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Evaluation of Different Phenotypic Methods Versus Polymerase Chain Reaction for Detection of Plasmid Mediated AmpC β-Lactamase-Producing Strains of *Proteus mirabilis*

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A B S T R A C T

In recent years, the prevalence of infections with multidrug resistant Enterobacteriaceae has steadily increased. Enterobacteriaceae producing AmpC β-lactamases (AmpCs) have become a major therapeutic challenge. The detection of AmpC-producing Proteus mirabilis is of significant clinical relevance, as this may lead to inappropriate antimicrobial regimens and therapeutic failure. The aim of this study is to evaluate and comparing routinely phenotypic methods in detection of resistance with molecular methods. From this study, it can be concluded that cephamycin-Hodge test is the most sensitive, specific, interpretable and efficient test for detection of AmpC β-lactamases in clinical isolates of Proteus mirabilis, compared to the molecular method.

Introduction

β-lactam antibiotics account for approximately 50% of global antibiotic consumption which has considerably increased the resistance in Gram negative bacteria. Amp Cβ-lactamase production is one of the commonest causes of resistance to β-lactam antibiotics among Gram negative bacteria (1).

Proteus mirabilis, is a major organism among normal flora and it causes a wide variety of intestinal and extra- intestinal diseases, such as bacteremia, pneumonia, and other infections as wound, chest and even meningitis (2, 3). As a result of antibiotics abuse, the problem of having different antibiotic resistant patterns among micro-organisms had extensively emerged.

The main cause of the emergence of such problem is being away from applying measures and guidelines of infection control regarding programs of antibiotics stewardship in hospitals. This had led to increase the

magnitude of the problem and also the spread of this problem worldwide (4).

Different methods of AmpC β-lactamases group C detection have been described. Screening tools include resistance to cephamycins and/or ceftazidime (5), retaining cefepime susceptibility (6), modified cefoxitin Hodge test (7) and Tris-EDTA disc test(8), inhibitor-based assays (e.g., using boronic acid compounds (9) or cloxacillin,(10) and rapid chromogenic assays (11). Those methods are not used for routine work in clinical microbiology laboratories and for the diagnosis of different AmpC β-lactamases (12).

There is a high need for simple methods to observe the resistance of plasmid AmpC β-lactamase. The aim of this study was to evaluate efficacy of different phenotypic methods compared to PCR as a gold standard test for rapid and accurate detection of AmpC β-lactamases.

Materials and Methods

This study was conducted on fifty clinical isolates of Proteus spp. isolated from different clinical specimens referred to Microbiology Central Laboratory of Helwan University Hospitals in the period from May to December 2015. Specimens studied were 21 pus specimens, 10 urine specimens, 9 wound swabs, 5 sputum specimens, 3 blood specimens, 1 endotracheal tube specimen and 1 stool specimen. All samples were collected under aseptic conditions, and isolates of proteus species were stored in aliquots on trypton soya broth (Oxoid, UK.) at -70°C till used.

Isolates were directly sub-cultured on blood and MacConkey agar plates using a sterile bacteriological loop. Incubation of plates was done at 37°C in aerobic condition. Plates were examined after overnight incubation for separate colonies. Isolates were identified by gram stain, culture characters and biochemical reactions. Antibiotic susceptibility testing was performed using susceptibility test disks (Becton Dickinson, Germany), and CLSI guidelines.

Susceptibility testing was performed on Muller- Hinton agar (bioMerieux, France), using overnight cultures at a 0.5 McFarland standard followed by incubation at 35 C for 16-18h.

Detection of AmpC B- lactamases

Phenotypic detection of AmpC*β*-Lactamase

Cephamycin Hodge test

Cephamycin Hodge test using cefoxitin disk 30 µg and E.coli reference strain ATCC 25922 (supplied by NAMRU-3) was done and interpreted according to Nassim *et al.,* (13).

Tris-EDTA (TE)-disk test

A suspension of the cefoxitin susceptible strain of E.coli ATCC 25922, and results were interpreted according to Singhal *et al.*, (14).

