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ORIGINAL ARTICLE

Prevalence of AmpC beta-lactamase and extended spectrum beta-lactamase co-producer in *Escherichia coli* and *Klebsiella* species in a teaching hospital

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Abstract

Introduction: Beta-lactamase producing bacterial infection has been on surge due to selection pressure and injudicious antibiotics usage. Organisms that co-produced more than one beta lactamase enzyme posed diagnostic challenges which may result in inadequate treatment. To date, there is no standardised guideline offering phenotypic detection of AmpC β-lactamase. The purpose of this study was to determine the prevalence of ESBLs, AmpC β-lactamase and co-producer organisms in a teaching hospital. Materials and Methods: Three hundred and four isolates of *E. coli* and *Klebsiella* sp. had been selected via convenient sampling. These isolates were identified using conventional laboratory methods and their antimicrobial susceptibilities were determined using disc diffusion method. Those isolates were then proceeded with ESBL confirmatory test, cloxacillin-containing Muller Hinton confirmatory test, modified double disk synergy test and AmpC disk test. Results: Out of 304 isolates, 159 isolates were *E. coli* and 145 were *Klebsiella* sp. The prevalence of organisms which co-produced AmpC β-lactamase and ESBL enzymes were 3.0%. Besides that, 39 cefoxitin resistant and three cefoxitin susceptible isolates (13.8%) were proven to produce AmpC β-lactamase through AmpC disk test. Through the CLSI confirmatory test, 252 (82.9%) isolates were identified as ESBLs producers and the prevalence increased slightly when cloxacillin-containing Muller Hinton were used. Only three ESBLs positive organisms were positive for modified double disk synergy test. Conclusion: Distinguishing between AmpC β-lactamase and ESBL-producing organisms has epidemiological significance as well as therapeutic importance. Moreover, AmpC β-lactamase and ESBLs co-producing organisms can lead to false negative ESBL confirmatory test. Therefore, knowing the local prevalence can guide the clinician in navigating the treatment.

Keywords: ESBLs, AmpC β-lactamase, *Escherichia coli*, *Klebsiella* sp., co-producing

INTRODUCTION

Beta-lactamases are enzymes produced by bacteria as a defense against the beta-lactam antibiotics in order to prevail. These enzymes are widely distributed among gram-negative and gram-positive bacteria and play its role by breaking the beta-lactam ring present in the structure of antibiotics. This results in resistant towards penicillin, cephalosporin, carbapenem and monobactam depending on types of beta-lactamases they produce.[1] First attempt to classify these enzymes was done by Ambler in 1991. According to Ambler molecular classification scheme, β-lactamases are classified into group A to group D based on their amino acid sequence.[2] After some time, Bush-Jacoby-Medeiros revamped the classification into four main groups (Group 1 to 4) with multiple subgroups based on their functional characteristics.[3] Later in 2009, Bush and Jacoby expanded and revised their classification depicting on hydrolytic and inhibitory profiles of key β-lactamases.[4]

AmpC β-lactamases was first discovered in *Escherichia coli* and classified into Class C under Ambler structural classification. Meanwhile, Bush and Jacoby assigned them

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into group 1.[4] Bacteria producing this enzyme are proven to have in-vitro resistance to penicillins, monobactams, all cephalosporins except cefepime and carbapenems. There are two types of AmpC β-lactamases which are plasmid-mediated (pAmpC) and chromosomally encoded inducible AmpC (cAmpC). Plasmid mediated AmpC β-lactamase are typically produced by *Escherichia coli*, *Klebsiella* sp, *Proteus mirabilis* and *Salmonella* sp.[5] Meanwhile organisms that have chromosomally encoded AmpC β-lactamases include *Enterobacter cloacae, Serratia marcescens*, *Citrobacter freundii* and *Morganella morganii*.[6] They are associated with multiple antibiotic resistance, leaving limited option of antibiotics choice.[7] Globally, CMY-2 is the most common AmpC gene responsible for the resistance mechanism.[8] To date, there is no recommended guideline by Clinical Laboratory Standard Institute (CLSI) on detection of AmpC β-lactamase.

Extended spectrum β-lactamases (ESBLs) were first reported in *Enterobacteriaceae* in 1983 and the plasmid from resistant strains were found to be transferable to other *Enterobacteriaceae*.[9] Essentially, ESBLs are mutant, plasmid-mediated β- lactamase that have the ability to hydrolyse all penicillins, oxyimino-cephalosporins and aztreonam but spare carbapenem and cephamycin, leading to treatment dilemma.[10] Nevertheless, it can be inhibited by clavulanic acid, sulbactam and tazobactam. Unflatteringly, ESBLs composed of many different enzymes family with multiple variants under each family. Naming a few would be CTX-M, TEM, SHV and OXA.[11] Accordingly, ESBLs fall under Group A for Ambler classification and Group 2be for Bush-Jacoby-Medeiros classification. These enzymes are rampantly found in gram negative bacteria especially *Enterobacteriaceae* and *Pseudomonas aeruginosa*.[12] The present study was to evaluate the prevalence of AmpC and extended spectrum β-lactamase (ESBL) co-producing strains of *E. coli* and *Klebsiella* sp. in a teaching hospital. At the same time, this study also determines the antimicrobial susceptibility pattern for AmpC β-lactamase producers, ESBLs and co-producers in both organisms of interest.

