Comparison of Five Methods for Detection of Extended Spectrum β -Lactamases in Gram Negative Enteric Bacteria

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Abstract

The presence of extended-spectrum β -lactamases in 55 isolates of Gram negative enteric bacteria isolated from lower respiratory tract infections, was investigated by using the Clinical Laboratory Standards Institute CLSI method which showed that 41.8% of the isolates produced this type of β -lactamases, and that *Klebsiella pneumoniae* isolates were the most producing species with a production rate of 61.1%, followed by *Escherichia coli* isolates 43.75%. Five confirmatory methods were used to detect these enzymes: ceftazidime agar method, double-disk synergy method, combination disk method, modified 3D extract method and enzymatic disks method. The study indicated that ceftazidime agar method was the best method in detecting extended-spectrum β -lactamases as it gave a detection rate of 95.7%, followed by the double-disk synergy method with a rate of 87%, then enzymatic disks method with a rate of 73.9%.

Keywords

Extended spectrum β -lactamases, Detection methods, Gram negative bacteria

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1. Introduction

Gram negative enteric bacteria represented by *Enterobacteriaceae* and *Pseudomonas aeruginosa* are one of the most antibiotic-resistant bacteria, especially to β -lactams [1,2]. As these species are opportunistic pathogens and common causes of hospital-acquired infections, their possession to such resistance to various β -lactams including broad spectrum ones increases their virulence and resistance to treatment, which makes infections caused by these bacteria more serious and sometimes life-threatening [3–5].

Bacterial species resist β -lactam antibiotics using different mechanisms, but the most common, important and widespread mechanism are β -lactamase enzymes, which act by opening the β -lactam ring necessary for the effectiveness of the antibiotics, thus posing a serious threat to the use of these antibiotics in treating infections caused by the bacteria producing these enzymes, particularly Gram-negative enteric bacilli [6–10].

Extended-spectrum β -lactamases ESBLs (serine enzymes of molecular class A) are the most common types of these enzymes, and are characterized by their ability to hydrolyze the third generation cephalosporins as well as the penicillins and early cephalosporins, but they remain sensitive to cephamycins, and they inhibited by clavulanic acid, which is often used in the detection of ESBL-producing bacteria [11–15].

The importance of these enzymes increases with the increase of their number and the persistence of the emergence of new types of them, noteworthy plasmid-mediated ESBLs has contributed to the widespread dissemination of ESBLs among different bacterial species all over the world [16–18]. As well, the large number of β -lactam drugs and the uncontrolled usage of them often assist in the selection of resistant bacterial strains under the influence of selective pressure [19–21].

Therefore, the present study aimed to investigate the presence of extended-spectrum β -lactamase enzymes in some Gram-negative bacterial species in more than one method and determine the most efficient method or methods.

2. Materials and methods

2.1. Bacterial isolates

Fifty five clinical bacterial isolates of Gram negative enteric bacteria isolated from lower respiratory

tract infections in Mosul city were used. Isolation and identification of isolates were done according to their morphology, Gram stain, cultural and biochemical characteristics [22,23]. Table 1 shows the bacterial species used and their numbers.

2.2. Preparation of crude enzymatic extracts

Fresh bacterial suspensions were prepared in trypton soy broth and incubated for 4 h at 35 °C. The cells were precipitated using a cooled centrifuge at a speed of 19470 g for 15 min and washed three-times with phosphate buffer saline. The precipitant was suspended in a solution of 0.2 M Sodium acetate (pH 5.5) and subjected to seven cycles of freezing and thawing [24–26]. The supernatant was obtained using a cooled centrifuge (19470 g) for 30 min. The extracts were kept at a temperature of –20 °C until they were used in the detection tests.

2.3. Detection of ESBLs using CLSI method

2.3.1. Initial screening test

Bacterial suspensions equivalent in turbidity to tube No. 0.5 of McFarland standards were prepared, inoculated on Mueller-Hinton agar as recommended by CLSI. Cefpodoxime, ceftriaxone, cefotaxime ceftazidime, and aztreonam disks were distributed on the surface of the agar, and incubated at 35 °C for 18-16 h. After the incubation period, the diameter of the inhibition zones were measured and compared with the CLSI table for this test. The isolates that gave a positive result for this test were confirmed by the phenotypic confirmatory test [27].

Table 1
Bacterial species used in the study.

