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ORIGINAL ARTICLE

Detection of β -Lactamase Activity in Various Clinical Bacterial Isolates by Three Different Methods and its Correlation with Drug Resistance.

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Abstract:

Background: β-lactams such as penicillins are the most widely used antibiotics, and β-lactamases are the greatest source of resistance to penicillins. *Aims and Objectives:* To study β-lactamase production in clinical isolates of family *Enterobacteriaceae, P. aeruginosa and* Staphylococci by three different methods and to correlate its potential with drug resistance; with an endeavour to evaluate convenient and economical method duly supported by relevant Minimum Inhibitory Concentration (MIC) studies. *Material and Methods:* Total 240 clinical isolates (Gram-negative bacilli-191, staphylococci-49) were subjected to antimicrobial susceptibility testing by Kirby-Bauer disk diffusion method and MIC for ampicillin and penicillin was determined by agar dilution method. β-lactamase was detected by broth acidometric, iodometric cell suspension and microbiological method. *Results:* Multidrug resistance was observed in more than 90% isolates. One hundred and ninety Gram-negative bacilli were resistant to ampicillin and 47 staphylococcal isolates were resistant to both penicillin and ampicillin. Though microbiological method gave highest positive results 210 (87.5%), iodometric method could detect β-lactamase in apparently sensitive isolates as well giving satisfactory [207 (86.25%)] comparable results. *Conclusion:* In view of the noted bacterial resistance, tests for β-lactamase should be carried out on a routine basis for an early implementation of appropriate antimicrobial therapy. Iodometric method is eminently convenient, economical and reliable method. Isolates showing MIC ≤0.125µg/ml for penicillin and MIC ≤8µg/ml for ampicillin should be checked for β-lactamase production.

Key words: β-lactam resistance, β-lactamase detection, iodometric method penicillin and ampicillin MICs.

Introduction:

The current era of antimicrobial chemotherapy began in 1935 with the discovery of sulphonamides. Introduction of penicillin in 1940 which ironically was discovered fortuitously in 1929 by Alexander Fleming has saved many lives. Infections that cause mortality and morbidity were treated successfully with penicillin and further by introduction of cephalosporins in the early 1960s [1]. Today among the wide array of antibiotics, β-lactams are the most varied and widely used agents accounting for more than 50% of all systemic antibiotics in use [2]. But after the extensive usage of penicillins and cephalosporins, bacteria have developed resistance via different mechanisms. β-lactamase enzyme production is the first developed and the most important mechanism of

resistance to β-lactams [1]. β-lactamases attack the amide bond in the β-lactam ring of penicillin and cephalosporins causing disruption of molecule and convert the antibiotic to inactive penicilloic acid [3]. An acyl-enzyme complex forms. By deacylation, the enzyme turns into its initial structure and binds other â-lactam antibiotics [1].

Disease caused by β-lactamase producing strains is refractile to therapy with β-lactam agents which are frequently used for empiric therapy leading to the serious treatment failures. *In-vitro* antimicrobial susceptibility shows that the isolates to be sensitive to β-lactams but when agent is used for treatment, it doesn't work. For this reason *in-vitro* determination of β-lactamase production is of clinical value in the management of patients with infectious diseases [4]. For instance, the simplest situation is seen with β-lactamase producing strains of *S.aureus*, where the presence of the enzyme almost certainly contributes towards resistance. In such case, infections due to β-lactamase producing isolates should not be treated with enzyme sensitive penicillins [3]. Since antibiotic susceptibility results may not be obtainable for two or three days after culturing, the availability of a rapid, reliable test for the detection of β-lactamase in the clinical laboratory would be an obviously useful tool and would prove to be extremely helpful in predicting β-lactam resistance [5]. Test results can also be available for blood and CSF isolates as soon as colonial growth is obtained on subculture plates and reports can be phoned to clinicians when these results are available before antimicrobial susceptibilities [5]. Hence a test for β-lactamase production is required which can be used to assay a wide variety of organisms suspected of producing the enzyme. Also, the test should be rapid in order to provide virtually immediate results after isolation of the pathogen [6].

