Resistant zone diameter break points were increased from <13 mm to <19 mm for imipenem and meropenem whereas it was increased from <15 mm to <19 mm for ertapenem (CLSI, 2011).

Molecular detection of carbapenemase productions

Molecular identification is standard technique for identification and differentiation of carbapenemase production. Polymerase Chain Reaction (PCR) based techniques followed by sequencing can give precise identification of carbapenemases. PCR can be performed either single or multiplex PCR techniques (Poirel *et al.,* 2011; Avlami, Bekris and Ganteris, 2010; Chen, Mediavalla and Endimiani, 2011). It can give results within 4-6 hours with high sensitivity and specificity. However these methods are difficult to be used in routine diagnosis due to high cost. (Naas, Cuzon and Bogaerts, 2011).

3. METHODS

The isolates analyzed in this study were from Teaching hospital, Batticaloa. Isolates that were resistant or intermediate resistant to any of carbapenem by using CLSI sensitivity testing at Microbiology laboratory of THB were collected within a six month period, from 1st of October 2015 to 30th March 2016 and sub cultured on Nutrient agar; incubated at 37 ^{0}C for 24 hours and stored at 4–8 ^{0}C.

All stored isolates were sub cultured on blood agar (Oxoid, UK) and MacConkey agar (Oxoid, UK) and incubated at 37 ^{0}C for 18–24 hours in air. A second subculture was performed prior to conducting any biochemical tests. Pure cultures of clinical isolates were identified using a set of biochemical tests (Cowan & Steel, 1993). Identification of enterobacteriaceae was confirmed by performing motility test, catalase test, Oxidase test and Oxidative Fermentative (OF) test. Identification of isolates into species level was confirmed by performing motility test, citrate utilization test, urease test, Hydrogen sulfide (H_2S) from Triple sugar iron agar (TSI), Methyl Red test (MR test at 37 ^{0}C), Voges-Proskauer test (VP test at 37 ^{0}C), Indole test and Tested for acid from sugars.

Antimicrobial Susceptibility Testing (ABST)

Susceptibility testing was performed using disc diffusion method on Muller Hinton Agar (MHA) (Oxoid, UK) according to CLSI guidelines (CLSI, 2015). Briefly, an overnight culture in blood agar was suspended into normal saline to a turbidity of 0.5 MacFarland standards and inoculated on MHA plates. Inoculum was spread over entire surface of MHA plate. A selected set of antibiotic discs (Mast diagnostics, UK) were applied to surface of agar within 15 minutes of inoculation.

Antimicrobial agents were chosen from carbapenems, cephems (Parenteral), monobactems, aminoglycosides, fluoroquinolones and folate pathway inhibitors. Susceptibility was tested against imipenem 10 µg, meropenem 10 µg, ertapenem 10 µg,

ceftazidime 30 µg, cefotaxime 30 µg, ciprofloxacin 5 µg, levofloxacin 5 µg, netilmicin 30 µg, aztreonam 30 µg, trimethoprim sulfamethaxazole 10/30 µg. Sensitivity zone diameters were interpreted according to CLSI, *Performance standard for antimicrobial susceptibility testing*. 26th ed. CLSI supplement M100S, 2015 (CLSI, 2015).

Phenotypic identification of resistance mechanisms

Phenotypic identification methods were done to identify β- lactamase producers. Isolates were screened for ESBL and carbapenamase production according to CLSI guidelines. In brief, MHA plates were inoculated according to standard disc diffusion methods. Cefotaxime 30 µg and Ceftazidime 30 µg were placed and incubated for 16–18 hours at 35 ^{0}C in ambient air. Inhibition zone less than 27 mm for Cefotaxime and 22 mm for Ceftazidime are criteria for performance of ESBL confirmation test. Carbapenemases producing enterobacteriaceae were screened as intermediate or resistant to one or more carbapenems and resistant to one or more third generation cephalosporins using current interpretative criteria (CLSI, 2015).

PCR

PCR was carried out for detection of selected genes on a Swift MaxPro® thermal cycler (ESCO healthcare, Singapore) by a multiplex PCR. PCR amplification of bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ for ESBL (Monstein *et al.,* 2007) and $bla_{\text{OXA-48}}$, bla_{KPC} and bla_{NDM} for carbapenemases (Poirel *et al.*, 2011) were performed as described previously.

4. RESULTS

Bacterial isolates

The total numbers of specimens received for processing at Microbiology laboratory during this period ranging from 1st Ocober 2015 to 30th March 2016 were 3071 blood cultures and 2671 urine cultures. Out of these, 768 (13 %) isolates were enterobacteriaceae. Of these, five isolates (0.65 %) were non susceptible to carbapenems. Out of this five, four isolates were collected from urine sample and one from blood cultures (Figure 1). Four out of five isolates were collected from inward patients and one from outpatient department.