MATERIALS AND METHODS

Study design

This prospective cross-sectional study was carried out in bacteriology laboratory of Hospital Canselor Tuanku Muhriz over thirteen months from 31st January 2020 to 30th January 2021. Convenient sampling method was used in which isolates that fulfilled the inclusion criteria were selected. The study was ethically approved by University Kebangsaan Malaysia (JEP-2019-871; FF-2020-045). Inclusion criteria were all non-repetitive strains of *Escherichia coli* and *Klebsiella* sp. isolated from various clinical specimens that were positive for ESBLs screening. A total of 304 isolates were included in this study.

Study Procedure

Clinical isolates of *E. coli* and *Klebsiella* sp. recovered from clinical samples such as urine, pus, wound swab, vaginal swab, blood, body fluids, cerebrospinal fluid, tissue, sputum and tracheal aspirate during routine testing were processed. All isolates were identified using conventional laboratory methods and their antimicrobial susceptibilities were determined using disc diffusion method. There was no speciation done on *Klebsiella* sp as isolate from non-sterile site were identified using biochemical methods. Adopting Clinical & Laboratory Standard Institute (CLSI) guideline, isolates that were positive for ESBL screening with zone inhibition of ≤ 22mm for ceftazidime or ≤ 27mm for cefotaxime were included in this study. These isolates were further subjected to ESBL confirmatory test (Figure 1). The organism that showed increase zone of inhibition by 5 mm or more when clavulanic acid was added compared to cephalosporin alone were classified as ESBL producer and the remaining were labelled as non-ESBL producers.[13] Non-ESBL producers were subsequently subjected to another confirmatory test using cloxacillin-containing Mueller-Hinton agar (CT+CLO) with similar interpretation as confirmatory test for ESBL.[14] In modified double disk synergy test (MDDST) (Figure 2), ESBL producers were identified when isolates exhibited synergism between cefepime and tazobactam.[15] Subsequently, all isolates were subjected to cefoxitin susceptibility test and AmpC disc test to detect AmpC β-lactamases (Figure 3). Isolates with zone diameters less than 18mm were considered AmpC positive. Negative control used was *E coli* ATCC 25922 while *Klebsiella pneumonia* ATCC 700603 was used as positive control for ESBL.[16]

RESULTS

Of the 304 isolates included in this study, 159

FIG. 1: ESBL confirmatory test. ESBL production was inferred if the zones produced by the disks with clavulanate acid (CAL and CTL) were ≥5 mm larger than ceftazidime (CAZ) and/or cefotaxime (CTX) alone.

were identified as *Escherichia coli* and 145 as *Klebsiella* sp. from various clinical samples. The majority (37.8%) were isolates from blood while 107 (35.2%) isolates from urine, 31 (10.2%) isolates from respiratory sample, 28 (9.2%) from swab specimen, 11 (3.6%) from tissue and 12 (3.9%) from body fluid.

A total of 252 (82.9%) and 42 (13.8%) out of

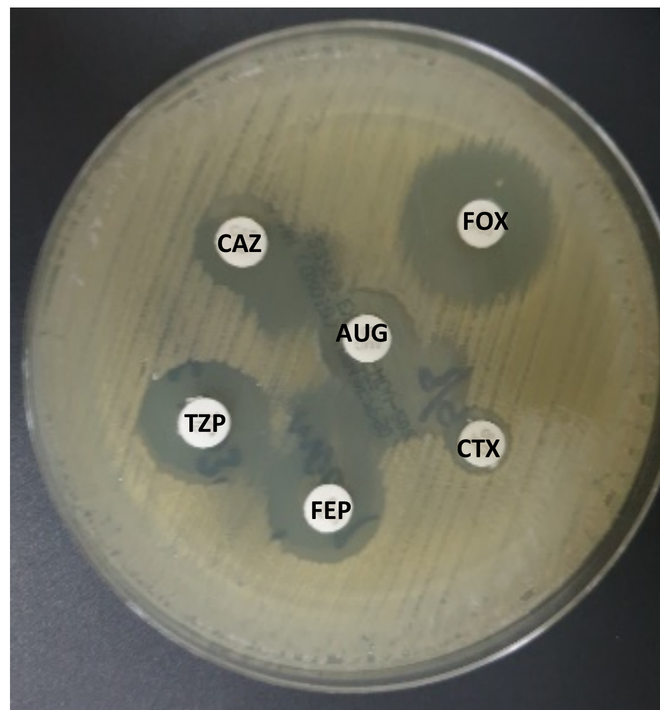

FIG. 2: Modified double disk synergy test (MDDST). ESBL producers were identified when isolates exhibited synergism between cefepime (FEP) and piperacillin/tazobactam (TZP).