Combination-disk test with boronic acid

Disks containing cefoxitin 30 µg and cefoxitin plus 400 µg of boronic acid were used and the test was done according to Song *et al.,* (15).

Molecular detection of AmpC*β*-Lactamases

Preparation of template DNA

Cells were harvested in a microcentrifuge tube by centrifugation for 10min at 5000xg

(7500rpm). The supernatant was discarded. The DNA Mini spin column was placed in a new 2ml collection tube, with added 500ul buffer in 2 steps successively using AW1 then AW2. Finally the DNA Mini spin column was placed in a clean 1.5ml microcentrifuge tube and 100ul buffer AE was pipette directly onto the DNA membrane centrifugation for 1 min (8000rpm) to elute.

Protocol for Real Time PCR

Real time PCR was performed for amplification of FOX-1geneusing the method described by Perez-Perez and Hanson (16). PCR was performed in a DNA thermal cycler (Biometra, Germany) with a final volume of 50ulin a 0.5-ml thin- walled tubes. For the detection of FOX-1 gene 5-AAC ATG GGG TAT CAG GGA GAT G-3 was used as a forward primer (corresponding to nucleotides 1475-1496) and 5-CAA AGC GCG TAA CCG GAT TGG-3 was used as a reverse primer (corresponding to nucleotides 1664-1644) expected amplicon size 190bp.The template DNA (≤ 500 ng/reaction) was added to the individual PCR tubes containing the master mix. The thermal cycler was programmed according to Alper *et al.,* (17).

Data analysis

Performances of various phenotypic tests in the detection of AmpC β-Lactamases were evaluated to their PCR results.

Interpretation

The greenish horizontal line in the graph of Figure 1 is the threshold line at which the fluorescence begins to be detected (The point at which the amplification plot crosses the threshold is the cycle threshold=Ct). The Tm of samples which were identical or close to that of positive control was considered the gene of target as shown in Figure 2.

Results and Discussion

In our study, out of 50 specimens, 21(42.0%) were negative by both cephamycin-Hodge test and PCR and 29(58.0%) out of 50 specimens were positive by PCR, 22(75.9%) of which were positive by both tests while 7 (24.1%) specimens were negative by cephamycin-Hodge test and positive by PCR. Agreement between both methods was 86.0%. There was a statistical significant agreement between them ($P < 0.05$) (Table 1 and Figure 3).

Out of 50 specimens, 21(42.0%) were negative by both Tris-EDTA disk test and PCR and 29(58.0%) out of 50 specimens were positive by PCR, 11(37.9%) of which were positive by both tests while 18(62.1%) specimens were negative by Tris-EDTA disk test and positive by PCR. Agreement between both methods was 64.0%. There was a statistical significant disagreement between them ($P < 0.05$) (Table 2 and Figure 4). Out of 50 specimens, 21(42.0%) were negative by both combination disk test with boronic acid and PCR and 29(58.0%) out of 50 specimens were positive by PCR, 17(58.6%) of which were positive by both tests while 12 (41.4%) specimens were negative by combination disk test with boronic acid and positive by PCR. Agreement between both methods was 76.0%. There was a statistical significant disagreement between them ($P < 0.05$) (Table 3 and Figure 5).

27 specimens (96.4%) were negative by both Cephamycin-Hodge test and Tris-EDTA disk test and 10 specimens (45.5%) were positive by both tests while 12 specimens (54.5%) were positive by Cephamycin-Hodge test and negative by Tris-EDTA disk test and 1 specimen (3.6%) was positive by Tris-EDTA disk test and negative by Cephamycin-Hodge test. There was a statistical significant difference between them ($P <0.05$) (Table 4 and Figure 6).

26 specimens (92.9%) were negative by both Cephamycin-Hodge test and combination-disk test with boronic acid and 15 specimens (68.2%) were positive by both tests while 7 specimens (31.8%) were positive by Cephamycin-Hodge test and negative by combination-disk test with boronic acid and 2 specimens (7.1%) was positive by combination-disk test with boronic and negative by Cephamycin-Hodge test. There was a statistical significant difference between them ($P < 0.05$) (Table 5 and Figure 7). 33 specimens (84.6%) were negative by both Tris-EDTA disk test and combination-disk test with boronic acid and 11 specimens (100.0%) were positive by both tests while 6 specimens (15.4%) were negative by Tris-EDTA disk test and positive by combination-disk test with boronic acid. There was a statistical significant difference between them ($P <0.05$) (Table 6 and Figure 8).