Bacterial species	No. of isolates
<i>Klebsiella pneumoniae</i>	18
<i>Escherichia coli</i>	16
<i>Pseudomonas aeruginosa</i>	14
<i>Serratia marcescens</i>	2
<i>Enterobacter aerogenes</i>	3
<i>Enterobacter cloacae</i>	1
<i>Proteus mirabilis</i>	1
Total	55

2.3.2. Phenotypic confirmatory test

The test was performed as recommended by CLSI. Mueller-Hinton agar plates were inoculated with fresh bacterial suspensions, and the following disks were distributed: ceftazidime, ceftazidime/clavulanic acid, cefotaxime, cefotaxime/clavulanic acid. After incubation at 35 °C for 18–16 h, the diameter of the inhibition zones was measured. An increase in the diameter of the inhibition zone (5 mm and more) in the case of antibiotic with clavulanic acid compared to its diameter in the case of antibiotic alone was considered as an indication of the presence of ESBLs [27–29].

2.4. Double disk synergy method

In this method, amoxicillin-clavulanic acid disk was placed in the center of inoculated Mueller-Hinton agar plates, Cefotaxime and Ceftazidime disks were placed 20–30 mm from the central disk. The plates were incubated at 35 °C for 18–16 h. The expansion of the inhibition zone towards amoxicillin-clavulanic acid disk was considered as evidence of ESBLs presence [30,31].

2.5. The combination disk method

Cefpodoxime 10 µg/disk was used in this method. The diameter of the inhibition zone given by this antibiotic is compared with that given by the same antimicrobial plus clavulanic acid at a concentration of 1 µg/disk. Bacterial isolate will be considered as a producer of ESBLs if the diameter of the inhibition zone in the presence of clavulanic acid has an increase equal to or greater than 5 mm than in its absence [32–34]. Because of the absence of ready-made disks containing cefpodoxime with clavulanic acid, a modification to the method was made by placing an amoxicillin-clavulanic acid disk, as a source of clavulanic acid, 20–30 mm from cefpodoxime disk, plates were incubated at 35 °C for 16–18 h, after which zone expansion was detected.

2.6. Modified three dimensional extract method

Mueller-Hinton agar plate was inoculated with ceftazidime-sensitive isolate, and then a ceftazidime disk was placed in the center of the plate. Four slits were made in the agar using a sterile scalpel starting at a distance of 5 mm from the edge of the disc toward the edge of the plate. Thirty microliters of crude enzymatic extracts were added to each slit, plates were incubated at 35 °C for 16–18 h. The emergence of

growth in the region where the slit intersects with the inhibition zone is an affirmative evidence of the occurrence of ESBLs in the isolate [24,35,36].

2.7. Ceftazidime agar method

Ceftazidime was added to Mueller-Hinton agar with a concentration of 4 µg/ml. Plates were inoculated with ceftazidime-sensitive isolate, after that wells were made with a diameter of 5 mm, to which 30 µl \ well of the crude enzymatic extracts were added. Plates were incubated at 35 °C for 16–18 h. The growth of the sensitive isolate around any of the wells is evidence of the presence of ESBLs in the enzymatic extract [6,37].

2.8. Enzymatic disks method

Mueller-Hinton agar plate was inoculated with a ceftazidime-sensitive isolate, and then a ceftazidime disk was placed in the center of the plate surrounded by disks with crude enzymatic extracts at a distance of 20–25 mm from the central disk. Plates were incubated at 35 °C for 16–18 h. The result was recorded by the emergence of growth around the enzymatic extract disks within the inhibition zone, which was considered as an evidence of the presence of ESBLs (ceftazidimases), which allowed the sensitive isolate to grow.

3. Results

3.1. Detection of ESBLs using CLSI method

Extended spectrum β-lactamases were detected according to the CLSI recommendations where initial screening test and phenotypic confirmatory test were performed (Fig. 1). Out of 55 clinical isolates 23 (41.8%) gave positive result. *Klebsiella pneumoniae* isolates had the highest percentage, while other species varied in possessing these enzymes and some gave negative results (Table 2).

3.2. Detection of ESBLs using confirmatory methods

In the present study, five additional methods were used to confirm the results of CLSI method in detecting ESBLs production in Gram negative enteric bacteria. These tests were performed on 23 isolates that gave positive results for the CLSI method (Table 2).

Table 3 shows the results of these tests in comparison to CLSI method, where ceftazidime agar method gave the best detection percentage (95.7%) in

Fig. 1. Detection of ESBLs using CLSI method. Right plate: Initial screening test. Disks from above clockwise: Aztreonam, Ceftriaxone, Cefpodoxime, Cefotaxime. Left plate: Phenotypic confirmatory test. Disks from above left clockwise: Ceftazidime, Ceftazidime/Clavulanic acid, Cefotaxime, Cefotaxime/Clavulanic acid.

Table 2
ESBLs production percentages using CLSI method.