Over the years several methods viz. acidometric method, iodometric method, chromogenic cephalosporin method [1] and microbiological method [3,7] have been developed to detect â-lactamase production of bacteria.

The present study was an attempt to detect β-lactamase production among clinical bacterial isolates by three different methods and to correlate its possible role in resistance of bacteria to antimicrobial drugs. We were interested in evaluating a convenient, economical, reliable and suitable method of β-lactamase detection which can be put up in day-to-day clinical microbiology laboratory practice. An effort was also being made in establishing the correlation between penicillin and ampicillin MICs and the production of β-lactamase i.e., in determining at what level an isolate could be assumed to be a potent β-lactamase producer.

Material and Methods:

Approval was obtained from our institutional review board. The study was carried out on 240 consecutive, non repeat common clinical isolates of *Enterobacteriaceae* family, *P.aeruginosa* and staphylococci derived from different clinical specimens of varied clinical syndromes. Isolates were identified by standard procedures. Antimicrobial susceptibility testing for routine antibiotics was carried out by Kirby-Bauer disk diffusion method and mini-

mum inhibitory concentration (MIC) was determined by agar dilution method for ampicillin for all the isolates, and for penicillin for staphylococcal isolates. Control organisms *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853 and *S.aureus* ATCC 25923 were included in the study. Isolates were tested further for β-lactamase detection by broth acidometric method as described earlier by Escamilla J *et al*. [8], iodometric cell suspension method as described by Sykes RB [3] and microbiological assay method as described by Bhat KG *et al*. [7]. A known β-lactamase producing laboratory strain of *S.aureus* and *S.aureus* ATCC 25923 were used as positive and negative controls respectively.

Results and Discussion:

Multi-drug resistance is a common phenomenon. In our hospital more than 90% of the bacterial isolates have shown variable drug resistance to three or more antimicrobial agents. In this study among Gram-negative isolates highest resistance was seen for ampicillin (90-100%), tetracycline (90-100%), co-trimoxazole (88-100%), ciprofloxacin (50-100%) and cefoperazone (60-80%). Staphylococcal isolates showed highest resistance to ampicillin (96-100%), penicillin (94-96%), tetracycline (77-96%) and gentamicin (85-87%) (Table -1). Resistance to such most commonly used antimicrobial agents has been reported by many authors [9 -13].

MIC studies revealed that 190 (99.47%) Gram-negative (n=191) isolates were resistant to ampicillin (MIC≥32µg/ml). Only one isolate was found to be sensitive (MIC≤8µg/ml) (Table - 2).

Among staphylococcal (n=49) isolates, 47 (95.91%) isolates were resistant to both penicillin (MIC≥0.25µg/ml) and ampicillin (MIC ≥0.5µg/ml) i.e., only two isolates were found to be sensitive to both antibiotics (Table - 3).

Table - 1. Antimicrobial resistance pattern of different organisms.

Organisms	Ampi	Cefo	Genta	Tetra	Cipro	Cotri	Amik
E.coli (n=42)	42	29	30	40	40	37	26
K.pneumoniae (n = 26)	26	21	25	25	26	23	22
Citrobacter spp. (n = 53)	53	41	50	51	50	53	47
P.aeruginosa (n = 40)	40	30	39	39	39	40	31
Proteus spp. (n = 20)	20	13	18	20	17	20	15
Salmonella spp. (n = 10)	09	06	04	09	05	09	02
COPS (n = 18)	18	14	12	14	08	17	12
CONS (n= 31)	30	22	27	30	15	30	25
Total (n=240)	238	176	205	228	200	229	180
Percentage	99.16	73.33	85.41	95	83.33	95.41	75

(Ampi-Ampicillin, Cefo-Cefoperazone, Genta-Gentamicin, Tetra-Tetracycline, Cipro-Ciprofloxacin, Cotri-Cotrimoxazole, Amik-Amikacin)

Table - 2. MIC of ampicillin in Gram-negative isolates.

