DETECTION OF AMP-C BETA-LACTAMASES IN URINARY ISOLATES OF ESCHERICHIA COLI THROUGH VARIOUS PHENOTYPIC METHODS

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ABSTRACT

Objective: To determine the prevalence of Amp C beta lactamases in urinary isolates of Escherichia coli (*E. coli*), and to evaluate and compare different phenotypic methods for its detection in a cost effective way.
Study Design: Descriptive study.
Place and Duration of Study: Department of Microbiology, Combined Military Hospital Lahore Pakistan, from Jan 2016 to Jun 2016.
Material and Methods: Modified Three Dimensional Test (M3DT) taken as Gold standard, Modified Hodge Test (MHT) (Cefoxitin), Nitrocephin test and three screening strategies for the detection of Amp C Beta-lactamases were tested on urinary isolates of *E. coli* collected during a period of 06 months.
Results: Modified Hodge Test, was found to be simple, highly specific and sensitive in detecting these enzyme producers. Collectively these tests detected 45.07% of *E. coli* to be Amp C producers.
Conclusion: Each of the three tests can be used as an acceptable phenotypic confirmatory tool when Amp C production in *E. coli* is suspected.

Keywords: Amp C beta-lactamases, MHT.

INTRODUCTION

Emerging therapeutic problem faced by most of the clinicians and microbiologist is the presence of Plasmid mediated Amp C beta lactamases in the microorganisms commonly isolated from various clinical specimens. They constitute an utmost resistance to all beta lactams drugs sparing carbapenems and cefipime as the only hope for survival[1].

Nucleotide sequencing suggests that genes encoding these enzymes derive from chromosomes of enterobacteraceae having several Amp C genes, which have been integrated into transferable genetic elements (plasmids) facilitating the spread to different micro-organisms[1].

In new microbiological set ups phenotypic methods with high sensitivity and specificity are required for better infection control practices. It not only improves clinical management of patient but also provide sound epidemiological data. Microbiologist are searching for standardised phenotypic methods readily available for detection. Reduced susceptibility to cefoxitin may be an indicator of Amp C activity in the enterobacteriaceae, but this resistance may also be generated by alterations to outer membrane permeability[2]. Susceptibility testing may also fail to reliably detect these strains, as the minimum inhibitory concentrations (MICs) of third-generation cephalosporins may fall below the currently recommended breakpoints from the clinical laboratory standards institute (CLSI)[3,4]. Optimum phenotypic detection method for Amp C activity have always been Enzyme extraction methods. However, these are labor-intensive and not suitable for routine clinical use. The use of disk approximation tests by Kirby-Bauer testing to detect inducible Amp C activity has also been described, using one antibiotic as an inducing substrate and a second antibiotic as a reporter substrate[5]. Boronic acid and cloxacillin are well considered as Inhibitors of the Amp C enzyme, incorporation of these substances into disk-based assays, increases the probability of detection and help reaching diagnostic finality[3,6].

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Surveillance of plasmid mediated Amp C beta lactamase producing organisms is problemetic. Moreover, no specific CLSI recommended method is available. Most of the tertiary care setups in Pakistan are looking for standardised phenotypic methods for its detection. Non availability of molecular techniques for every organism in tertiary care units require several phenotypic detection methods liable to discriminate between acquired and chromosomal Amp C enzymes in organisms that produce endogenous Amp C enzymes.

This prospective study was set out to determine the prevalence of Amp C beta lactamases in urinary isolates of Escherichia coli *(E.coli)*, and to evaluate the sensitivity, specificity and over all efficiency of a variety of phenotypic methods based on simple techniques for the detection of Amp C beta lactamases in these isolates.

MATERIAL AND METHODS

A total of 71 Escherichia coli recovered during a period of Jan 2016 to June 2016 from a total of 945 non-repetitive urine samples. Sampling technique was non probability convenience. Identification of all the isolates after an overnight incubation at 37°C on cysteine lactose electrolyte deficient (CLED) agar was done by colony morphology, gram's staining and standard biochemical methods i.e API 20E. The isolates were tested for antimicrobial susceptibility through modified Kirby-Bauer disk diffusion method according to CLSI guidelines (2015)[7].

To label the isolates as presumptive Amp C producers, three screening strategies were used: reduced susceptibility to third generation cephalosporins, reduced susceptibility to cefoxitin less than 18 mm zone of inhibition, and a combination of reduced susceptibility to third generation cephalosporins and cefoxitin[8]. The inhibition zone sizes and MICs were interpreted as per the CLSI guidelines[7] (fig-1).

All the screen positive isolates after confirmation by MICs were subjected to different phenotypic tests. Modified Three Dimensional Test (M3DT)[9], Modified Hodge Test with cefoxitin[10] and Nitrocefin test[11] for the detection of Amp C beta lactamases in these isolates.

A known *E. coli* strain was used as Amp C positive control and *E. coli* ATCC 25922 as a negative control with each batch of tests.

