

ORIGINAL ARTICLE

# Rapid Simultaneous detection of AmpC and ESBLs among *Enterobacteriaceae* using MastD68C detection set and possible therapeutic options

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## ABSTRACT

### Key words:

Enterobacteriaceae,  
Extended spectrum  $\beta$ -  
lactamases,  
AmpC  $\beta$ -lactamases,  
MAST D68C test,  
Tigecycline,  
Doripenem,  
Colistin and Temocillin

**Background:** Extended spectrum  $\beta$ -lactamases (ESBLs) and AmpC  $\beta$ -lactamases are enzymes produced by a variety of Gram-negative bacteria which confer an increased resistance to commonly used antibiotics and represent a substantial clinical threat. Several phenotypic tests have been recommended for screening and confirmation of ESBL- and AmpC-producing organisms. However, a comprehensive diagnostic algorithm integrating both screening and confirmation has not been established. **Objectives:** This study aimed to detect ESBL and/or AmpC production by using MastD68C ESBL and AmpC detection set as a single phenotypic method and to study its sensitivity and specificity comparing to other methods. Evaluate the effect of novel antibiotics namely tigecycline and doripenem, as well as the efficacy of old reviving antibiotics as colistin and temocillin against ESBL- and AmpC-producing *Enterobacteriaceae*. **Methodology:** Hundred *Enterobacteriaceae* isolates were screened for ESBL production using disc diffusion method and confirmed by combination disc diffusion test. Screening of AmpC production was done by ceftioxin disc test, disc approximation test and confirmation was done by AmpC disc test. Isolates screened positive for ESBL were investigated for their susceptibility to temocillin, tigecycline, colistin and doripenem by E-test. **Results:** Among the 100 *Enterobacteriaceae* isolates, 45 were screened positive for ESBL-production using the disc diffusion test and 36 were confirmed by the combination disc test. Nine isolates were screened for AmpC-production using the ceftioxin disc test and 5 isolates were confirmed as AmpC producers by AmpC disc test. Using MAST D68C set, 35 isolates were ESBL producers, 2 were AmpC producers, one isolate was both ESBL and AmpC producer. All isolates were sensitive to tigecycline and doripenem. Forty-three isolates were sensitive to colistin, while, thirty-seven isolates were sensitive to temocillin. **Conclusion:** MAST D68C test appears to be a promising way to detect isolates producing ESBL and/or AmpC. Tigecycline, doripenem, temocillin and colistin revealed excellent activity against ESBL- and AmpC- producing *Enterobacteriaceae*.

## INTRODUCTION

Resistance to broad spectrum  $\beta$ -lactams, mediated by ESBL and AmpC  $\beta$ -lactamase enzymes among *Enterobacteriaceae* is an increasing problem worldwide. Presence of isolates that harbor these enzymes in clinical infections can result in treatment failure if one of the  $\beta$ -lactam drugs, including extending

spectrum cephalosporins, is used<sup>1</sup>. The co-existence of AmpCs and ESBLs in the same strain may result in false negative tests for the detection of ESBLs by the current CLSI criteria<sup>2</sup>.

Infections caused by such resistant organisms can prolong hospital stay and result in intensive care unit (ICU) admission. Also inappropriate treatment of these complex infections can increase mortality and morbidity. Whereas, rapid detection of these enzymes allows for de-escalation to more targeted therapy and it is also an important infection control issue<sup>3</sup>. Several phenotypic tests have been recommended for screening

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and confirmation of ESBL- and AmpC-producing organisms. However, a comprehensive diagnostic algorithm integrating both screening and confirmation has not been established. Therefore there is a requirement for a simple and reliable diagnostic test for confirmation of AmpC and ESBL production<sup>3</sup>. Strains with ESBL and/or AmpC genes are often resistant to multiple agents, making the selection of an effective antibiotic difficult.  $\beta$ -Lactam/ $\beta$ -lactamase inhibitor combinations and most cephalosporins and penicillins should be avoided because of in vitro resistance, so it is not surprising that antibiotic choice for infections with such organisms is seriously reduced<sup>4</sup>.