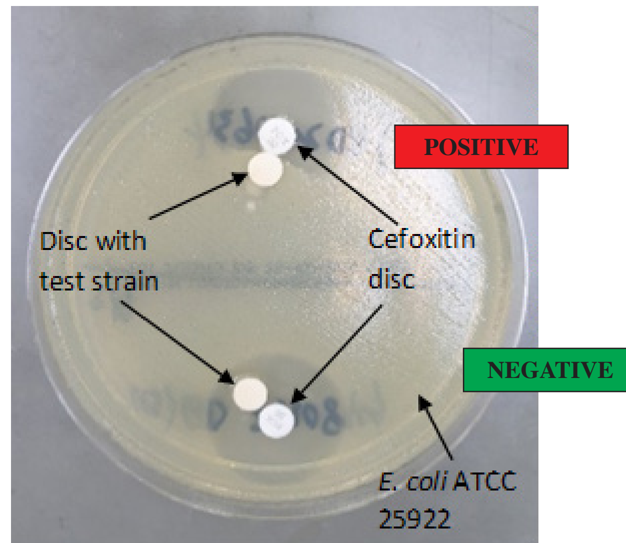

FIG. 3: AmpC disk test. Indentation or flattening of the cefoxitin zone of inhibition is seen in the vicinity of the disk with positive strain, while there is an undistorted zone of inhibition near the negative strain.

304 isolates were ESBL and AmpC producers respectively (Figure 4). The gender and sample type distributions for sole ESBL and AmpC beta-lactamase producers is shown in Figure 5 and Figure 6 respectively. Only nine isolates were identified to produce both enzymes (3.0%)

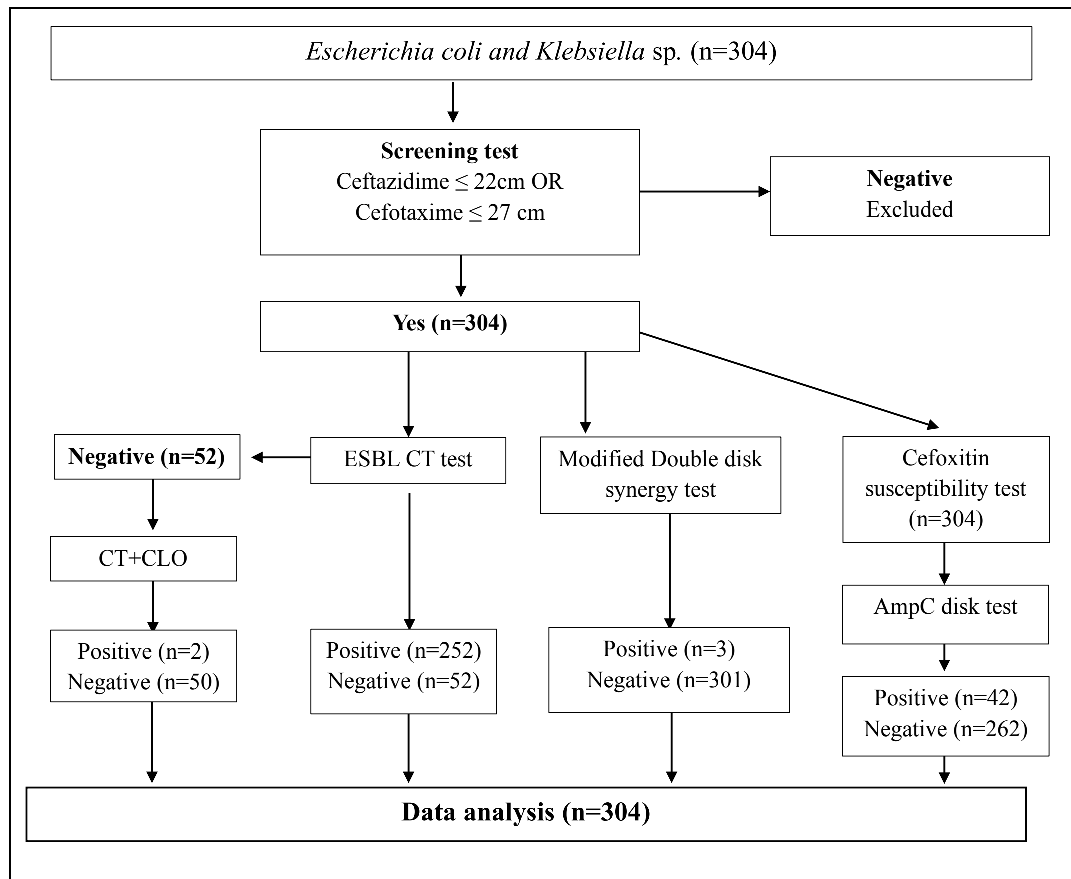

FIG. 4: Flow chart showing the results of different tests done on the isolates.