A 10-15 mg of bacterial wet weight was obtained in a pre weighed micro centrifuge tube from an overnight growth of *E. coli* on Mueller Hinton Agar (MHA) plate. Peptone water was used to suspended the bacterial mass and a bacterial pellet was obtained by centrifugation at 3000 rpm at 4°C for 15 minutes. By repeated freezing and thawing of the bacterial pellet, crude enzyme extract was prepared. Lawn culture of *E. coli* ATCC 25922 was prepared on MHA plates and cefoxitin disc of 30µg was placed in the centre of the plates. Using a sterile surgical blade, linear slits of 3cm were cut up to 3mm away from cefoxitin disc. A small circular well was made at the other end of the slit, which was loaded with 30-40µl of the enzyme extract. The plates were kept upright for 5-10 minutes until the liquid dried and incubated overnight at 37°C. Any extension of growth of *E coli* (control) into the zone of inhibition due to neutralization of cefoxitin by the enzyme at the point where the slit inserted the zone of inhibition of cefoxitin was considered a positive M3DT test and interpreted as evidence for the presence of Amp C beta-lactamases[9].

This was performed as described by Yong *et al*[10] (fig-2).

This test, which utilizes a chromogenic oxyimino cephalosporin, was performed as described by manufacturer[11] (fig-3).

The performance of all the phenotypic tests was evaluated using M3DT as a "Gold standard" method. All the tests were repeated twice and the results were subjected to statistical analysis with SPSS version 21 software (SPSS Inc., Chicago, IL), using chi-square test. Sensitivity, specificity and overall efficiency of the tests were calculated using appropriate formulae.

RESULTS

Out of 945 urine samples, growth was obtained in 177 urine samples, 713 samples yielded no growth and 55 samples yielded growth of mixed organisms (later put into the category of repeat sample). Among these 177 samples, *E coli* were isolated in 71 urine samples as shown in table-I.

Amongst 71 *E. coli* isolates, 33 *E. coli* were screened positive for Amp C production[8]. Out of these 33 Amp C positive isolates, 19 had shown high sensitivity and specificity of 100% and 97.44%, applying "Chi square test". All isolates had their Break point MICs within the CLSI defined range.

Tests applied on 32 Amp C beta lactamases producing isolates were MHT (Cefoxitin) and Nitrocefin Test and compared with M3DT, Both the tests detected Amp C β-lactamases in 31/32 cases (table-II).

The ability to detect Amp C by these methods against M3DT as "Gold standard" using

Table-I : Percentage of organisms isolated from urine samples for C/S.

Organism isolated (n=177)	Positive isolates (n=177) (%)
Staph saprophyticus	04 (2.25)
Klebsiella oxytoca	03 (1.69)
Klebsiella pneumoniae	09 (5.08)
Pseudomonas aeruginosa	12 (6.7)
Acinetobacter jhonsonii	03 (1.69)
E. coli	71 (40.1)
Candida species	38 (21.4)
Staphylococcus aureus	05 (2.82)
Enterococcus fecalis	21 (11.86)
Enterococcus facium	07 (3.95)
Proteus mirabilis	04 (2.25)

Table-II: Test performance of MHT and Nitrocefin Test for Amp C beta lactamase detection n=32, taking M3DT as "Gold standard".

Methods	Total no of isolates (n=19)		Sensitivity Against M3DT	Specificity Against M3DT	Efficiency Against M3DT
MHT	31/32		96.88%	100%	98.5%
Nitrocefin test	31/32		96.88%	100%	98.5%
M3DT	32/33	Gold Stadard			
3 Screening Strategies	33				

presence of Amp C beta lactamase alone, while 14 isolates were co -producers i.e presence of Amp C beta lactamases along with ESBLs (Extended spectrum beta lactamases).

The M3DT test "Gold Standard" including the control allowed us to classify 32 of the isolates as Amp C beta lactamase producers and 39 as non producers. It had not detected Amp C in one screened positive isolate. Thus, three screen strategy when compared with M3DT had shown Chi square test was as follows; MHT (cefoxitin) and Nitrocefin test had an overall sensitivity of 96.88% and specificity of 100%, while positive predictive value (PPV) was 100% and negative predictive value (NPV) was found to be 97.50%. Overall efficiency of the tests was 98.5% respectively (table-II)

DISCUSSION

The greatest challenge posed to micro-biological laboratories is the detection of Amp C

beta-lactamases in *E. coli* during routine work. For all practical reasons, it is not feasible to routinely test all *E. coli* isolates for Amp C production in detail. In our study, we selected 32 clinical isolates of *E. coli* collected during a period of 6 months from urine samples for putative Amp C production based on reduced susceptibility to oxyimino-cephalosporins (ceftriaxone, ceftazidime) and cephamycins (cefoxitin)[12].