Carbapenems are considered to be the treatment of choice against serious ESBL and AmpC associated infections. This is mainly because they are not inactivated by these enzymes *in vitro*, and have demonstrated adequate effectiveness for the treatment of serious Gram-negative infections at various body sites. Unfortunately, resistance has emerged in many bacteria treated with carbapenems<sup>5</sup>. Doripenem, the newest addition to the carbapenem class of antibiotics, was approved by the Food and Drug Administration (FDA) to treat intra-abdominal and urinary tract infections caused by ESBL- and AmpC-producing *Enterobacteriaceae*<sup>6</sup>. Other therapeutic alternatives include tigecycline which had good activity against most ESBL-producing and AmpC-hyperproducing *Enterobacteriaceae*, many of which are also multi-resistant to quinolones, aminoglycosides and classical tetracyclines<sup>7</sup>. Temocillin (the 6- $\alpha$ -methoxy derivative of ticarcillin) a modification which increases stability to  $\beta$ -lactamases including AmpC and extended-spectrum types, has been re-launched in the UK. It has been used as a potential alternative to carbapenems particularly against urinary tract infections caused by ESBL producers and other cephalosporin-resistant strains<sup>8</sup>. Colistin, an intravenous formulation of a polymyxin, has fairly reliable *in vitro* activity against the ESBL and AmpC  $\beta$ -lactamase-producing *Enterobacteriaceae*, and it might be useful in the treatment of co-infection with these organisms<sup>9</sup>. The purpose of this study was to detect ESBL and/or AmpC production by using MastD68C ESBL and AmpC detection set as a single phenotypic method and to study its sensitivity and specificity comparing to other methods. Also to evaluate the effect of novel antibiotics namely tigecycline and doripenem, as well as the efficacy of old reviving antibiotics as colistin and temocillin against ESBL- and AmpC-producing *Enterobacteriaceae* clinical isolates.

## METHODOLOGY

### Bacterial isolates and Specimens:

The study was conducted on 100 *Enterobacteriaceae* isolates out of 177 Gram negative bacilli isolated from 550 clinical specimens were

isolated from patients attending outpatient clinic attendants and inpatients admitted to Theodor Bilharz Research Institute (TBRI) during the period from September 2013 to January 2014. Specimens included urine (n=69), sputum (n=13), pus (n=9), blood culture (n=3) and ascetic fluid (n=6). Blood and ascetic fluid cultures were done using BACTEC 9010 (Becton, Dickinson).

All clinical samples including positive blood culture bottles and ascetic fluid samples were plated onto MacConkey agar and blood agar. In addition urine samples were cultured on CLED agar (Biorad).

Bacterial identification was done by colony morphology, Gram stain and using API20E (Bio-Mérieux, France).

### Detection of $\beta$ -lactamases:

**a) ESBLs detection:** ESBLs were screened in *Enterobacteriaceae* according to zone diameters described in CLSI guidelines; ceftazidime  $\leq$  22 mm, cefotaxime  $\leq$  27 mm, ceftriaxone  $\leq$  25 mm, aztreonam  $\leq$  27 mm, cefpodoxime  $\leq$  22 mm and were confirmed by combination disc diffusion method using; ceftazidime discs (CAZ; 30 $\mu$ g) with and without clavulanate (10 $\mu$ g) on Mueller-Hinton agar (MHA) (Biorad). A greater than or equal to 5 mm diameter difference between the antibiotic zone alone and the combined disc with clavulanate confirmed an ESBL producing organism<sup>10</sup>.

**b) AmpC detection:** AmpC was screened by 2 methods;

**i)** using cefoxitin (FOX;30  $\mu$ g) (Biorad) as a resistance marker; inhibitory zones less than 18 mm may indicate AmpC production<sup>11</sup> and **ii)** using disc approximation test in which 10- $\mu$ g imipenem, 30- $\mu$ g cefoxitin, and 20/10- $\mu$ g amoxicillin-clavulanate discs were used as inducing substrates and 30- $\mu$ g ceftazidime disc as substrate. Discs were applied at a distance of 20 mm, and any obvious blunting or flattening of the zone of inhibition between the ceftazidime disc and the inducing substrates was interpreted as a positive result for AmpC<sup>12</sup>.

AmpC production was confirmed using AmpC disc test previously described by<sup>13</sup>, in which an inoculum of *E. coli* ATCC 25922, which is completely susceptible to cefoxitin antibiotic, was inoculated on a MHA plate. Whereas the test strain was inoculated on sterile filter paper discs (5 mm) that were moistened with sterile saline (10 $\mu$ L). The disc was placed almost touching a 30 $\mu$ g FOX disc on the inoculated plate. The plate was incubated overnight at 37°C. Any indentation or flattening of the zone of the cefoxitin inhibition zone in the vicinity of the test disc indicated the release of AmpC  $\beta$ -lactamases in the external environment that reduced susceptibility to cefoxitin antibiotic.

**c) Detection of ESBLs in AmpC positive isolates:** A modified double disc synergy test (MDDST) was

done to detect co-production of both enzymes as described previously<sup>14</sup>. In which antibiotic discs of cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), cefepime (FEP; 30 µg), aztreonam (ATM; 30 µg), amoxicillin-clavulanic acid (AMC; 20/10 µg) and piperacillin/tazobactam (TZP; 100/10µg), (Bio-Rad, France) were used. CAZ, CTX, ATM, TZP and FEP were arranged in proximity (30mm centre to centre) with the AMC disc. The TZP disc was always put in proximity with the FEP disc. The plates were incubated for 18-24 h at 37°C. Any test organism that showed synergy between the AMC disc and any cephalosporin disc or the ATM disc and/or between TZP and FEP was interpreted as positive ESBL-production. Synergy was detected in the form of "bouchon-champagne", "key-hole appearance" or "lens appearance" between the expected discs.