	MIC (µg/ml)					
Organisms	08	16	32	64	128	>128
E.coli (n = 42)	-	-	01	04	03	34
K.pneumoniae (n = 26)	-	-	-	01	02	23
Citrobacter spp. (n = 53)	-	-	01	05	04	43
P.aeruginosa (n = 40)	-	-	02	05	03	30
Proteus spp. (n = 20)	-	-	-	03	02	15
Salmonella spp. (n = 10)	01	-	-	-	02	07
Total (n=191)	01	-	04	18	16	152

Table - 3. MIC of penicillin and ampicillin in staphylococcal (n=49) isolates.

Antibiotics	0.06	0.125	0.25	0.5	1	>1
Penicillin (%)	2(4.08)	-	1(2)	2(4.08)	6(12.24)	38(77.55)
Ampicillin (%)	-	1(2)	-	1(2)	4(8.16)	42(86.71)

Our results were consistent with earlier studies. Kumar MS et al. [2] and Siddiqui MN et al. [14] reported MIC ≥256µg/ml and ≥500µg/ml for ampicillin in *Enterobacteriaceae* isolates. Rosenblatt JE et al. [5] reported MIC≥0.5µg/ml for penicillin in 84% (n=100) staphylococcal isolates.

Out of total 240 isolates microbiological assay method yielded 210 (87.50%) positive results for β-lactamase detection, followed by 208 (86.66%) by acidometric method and 207 (86.25%) by iodometric method (Table - 4).

Table - 4. β-lactamase production by different organisms.

Organisms (n=240)	Iodometric	Acidometric	Microbiological
E.coli (n = 42)	38	34	30
K.pneumoniae (n = 26)	21	22	20
Citrobacter spp. (n = 53)	45	51	49
P.aeruginosa (n = 40)	31	39	39
Proteus spp. (n = 20)	13	07	18
Salmonella spp. (n = 10)	10	09	08
COPS (n = 18)	18	16	16
CONS (n = 31)	31	30	30
Total positive	207 (86.25%)	208 (86.66%)	210 (87.50%)
Negative	33 (13.75%)	32 (13.33%)	30 (12.50%)

Our results were in well agreement with other studies like Bhat KG *et al*. [7] who reported 46 (94%) *S.aureus* (n=50) isolates positive by microbiological method. Similarly Doern GV et al. [4] reported 58 (73.38%) *B.catarrhalis* (n=74) isolates positive by acidometric and iodometric method.

In the present study, if the number of positive results yielded by different methods is used as assay sensitivity then, microbiological method is more sensitive than acidometric method and acidometric method is more sensitive than iodometric method.

Highest positivity rate with microbiological assay could be explained by the inducible nature of β-lactamase. In this test, the bacteria will be in contact with penicillin for a long time and β-lactamase can be induced [7]. But one disadvantage is that it is time consuming, one has to wait for 18-24 hours to get the results. So this method is time consuming while considering the urgency of reports in clinical microbiology laboratory.

Acidometric method though it gave higher positive results than iodometric method, it has shortcomings for routine use as it requires accurate pH adjustment while preparing reagent and the reagent prepared is not stable for longer time. It may remain stable for about 7-8 days, after which fresh reagent has to be prepared every time. Further, the test is inoculum dependent and requires large inoculum to obtain satisfactory results [3, 6].

Iodometric method gave satisfactory results and are comparable to other two methods. The iodine reagent is easy to prepare, does not need any pH adjustment and once prepared it remains stable for many months under refrigeration temperature. Penicillin-PBS solution once prepared is also stable for many days. Gram's iodine is readily available in most of the microbiology laboratories. In addition, the penicillin powder is inexpensive and stable in dry form for at least six months or more. Test is not inoculum dependent, few colonies suffice as the inoculum. Only thing is that, fresh starch solution has to be prepared and pre-incubation period of one hour is needed before test interpretation [6]. By considering all these advantages, it is very clear that this method is most convenient for routine use in clinical microbiology laboratory. Many authors have reported the efficacy of iodometric method over other methods. Kilic E *et al*. [1] stated that iodometric tests were found to be easy to perform, cheap and effective in detecting staphylococcal β-lactamase. Likewise Oberhofer TR *et al*. [6] have reported that iodometric paper strip method proved to be an extremely reliable, accurate, low-cost, and easy-to-use test for screening primary isolates of *Haemophilus, Neisseria, Branhamella*, and *Staphylococcus* species in clinical laboratories.