Bacterial species	Number of tested isolates	Positive isolates No. (%)
<i>K.pneumoniae</i>	18	11 (61.1)
<i>E.coli</i>	16	7 (43.75)
<i>P.aeruginosa</i>	14	4 (28.6)
<i>S.marcescens</i>	2	1 (50)
<i>E.aerogenes</i>	3	0 (0)
<i>E.cloacae</i>	1	0 (0)
<i>Pr.mirabilis</i>	1	0 (0)
Total	55	23 (41.8) ^a

^a Positive isolates were selected for confirmatory detection methods.

comparison to CLSI method, followed by double disk synergy method (87%), and enzymatic disks method (73.9%), while both the combination disk method and the modified three-dimensional extract method had the lowest percentage (56.5% for each) as depicted in Figs. 2–5.

Table 3
ESBLs production percentages using confirmatory methods.

Bacterial species	Isolates tested	Positive isolates NO. (%)					
		CLSI method	Double disk synergy method	Combination disk method	Modified 3D extract method	Ceftazidime agar method	Enzymatic disk method
<i>P.aeruginosa</i>	4	4 (100)	3 (75)	1 (25)	2 (50)	3 (75)	2 (50)
<i>K.pneumoniae</i>	11	11 (100)	10 (90.9)	7 (63.6)	6 (54.6)	11 (100)	9 (81.8)
<i>E.coli</i>	7	7 (100)	6 (85.7)	5 (71.4)	5 (71.4)	7 (100)	6 (85.7)
<i>S.marcescens</i>	1	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)
Total	23	23 (100)	20 (87)	13 (56.5)	13 (56.5)	22 (95.7)	17 (73.9)

4. Discussion

Most of the Gram-negative enteric bacteria have at least one of the β -lactamases that protects them from the lethal action of β -lactam antibiotics. Some of these bacteria may have several types of these enzymes that differ in their properties and spectrum of antimicrobials they work on, which give them an advantage over other bacteria [16,18].

Extended spectrum β -lactamases are major group of β -lactamases that are responsible for the worldwide bacterial resistance to β -lactam antibiotics especially in Gram negative enteric bacteria, which makes the detection of these enzymes of significant importance for proper treatment to achieve and bacterial resistance to defeat [1,21]. Therefore, our study concentrated on the detection methods developed and used for screening bacterial isolates for ESBLs production in order to investigate their efficiency and determine the best method in comparison to the CLSI method.

Fig. 2. Detection of ESBLs using double disk synergy method.

We found that out of 55 isolates tested by CLSI method 23 (41.8%) gave positive results, and that *K. pneumoniae* isolates had the highest percentage, followed by *Escherichia coli*, *P. aeruginosa*, *Serratia marcescens* (see Table 2). These results indicated the high production of ESBLs enzymes by these species, which are in agreement with many studies and researches including CLSI documents [6–8,10,27,38], which clearly stated the importance and significance of these enzymes as they represent a real threat to infection treatment with β -lactams, especially if we consider that these enzymes almost been plasmid-mediated with high dissemination capabilities to other species.

Fig. 4. Detection of ESBLs using modified 3D extract method.

Extended-spectrum β -lactamase production rates vary between bacterial species as well as between different countries. Also, the antibiotics amount used plays an important role in determining these rates and selecting resistant strains, as well as the occurrence of R-plasmids. It is crucial to prescribe β -lactam antibiotics (especially new ones) only after confirming the sensitivity of the pathogenic bacteria in order to reduce the spread of resistant strains, with the importance of testing bacteria for ESBLs production and not just for traditional sensitivity tests, where CLSI recommendations indicate that ESBLs-positive isolates must be recorded as resistant to all third generation cephalosporins, as well as for aztreonam, and it is not recommended to give these antibiotics as a treatment

Fig. 3. Detection of ESBLs using combination disk method.

Fig. 5. Detection of ESBLs using ceftazidime agar method.

regardless of the results of traditional sensitivity tests [16,27].

It is noteworthy that some bacterial isolates may have several enzymes of more than one type of β -lactamases, these enzymes may differ from each other in their inhibitory properties and substrate profile [10,18,39]. Thus, when conducting detection tests, the properties of some of these enzymes may mask others so that they block them and give false negative results or vice versa, as is the case with AmpC enzymes and ESBL enzymes where AmpC enzymes, unlike ESBLs, are not inhibited by clavulanic acid but when both types are present in the same isolate, the isolate may not appear to be sensitive to clavulanic acid due to the presence of AmpC enzyme and thought to be non ESBLs producer, and that what Yang et al. [40] mentioned in their research where they reported that some *K. pneumoniae* strains had four enzymes, others had three enzymes, and others contained only one enzyme, and found that some of these enzymes were AmpC and others were ESBLs.

Detecting the types of β -lactamase is necessary and essential for identifying the bacteria that possess these enzymes, controlling them and preventing or reducing their spread [16,21]. However, this process is not that easy because of the overlap of many factors and the abundance of enzymatic types. Many methods have been developed for the detection of these enzymes, although many have proven effective, yet no method have been developed to detect the types of these enzymes in different bacterial species with good efficiency.