- d) Detecting ESBL and/or AmpC production using the Mast D68C ESBL and AmpC detection set (MAST group):** An 0.5 McFarland suspension of the test isolate was inoculated evenly on MHA plate, then each one of four discs (A–D) supplied by the kit were placed onto the agar in accordance with the manufacturer's instructions (Mast Group). In which disc A contained cefpodoxime (10 mg), disc B contained cefpodoxime (10 mg) and an ESBL inhibitor, disc C contained cefpodoxime (10 mg) and an AmpC inhibitor and disc D contained cefpodoxime (10 mg) and both AmpC and ESBL inhibitors. MHA plates were incubated at 35°C for 18–24 hr.

A zone difference of  $\geq 5$  mm between disc B and disc A, or between disc D and disc C, was taken to indicate the presence of an ESBL. A zone difference of  $\geq 5$  mm between disc C and disc A, or between disc D and disc B, was taken to indicate the presence of a *bla*<sub>AmpC</sub>, whereas a zone difference of  $\geq 5$ mm between disc D and disc C, but  $< 4$  mm difference between disc A and disc B, was taken to indicate the presence of both a *bla*<sub>AmpC</sub> and an ESBL. When all zones differ by  $\leq 2$  mm this indicated the absence of both a *bla*<sub>AmpC</sub> and an ESBL.

#### Antimicrobial susceptibility testing:

It was performed for the screened positive ESBL and AmpC-producing *Enterobacteriaceae* isolates by Kirby-Bauer disc diffusion method using trimethoprim sulphamethaxole (SXT; 23.5µg sulphamethaxole, 1.25µg trimethoprim), ciprofloxacin (CIP; 5µg), gentamycin (GM; 10µg), amikacin (AK; 30µg), levofloxacin (LVX; 5µg), imipenem (IPM; 10 µg) and interpretation of results was according to<sup>10</sup>.

Minimum inhibitory concentrations (MICs) in the form of E-test strips (AB Biodisc, Sweden) were used to determine MICs of all isolates to old reviving antibiotics; temocillin and colistin, and to novel commercially available antibiotics; tigecycline and doripenem following the manufacturer's

recommendations and interpretation of results was according to<sup>15</sup>.

#### Statistical analysis:

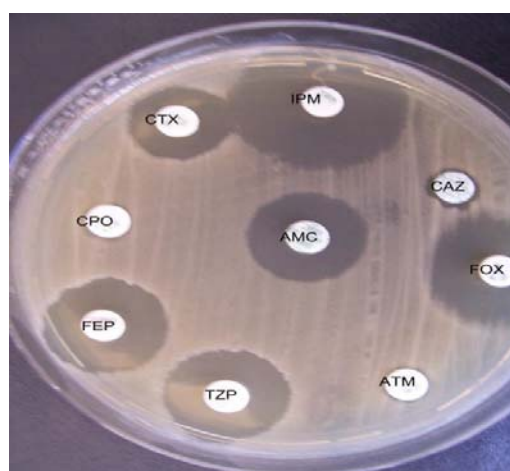
Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). Agreement between the different studied techniques was done using kappa statistic. Accuracy was represented using the terms sensitivity, specificity, positive predictive value, negative predictive value. A probability value (P value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2010 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 16 for Microsoft Windows.

## RESULTS

#### Detection of ESBL production:

Forty five isolates out of the 100 *Enterobacteriaceae* were screened positive for ESBL by the disc diffusion screening method. These 45 isolates include 32 *E. coli*, 12 *K. pneumoniae* and only one *E. cloacae* isolate. So ESBL was detected in 32 out of 77 (41.5%) of *E. coli* isolates while it was detected in 12 out of 22 (54.5%) of *K. pneumoniae* isolates (Fig 1).

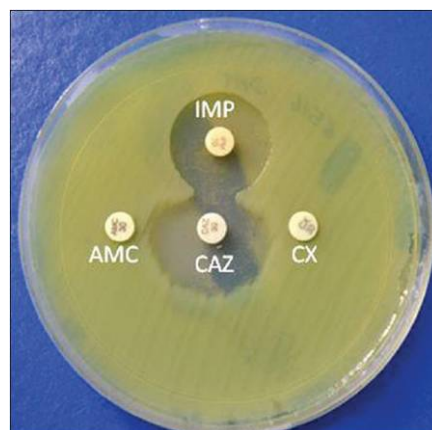
The 45 isolates screened positive for ESBL production by disc diffusion method were subjected to combination disc confirmatory test to detect ESBL production. The combination disc test confirmed 36 isolates as ESBL producers. This confirmed ESBL isolates were 29 *E. coli* (29/77; 37.6%) and 7 *K. pneumoniae* (7/22; 31.8%) (Fig 2).



