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PCR-dipstick DNA Chromatography for detection of Carbapenemase Producing Carbapenem-Resistant Enterobacteriaceae

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Abstract

Carbapenemase producing carbapenem-resistant Enterobacteriaceae (CP-CRE) are an epidemiologically important emerging public health threat. Early and reliable detection of CP-CRE is important for containment of these pathogens. The aim of this study was to evaluate and observe the sensitivity of polymerase chain reaction (PCR)-dipstick DNA chromatography (GeneFields CPE) for detection of carbapenemase genes (NDM, OXA-48, KPC, IMP) in comparison to conventional PCR. In this cross-sectional study, a total of 145 carbapenem resistant Enterobacteriaceae isolates were subjected to test for carbapenemase by phenotypic mCIM and genotypic- conventional PCR and PCR-dipstick DNA Chromatography method. In PCR dipstick DNA chromatography assay, after multiplex PCR a DNA-DNA hybridization-based detection system is applied where amplified products can be easily interpreted by observing a blue line in DNA strip visually within 15 minutes without gel electrophoresis. All the CP-CRE isolates (100%) were positive for either NDM or OXA-48 by PCR-dipstick DNA Chromatography assay, whereas 142 (97.9%) isolates were detected by conventional PCR. The positivity rate of NDM, OXA-48 and both NDM and OXA-48 among CP-CRE isolates were 73.8%, 51.7% and 25.5% respectively. The sensitivity, specificity of PCR-dipstick DNA Chromatography assay for detection of NDM and OXA-48 were 100.0%, 92.7% and 98.6%, 97.2% respectively. This assay showed excellent agreement with conventional PCR for detection of CP-CRE. PCR-dipstick DNA chromatography may be an excellent and robust diagnostic tool in terms of rapidity and sensitivity for the detection of CP-CRE.

Keywords: PCR-dipstick DNA Chromatography; carbapenemase; NDM; OXA-48; Carbapenemase producing carbapenem-resistant Enterobacteriaceae.

1. Introduction

Carbapenems are used as the last treatment options to treat the infections caused by multidrug-resistant bacteria specially ESBL-producing Enterobacteriaceae, whose use as empiric regimens resulted in the emergence of Carbapenem-resistant Enterobacteriaceae (CRE). Infections caused by CRE are associated with high morbidity and mortality due to limited therapeutic options [1,2,3]. The main two mechanisms of carbapenem resistance in Enterobacteriaceae include (i) production of carbapenem-hydrolyzing enzymes (i.e. serine carbapenemase and /or metallo-β-lactamase) that are capable of degrading carbapenems due to acquisition of carbapenemase genes (carbapenemase producing CRE [CP-CRE]) and (ii) over expression of β-lactamases(ESBL, AmpC) combined with

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alteration in membrane permeability due to loss of porin channels or up-regulation of efflux pumps (non-CP-CRE) [3,4,5]. The most prevalent carbapenemases in terms of geographical spread are – Class A (KPC) and Class D (OXA-48) serine-carbapenemases and Class B (NDM, IMP, VIM) metallo- β-lactamases [2,3]. The virulent nature of infections, greater degree of antimicrobial resistance, increased healthcare cost and higher mortality rates (40%–50%) due to CP-CRE infections are now become a great threat to public health in comparison to non CP-CRE infections [4,6,7].

Reliable detection of CP-CRE is important to prioritize newer antibiotics for the treatment of these difficult-to-treat infections and for implementation of infection prevention and control strategies to minimize the spread of these bacteria both in the community and in healthcare facilities [4,6]. Among different phenotypic methods for detection of carbapenemase, mCIM has shown an excellent sensitivity and specificity for the detection of CPE isolates but does not provide enough information regarding the specific carbapenemase genes which can be detected by molecular methods [1,4,8]. In this study, a new molecular method, PCR-dipstick DNA Chromatography was evaluated considering conventional PCR as gold standard for detection of carbapenemase genes along with phenotypic confirmation of carbapenemase by mCIM method. In PCR- dipstick DNA Chromatography method, DNA-DNA hybridization of amplified products of multiplex PCR to their probe in a DNA strip occurs without denaturation and interpretation of results by visual observation can be done within 15 minutes without gel electrophoresis [9].

2. Material and Methods

2.1. Bacterial isolates

A total of 145 nonduplicated clinical strains of Enterobacteriaceae isolated from Microbiology laboratory of Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh between October 2018 to May 2019, which showed resistance to at least one of the tested carbapenems (meropenem, imipenem and ertapenem) according to the disk diffusion break point criteria of CLSI M100- Ed-29 guideline were studied [10]. Enterobacteriaceae were isolated from different clinical specimens such as blood, wound swab, tracheal aspirate, urine, pus, sputum, throat swab, bile and drain fluid.