Identification of Gram negative isolates

Among five carbapenem resistant isolates two were identified as *Klebsiella pneumoniae* subspecies aeruginosa (40 %), one was *Escherichia coli* (20 %), one was *Enterobacter cloacae* (20 %) and one was *Enterobacter sakazakii* (20 %).

Antibiotic susceptibility testing

All isolates were intermediate resistant or resistant to imipenem, meropenem, ertapenem, ceftazidime, cefotaxime, ciprofloxacin, levofloxacin and aztreonam by disc diffusion tests. Isolate No. 27, *Escherichia coli* showed intermediate resistant to imipenem and meropenem. Two isolates, *Escherichia coli* and *Enterobacter cloacae* were susceptible to Netilmicin. Only one isolate was susceptible to Trimethoprim sulfamethaxazole. In addition, all of these isolates were found to be MDR, conferring resistant to more than three classes of antibiotics (Magiorakos *et al*., 2012).

Table 1. Sensitivity pattern of isolates

Antibiotic disc	Isolate No. 27 *E.coli*		Isolate No. 147 *E.cloacae*		Isolate No. 469 *K.pneumoniae*		Isolate No. 558 *K.pneumoniae*		Isolate No. 574 *E.sakazakii*	
Imipenem 10µg	19mm	IR	17mm	R	14mm	R	13mm	R	14mm	R
Meropenem 10µg	19mm	IR	16mm	R	9mm	R	11mm	R	12mm	R
Ertapenem 10µg	12mm	R	14mm	R	6mm	R	7mm	R	7mm	R
Cefotaxime 30µg	6mm	R	6mm	R	6mm	R	6mm	R	6mm	R
Ceftazidime 30µg	6mm	R	12mm	R	6mm	R	6mm	R	6mm	R
Ciprofloxacin 5µg	6mm	R	14mm	R	6mm	R	6mm	R	6mm	R
Levofloxacin 5µg	6mm	R	19mm	R	6mm	R	6mm	R	6mm	R
Aztreonam 30µg	6mm	R	10mm	R	6mm	R	6mm	R	8mm	R
Cotrimaxazole 25µg	6mm	R	6mm	R	21mm	S	6mm	R	6mm	R
Netilmicin 30µg	12mm	S	12mm	S	6mm	R	7mm	R	11mm	R

Phenotypic identification of β- Lactamase and Carbapenemase productions

All isolates fulfilled screening criteria for ESBL production and showed increase of more than 5 mm in zone diameter for either antimicrobial agent tested in combination with clavulanic acid compared to zone diameter of Cefotaxime and Ceftazidime alone. Therefore, all isolates were confirmed to be ESBL producers. All five isolates were found to be positive for carbapenemases production in screening test and MHA test.

Phenotypic identification of MBL production

Overall, EDTA presented with weak bactericidal activity, they showed lowest level of inhibition of bacterial growth (No zone or less than 1 mm). *Escherichia coli* (27) showed enhancement of phantom zone in EDTA/DDST with imipenem. Other four isolates did not show any enhancement of zone in EDTA/DDST with imipenem. *Escherichia coli* showed

better phantom zone in 20 mm and 15 mm distance between imipenem and EDTA disc. It also showed highest degree of phantom zone in imipenem EDTA synergy test using 300 mM EDTA with 20 mm distance between imipenem disc and EDTA disc. Two isolates, *Escherichia coli* (27) & *Klebsiella pneumoniae* (558) showed enhancement of zone in EDTA/DDST with meropenem. *Escherichia coli* showed better enhancement of zone between 15 mm–20 mm. *Klebsiella pneumoniae* showed better phantom zone in 500 mM & 300 mM EDTA concentrations.

Detection of genetic determinants of resistance by PCR

The molecular analysis detected that four out of five isolates were positive for genetic determinants of ESBLs producers. Two strains harboured bla_{SHV} genes, three harboured $bla_{CTX\text{-}M}$ and bla_{TEM} genes. PCR detection identified genetic determinants of carbapenamases in four out of five clinical isolates. Among isolates, three strains harboured bla_{OXA} gene and one isolate harboured bla_{NDM} gene. bla_{KPC} gene was not identified in any of isolates.