**Fig. 1:** Screening for ESBL production by the disc diffusion test: an ESBL producing *E.coli* isolate showing resistance to antibiotics; ceftazidime (CAZ), cefpodoxime (CPO), cefotaxime (CTX) and aztreonam (ATM).



**Fig. 2:** Confirmation of ESBL production by the combined disc method: An ESBL producing isolate showing >5 mm difference in zone diameter between ceftazidime (CAZ) and ceftazidime plus clavulanic acid (CCAZ).



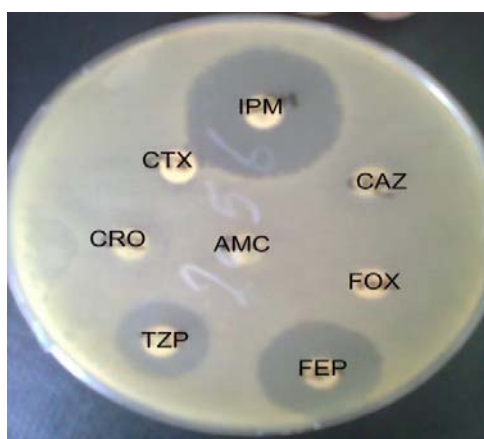
**Fig 4:** Screening for AmpC production by disc approximation test. An *E. cloacae* isolate showing flattening of zone of ceftazidime towards imipenem disc (inducing substrate).

**Detection of AmpC-producing isolates:**

Nine out of the 100 *Enterobacteriaceae* isolates were screened positive for AmpC production by cefoxitin disc test. They include 3 *E. coli*, 5 *K. pneumoniae* and one *E. cloacae* (Fig 3). Only one out of the 100 *Enterobacteriaceae* isolates was screened positive for AmpC by disc approximation test. This isolate was *E. cloacae* (Fig 4). Five out of the 9 isolates were confirmed to be AmpC producers by AmpC disc test (Fig 5).



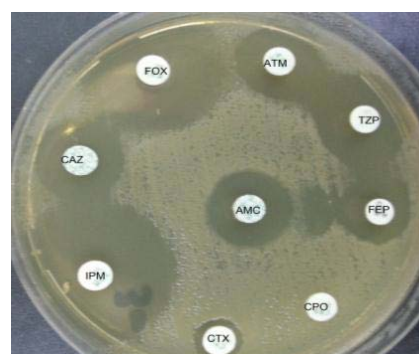
**Fig. 5:** Positive AmpC disc test confirming AmpC production in an *E. coli* isolate showing indentation in the cefoxitin (FOX) inhibitory zone.



**Fig. 3:** Screening for AmpC production by the cefoxitin disc test. An *E. coli* isolate showing cefoxitin (FOX) zone diameter of 6 mm (less than 18 mm) i.e. resistant to cefoxitin according to CLSI (2013)

**Detection of ESBLs in AmpC positive isolates:**

Modified double disc synergy test was done to detect ESBL production in case of co-production of AmpC enzyme (Fig 6). It gave positive ESBL result with 21 isolates, including one *E. coli* isolate which was negative for ESBL by combined disc confirmatory method but was positive for AmpC by AmpC disc test indicating co-production of both enzymes.



**Fig. 6:** Modified double disc synergy test showing the *E. coli* isolate coproducing ESBL and AmpC where synergy is seen between cefepime (FEP) and amoxicillin-clavulanic acid (AMC) and also between FEP and piperacillin/tazobactam (TZP) but not between AMC and 3<sup>rd</sup> generation cephalosporins.



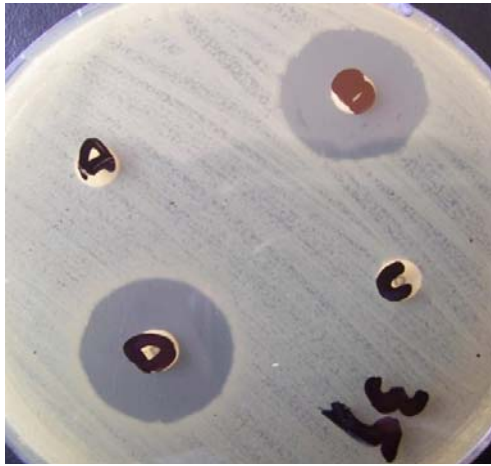
So confirmatory tests showed that ESBL was found in 36 isolates by combination disc, whereas AmpC production was found in 5 isolates by AmpC disc test and one *E. coli* isolate gave positive result for both MDDST and AmpC disc test confirming co-production of both enzymes.