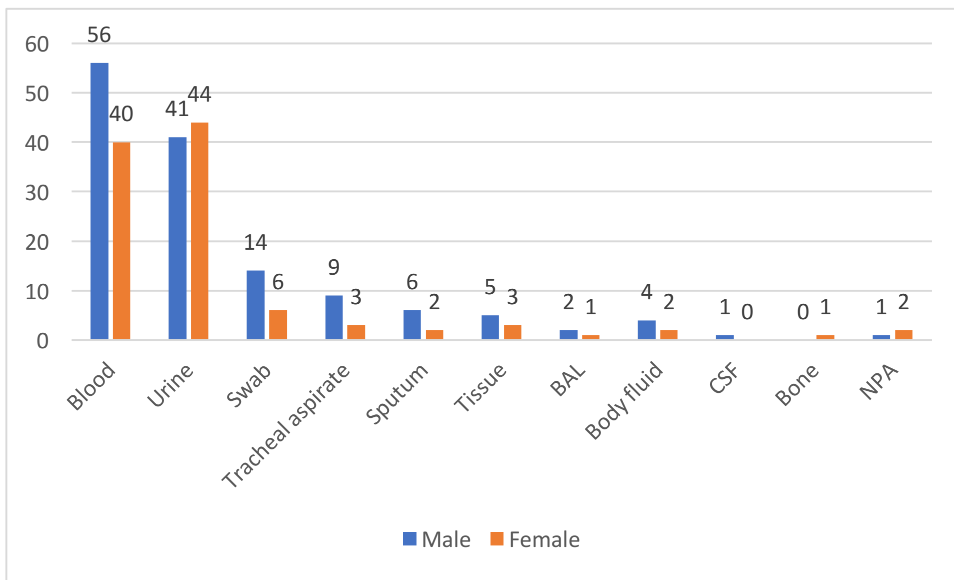

FIG. 5: Gender and sample type distributions for sole ESBL producers

(Table 1). Three of them were *E. coli* (1.0%) and the rest were *Klebsiella* sp (2.0%). All the ESBL producers were identified using CLSI confirmatory test while AmpC β-lactamase were identified using AmpC disk test. Nineteen isolates did not produce either AmpC β-lactamase or ESBL.

Table 2 shows the results of screening of AmpC β-lactamase using cefoxitin disk and presence of the enzyme by AmpC disk test. Out of 77 isolates that were non susceptible to cefoxitin, only 39 isolates were positive for AmpC disk test. Nevertheless, three cefoxitin susceptible isolates gave positive AmpC disk test resulting in total of 42 isolates positive for AmpC β-lactamase (13.8%).

Table 3 shows comparison between CLSI CT and MDDST in detecting ESBL enzymes. Using modified double disc synergy test (MDDST), three out of 252 confirmed ESBLs organisms exhibited positive phenotypic features, accounting for 1.2%. MDDST might not be a good option for phenotypic ESBL detection as the majority of the ESBL isolates could not