2.2. The phenotypic Modified Carbapenem Inactivation method (mCIM)

The mCIM was performed and interpreted following CLSI M100-Ed29 guideline [10].

2.3. The PCR-dipstick DNA Chromatography (Gene Fields CPE, KURABO, Japan)

The bacterial DNA was extracted by following previously described boiling method of DNA extraction [11] and was stored at -20ºC for further PCR analysis. GeneFields CPE kit was applied for detection of carbapenemase genes according to the manufacturer's instructions. GeneFields CPE kit contains PCR Oligomix (containing primers for KPC type, NDM type, OXA-48, IMP type carbapenemase genes) for multiplex PCR and coloring buffer, DNA strip and judgement card for documentation of amplified products by DNA Chromatography. At first, multiplex PCR was done and to make a final reaction volume of 30µl for PCR, 2 µl of undiluted extracted DNA from each separate sample was added to the 0.2 ml PCR tube containing a 28µl of reaction mixture comprised of 15µl of master mix (HELINI, India), 0.2µl of taq polymerase (Fire pol, Solis BioDyne, Germany), 5µl PCR Oligomix and 7.8 µl of nuclease free water. PCR reaction in the thermal cycler was started within 10 minutes of preparation of the reaction solution and run at 98ºC for 3 minutes, then for 35 cycles of 98ºC for 30 seconds, 62ºC for 30 seconds and 72 ºC for 30 seconds, followed by a final step at 72 ºC for 7 minutes. For documentation of carbapenemase genes amplified in multiplex PCR by DNA Chromatography, 5 µl of the amplified product was mixed with 11 µl of coloring buffer containing blue avidin beads and 5 µl of sterilized nuclease-free water to make a final volume 21 µl in a 1.5-ml of microcentrifuge tube. The DNA strip was immersed in the mixture upto the bottom of 1.5 ml tube and incubated in room temperature for 15 minutes (figure 1). For amplification of any of four carbapenemase genes, biotin on the 5´ end of reverse primer of PCR amplicon was bound to avidin beads in coloring buffer. The reaction mixture was carried upward through capillary action during which the tag-linker sequence on the 5´ end of each forward primer of the PCR product was hybridized to its respective probe in DNA strip and formed a blue line within 15 minutes. The result was interpreted within 15 minutes by comparing the blue line in the DNA strip with the judgment card provided by the company.

2.4. Conventional PCR

Separate conventional PCR was done by using primers [12,13,14,15] targeting 813bp [12] ,743bp [13], 887bp [14] and 188 bp [15] for NDM, OXA-48, KPC and IMP carbapenemase genes respectively.

2.5. Statistical Analysis

All the data were analyzed using IBM SPSS statistics processor (Version-23). Descriptive analysis of all relevant variables was done by using frequency and percentage. Sensitivity, specificity and diagnostic accuracy of PCR-dipstick DNA chromatography (GeneFields CPE) assay were calculated in comparison to conventional PCR as gold standard. Cohen's kappa coefficient was calculated to check the agreement between PCR-dipstick DNA chromatography (GeneFields CPE) and conventional PCR for detection of carbapenemase genes.

3. Results

3.1. Characteristics of CP-CRE isolates

Out of 145 CP-CRE isolates, 76.6 % (111) were *Klebsiella* spp, 15.8% (23) were *E. coli* and 7.6% (11) were *Enterobacter* spp. Hundred percent (145) of CP-CRE isolates were detected by PCR dipstick DNA chromatography (GeneFields CPE) assay followed by 99.3% (144) by mCIM and 97.9% (142) by conventional PCR. Indeterminate result of mCIM (0.7%) was observed only in one CP-CRE isolate which was positive for carbapenemase gene by both conventional PCR and PCR-dipstick method.

Out of 107 NDM carrying CP-CRE isolates, 97.2 % (104) were detected by conventional PCR and 100% (107) were detected by PCR-dipstick method. In case of 75 OXA-48 positive isolates, 97.3% (73) were detected by conventional PCR and 98.6% (74) were detected by PCR-dipstick method. Interestingly, thirty seven CP-CRE isolates carried both NDM and OXA-48 genes of which 94.6% (35) were detected by conventional PCR and 97.3% (36) were detected by PCR-dipstick method. No KPC and IMP genes were detected among CP- CRE isolates by any of these two methods (Table 1).