Amp C is distinguished from many other beta-lactamases as it hydrolyses cephamycin. We used that non-susceptibility to cefoxitin, as a useful screening tool for Amp C detection, with a sensitivity of 96.88% using MIC and disc-based method according to Coudron *et al*[13]. However he suggested that, use of a cefoxitin inhibitory zone size less than the CLSI breakpoint (or a higher MIC) improved specificity with marginal loss of sensitivity, same was proved in my study as well. Further more, other mechanisms of cephamycin resistance (e.g. porin deficiency, carbapenemases) weakens the specificity of a single screening approach[14]. The use of combined screening tests like reduced susceptibility to third generation cephalosporins and cefoxitin, both had an added benefit of improved specificity, and proved to be an optimal screening test combination in our study.

Although there are no CLSI guidelines for phenotypic methods to screen and detect Amp C, a positive three dimensional test (M3DT) with cefoxitin demonstrates hydrolysis of cefoxitin and differentiates between Amp C production and reduced outer membrane permeability. In a study done by Jaspal *et al* 58.4% isolates were cefoxitin resistant while three dimensional test was positive in 46.1%[15]. Another study by Tanushree *et al* 71 screen positive isolates of *E. coli*, 29 (40.8%) were identified as Amp C producers by modified three dimensional test and Amp C disk test. He took PCR as Gold standard which detected 25 isolates to be Amp C β-lactamase producer out of 71 screen positive *E coli*[16]. Interestingly, our results bit vary from this study may be because of strict selection criteria as 32/33 isolates gave positive M3DT and selected as gold standard test. As being cost effective this test could easily be incorporated into small microbiological setups for accurate identification of Amp C beta lacta-mases.

The overall good performance of the Nitrocefin test (oxoid, Basingstoke, UK), was most probably because of strict selection criteria for Amp C producers in our study. Although

Figure-1: Three Screening Strategies: Reduced susceptibility to third generation Cephalosporins, reduced susceptibility to Cefoxitin less than 18 mm zone of inhibition, and a combination of reduced susceptibility to third generation Cephalosporins and Cefoxitin.

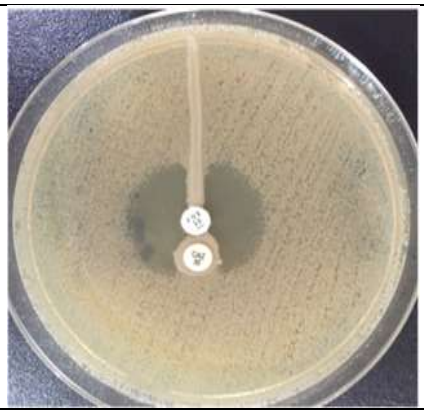

Figure-2: Positive Modified Hodge Test (Cefoxitin) with positive third generation disc test for Amp C detection.

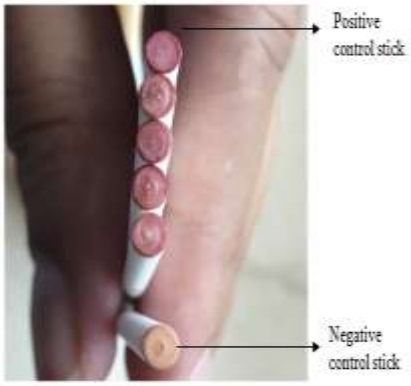

Figure-3: Positive Nitrocefin test (Oxoid) with Positive sticks along with negative control stick.

high sensitivity and specificity is inconsistent with findings of other studies e.g Montgomery *et al*[17], yet further studies are required including isolates having both plasmid mediated Amp C and chromosomal mediated Amp C.

In other studies the modified Hodge test has been compared with other methods of detecting Amp C expression such as the boronic acid test and EDTA disc test. The results of modified Hodge test was, if anything, less sensitive than the inhibitor based methods e.g boronic acid test[18]. Although we found a very good correlation between the nitrocefin test and the modified hodge test in a subset of our isolates. It appears as the modified hodge test and nitrocefin test identified more positives. This is most probably because of strict selection criteria and only plasmid mediated Amp C producers were added.

Thus, it is possible that different methods would enhance the capability of a microbiology lab to identify more accurately, isolates with Amp C; however, this has been proved that in our microbiological setups no matter how small they are, the ability to detect the strongest positive Amp C-producing strains is virtually 90% among the different methods.

LIMITATION OF STUDY

1. M3DT was taken as "Gold Standard" test, as PCR facility was not available.

2. Both, the isolates purely producing Amp C β-lactamases and co-producers (Amp C, ESBL β-lactamases producers) were added in the study.

CONCLUSION

Each of the three tests can be used as an acceptable phenotypic confirmatory tool when Amp C production in *E. coli* is suspected.

A simple disc-based protocol utilizing cefoxitin and oxyimino-cephalosporins non-susceptibility as screening methods, then using M3DT, nitrocefin test or MHT for confirmation, could conveniently be incorporated into laboratory workflow. It would detect Amp C β-lactamases in *E. coli* with a sensitivity of 96.88% and a specificity of 100%.

CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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