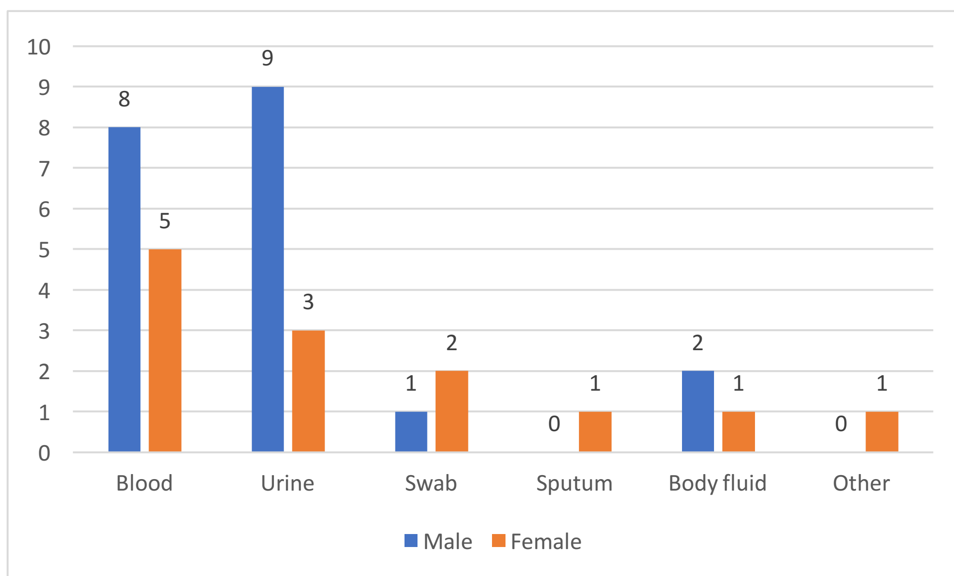

FIG. 6: Gender and sample type distributions for sole AmpC beta-lactamase producers

TABLE 1: Distribution of ESBL, AmpC and co-producer in E.coli and Klebsiella sp.

Organisms	ESBL only		AmpC only		Co-producer	
	Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)
E.coli	124	40.8	19	6.3	3	1.0
***Klebsiella* sp.**	119	39.1	14	4.6	6	2.0
Total	*243*	*79.9*	*33*	*10.9*	*9*	*3.0*

TABLE 2: Comparison of cefoxitin susceptibility and AmpC disk test

AmpC test / Cefoxitin <18mm	Positive		Negative		TOTAL
	Number	**Percentage (%)**	**Number**	**Percentage (%)**	
Positive	39	50.7	38	49.4	77
Negative	3	1.3	224	98.9	227
Total	*42*		262		304

TABLE 3: Comparison of percentage positivity for ESBL using CLSI CT and MDDST

Organism/Phenotype		MDDST positive	MDDST negative
E.coli	CT positive	2	125
	CT negative	0	32
Klebsiella sp.	CT positive	1	124
	CT negative	0	20
Total		**3**	**301**

reproduce positive result. However, none of the isolates with negative ESBL confirmatory test were shown to have positive MDDST. On top of that, only two out of three isolates were shown to co-produce AmpC β-lactamase enzyme. Therefore, guidelines offered by CLSI remained the best option for ESBL detection in lieu of molecular detection.

When ESBL confirmatory test for the 52 ESBL-negative isolates were repeated using cloxacillin-containing Muller Hinton agar, only two isolates were detected to be positive. Nevertheless, only the 252 isolates were considered as ESBL producers based on standardised guidelines.

The majority of the AmpC β-lactamase producer were resistant to penicillins, second and third generations cephalosporins but susceptible to fourth generation cephalosporin, cefepime in vitro (Figure 8). Similarly, all ESBLs were resistant to penicillin and oxyimino-cephalosporins but susceptible to carbapenem group (Figure 9). Although 67.9% ESBL isolates were susceptible to piperacillin-tazobactam, caution must be taken when piperacillin-tazobactam is chosen as explained later under Discussion.

DISCUSSION

Many clinical microbiologists appear to be unaware of the presence of plasmid mediated AmpC β-lactamase enzymes in resistant isolates due to absence of standard method of detection to date. Without knowing the presence of this hydrolysing enzyme, clinicians may prescribe susceptible looking cephalosporins only to meet treatment failure.[17] To make matters complicated, phenotypic confirmation of ESBLs may appear to be false negative when the organism co-produce AmpC β-lactamase as the latter enzyme is weakly inhibited by clavulanate.[18] For epidemiological and infection control purpose, detection of AmpC and ESBL are crucial to determine disease