Agreement between PCR results and phenotypic methods were 86%, 64% and 76% for cephamycin- Hodge test, Tris- EDTA test and combination disk test respectively with statistical significant difference between them ($P <0.05$).

Table.1 Correlation between Cephamycin-Hodge test and PCR as a reference method

			PCR		Total
			Negative	Positive	
Cephamycin-Hodge Test	Negative	Count	21	7	28
		%	42.0%	24.1%	56.0%
	Positive	Count	0	22	22
		%	0.0%	75.9%	44.0%
Total		Count	21	29	50
		%	100.0%	100.0%	100.0%

Table.2 Correlation between Tris-EDTA disk test and PCR as a reference method

			PCR		Total
			Negative	Positive	
Tris-EDTA disk Test	Negative	Count	21	18	39
		%	42.0%	62.1%	78.0%
	Positive	Count	0	11	11
		%	0.0%	37.9%	22.0%
Total		Count	21	29	50
		%	100.0%	100.0%	100.0%

Table.3 Correlation between combination- disk test with boronic acid and PCR as a reference method

			PCR		Total
			Negative	Positive	
Combination disk Test with BA	Negative	Count	21	12	33
		%	42.0%	41.4%	66.0%
	positive	Count	0	17	17
		%	0.0%	58.6%	34.0%
Total		Count	21	29	50
		%	100.0%	100.0%	100.0%

Table.4 Correlation between Cephamycin-Hodge test Tris-EDTA disk test

			Cephamycin-Hodge Test		Total
			Negative	Positive	
Tris-EDTA disk T	Negative	Count	27	12	39
		%	96.4%	54.5%	78.0%
	Positive	Count	1	10	11
		%	3.6%	45.5%	22.0%
Total		Count	28	22	50
		%	100.0%	100.0%	100.0%

Table.5 Correlation between cephamycin-Hodge test and combination-disk test with boronic acid

			Cephamycin-Hodge Test		Total
			Negative	Positive	
Combination-disk Test with BA	Negative	Count	26	7	33
		%	92.9%	31.8%	66.0%
	Positive	Count	2	15	17
		%	7.1%	68.2%	34.0%
Total		Count	28	22	50
		%	100.0%	100.0%	100.0%

Table.6 Correlation between Tris-EDTA disk and combination- disk test with boronic acid

			Tris-EDTA disk Test		Total
			Negative	Positive	
Combination disk Test with BA	Negative	Count	33	0	33
		%	84.6%	0.0%	66.0%
	Positive	Count	6	11	17
		%	15.4%	100.0%	34.0%
Total		Count	39	11	50
			100.0%	100.0%	100.0%

Fig.1 Results of syber Green real time PCR in amplification plot with cycles number on X axis and florescence on Y axis