In the current study, five methods have been used to detect and confirm the presence of ESBLs in Gram negative enteric bacteria. Detection tests were performed on 23 isolates that gave a positive detection result using CLSI method. Table 3 shows the results of these confirmatory tests in comparison to CLSI method, where they varied in detection rates they gave; ceftazidime agar method was the most efficient method followed by the double disk synergy method and enzymatic disks method, while the combination disk method and the modified 3D extract method had a lower efficiency of detecting these enzymes.

Ceftazidime agar method was not included in the published methods for detecting ESBLs enzymes as far as we were aware, but we relied on the principle of the cefoxitin agar method introduced by Nasim et al. [37] to detect AmpC β -lactamases, and we modified it to be used to detect ESBLs instead of AmpC enzymes. This method has been shown to be efficient in detecting ESBLs compared to other methods. We think that the

crude enzymatic extracts act by hydrolyzing ceftazidime in the media, allowing the sensitive isolate to grow around the well, which is considered as an evidence for the presence of ESBLs enzymes and that it is the mechanism used in the resistance.

For more certainty, the same method was used with amoxicillin-clavulanic acid instead of ceftazidime, where clavulanic acid inhibits ESBLs enzymes, if present, in the enzymatic extracts and therefore the sensitive isolate does not grow. Growth appearance suggests the presence of enzymes that are not affected by clavulanic acid such as AmpC enzymes or inhibitor-resistant enzymes which hydrolyze amoxicillin and allow the sensitive isolate to grow [15,34]. Although this method is time-consuming and precision-requesting, it has proved its efficiency in detecting ESBLs enzymes compared with other methods, as well as the usage of crude enzymatic extracts which excludes other resistance mechanisms like impermeability or modifications in penicillin-binding proteins, and restricts it to β -lactamase enzymes, and this is what distinguishes these methods from others.

The results also revealed the efficiency of double disk synergy method where it gave a detection rate of 87% (Table 3). This method is one of the most widely used methods in clinical laboratories to detect ESBLs enzymes for its simplicity and ease of performing [32,35]. However, it may sometimes give false results; the distance between the two disks is of great importance, where Sanders et al., 1996 [30] indicated that the distance between the disks should be 30 mm, while Livermore and Brown, 2001 [34] reported that the distance between the disks should be between 25 and 30 mm, this distance is so critical because it should allow the antibiotic to diffuse to reach clavulanic acid in order synergism to occur between them, which leads to the expansion of inhibition zone to give the positive result, if the distance is too large to allow such synergism to occur, the expansion does not occur and the result is negative despite the presence of ESBLs enzymes.

Enzymatic disks method is based on the same principle as ceftazidime agar method, with the use of disks instead of wells to contain the enzymatic extracts. It also proved to be of good efficiency, although less than that of ceftazidime agar method, possibly because the amount of extract taken by the disk is less than that of the well. The combination disk method detected ESBLs-producing isolates with a rate of 56.5% (Table 3), this method relies on the use of cefpodoxime instead of ceftazidime or cefotaxime. Some studies have indicated that this antibiotic has a higher

sensitivity to detect ESBL enzymes; the researchers Carter et al. [32]; Livermore and Brown [34], for instance, reported that this method has detected 100% of ESBL-producing *K.pneumoniae*. The results of the present study are not entirely consistent with that, we found that this method has detected ESBL-producing *K. pneumoniae* by 63.6% as shown in Table (3), and this may be due to the difference in the disks used. We used amoxicillin-clavulanic acid as a source of clavulanic acid instead of the cefpodoxime-clavulanic acid disk, because of the lack of the latter.

The modified 3D extract method gave a similar detection rate to that of the combination disk method (56.5%). This method needs precision when performing and when results are read and interpreted, Thomson and Sanders [35] reported that a slight distortion in the inhibition zone at the intersection of the slits is a positive result, as the enzymatic breakdown of the antibiotic depends on the amount of the enzyme present in the extract and the amount added, and should be added accurately so that the quantity does not exceed the size of the slit. In addition, some bacteria may produce the enzymes in small quantities that they are not enough to breakdown the antibiotic to give a positive result, especially in this method where the extracts are added in the slits which represent more area than the wells where the enzymes are concentrated in less area so its effect appears even if its concentration is low.

5. Conclusion

ESBLs occurrence in Gram negative enteric bacteria was detected using CLSI method. Five confirmatory detection methods were further used, in comparison to CLSI method, in order to determine the most efficient method. The present study found that ceftazidime agar method is more efficient method than the others, followed by double disk synergy method and enzymatic disk method. The results also indicated the efficiency of cell-free enzymatic extracts dependent methods in detecting ESBLs.

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