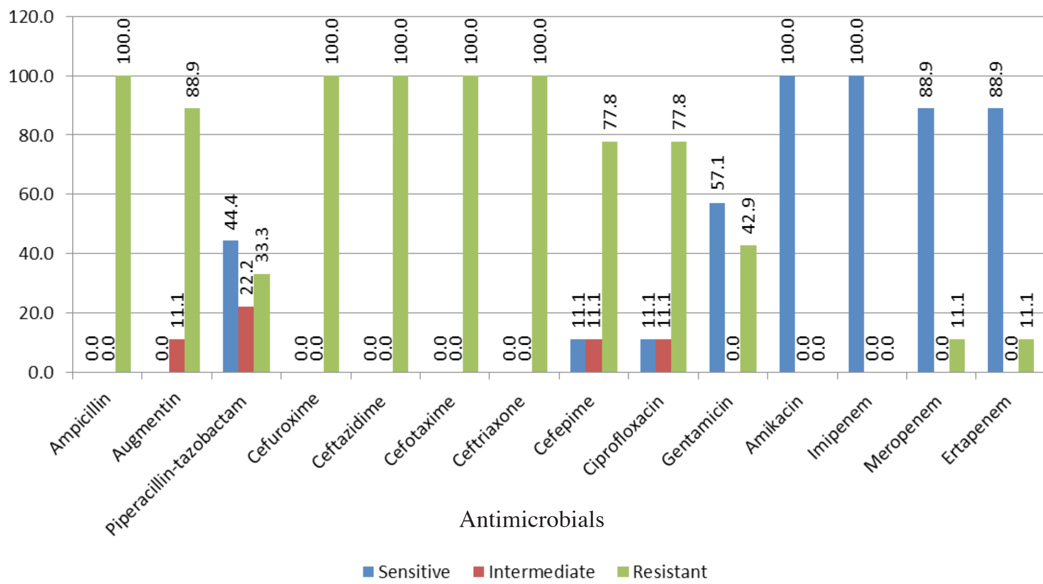

FIG. 7: Antimicrobial susceptibility profile of ESBL and AmpC β-lactamases co-producing isolates.

burden. In addition, there is lack of local data on the prevalence of ESBL and AmpC β-lactamase leading to treatment dilemma.

Our study discovered that 3.0% (9/304) ESBLs-screened positive organisms were ESBLs and AmpC β-lactamase co-producers. This finding was comparable to findings in Egypt of those patient who have urinary tract infection (3.8%).[8] In other part of the world, study done by tertiary hospital in India recorded as high as 11.9%.[19] Similarly, Iran also reported 30% of cefoxitin-resistant isolates simultaneously exhibit ESBL and AmpC β-lactamase activity. On another note, north Lebanon reported 2.8% carriage rate of ESBLs and AmpC β-lactamase co-producer among nursing home residents.[20]

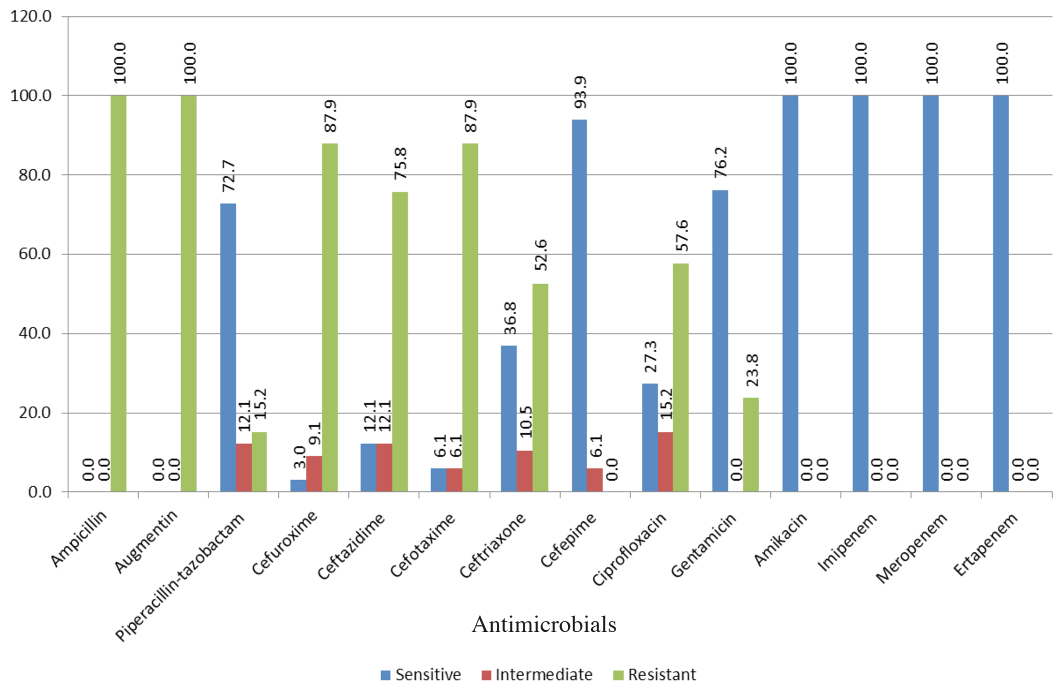

FIG. 8: Antimicrobial susceptibility profiles of AmpC β-lactamase producers.

