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**GENOTYPIC CHARACTERISATION OF CARBAPENEM RESISTANT
ESCHERICHIA COLI ISOLATES FROM BLOOD STREAM INFECTION
AND FALLACIES ASSOCIATED WITH MODIFIED HODGE TEST TO
DETECT PRODUCTION OF CARBAPENEMASES**

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ABSTRACT

The infections caused by *Enterobacteriaceae* are community and hospital-acquired. This pilot study was done to characterise *Escherichia coli* isolates from blood stream infection and identify the genes responsible for carbapenemase production and correlate the results with MHT. Twenty consecutive isolates of *E.coli* resistant to imipenem (10 µg)/meropenem (10 µg) isolated from individuals with blood stream infection were selected for testing. Antimicrobial susceptibility results of these isolates were interpreted according to CLSI breakpoints. These isolates were retrieved for further characterisation with MHT and molecular detection of various genes. Among a total of 20 isolates, all were resistant to both imipenem and meropenem by disk diffusion screening test. The rates of resistance for other antimicrobials were 100% for cefpodoxime, cefaperazone/sulbactam, piperacillin/tazobactam, 90% for gentamicin; and 50% for amikacin and netilmicin. However, all the isolates were susceptible to colistin and tigecycline. Among the 20 isolates tested, 7 were positive with MHT. In conclusion accurate and timely identification of genes conferring resistance to carbapenems serves as an important tool in instituting therapy and infection control measures. Conventional multiplex PCR is a sensitive technique to detect multiple genes.

INTRODUCTION

Gram negative enteric bacilli are a leading cause for sepsis in children and adults (Viswanathan *et al.*, 2012; Kang *et al.*, 2005; and Adams-Sapper *et al.*, 2012). The infections caused by *Enterobacteriaceae* are community and hospital-acquired. These bacteria have the potential to spread in the hospital environment and also across continents (Nordmann *et al.*, 2011). Irrational use of antibiotics coupled with rapid emergence of drug resistance in bacteria is a major problem faced by health care setting around the world (Zhang *et al.*, 2011). The production of beta lactamase is the most common mechanism of resistance to drugs especially in gram negative bacteria. Carbapenemase is one of the beta lactamases conferring resistance to carbapenem group of drugs making it difficult to treat since the treatment options are lesser. Detection of mechanisms of resistance to carbapenems plays an important role in understanding the epidemiology and spread of resistance. This further helps to develop policies for antimicrobial therapy and surveillance (Cohen Stuart and Hall, 2010).

This pilot study was done to characterise *Escherichia coli* isolates from blood stream infection and identify the genes responsible for carbapenemase production and correlate the results with MHT. Multiplex PCR was performed to detect various genes responsible for carbapenemase production that are NDM, KPC, SPM, VIM, OXA-48, IMP and GES and compared with the MHT results.

MATERIALS AND METHODS

Twenty consecutive isolates of *E.coli* resistant to imipenem (10 µg)/meropenem (10 µg) isolated from individuals with blood stream infection were selected for testing. Antimicrobial susceptibility results of

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these isolates were interpreted according to CLSI breakpoints (Clinical and Laboratory Standards Institute, 2013). These isolates were retrieved for further characterisation with MHT and molecular detection of various genes.

Modified Hodge test (MHT) was performed on all the isolates. A 0.5 Mc Farland broth of *E. coli* ATCC 25922 in saline was prepared, diluted 1 in 10 and inoculated on Mueller Hinton Agar plate as lawn culture. Ertapenem disk (10µg) was placed in the centre of the plate. Three to five colonies of the test strain and positive (known strain of *Klebsiella pneumoniae* producing KPC) control strains were streaked perpendicular to the disk starting from the edge of the disk to the periphery. The plate was incubated at 37°C for 16-20 hours. An enhanced growth of lawn culture around the streak at the intersection of the streak and zone of inhibition (clover leaf pattern) was considered a positive test (Clinical and Laboratory Standards Institute, 2013).

Conventional multiplex PCR was performed on all the 20 isolates irrespective of the results of the MHT. Separate clean rooms were used extraction of DNA and PCR reaction and the amplicons were visualised on agarose gel in a third room to avoid contamination. Positive control strains for PCR were obtained. (Courtesy IMHA.inc, Chicago). PCR grade water was used as the negative control. Controls were included with every run of the PCR. The test isolates and control strains were grown on blood agar plates and identification of colonies confirmed with preliminary screening media and biochemical reactions. Five to six similar looking colonies were selected and inoculated into 2ml of sterile saline. The tubes were vortexed, placed on a heating block at 100°C for 10 minutes and centrifuged. The extracted DNA was used for the PCR reactions.