Table 1 Detection of carbapenemase gene by conventional PCR and PCR-dipstick DNA chromatography among CP-CRE isolates

Carbapenemase encoding gene	Methods	CP-CRE isolates n (%)
NDM (n = 107)	Conventional PCR	104 (97.2)
	PCR-dipstick	107 (100.0)
OXA-48 (n = 75)	Conventional PCR	73 (97.3)
	PCR-dipstick	74 (98.6)
Both NDM, OXA-48 (n = 37)	Conventional PCR	35 (94.6)
	PCR-dipstick	36 (97.3)

Note: No KPC and IMP genes were detected among any species of CP-CRE isolates

Among NDM positive CP-CRE isolates 67.3% (72) were *K. pneumoniae*, 21.5% (23) *E. coli*, 4.7% *E. aerogenes*, 4.7% *E. cloacae* and 1.7% were *K. oxytoca*. On the other hand, 89.3% (67) of OXA-48 positive isolates were *K. pneumoniae*, 5.3% (4) *E. coli* and 5.3% (4) were *E. aerogenes*.

Majority of NDM and OXA-48 co-producing isolates were *K. pneumoniae* (81.1%) followed by 10.8% *E. coli* and 8.1% *E. aerogenes*. All the *K. oxytoca* (2) and *E. cloacae* (5) isolates were positive for NDM but none of them were positive for OXA-48 (Table 2).

Table 3 shows the sensitivity, specificity and diagnostic accuracy of PCR-dipstick method for detection of NDM and OXA-48 among different species of CP-CRE isolates considering conventional PCR as gold standard. PCR-dipstick method showed 100% (CI 97.4 – 100.0) sensitivity and 97.9 (CI 94.1-99.6) of diagnostic accuracy for detection of any of carbapenemase genes (NDM /OXA-48) among CP-CRE isolates. The sensitivity, specificity, diagnostic accuracy of PCR-dipstick for detection of NDM and OXA-48 were 100 %, 92.7%, 97.9% and 98.6%, 97.2%, 97.9% respectively.

Cohen's kappa (k) value was calculated to see the agreement between PCR dipstick DNA Chromatography (GeneFields CPE) and conventional PCR for detection of carbapenemase genes which was 0.948 and 0.958 for detection of NDM and OXA-48 respectively that indicates excellent agreement between these two methods (Table 4).

Table 2 Distribution of carbapenemase genes among CP-CRE isolates

Carbapenemase gene type	**No. of isolates**				
	K. pneumoniae **n (%)**	***K. oxytoca*** **n (%)**	***E. coli*** **n (%)**	***E. aerogenes*** **n=6(%)**	***E. cloacae*** **n=5 (%)**
NDM (n=107)	72 (67.3)	2 (1.7)	23 (21.5)	5 (4.7)	5(4.7)
OXA-48 (n= 75)	67 (89.3)	0 (0)	4 (5.3)	4 (5.3)	0 (0)
Both OXA-48, NDM (n=37)	30 ((81.1)	0 (0)	4(10.8)	3 (8.1)	0 (0)

Note: Percentage was calculated on respective carbapenemase genes

Table 3 Sensitivity, specificity and accuracy of PCR-dipstick DNA chromatography in comparison to conventional PCR for detection of carbapenemase gene among CP-CRE isolates

CP-CRE isolates	**Carbapenemase Gene**	**True positive**	**False positive**	**False negative**	**True negative**	**Sensitivity (95% CI) %**	**Specificity (95 %CI) %**	**Accuracy (95%CI) %**
All CP-CRE isolates (n=145)	Any carbapenemase gene	142	3	0	0	100.0 (97.4-100.0)	0.0 (0.0-70.8)	97.9 (94.1-99.6)
	NDM	104	3	0	38	100.0 (96.5-100.0)	92.7 (80.1-98.5)	97.9 (94.1-99.6)
	OXA-48	72	2	1	70	98.6 (92.6-99.9)	97.2 (90.3-99.7)	97.9 (94.1-99.6)
	Both NDM, OXA-48	34	2	1	108	97.1 (85.1-99.9)	98.2 (93.6-99.8)	97.9 (94.1-99.6)
Klebsiella spp. (n=111)	NDM	71	3	0	37	100.0 (94.9-100.0)	92.5 (79.6-98.4)	97.3 (92.3-99.4)
	OXA-48	65	1	1	44	98.5 (91.8-99.9)	97.8 (88.2-99.9)	98.2 (93.6-99.8)
Enterobacter spp. (n=11)	NDM	10	0	0	1	100.0 (69.2-100.0)	100.0 (2.5-100.0)	100.0 (71.5-100.0)
	OXA-48	4	0	0	7	100.0 (39.76-100.0)	100.0 (59.1-100.0)	100.0 (71.5-100.0)
E. coli (n=23)	NDM	23	0	0	0	100.0 (85.2-100.0)	-	-
	OXA-48	3	1	0	19	100.0 (29.2-100.0)	95.0 (75.1-99.9)	95.7 (78.1-99.9)