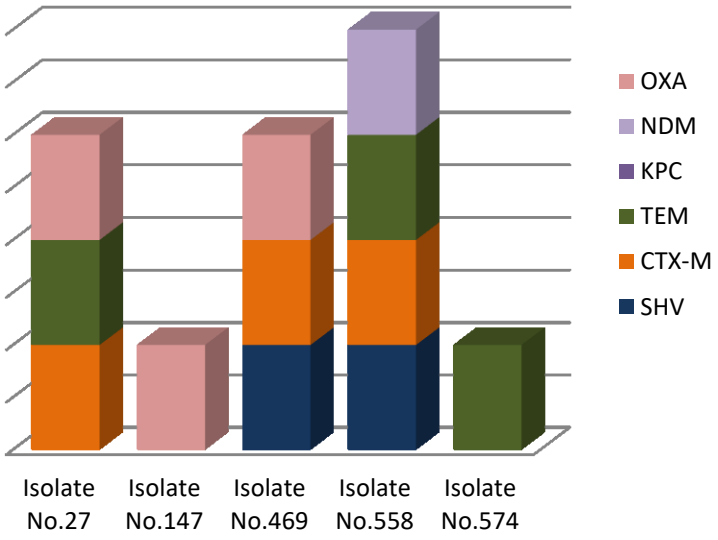

Figure 1. Distribution of combinations of genetic determinants of ESBL and carbapanamase genes among 5 isolates.

Prevalence

Total of 768 enterobacteriaceae were isolated in THB during study period and out of these 5 were confirmed CRE. Overall frequency of CRE was 0.65 %. CTX–M, TEM and OXA were found in 3 out of 5 isolates whereas 2 isolates had SHV and one had NDM. KPC was not found in any of tested isolates.

5. DISCUSSION

Even though CRE has been reported from Sri Lanka, molecular approaches for detection of *bla* genes are still limited. We only have a few reports characterizing carbapenem resistance genes at a molecular level in Sri Lanka. There are several reported outbreaks with carbapenem resistant Gram negative organisms recent past in General Hospitals of Sri Lanka (Jayatilleka, 2014).

We isolated five CRE isolates (0.65 %) during period of six months. This is comparable with previous studies. In Saudi Arabia, they collected 10 isolates of CRE (0.74 %) during periods of 15 months (Amina, 2015). On the other hand this prevalence is lower than data presented in India where they found 57 CRE out of 465 (12.26 %) enterobacteriaceae during period of 12 months from a tertiary care hospital (Pravin and Michelle, 2013). In one study from a tertiary care hospital in North-East India reported 29 (30 %) carbapenemase producing Gram negative isolates were collected using disc diffusion test during periods of 24 months (Henkhoneng Mate *et al*., 2014).

A very recent study in Sri Lanka isolated 22 carbapenem resistant *Klebsiella pneumoniae* from accident and trauma wards of National Hospital of Sri Lanka which is largest teaching hospital in Sri Lanka ((Jarrad *et al*., 2014). This study was conducted for period of 4 months. High number of CREs isolated in this study compared to our study is probably due to fact that study was done at national tertiary care hospital which receives patients all over from Sri Lanka.

Carbapenemase productions are emerging cause of carbapenem resistance among Gram negative bacteria. In our study class B and class D carbapenemases were identified. Four out of five phenotypically confirmed CRE harboured carbapenemase genes. In one isolate, *Enterobacter sakazakii* carbapenemase genes tested were negative. However, it can be explained by other mechanism of carbapenem resistance in this isolate for example porin loss.

The study has demonstrated that OXA and NDM are two types of genes seen among CRE isolates in THB. This data is comparable to a recent study done in western province in Sri Lanka in which they found similar presence of carbapenem resistant genes (OXA-181, NDM-1) among carbapenem resistant *Klebsiella pneumoniae.* A high prevalence of OXA gene (3 out of 5 isolates) was detected among CRE in THB. High prevalence of OXA genes in our study may also be explained by fact that travel between India and Eastern part of Sri Lanka is increasing specially for medical purposes.

The results of this study show association of other resistance determinants. The bla_{OXA} is commonly associated with $bla_{CTX\text{-}M}$ allele in present study. Combinations of different genes in single strains were observed. In present study NDM gene was associated with cephalosporin resistant genes such as SHV, CTX-M and TEM. Capability of NDM-1 to associate with other resistance genes raises serious concerns.

6. CONCLUSION

Most of CRE were from Urine culture specimens. MDR carbapenemase producing enterobacteriaceae are present at Teaching hospital, Batticaloa. Presence of OXA and NDM genes indicate MBL producing MDR enterobacteriaceae are present in Eastern region. One out of five isolates was from OPD urine sample which was positive for OXA gene. This is an alarming fact that carbapenemase producing isolates are present in community.

7. LIMITATION

Major limitation of study was low numbers of samples were studied. Study period couldn't be extended due to restricted allocation of time. We only considered clinically significant isolates and this likely underestimates burden of colonized patients, which may effectively spread CRE through regions.

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