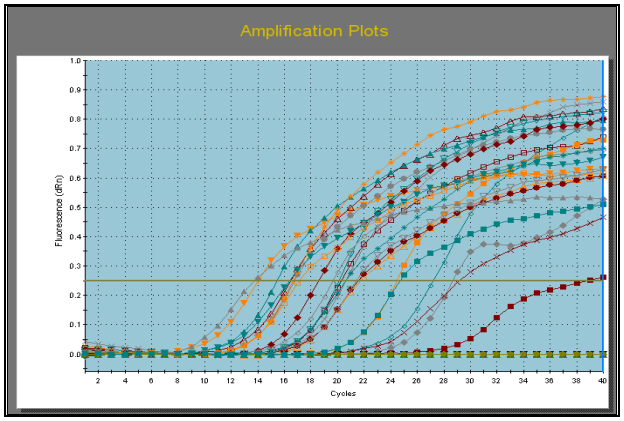


Fig.2 Results of melting curve, average Tm= 77.13°C -77.72°C

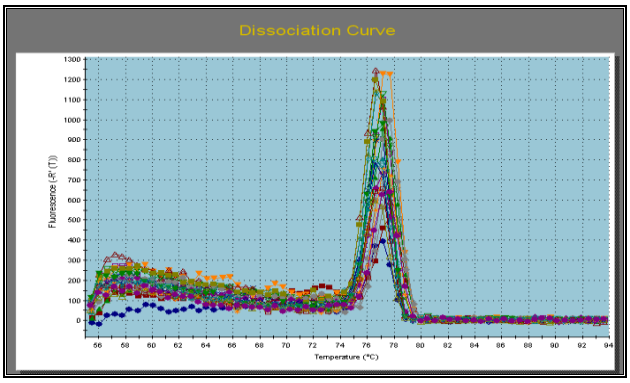


Fig.3 Evaluation of cephamycin-Hodge Test Vs PCR

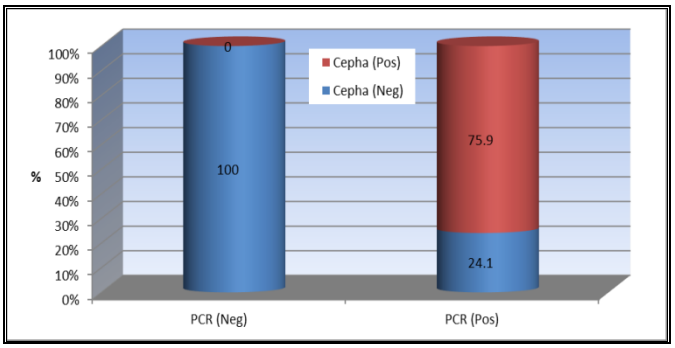


Fig.4 Evaluation of Tris-EDTA Disk Test Vs PCR

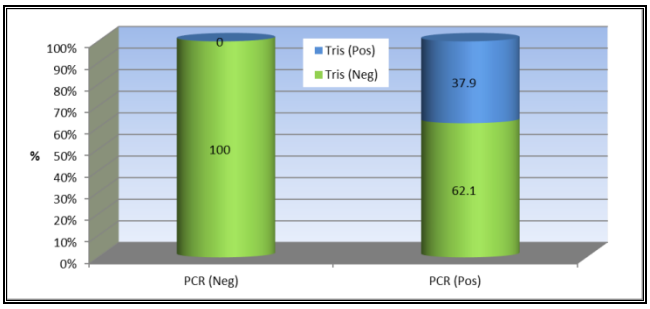


Fig.5 Evaluation of combination Disk Test with BA Vs PCR

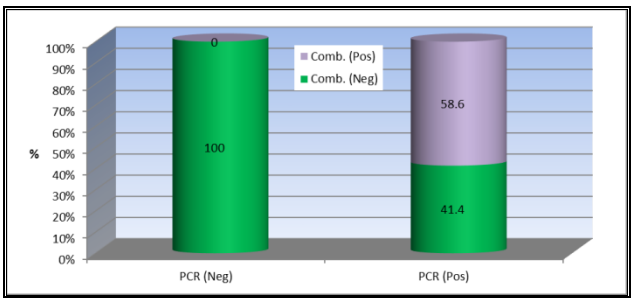


Fig.6 Correlation between cephamycin-Hodge test and Tris-EDTA disk test

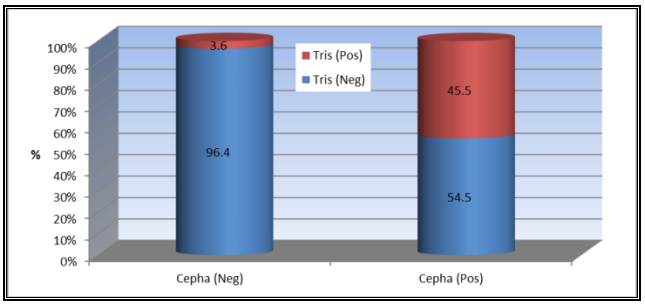


Fig.7 Correlation between cephamycin- Hodge test and combination-disk test with boronic acid