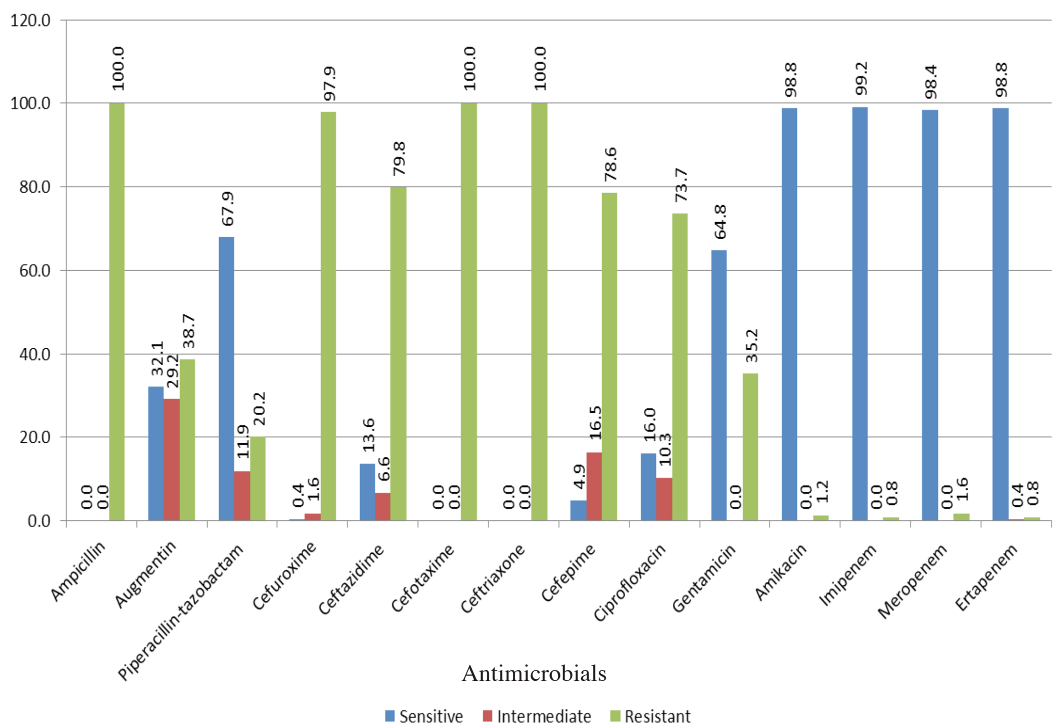

FIG. 9: Antimicrobial susceptibility profiles of ESBL producers.

A total of 252 (82.9%) isolates in this study were ESBL producers. This finding was comparable to previous study whereby 86.5% of ESBL-screened positive *E. coli* were ESBL producers.[21] However, other local study showed only 54% ESBL producers from presumptive ESBL based on phenotypic study.[22]

In this study, seventy-seven out of 304 isolates were suspected to produce AmpC enzyme based on cefoxitin disc screening. Among the seventy-seven cefoxitin-resistant isolates, 39 isolates (50.6%) were shown to display the phenotypic detection of AmpC β-lactamase enzyme. In Egypt, 51.4% cefoxitin-resistant isolates were reported to harbour AmpC genes which were detected through multiplex PCR[8]. Additionally, three cefoxitin-susceptible isolates also appeared to be positive for AmpC enzyme resulting in total 42 out of 304 (13.8%) isolates positive for AmpC β-lactamase enzymes. In 2006, Deshpande et al reported up to 26.2% ESBL screen positive *E. coli* isolated from 30 North American medical centres harboured AmpC enzyme.[23]

The positive screening yet negative AmpC test can be attributable to other mechanism such as efflux and porin loss.[24] Not all AmpC β-lactamase producing enzyme will be resistant to cefoxitin. Novel enzyme ACC-1 can be exceptionally susceptible to cefoxitin in vitro and this enzyme possibly present in the three cefoxitin susceptible but positive AmpC disk test isolates.[25] In addition, AmpC disk test can help to distinguish cefoxitin insusceptibility secondary to β-lactamase enzyme from other mechanism such as porin mutation. Nevertheless, AmpC disk test could not differentiate plasmid mediated AmpC enzyme from upregulated chromosomally mediated AmpC β-lactamase for *E. coli*.[16]

The effect of concurrent ESBL and AmpC gene expression may adversely affect the performance of current ESBL screening and confirmatory testing, as the two enzyme groups have overlapping hydrolysis spectra, except that AmpC enzymes are not inhibited by clavulanate, sulbactam, or tazobactam. On the other hand, ESBLs can be inhibited by clavulanate but unable to hydrolyse cephamycin. Two cephalosporins are used in ESBLs confirmatory test because some ESBLs are best detected with ceftazidime and others with cefotaxime, for instance CTX-M enzymes.[26] Furthermore, certain OXA type ESBLs are poorly inhibited by clavulanate, giving false negative result.[27]

Modified double disc synergy (MDDST) test as its name implies was modified to increase the sensitivity of ESBLs detection in isolates that co-produce AmpC β-lactamase. The modifications employed the use of 4th generation cephalosporin

(cefepime) and piperacillin-tazobactam. It also emphasised on the optimum distance between the cefepime and clavulanate in which shorter distance improve the sensitivity rate.[28] Cefepime is stable to high level AmpC β-lactamase and tazobactam is less likely to induce AmpC enzyme, therefore it will not interfere with ESBLs interpretation. This test was also found to have high specificity of up to 100%.[29] Despite all these promising findings, our study could not reproduce the same outcome. Most of the time, the zone of inhibition for piperacillin-tazobactam was wide enough and causing it to overlap with the zone of inhibition of cefepime. Moreover, the synergistic effect is subjective to observer and therefore could be easily misinterpreted by different users. The optimum distance is yet to be determined hence becomes one of the deterrence factors for it to become standard test.