**Detecting ESBL and /or AmpC Production by the new kit Mast D68C:**

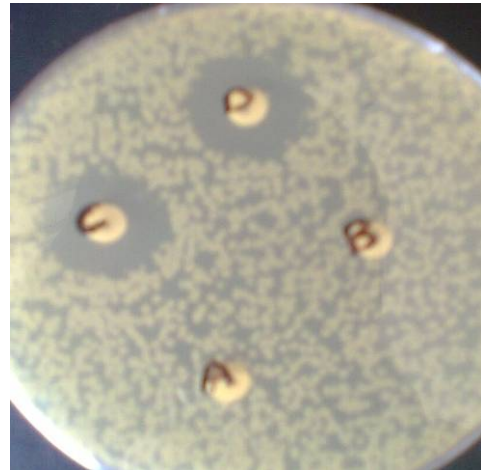
Thirty five (77.8%) isolates were positive for ESBL production (Fig 7), 2 (4.4%) isolates were positive for AmpC production (Fig 8), one (2.2%) *E. coli* isolate was positive for both ESBL and AmpC (Fig 9) and 7 (15.6%) isolates were negative for both ESBL and AmpC production (Table 1).

**Table 1: Result of Mast D68C ESBL and AmpC detection set in relation to type of isolate.**

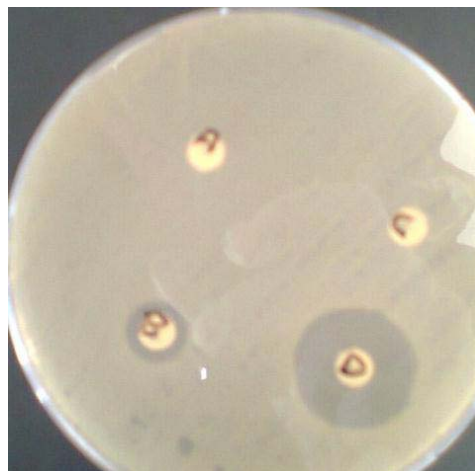
	Positive ESBL	Positive AmpC	Positive ESBL and AmpC	Negative for ESBL and AmpC
<i>E. coli</i>	28	1	1	2
<i>K. pneumonia</i>	7	0	0	5
<i>E. cloacae</i>	0	1	0	0
<b>Total</b>	<b>35</b>	<b>2</b>	<b>1</b>	<b>7</b>



**Fig. 7:** Detection of ESBL production by Mast D68C set showing ESBL positive result: B-A and D-C >5mm and the differences of each of B and D and A and C is < 4mm. A: Cefpodoxine (CPD) 10 µg, B: CPD10 + ESBL inhibitor, C: CPD10 + AmpC inhibitor, D: CPD10 + ESBL inhibitor + AmpC Inhibitor.



**Fig. 8:** Detection of AmpC production by the Mast D68C set showing AmpC positive result: C - A and D - A > 5mm and the differences of each of A and B and C and D are < 4mm



**Fig. 9:** Detection of ESBL and AmpC co-production by the Mast D68C set. The difference between the zones of D and C (D-C) is >5mm and the differences of the zones of each of A and B < 4mm.

The performance of the new kit in detection of ESBL producers was compared to combined disc diffusion method as a confirmatory method by CLSI (2013) (Table 2). There was a significant correlation between the two tests.

**Table 2: Performance of Mast D68C set in detection of ESBL production in relation to combined disc diffusion method in the 45 isolates screened positive for ESBL.**

		Combined disc		Sensitivity	Specificity	PPV	NPV
		Positive (n= 36)	Negative (n= 9)				
<b>Mast D68C</b>	<b>Positive</b>	35	1	35/36 (97.2%)	8/9 (88.8%)	35/35 (100%)	9/10 (90%)
	<b>Negative</b>	1	8				

PPV= Positive predictive value; NPV= Negative predictive value.

Results of the new kit Mast D68C was compared in relation to AmpC disc test regarding detection of AmpC production. (Table 3).

**Table 3: Performance of Mast D68C set in detection of AmpC production in relation to AmpC disc test in 9 isolates screened positive for AmpC.**

		AmpC disc test		Sensitivity	Specificity	PPV	NPV
		Positive (n= 5)	Negative (n= 4)				
<b>Mast D68C</b>	<b>Positive</b>	3	0	3/5 (60%)	4/4 (100%)	3/3 (100%)	4/6 (66.7%)
	<b>Negative</b>	2	4				

The kit showed ESBL and AmpC co-production result in only one *E. coli* isolate recovered from urine sample, this isolate gave ESBL negative result by combined disc test but was positive for ESBL production by MDDST and AmpC production by AmpC disc test.

**Antimicrobial susceptibility testing:**

All 45 (100%) isolates were sensitive to IPM while 39 (86%) isolates were resistant to SXT, CIP and LVX, whereas 22 (48.9%) were resistant to GM and 11 (24.4%) were resistant to AK.

Determination of MIC of novel and old reviving antibiotics showed that all 36 confirmed ESBL-producing isolates were sensitive to both novel drugs tigecycline and doripenem (100%). Regarding colistin; 34 isolates (94.4%) were sensitive and 2 (5.6%) were intermediately sensitive: Whereas for temocillin; 32 isolates (88.9%) were sensitive, 3 (8.3%) were intermediately sensitive and only one was resistant (2.8%).