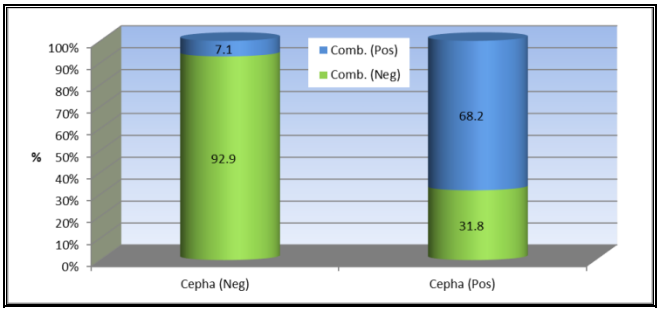


Fig.8 Correlation between Tris- EDTA disk test and Combination- disk test with boronic acid

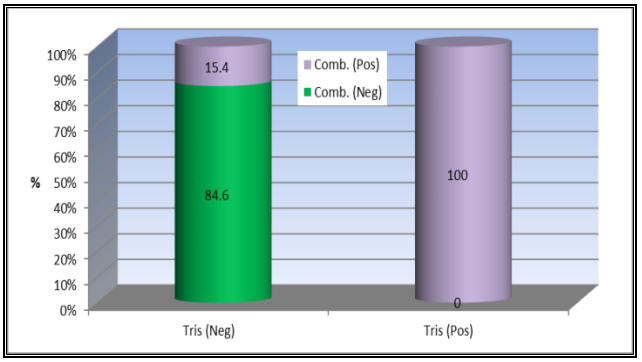
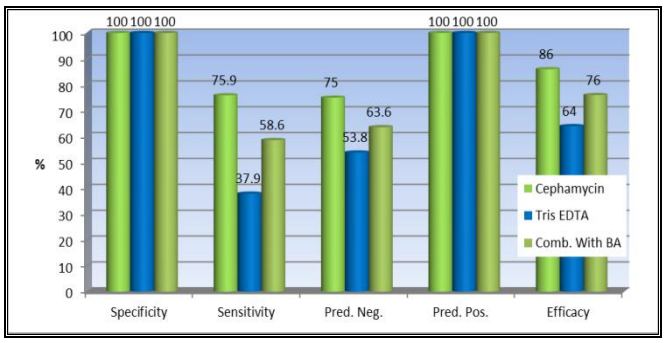


Fig.9 Comparison between all studied methods as regards evaluation criteria



Specificity of Cephamycin-Hodge test, Tris-EDTA disk test and combination-disk test with boronic acid was (100.0%, 100.0% & 100.0%) respectively, sensitivity was (75.9%, 37.9% & 58.6%) respectively, negative predictive value was (75.0%, 53.8% & 63.6%) respectively, positive predictive value was (100.0%, 100.0% &100.0%) respectively and the efficacy was (86.0%, 64.0% & 76.0%) respectively (Figure 9).

In the present study, combination-disk test with BA detected AmpC producing strains in 17(58.6%) out of 29 PCR-positive isolates. In contrast to our results, Coudron 2005 (18) screened 271 clinical isolates for cefoxitin susceptibility by the standard disk diffusion method (128 Klebsiella spp., 115 *E. coli* and 28 *P. mirabilis* isolates). Screen-positive isolates were tested for the presence of the AmpC β-lactamase by a three-dimensional method, boronic acid disk test and PCR. He found that 55 out of 271 isolates were AmpCPCR-positive, and the boronic acid disk test detected 54 of the isolates (13 *Klebsiella*, 38 *E. coli* and 3 *P. mirabilis*). For this reason, he concluded that the boronic acid disk test is the recommended method as sensitivity was 90% and specificity was 98.2% and it is a practical and efficient method that uses current CLSI methodology to detect plasmid-mediated AmpC β-lactamase (PABL) in organisms that usually do not harbor genes for these enzymes. It is unclear why the disk test missed one of the AmpC-producing Proteus mirabilis isolates, although it may be due to swarming phenomena, which is often seen with this organism on agar media.

