Comparative Study of Two Beta - Lactamase Test Methods in Five Different Microorganisms Isolated from Azadi Hospital in Kirkuk , Iraq

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Abstract- Infections caused by beta lactam antibiotic resistant bacteria are common in hospitals and community and continue to be a major cause of morbidity and mortality worldwide. Hence, rapid laboratory diagnosis is necessarry to counter the effect of these bacteria. Several tests have been developed for screening for beta lactamases bacteria. The aim of the present study was to compare the validity of two tests used for this purpose. *Staphylococcus aureus*, *Streptococcuspyogenes*, *Bacillus subtilis*, *E.coli* and *Klebsiellapneumoniae* were isolated from different clinical samples from Azadi teaching hospital, Kirkuk, Iraq and screened for beta lactamases production by antibiotic disk diffusion test and iodemetric test. Both tests seem to have same reliability when screening for penicillinase producing bacteria. However, when cephalosporin resistance is concerned, antibiotic disk diffusion test is recommended.

Index Terms- Beta lactamases screening, antibiotic disk diffusion test, iodemetric test, Azadi Teaching Hospital.

I. INTRODUCTION

Infections caused by beta lactam antibiotic resistant bacteria are common in hospitals and community and continue to be a major cause of morbidity and mortality worldwide (Alekshun and Levy, 2007). Beta lactam antibiotics such as penicillins and cephalosporins are the most frequently prescribed antimicrobial agents in treating gram positive and gram negative infections in human medicine (Bradford, 2001). Extended Specturum beta lactamases (ESBLs) are beta-lactamases produced by some bacteria and have the ability to hydrolyze beta lactam in penicillins and cephalosporins with an oxyamino side chain such as ceftriaxone, cefotaxime and ceftazidime. ESBL producing bacteria may look susceptible to some extended spectrum cephalosporins, however, treatment with such antibiotics has been associated with high failure rate. A broader set of beta-lactam antibiotics such as the oxyimino monobactam aztereonam are also susceptible to hydrolysis by these enzymes. Therefore, antibiotic options in the treatment of ESBLs producing bacteria are extremely narrow. Carbapenems are the treatment ofchoice for serious infections due to ESBL producing bacteria, although carbapenem resistant isolates have also recently been reported. One approach to counter bacterial beta-lactamases is the delivery of a combination of a beta-lactamase inhibitor with beta-lactam antibiotic. Example of such inhibitors are clavulanic acid, sulbactam and tazobactam (Maiti *et al*.,1998). These inhibitors are of biological origin so they are nontoxic. Although these compound have only weaken antibacterial properties; they are potent inhibitors of many β-lactamases found in clinical isolates (Wise *et al*., 1978). Ampicillin-sulbactam is a parenteral formulation that expands the spectrum of ampicillin to include most of beta-lactamase producing strains (Bush and Johnson 2000).The *in vitro* activity of this compound in combination with other penicillins is described. Amoxicillin is another example of such combination. It is a mixture of ampicillin and clavulanic acid and is avaliable in oral and parentral formulation. This combination has many advantages over ampicillin alone. It has a broader antimicrobial activity, better absorbed from GIT and therefore causing less diarrhea, and longer duration of action (8hrs) compared to 6 hrs in ampicillin. In this study research, the validity of two different methods used for the screening of beta lactamase producing bacteria were compaired namely simple diffusion test and iodometric method.

II. MATERIALS AND METHODS

Five different gram positive and gram negative bacteria were tested for beta lactamases production. These microorganisms were *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, E.coli and Klebsiellapneumoniae*. The microorganisms were isolated from different clinical samples obtained from Azadi Teaching Hospital during the period from June / 2015 to March / 2016. Isolation of pathogenic bacteria and identification of species was performed by standard methods using of API-20 system (McFadden 2000).

The isolates were tested for their antimicrobial susceptibilities to penicillins and 3rdgeneration cepahalosporins by Kirby - Bauer antibiotic disc diffusion method as recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines (2000). Using a fresh and pure culture, a suspension of the test organism equal to 0.5 McFarland Standard were spread over the entire area of Mueller Hinton agar (MHA). With a sterile forceps the antibiotic discs were placed onto the inoculated MHA plate, ensuring sufficient space between individual discs to allow for proper measurement of inhibition zones. The plates were then incubated at 37°C for 18-24. The following oxoid antibiotic disks were used: Amoxillin (30µg),penicillin G (10 µg), ceftriaxone (30µg) and cefotaxime (30µg). Area of inhibition arround the disks were

measured by a roler, recorded in mm and labelled as sensitive (S) and resistant (R). The results were intrepreted according to CLSI guidelines as follows :

Table 1 : CLSI guidelines for measuring areas of inhibition

Antibiotic disk	Area of inhibition (mm)	Interpretation
Cefotaxime (30µg)	⩽ 27 mm ★★	Resistant (Suggestive of ESBLs)
Ceftriaxone (30µg)	⩽ 25 mm ★	Resistant (Suggestive of ESBLs)
Amoxicillin (30µg)	⩽ 13 mm ★	Resistant (Suggestive of ESBLs)
Penicillin G (10µg)	⩽ 22 mm ★	Resistant (Suggestive of ESBLs)