Multiplex PCR was performed in two separate reactions; reaction 1 (SPM, KPC, OXA-48 and VIM) and reaction 2 (NDM, GES and IMP). Primers were obtained from (Sigma Aldrich, Bengaluru). The protocols used were according to those in the references shown in the tables. Amplification was done in VERITI 96 well Thermal cycler (Applied Biosystems, CA, USA) using the following conditions; 1 cycle at 95°C for 15 minutes, 30 cycles of denaturation, annealing and extension at 94°C for 30 seconds, 59°C for 90 seconds and 72°C for 90 seconds and a final extension of 1 cycle at 72°C for 10 minutes. Amplicons were visualized in a 2% agarose gel containing ethidium bromide under UV light using the Gel Doc XR (Bio-Rad, Hercules, CA, USA). The bands obtained for individual isolates were compared with the bands obtained for the control strains.

RESULTS

Among a total of 20 isolates, all were resistant to both imipenem and meropenem by disk diffusion screening test. The rates of resistance for other antimicrobials were as follows; 100% for cefpodoxime, cefaperazone/sulbactam, piperacillin/tazobactam, 90% for gentamicin, 50% for amikacin and netilmicin. However, all the isolates were susceptible to colistin and tigecycline.

Among the 20 isolates tested, 7 were positive with MHT. Among these, 4 of them harboured the genes for carbapenemase production as detected by PCR. This included 2 NDM, 1 IMP and 1 OXA-48 genes. Four of the isolates which tested negative with MHT were found to be positive for the NDM gene by PCR thus giving a false negative result with MHT. However, a total of 3 isolates were negative for the genes mentioned above thus giving a false positive result with MHT. Thirteen isolates gave a negative result with MHT among which 9 of them did not have any of the carbapenemase genes by PCR. A total of 8 out of 20 isolates gave a positive result with PCR for the genes tested. The most common gene conferring resistance to carbapenems for *E. coli* in the study was NDM (6 out of 20 isolates). We found one isolate each positive for IMP and OXA-48 genes.

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Table 1: Results obtained in the study for carbapenem resistant isolates by MHT and molecular characterisation for various genes using multiplex PCR and the possible mechanisms of resistance

	No. of samples positive with MHT	No. of samples negative for MHT	Remarks	References
No. of samples positive for NDM gene	2	4	False negative MHT could be due to low sensitivity in detecting carbapenemase producers harbouring the NDM gene (11%)	(Clinical and Laboratory Standards Institute, 2013)
No. of samples positive for IMP gene	1	-	The test has better sensitivity for OXA-48 and KPC producing organisms. In this study we found that MHT was positive for two isolates which were positive for OXA-48 and IMP genes.	(Girlich, 2012)
No. of samples positive for OXA-48 gene	1	-		
No. of samples negative for PCR	3	-	False positive MHT could be due to excess production of ESBL's by organisms which tends to give a resistant zone when tested with carbapenems since these enzymes have a weak activity against carbapenems	(Cohen Stuart and Hall, 2010) (Carvalhaes, 2010)
No. of samples negative for PCR	-	9	Resistance to carbapenems by disk diffusion but negative with both PCR and MHT could be due to porin loss (OMP defects which do not allow the antibiotic to enter the cell); efflux pumps which leads to a reduced concentration of the antimicrobial agent within the bacterial cell; production of Amp C beta lactamase production (which was not tested for in the current study)	(Nordmann, 2012)
Total	7	13		

DISCUSSION

Emergence of drug resistant Enterobacteriaceae continues to be a major problem leading to mortality worldwide (Nordmann *et al.*, 2011). Hospital acquired infections mainly blood stream infection caused by carbapenem resistant organisms is a major challenge since treatment options are minimal and clinical failure is rampant in such cases (Viswanathan *et al.*, 2012; Kang *et al.*, 2005; and Nordmann *et al.*, 2011). Identification of carbapenem resistance *in-vitro* thus has a very important role in guiding treatment options.

Carbapenemases are a versatile group of β -lactamases that are characterised by their resistance to virtually all β -lactam antibiotics, including the cephalosporins and carbapenems. These resistant isolates are co-resistant to other classes of drugs like fluoroquinolones, aminoglycosides and co-trimoxazole. There are three important classes of carbapenemases Ambler Class A serine beta lactamases (KPC, SME, NMC-A,

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IMI, PER, GES, SFO, SFC, IBC), Class B metallo beta lactamases (VIM, GIM, SIM, NDM, IMP, SPM) and Class C oxacillinases (OXA, PSE) (Cohen Stuart and Hall, 2010).