All 5 confirmed AmpC producers including the co-producing isolate were sensitive to tigecycline, colistin and doripenem. As regard temocillin 3 isolate including the co-producing isolate were sensitive, one isolate was intermediately sensitive and one was found to be resistant (Figs 10,11,12,13 and Table 4).



**Fig. 10: Tigecycline E-test showing a sensitive isolate (MIC = 0.064 µg/ml)**



**Fig. 11: Doripenem E-test showing a sensitive isolate (MIC= 0.047 µg/ml).**



**Fig. 12:** Colistin E-test showing a sensitive isolate (MIC = 0.094 µg/ml)



**Fig. 13:** Temocillin E-test showing a resistant isolate (MIC = 48 µg/ml).

**Table 4: MIC of tested antibiotics.**

Isolates	Tigecycline	Doripenem	Colistin	Temocillin
<b>Screen positive ESBL and AmpC (n=45)</b>	S=45(100%) I=0 R=0	S=45(100%) I=0 R=0	S=43(95.5%) I=2(4.4%) R=0	S=37(82.2%) I=5(11%) R=3(6.6%)
<b>Confirmed ESBL (n=36)</b>	S=36(100%) I=0 R=0	S=36(100%) I=0 R=0	S=34 (94.4%) I=2 (5.6%) R=0	S=32(88.9) I=3(8.3%) R=1(2.8%)
<b>Confirmed AmpC (n=5)</b>	S=5(100%) I=0 R=0	S=5(100%) I=0 R=0	S=5(100%) I=0 R=0	S=3(60%) I=1(20%) R=1(20%)

## DISCUSSION

It is important to know the prevalence of ESBL and/or AmpC producing organisms so that judicious use of antibiotics could be done<sup>16</sup>. This study aimed to detect ESBL and/or AmpC production by using MastD68C ESBL and AmpC detection set as a single phenotypic method and to study its sensitivity and specificity comparing to other methods. In our study, the prevalence of ESBL-producing isolates among the 100 *Enterobacteriaceae* isolates using combined disc confirmatory method was 36%. This result was comparable to other studies previously done at TBRI, where Badawi et al.<sup>17</sup> and Fam et al.<sup>18</sup> reported that ESBL producing *Enterobacteriaceae* represented 23.8% and 29.9%, respectively of the total organisms recovered from tested specimens. However, other Egyptian studies conducted at Egyptian critical care centre at Kasr El Aini Hospital, Assiut University hospital and Banha University hospital reported higher prevalence rates of 62.5%, 52.2% and 53.3%, respectively<sup>19,16</sup>. This difference in the prevalence rates might be attributed to different antibiotic policies which may aid in selection of certain antibiotic resistant pathogens than another, and/or strict implementation of infection control measures. Similar spread of ESBL-producing organisms is found globally. In Europe,

ESBL-producing *Enterobacteriaceae* has been spreading at an alarming rate. Although there is extensive difference between European countries, almost every European country has experienced outbreaks with ESBL-producing organisms. A rate of 10% in Hungary, Poland, Romania, Russia and Turkey to 45 % in Spain and Portugal was reported<sup>14</sup>. Also outbreaks of infections with ESBL-producing organisms have been reported from Africa. In Nigeria, ESBL production rate was 66.7%<sup>20</sup>. In Asia, ESBLs studies have shown elevated levels of ESBL phenotypes at a rate of 20% to 50%<sup>21</sup>. Also a study from India by Sasirekha<sup>22</sup> reported that ESBL producers was 53.4%.

In our study, screening of ESBL production using disc diffusion test revealed 45 ESBL positive isolates of which 36 isolates (80%) were confirmed as ESBL producers and nine isolates were non confirmed. This result was in agreement with Steward et al.<sup>23</sup>, Muzahed et al.<sup>24</sup> and Sridhar et al.<sup>25</sup> who reported 84%, 96% and 84.3%, respectively, but was higher than those reported by Yushau et al.<sup>20</sup> and Idowu et al.<sup>26</sup> which were 54% and 35.3%, respectively.

In our study 5 of the 9 non-confirmed ESBL producing isolates were confirmed to be AmpC producers. Philippon et al.<sup>27</sup> stated that the emergence of plasmid-borne AmpC β-lactamases, which are not inhibited by clavulanic acid, in members of the *Enterobacteriaceae* is likely to explain at least some of

the strains that have a positive screening test but a negative confirmation test. Moreover, the high detection rate of enzymes capable of inactivating third-generation cephalosporins in screen-positive, non-confirmed strains should present a clear warning perhaps to the existence of as-yet-undescribed  $\beta$ -lactamases and that the screening test itself was more meaningful than the confirmation test<sup>28</sup>.