In a study performed by Hemalatha and his colleagues in 2007 (19), a total number of 76 clinical isolates (67 *E. coli* and 9 *Klebsiella pneumoniae*) were screened for AmpC production by disk diffusion method using cefoxitin (30µg) disks and confirmed by inhibitor based test using boronic acid as inhibitor. A total of 36 of 76 isolates (47.3%) screened were harboured AmpC enzymes, of which a majority 31 (86.1%) co-produced ESBL enzymes. Pure AmpC production was seen in 7 out of 76 (9.2%) of isolates only. The inhibitor based test was useful in identifying cefoxitin susceptible AmpC producers and could also effectively differentiate ESBL from AmpC producing isolates.

In our study we found that the sensitivity of combination-disk test with boronic acid was 58.6%. Hemalatha and his colleagues 2007 (19) found that the sensitivity was 47.3%. The detection failure could be due to very high levels of AmpC expression and detection could possibly be improved by increasing the concentration of the enzyme inhibitor (boronic acid).

In our study, cephamycin-Hodge test showed higher sensitivity (75.9%), specificity (100.0%) and efficacy (86.0%) than Tris-EDTA disk test (37.9%, 100.0% and 64.0% respectively) and combination-disk test with boronic acid (58.6%, 100.0% and 76.0% respectively).

In agreement with our results, Shanthi *et al.*, 2012, (20) screened 77 isolates, K. pneumonia (n = 52) and *E. coli* (n = 25) for AmpC production by disk diffusion and MIC determination using cefoxitin. These isolates were then subjected to cefoxitin Hodge test and boronic acid inhibitor method. The presence of AmpC genes was confirmed by multiplex PCR. The Boronic acid Inhibition (BAI) test was positive in 55 isolates which included 19 *E. coli* and 36 *K. pneumoniae*. Hodge test using cefoxitin was positive in 40 isolates which included 20 of each *E. coli* and *K. pneumoniae*. Multiplex PCR detected plasmid Amp C in 23 isolates, of which 12 were *K. pneumoniae* and 11 were *E. coli*. Considering PCR as the gold standard, they compared the sensitivity and specificity of the

phenotypic tests employed. The Hodge test fared better in terms of sensitivity and specificity when compared with the inhibitor-based test (78.2% and 59.2% vs 65.2% and 25.9%). They concluded that PCR and isoelectric focusing remain the gold standard for detection of AmpC. The phenotypic tests for Amp C detection have low sensitivity and specificity as multiple Amp C types coexist in many isolates and presence of β-lactamases and/or porin defects render the phenotypic tests unreliable since many of these mechanisms mask each other.

In contrast to our results, Lee *et al.*, 2009 (21) studied 276 clinical isolates of *E. coli* (N=97), *K. pneumoniae* (N=136), and *P. mirabilis* (N=43). Multiplex PCR was performed to detect the PABL genes. Further, 3 phenotypic detection methods cephamycin-Hodge test, Tris-EDTA (TE) disk test, and combination-disk test with 3-aminophenylboronic acid (BA) were performed using cefoxitin and cefotetan disks. They found that PABL genes were detected by multiplex PCR in 122/276 isolates, including 14/97 *E. coli*, 105/136 *K. pneumoniae*, and 3/43 *P. mirabilis* isolates. A total of 93 PABL-producing strains were positive with the cephamycin-Hodge test, but 29 PABL-producing strains were not detected by this method. By TE -disk test, 98 strains were determined as PABL-producers, while 24 strains were false-negatives. Combination-disk test with BA detected 120 strains of the 122 PABL-producers, but 12 PABL non-producers were also tested positive by this method. The combination-disk test with BA showed sensitivity (98.4%), specificity (92.2%), and efficiency (96.3%) and the cephamycin-Hodge showed (76.2%, 96.1%, and 88.6%, respectively) and the TE-disk test (80.3%, 91.6%, and 87.9%, respectively). They concluded that the combination-disk test with BA is a simple, efficient, and interpretable test that can be applicable in clinical laboratories for detection of PABLs in clinical isolates of *E. coli*, *K. pneumoniae*, and *P. mirabilis*.

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