Table 4 Agreement between PCR-dipstick DNA chromatography and conventional PCR for detection of NDM and OXA-48 among CP-CRE isolates

Carbapenemase Gene	True positive	False negative	False positive	True negative	Total	k value	p value
NDM	104	0	3	38	145	0.948	0.001
OXA-48	72	1	2	70	145	0.958	0.001

Note: Cohen's kappa (k) value was calculated to see the agreement between PCR dipstick

4. Discussion

Since the first reports of CRE isolates in the early 1990s, there is a gradual increase in dissemination of CP-CRE isolates globally [16]. Among the clinically and epidemiologically important carbapenemases: KPC is endemic to regions in China, Italy Israel, England, Greece, Romania, Brazil, Argentina and Colombia whereas (NDM-1) is endemic to the Indian subcontinent (India, Pakistan, Sri Lanka and Bangladesh) with recent increases in the rest of the world. Endemicity of OXA-48-like carbapenemases is observed in the Middle East, India and North Africa where as sporadic cases are reported in other countries with history of recent travel to endemic regions [16,17]. In this study, the distribution rates for NDM and OXA-48 among the CP-CRE isolates were 73.8% and 51.7% respectively, while 25.5 % CP-CRE isolates carry both NDM and OXA-48. At present, carbapenemase resistance mechanism characterization is not recommended for the guidance of therapeutic decisions, but it can identify CP-CRE which is important for infection control and epidemiologic purposes [18, 19]. Phenotypic method, mCIM has excellent sensitivity (99%) and the specificity (100%) for detection of carbapenemase production [19]. In this study, mCIM method showed 99.3% sensitivity for identification of CP-CRE isolates but it requires overnight incubation and does not provide enough information regarding specific carbapenemase genes which is important for epidemiological studies of carbapenemases in a defined area or regions [4]. PCR-dipstick DNA Chromatography (GeneFields CPE) assay showed 100 % sensitivity and 92.7% specificity for detection of NDM whereas 98.6% sensitivity and 97.2% specificity for OXA-48 from cultured bacteria and results can be generated within a single work shift. In previous study this assay showed 100% sensitivity for both NDM, OXA-48 with specificity of 96.4% and 99.3% for detection of NDM and OXA-48 from cultured bacteria respectively. This assay showed overall high sensitivity (100.0%) and accuracy (97.9%) for detection of any carbapenemase genes in comparison to previous study with 77.4% sensitivity and 86.2% accuracy from cultured bacteria [8]. On the other hand in another study, 93.3% sensitivity and 99.1% specificity of this assay for the detection of carbapenemases directly from stool specimens were reported [9]. In addition, this assay successfully detected carbapenemases in double carbapenemase-producing isolates with 97.1% sensitivity, 98.2% specificity and 97.9% accuracy. Among four targeted carbapenemases, only NDM and OXA-48 were detected by molecular methods which highlight the high burden of NDM and OXA-48 producing CP-CRE isolates in this hospital settings. Cohens kappa statistics showed excellent agreement between Conventional PCR and PCR-dipstick DNA Chromatography (GeneFields CPE) assay for detection of CP-CRE isolates. Findings of this study reflects that PCR-dipstick DNA Chromatography (GeneFields CPE) possesses superior characteristics in terms of sensitivity, rapidity and robustness for detection of CP-CRE isolates.

5. Conclusions

The world-wide dissemination of CP-CRE is now become a great threat to public health. Rapid and accurate detection of CP-CRE is important to guide the antibiotic treatment decisions and to minimize their spreads. Microbiology laboratories are integral to the control of CP-CRE and should identify the underlying carbapenem resistant mechanism. PCR-dipstick DNA chromatography (Gene Fields CPE) can detect multiple genes simultaneously and results can be interpreted visually within short period of time without using any expensive/sophisticated equipment. This assay can be used for rapid and reliable detection of CP-CRE where precise identification of carbapenemase type is crucial, especially for epidemiologic studies.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors report no conflict of interest in this work.

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