In the present study, the frequency of confirmed ESBL-producing organisms was higher among *E. coli* (29/77; 37.6%) than *K. pneumoniae* (7/22; 31.8%). Similar findings were found by Tsering et al.<sup>29</sup> and Rubio-Perez et al.<sup>30</sup> where *E. coli* accounted for 41.9% and 72% against *K. pneumoniae*; 24.6% and 18%, respectively. Whereas in a former study at TBRI, Fam and El-Damarawy<sup>31</sup> reported higher rates of ESBL-producers among *K. pneumoniae* isolates (55.3%) compared to *E. coli* (35.7%). Other studies in the United States and India also reported higher rates of ESBL producers among *Klebsiella* spp. than *E. coli*<sup>32</sup>.

In the present study, the prevalence of AmpC-producing isolates among 100 *Enterobacteriaceae* isolates was 5%. This result was comparable to a study done at Zagazig University Hospitals that reported AmpC in 2.6% of studied *Enterobacteriaceae* isolates<sup>33</sup>, whereas a higher prevalence rate of 28.3% was reported at TBRI by Fam et al.<sup>34</sup>. This may be due to difference in methods of detection of AmpC, as in our study we used phenotypic methods only while Fam et al. used genotypic methods. Different prevalence rates of AmpC-producing organisms are found globally; 2.7% in China<sup>35</sup>, 0.43% in Spain<sup>36</sup>, 15.9% in India<sup>37</sup>. This difference might be attributed to the lack of phenotypic test recommended by CLSI for AmpC detection<sup>10</sup>, the differences in the study population and the epidemiological differences in various geographic regions.

In our study, screening of AmpC production by cefoxitin disc test revealed that 9 isolates (3 *E. coli*, 5 *K. pneumoniae* and one *E. cloacae*) were resistant to cefoxitin, while by disc approximation test, only one *E. cloacae* isolate gave AmpC positive result. By the confirmatory AmpC disc test 5 out of the 9 isolates (3 *E. coli*, one *K. pneumoniae* and one *E. cloacae*) were AmpC producers.

Silke et al.<sup>38</sup> assessed cefoxitin as a primary screening marker in relation to PCR for the detection of AmpC production. The sensitivity of cefoxitin was 97.4% and the specificity was 78.7%.

Several factors may explain resistance to cefoxitin in the AmpC-negative isolates by confirmatory test: First, it may arise due to porin channel alterations and mutations<sup>27, 11</sup>. Second, cefoxitin-resistance phenotype in *E. coli* can result from over expression of the chromosomal AmpC gene due to mutations in the promoter and/or attenuator regions resulting in

alterations in the permeability of the cell to cefoxitin or a combination of all these factors<sup>39</sup>.

In this study, detection of AmpC enzymes by disc approximation test showed poor results as it was only able to detect AmpC production in one isolate (*E. cloacae*). This may be due to the fact that this organism possesses inducible chromosomal AmpC  $\beta$ -lactamases<sup>40</sup>.

In our study the percentage of confirmed AmpC producers were more among *K. pneumoniae* isolates (1/22; 4.5%) than *E. coli* isolates (3/77; 3.9%). Coudron et al.<sup>41</sup> and Yilmaz et al.<sup>42</sup> revealed similar observation where AmpC producing organisms were 1.6%, 10% among *K. pneumoniae* and 1.1%, 0.9% among *E. coli*, respectively. Whereas Pitout et al.<sup>43</sup> and Sridhar et al.<sup>25</sup> reported opposite observation where AmpC producing organisms were more among *E. coli* (9%, 5.2%) than *K. pneumoniae* (1.1%, 3.5%), respectively. isolate (*E. cloacae*). This may be due to the fact that this organism possesses inducible chromosomal AmpC  $\beta$ -lactamases<sup>40</sup>.

In our study one *E. coli* isolate (1/45; 2.2%) was positive for both ESBL and AmpC. It was positive for AmpC by the cefoxitin screening test and the confirmatory AmpC disc test, whereas this isolate was negative for ESBL by the combined disc diffusion confirmatory method and positive for ESBL by MDDST (in which FEP and TZP were utilized in approximation), implying that the use of this phenotypic method could overcome the masking effect of AmpC enzyme in case of co-production<sup>44</sup>. Similar observation has been reported by Khan et al.<sup>45</sup> and Sridhar et al.<sup>25</sup> as they reported 3%, 2.9%, respectively as positive for both ESBL and AmpC. This rate was comparable to other studies done in western parts of the world; (6%) in Spain<sup>36</sup> and (4.9%) in Minnesota in USA<sup>46</sup>.

Although MDDST is considered globally to be a reliable simple method for detection of ESBL and it is described mainly to detect masked ESBL in case of co-production of AmpC enzymes<sup>14</sup>, it failed to detect ESBL in all ESBL isolates. This may be explained by the fact that in the MDDST the distance between antibiotic discs is not standardized. This finding was also reported by Jabeen et al.<sup>47</sup>. So we recommend the implementation of both methods; combined double disc and MDDST in microbiology laboratories for better and accurate detection of ESBL.