Any isolate giving a resistant zone size with the screening test which is the disk diffusion test with imipenem/meropenem (10µg) should be subjected to a genotypic or phenotypic confirmatory test (Cohen Stuart and Hall, 2010). These tests are important since there are various mechanisms conferring resistance to carbapenems.

Phenotypic tests detect the various classes of carbapenemases and include the following. Modified Hodge Test detects the presence of all carbapenemases. But there are fallacies associated with MHT in that it is less sensitive for detection of isolates harbouring the NDM gene (Clinical and Laboratory Standards Institute, 2013). Better assays have been developed known as inhibitor based disk diffusion assays for various resistance mechanisms such as metallo beta lactamases, KPC and Amp C beta lactamases. All metallo beta-lactamases are inhibited by dipicolinic acid, KPC is inhibited by boronic acid but not by cloxacillin and Amp C is inhibited by both boronic acid and cloxacillin (Neo-Sensitabs, Rosco Diagnostica, Denmark). Not all resistant mechanisms can be detected by phenotypic tests.

Conventional multiplex PCR is a rapid and sensitive method for the same (Monteiro, 2012). A multiplex format carries the advantage of giving an accurate result of detecting multiple genes at the same time (Dallenne *et al.*, 2010). Molecular assays are more expensive than inhibitor based disk diffusion tests but are more sensitive.

Molecular characterisation is necessary to detect the genes responsible for conferring resistance in particular geographic areas. NDM is the most prevalent in the Indian subcontinent (Nordmann *et al.*, 2012). KPC is spread worldwide (Nordmann *et al.*, 2012).

Hence in the current study we characterized the genes responsible for conferring resistance to carbapenems in *E.coli* isolates. We compared the results with MHT and found several discrepancies.

NDM was the common gene conferring resistance. However, we found that among a total of 6 isolates positive for the NDM gene, 4 of them gave a false negative result with MHT. The reason for this is a well known fact that MHT has a low sensitivity of 11% in detecting carbapenemase producers harbouring the NDM gene while it performs better for OXA-48 and KPC producing organisms (Clinical and Laboratory Standards Institute, 2013 and Girlich *et al.*, 2012). In this study we found that MHT was positive for the isolates which were positive for OXA-48 and IMP genes thus strengthening the fact that MHT performs poorly with organisms harbouring the NDM gene.

Among the 20 isolates, 3 gave a false positive result with MHT. The reasons could be excess production of ESBL's. These false positive results can be expected in our set up where the prevalence of ESBL's is rampant especially with the organism *E. coli*. Since these enzymes have a weak activity against carbapenems, they tend to give a resistant zone when tested with carbapenems (Cohen Stuart, Leverstein-Van Hall, 2010; and Carvalhaes *et al.*, 2010).

We found that a majority of isolates (9 out of 20) were negative with PCR and MHT though they had a resistant zone with carbapenems. The reasons for this could be other resistant mechanisms such as porin loss (OMP defects) and efflux pumps which reduce the concentration of antimicrobials within the bacterial cell. One more reason could be production of Amp C beta lactamase production (which was not tested for in the current study).

In conclusion accurate and timely identification of genes conferring resistance to carbapenems serves as an important tool in instituting therapy and infection control measures. Conventional multiplex PCR is a sensitive technique to detect multiple genes (Monteiro *et al.*, 2012). Phenotypic tests can serve as an inexpensive alternative when molecular techniques cannot be used.

Source of Support

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On behalf of all the contributors I (AS) herewith declare that all the authors have substantially contributed in the preparation of this manuscript and are not having any conflict of interest.