Addition of cloxacillin into MHA has been shown to increase the sensitivity of ESBLs detection by Garrec *et al*.[30] Cloxacillin has the ability to inhibit AmpC β-lactamase enzyme and hence presumably able to increase the sensitivity of ESBLs detection. However, some researchers refute the findings and claim that the method only increases the specificity but not the sensitivity.[31] This finding was echoed by Kałużna *et al*. in 2014.[32] In this present study, cloxacillin-supplemented MHA noticeably increase the zone of inhibition when compared to MHA alone. There were two isolates that was positive for ESBLs on cloxacillin-supplemented MHA but not on regular MHA, however both isolates did not produce AmpC β-lactamase. The fact that some strains fail to grow on this medium further restrict its use as standard test. Nevertheless, only positive CLSI confirmatory test isolates were considered as ESBL producers in this study.

In this study, 44% *E. coli* and 23% *Klebsiella* sp. ESBLs isolates were susceptible to piperacillin-tazobactam (TZP) in-vitro. A study showed that TZP may be an effective treatment for urinary tract infection as this drug is highly concentrated in urine compared to plasma.[33] This is further supported by Spanish group findings that patient with urinary source of ESBLs infection had favourable outcome with TZP treatment, irrespective of TZP MIC.[34] Nevertheless, it is strongly recommended to use carbapenem for ESBLs treatment as carbapenem was shown to have lower mortality compared to TZP treatment.[35] In essence, carbapenem remains the "gold standard" for the treatment of infections caused by ESBL-producing organisms.

In term of non-beta-lactam drugs, it is worth to note that these organisms showed highest susceptibilities towards aminoglycoside particularly amikacin. However, only half of the isolates demonstrate susceptibilities towards ciprofloxacin. This antibiogram pattern appear to have similar findings reported by Rai *et al*.[36] Different mechanism of resistance plus the frequency of antibiotics usage might contribute to these findings. Single dose aminoglycosides can be an alternative when come to uncomplicated cystitis. Meanwhile, fluoroquinolones can be oral step down for both ESBL and AmpC beta-lactamase producer provided susceptibility to this antibiotic is confirmed.

Having positive ESBLs screening alone is sufficient to report the isolates as resistant to extended spectrum cephalosporins. This suggestion was preceded by finding of high percentage of isolates displayed negative ESBL confirmatory test despite harbouring beta-lactamase gene.[37] In fact, this approach has been since adopted by Clinical and Laboratory Standard Institute who stopped recommending routine ESBLs confirmatory test unless for infection control purpose. Nevertheless, this recommendation must be taken as pinch of salt as some clinicians might treat patient with antimicrobials that appear to be susceptible in-vitro when the status of ESBLs is unknown. This will then lead to unfavourable outcome, hence the need to detect the ESBLs enzyme production.[38] Another dilemma that are faced by clinician are false negative confirmatory test making the risk of over treating or under treating inevitable. It is indeed a tough call for clinician as most laboratories do not offer genotypic testing for resistant organisms.

It is important to keep in mind that genotypic resistance gene does not necessarily equate to gene expression, hence enzyme production.[39] In regards with that, IDSA guidelines has classified *Enterobacterales* into moderate to high risk and low risk organism, recommending the best treatment options depending on the risk.[40] As many laboratories have transitioned antimicrobial susceptibilities tests into analyser-generated MIC, it is important to alert on the possibilities of the AmpC beta-lactamase producer organism by screening the cefoxitin susceptibility test. Subsequently, AmpC disk test can be performed to deduce the presence of this enzyme as automated MIC reading is unable to do so. Therefore, disk based phenotypic testing

is still useful when need arise in order to choose right antimicrobials.

CONCLUSION

Distinguishing between AmpC β-lactamase and ESBL-producing organisms has epidemiological significance and has therapeutic importance as well. Essentially, genotypic confirmation of beta-lactamase is the most favourable method but not economical and feasible in all settings. Hence, knowing the local prevalence can guide the clinician in terms of treatment. AmpC disk test has been a great complement in detecting AmpC beta-lactamase, but this method has not been standardized into any guideline, making it not widely used. Since none of the mentioned tests are infallible, clinical correlation and judgment still precede the laboratory result.

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Authors' contributions: All authors contributed equally.

Conflicts of interest: All authors have no conflicts of interest to declare.

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