The previous findings indicate that *E. coli* and *K. pneumoniae* producing AmpC  $\beta$ -lactamases and ESBLs has been increasingly reported worldwide<sup>45,32</sup>. This creates a requirement for laboratory testing methods that can accurately detect the presence of these enzymes in clinical isolates<sup>48,45</sup>.

In this study the phenotypic MAST D68C ESBL and AmpC detection set was used to detect the presence of ESBL and AmpC within the 45 isolates that were screened positive for ESBL. Among these isolates, 35



(77.8%) were ESBL producers, 2 (4.4%) were AmpC producers, 1 (2.2%) was positive for both ESBL and AmpC production, whereas 7 (15.6%) isolates were neither ESBL nor AmpC producers. This result was compared to a study done at Zagazig University Hospitals that used the same kit, they reported 65.8% were ESBL producers, 2.6% were AmpC producers, and 31.6% were neither ESBL nor AmpC producers<sup>33</sup>. Another study done by Lorenz et al.<sup>49</sup> reported that 87% were ESBL producers, 0.4% were AmpC producers, and 4% were neither ESBL nor AmpC producers.

In this study the performance of MAST D68C test in detection of ESBL and AmpC producers was assessed in relation to combined disc diffusion method and AmpC disc test as a reference method<sup>10,13</sup>. Regarding ESBL, MAST D68C test gave 97.2% sensitivity and 88.8% specificity, while in case of AmpC, MAST D68C test showed 60% sensitivity and 100% specificity. This result was compared to a study done at Zagazig University Hospitals using multiplex PCR as a reference method, MAST D68C test was of 92% sensitivity and 86.7% specificity for both ESBL and AmpC<sup>33</sup>. Another study in Australia evaluating MAST D68C test using multiplex PCR as a reference method reported (96%) sensitivity and (98%) specificity for both ESBL and AmpC<sup>50</sup>. Also Coyle et al.<sup>51</sup> assessed MAST D68C test using multiplex PCR as a reference method reported (83%) sensitivity and (100%) specificity for both ESBL and AmpC. MAST D68C test offers laboratories a simple, reliable and low cost means of identification and detection of ESBL and AmpC. The presence of an ESBL and/or AmpC is easily determined by zone size comparison when simultaneously tested with antibiotic and antibiotic plus inhibitor combinations. Furthermore, it allows the detection of ESBL and/or AmpC in one step. It also reduces the need for unnecessary confirmations which consume time and money. Moreover it is an excellent way to detect isolates co-producing ESBL and AmpC<sup>33</sup>.

In our study, the 45 isolates screened positive for ESBL were tested by different antibiotics as GM, AK, CIP, LVX, SXT and IPM. All isolates were sensitive to imipenem (100%). This was relatively in agreement with Kiffer et al.<sup>52</sup> and Ahmed et al.<sup>53</sup>, where they recorded susceptibility among their ESBL-producing isolates to imipenem of 99.1% and 100%; respectively.

In this study, our isolates showed high resistance (86.7%) to CIP and LVX which was comparable to a study done in Thailand by Thamlikitkul<sup>54</sup> who reported 90 % resistance to both CIP and LVX. However, a study done in Spain by Mata et al.<sup>36</sup> reported lower resistance rate to CIP and LVX (51.3% and 36.7%), respectively. This resistance may be due to the increased use of these agents for common infections as urinary and respiratory tract infections and the over counter availability of antibiotics for use without prescription.

In the current study, the 45 isolates that were screened positive for ESBL were tested for susceptibility to doripenem and tigecycline using the E-test. All of our studied isolates were susceptible to doripenem and tigecycline (100%), these results were in agreement with<sup>55,6</sup>.

In this study, temocillin and colistin were tested on the 45 isolates that were screened positive for ESBL by using E-test. Regarding temocillin, 32 (88.9%; 32/36) confirmed ESBL isolates were sensitive, 3 (8.3%; 3/36) were intermediately-sensitive and one (2.8%; 1/36) was resistant, while for confirmed AmpC isolates 3 (60%; 3/5) were sensitive, one (20%; 1/5) was intermediately-sensitive and one (20%; 1/5) was resistant. A study done by Livermore and Tulkens<sup>56</sup> reported 88 % sensitivity of temocillin to both ESBL and AmpC.

While for colistin, all confirmed AmpC isolates and 94.4% of confirmed ESBL isolates were sensitive to colistin. A study done by Warunee et al.<sup>57</sup> in Thailand reported 92 % sensitivity of ESBL-producing isolates to colistin. Another study done by Zohreh et al.<sup>9</sup> in Iran showed that colistin was 100 % sensitive to both ESBL and AmpC.

In conclusion, Mast D68C set appears as a promising method for the presumptive identification of ESBL- and AmpC-producing *Enterobacteriaceae* as well as isolates co-producing ESBL and AmpC. Tigecycline and doripenem as well as old reviving compounds as temocillin and colistin revealed excellent activity against all ESBL- and AmpC-producing *Enterobacteriaceae* and can be used as alternative drugs of choice to alleviate the burden on carbapenems.

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