★*E.coli and Klebsiellapneumoniae*

★★*Other gram negatives.*

Regarding iodometric test, a starch indicator was prepared by dissolving 1 gm of starch powder in 100 ml of boiling water (Bush et al., 1995). The iodine reagent prepared by dissolving 2 gm of KI (Porassium iodide) , 1 gm of iodine in 100 ml of distilled water (D.W). The solution was then stirred vigorously for 15min and stored in brown bottle (Collee et al., 1996). A solution of 10 000 units of benzylpenicillin per ml was prepared in phosphate buffer pH 7 0. This is done by dissolving 0.6 g of penicillin G powder in 60 ml of phosphate buffer saline (PBS). The volume was made up to 100 ml with D.W and then sterilized by Millipore (0.22µm) filters (Bush et al.,1995). The test was conducted by adding a loop full of overnight grown culture (of the tested bacteria) to 0.5 -1 ml of the penicillin G solution. The tube was left at 37°C for 30 min. Thereafter, 2 drops of the starch solution were added followed by 1 drop of iodine reagent. A colour change from dark blue to colourless was noted over the next 10 minutes. The change of the black color - to colorless solution indicated a positive test (Bush et al.,1995).

III. RESULTS

The results of antibiotic sensitvity test were summarized in **Table 2**. All the five different bacteria were resistant to amoxicillin and penicillin G. This might point out to the capability of all the studied bacteria to produce beta lactamase enzymes as indicated by the ability of the bacteria to grow in the medium despite the presence of antibiotic disks. Since some penicillin resistant bacteria are also resistant to beta lactam cepahalosporins, therefore cefotaxime and ceftriaxone as examples for 3rd generation cephalosporins were also studied. Similar results were also obtained with ceftriaxone antibiotic disk. However, when using cefotaxime disk, four of the five penicillin - resistant studied bacteria *(Klebsiellapneumoniae, Bacillus subtilis, E.coli*and *Staphylococusaureus)* seem to be resistant according to CELSI guidelines. Only *Streotococcus pyogenes* appeared to be sensitive to cefotaxime with area of inhibition ~30 mm.

Table 2: Results of antibiotic disk diffusion test

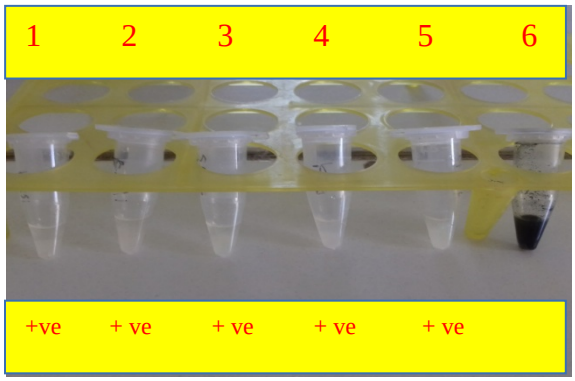
MO	Cefot.	PG	Ceftr.	Amox.
S.*pyogenes*	30 (S)★	00 (R)	00 (R)	00 (R)
K. pneumoniae	16 (R)★	00 (R)	12 (R)★	00 (R)
E.coli	00 (R)	00 (R)	00 (R)	00 (R)
B. subtilis	10 (R)★	00 (R)	00 (R)	00 (R)
Staph. aureus	00(R)	00 (R)	00 (R)	00 (R)
% of resistants	80 %	100%	100%	100%

★ *Area of inhibition in mm*

S: Sensitive

R: Resistant

Next, iodometric method was performed on same bacteria in order to compare the validity of this test with antibiotic diffusion method. The results showed that iodemetric method results were similar to antibiotic diffusin test were all the five studied bacteria appeared to be resistant to peniciilin (positive iodometric test , **Figure 1**).



1: S.pyogenes ; 2: S.aureus ; 3: B.subtilis ; 4: K.pneumoniae; 5: E.coli ; 6: starch / iodine complex

Figure 1: Results of iodometric test. Loopfuls of overnight growth culture of *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, E.coli and Klebsiellapneumoniae*were incubated penicillin G solution at 37°C for 30 minutes in separate tubes respectively. Thereafter , two drops of starch solution and one drop of iodine were added to each tube. If the mictorganism is penicillinase producer it changes the penicillin G to penicilloicacid . The latter reduces the iodine in starch / iodine complex . Iodine reduction changes starch / iodine complex from dark blue to colourless solution indicating a positive test. Simmilar to results of antibiotic disk diffusion test, all the five studed bacteria were also positive in iodometric test.