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REFERENCES

- Adams-Sapper S, Sergeevna-Selezneva J, Tartof S, Raphael E, Diep BA, Perdreau-Remington F, et al., (2012).** Globally dispersed mobile drug-resistance genes in gram-negative bacterial isolates from patients with bloodstream infections in a US urban general hospital *Journal of Medical Microbiology* **61**(7) 968–74.
- Carvalhoes CG, Picão RC, Nicoletti AG, Xavier DE, Gales AC (2010).** Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results *Journal of Antimicrobial Chemotherapy* **65**(2) 249–51.
- Clinical and Laboratory Standards Institute (2013).** Performance Standards for Antimicrobial Susceptibility Testing; *Twenty Third Informational Supplement*. M100-S23 ed. Wayne, PA, USA
- Cohen Stuart J and Leverstein-Van Hall MA (2010).** Guideline for phenotypic screening and confirmation of carbapenemases in Enterobacteriaceae. *International Journal of Antimicrobial Agents* **36**(3) 205–10.
- Dallenne C, Da Costa A, Decré D, Favier C, Arlet G (2010).** Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy* **65**(3) 490–5.
- Ellington MJ, Kistler J, Livermore DM, Woodford N (2007).** Multiplex PCR for rapid detection of genes encoding acquired metallo-beta-lactamases. *Journal of Antimicrobial Chemotherapy* **59**(2) 321–2.
- Girlich D, Poirel L, Nordmann P (2012).** Value of the Modified Hodge Test for Detection of Emerging Carbapenemases in Enterobacteriaceae. *Journal Clinical Microbiology* Feb;50(2):477–9.
- Kang C-I, Kim S-H, Park WB, Lee K-D, Kim H-B, Kim E-C, et al., (2005).** Bloodstream infections caused by antibiotic-resistant gram-negative bacilli: risk factors for mortality and impact of inappropriate initial antimicrobial therapy on outcome *Antimicrobial Agents and Chemotherapy* **49**(2) 760–6.
- Monteiro J, Widen RH, Pignatari ACC, Kubasek C, Silbert S (2012).** Rapid detection of carbapenemase genes by multiplex real-time PCR *Journal of Antimicrobial Chemotherapy*. 2012 Jan 9;dkr563.
- Nordmann P, Dortet L and Poirel L (2012).** Carbapenem resistance in Enterobacteriaceae: here is the storm! *Trends in Molecular Medicine* **18**(5) 263–72.
- Nordmann P, Naas T, Poirel L (2011).** Global spread of Carbapenemase-producing Enterobacteriaceae. *Emerging Infectious Diseases* **17**(10) 1791–8.
- Poirel L, Dortet L, Bernabeu S, Nordmann P (2011).** Genetic features of blaNDM-1-positive Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy* **55**(11) 5403–7.
- Queenan AM, Bush K (2007).** Carbapenemases: the versatile beta-lactamases. *Clinical Microbiology Review* **20**(3) 440–458.
- Viswanathan R, Singh AK, Basu S, Chatterjee S, Sardar S, Isaacs D (2012).** Multi-drug resistant gram negative bacilli causing early neonatal sepsis in India. *Archives of Disease in Childhood. Fetal and Neonatal Edition* **97**(3) F182–187.
- Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW and Steward CD et al., (2001).** Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **45**(4) 1151–61.
- Zhang R, Cai J-C, Zhou H-W, Nasu M, Chen G-X (2011).** Genotypic characterization and in vitro activities of tigecycline and polymyxin B for members of the Enterobacteriaceae with decreased susceptibility to carbapenems *Journal of Medical Microbiology* **60**(12) 1813–9.

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ANNEXURE:

Table 1: Primer sequences of the genes detected using multiplex PCR reaction 1

Primer	Primer sequence	PCR product size (bp)	Reference
SPM-F	AAA ATC TGG GTA CGC AAA CG	271	Ellington <i>et al.</i> , 2007
SPM-R	ACA TTA TCC GCT GGA ACA GG		
KPCy-F	TGT CAC TGT ATC GCC GTC	1011	Yigit <i>et al.</i> , 2001
KPCy-R	CGG GTT GGA CTC AAG ACG		
OXA-48A	TATATTGCATTAAGCAAGGG	800	Poirel <i>et al.</i> , 2011
OXA-48B	CACACAAATACGCGCTAACC		
VIM-F	GATGGTGTGTTGGTCGCATA	390	Dallenne <i>et al.</i> , 2010
VIM-R	CGAATGCGCAGCACCAG		

Table 2: Primer sequences of the genes detected using multiplex PCR reaction 2

Primer name	Primer sequence	PCR product size (bp)	Reference
IMP2-F	GGAATAGAGTGGCTTAAYTCTC	232	Poirel <i>et al.</i> , 2011
IMP2-R	CCAAACYACTASGTTATCT		
NDM-F	GGT TTG GCG ATC TGG TTT TC	984	Poirel <i>et al.</i> , 2011
NDM-R	CGG AAT GGC TCA TCA CGA TC		
GES-F	AGTCGGCTAGACCGGAAAG	399	Dallenne <i>et al.</i> , 2010
GES-R	TTTGTCCGTGCTCAGGAT		