IV. Discussion

Beta-lactam antibiotics are among the safest and most frequently prescribed antimicrobial agents worldwide (Nagham and Zainab, 2016). The emerergence of resistance to these agents appeared in the past 2-decades and resulted in a major clinical crisis (Randegger and Hàchler, 2001, Poole, 2004).To avoid the increasing threatening of extended spectrum beta lactamases (ESBLs) producing strains rapid beta-lactamase tests can yield clinically relevant information for the most suitable antibiotic therapy and can help in preventing further resistance mutations. Several test methods were developed to detect β lactamase producion by bacteria. These include **1)** Chromogenic method which based on the principle that hydrolysis of certain beta lactam antibiotic leads to a distinct color change from a light yellow to adeep red color (Shrestha and Rana, 2014). **2)** Acidimetric method uses a pH indicator color change from purple pink to yellow to detect the formation of at least one extra carboxyl group produced during the hydrolysis of beta lactam antibiotic by beta lactamase (Shrestha and Rana, 2014). **3)** Iodometric method detects the loss of blue color from a blue starch / iodine complex caused by the removal of iodine from the complex by the reducing action of a beta lactamase hydrolysis product (Shrestha and Rana, 2014) **4)** Antibiotic disk diffusion test depends on measuring the growth inhibition zone arrounfd antibiotic disks and compairing it with thkse reported by Clinical and Laboratory Standards Institution (CLSI) guidelines.

The aim of the current study was to compair the validity of two methods, namely antibiotic disk diffusion and iodometric methods, as screening tests for the detection of ESBLs. In order to do so, the two methods were carried out on the same microorganisms collected from Azadi Teaching Hospital in Kirkiuk. These microorganisms were *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, E.coli and Klebsiellapneumoniae.* Isolation of the above bacteria was done from different clinical samples including blood, pus , urine and secretions.

As previously mentioned, antibiotic disk diffusion test depends on measuring the growth inhibition zone arround antibiotc disk .Our results reveals that all the five isolated microorganisms were resistant to penicillin G, amoxicillin and ceftriaxone as indicated by the growth of microorganisms arround the disks despite the presence of antibiotics **(Table 2)**. With the exception of *S.pyogenes*, all the studied microorganisms seem to be resistant to Cefotaxime. This resistance might be attributed to the beta lactamase production by these microorganisms which destroys beta lactam ring of penicillins and 7- amino cephalosporonic acid ring of cephalosporins respectively. Since these rings are the site of antimicrobial activity of these drugs, thefore destruction of these rings renders microorganisms resistant to these antibiotics. These results are expected in regard to *S. aureus, K.pneumoniae, B.subtilis*and *E.coli* since all these microorganisms are known to produce beta lactammase enzymes as indicated in leterature. However, the results of *S.pyogenes*seems to be surprising and propably novel. There is a wide agreement in leterature that most strains of *S. pyogenes* are uniformly suceptible to penicillins and cephalosporins. Moreover, cases of failure of response to penicillin therapy, as in some cases of pharyngiotonsilitis, were usually attributed to the presence of other beta - lactamase bacteria. In our study *S. pyogenes*appears to be resistant to penicillin G, amoxicillin and ceftriaxone and only sensitive to cefotaxime. Although this results are apparently strange but it is not without explanation**.** Interestingly, there is some recent evidence in leterature that supports the emergence of truley penicillin resistant ***S.pyogenes.*** Salwa et al (2014) has isolated some strains of penicillin resistant *S.pyogenes*in Egypt. Although it is a low number (only 4%) but it is avery seriuos clinical finding which should raise an alarm about the emergence of resistant strains. When using penicillin G, ampicillin and ceftriaxone disks for screening for beta lactamase producers all the isolates were resistant with 100 % sensitivity rate. On the other hand, when using cefotaxime disk for screening anly 4 out of the five studied bacteria (apart from S.pyogens) were resistant with 80 % sensitivity rate. Based on these result, we recommend ceftriaxone over cefotaxime when using cephalosporin for screening for the five studied microorganisms.

In comparison to antibiotic diffusion method, reading of iodometric method results depends on the visual disapperance of dark blue colour from starch / iodine complex. The test based on the fact that hydrolysis of beta lactam ring of penicillin by beta lactamases producing bacteria yieldspenicilloic acid. The latter reduces the iodine in starch / iodine complex leading to decolourisation of medium. Our results of iodometric test are comparable to those of antibiotic diffusion test where all of the five studied bacteria were resistant to penicillin (positive iodometric test) with 100 % sensitivity rate in all 5 studied bacteria (**Figure 1).**

V. CONCLUSION

In conclusion, both of the antibiotic diffusion and iodometric tests appear to be reliable methods for screening for penicillinase production in *Streptococcus pyogenes, Staphylococcus aureus, Bacillus subtilis, E.coli and Klebsiellapneumoniae*. Both these tests have been reported to be 100% correlated with resistance to penicillin and amoxicillin. However, when screening for cephalosporin resistance, antibiotic diffusion test should be used

since in iodometric test only penicillin is used as the substrate and, therefore, the test is equipped to detect penicillinases only. i.e. it does not detect cephalosporinases. In regard to *S. pyogenes* , ceftriaxone disk seems to be more sensitive than cefotaxime disk for the detection of cepahlospirin resistance. No such difference was found for the other four studied bacteria. Interesting and clinically important result was found by the discovery of penicillin resistant strains of *S.pyogenes*. More studies are recommended to confirm such finding.

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