Table -5 shows correlation of disk diffusion testing and MIC results of penicillin with β-lactamase production. Out of 49 staphylococcal isolates 47 isolates were resistant (MIC $\geq$0.25µg/ml) to penicillin and remaining 02 were sensitive (MIC$\leq$0.12µg/ml). Iodometric method gave positive results for all 49 isolates (100%). This method detected β-lactamase in apparently sensitive isolates (n=2) too. Acidometric method gave positive results for 46 (93.87%) resistant isolates only; however, it could not detect β-lactamase in sensitive isolates. Microbiological method detected

β- lactamase in 45 resistant isolates and 01 sensitive isolate i.e., total positivity by this method was 46 (93.87%).

Table - 5. Correlation of penicillin susceptibility and β-lactamase production.

Penicillin	Iodometric	Acidometric	Microbiological
Sensitive = 2 (MIC ≤ 0.125 μg/ml)	02	00	01
Resistant = 47 (MIC ≥ 0.25 μg/ml)	47	46	45
Total = 49	49	46	46
Percentage	100	93.87	93.8

Similar kind of results have been reported by other authors also [1,5,6,15,16]. Oberhofer TR *et al*. [6] reported all 169 (81.6%) resistant *S.aureus* (n=207) isolates positive for β-lactamase test by iodometric method. He observed similar findings in *S.epidermidis, Haemophilus* spp., *Neisseri*a spp. and *B.catarrhalis* isolates, while Kilic E *et al*. [1] reported 69 (21.4%) sensitive isolates of *Staphylococcus* spp. (n=323) to be β-lactamase producers by acidometric and iodometric methods.

Gill VJ *et al*. [16] correlated β-lactamase detection by acidometric method with MIC of penicillin in staphylococcal isolates. He reported 156 (67%) *S.aureus* (n=234) isolates with MIC≥1μg/ml, 71 (30%) isolates with MIC 0.05-1μg/ml and 7 (3%) isolates with MIC ≤0.05μg/ml were β-lactamase producers. And in the same study out of 213 *S.epidermidis* isolates, 135 (64%) isolates with MIC ≥1μg/ml, 75 (35%) with MIC 0.05-1μg/ml and 3 (1%) with MIC≤0.05μg/ml were β-lactamase producers. These results showed that from 1 to 3% of isolates can be very susceptible to penicillin at MICs≤ 0.05μg/ml and yet be β-lactamase producers. He suggested that laboratories should at least check all staphylococcal isolates for which MICs are ≤0.05μg/ml. There should be some mechanism in the laboratory reporting system to indicate that staphylococcal isolates for which penicillin MICs are ≤0.05μg/ml but are positive for β-lactamase production should be considered penicillin resistant. Chemical testing of isolates with MICs ≥0.05μg/ml would not be necessary since these all appear to be β-lactamase producers. The reporting system should specify that isolates for which penicillin MICs are ≥0.05μg/ml should be considered β-lactamase producers which are resistant to penicillin. Kasse M *et al*. [17] stated that, an erroneous report of penicillin susceptibility could result in potentially inadequate therapy of *S.aureus* infections. A penicillin MIC of ≤ 0.12 mg / L is formally in the sensitive range, but the CLSI recommends that additional testing should be performed.

Table - 6 shows correlation of disk diffusion testing and MIC results of ampicillin with β-lactamase production. Out of total 240 isolates, 238 isolates were resistant to ampicillin and 02 isolates were sensitive. Iodometric method gave positive results in 205 resistant and 02

sensitive isolates. Acidometric method detected β-lactamase in 208 resistant isolates only. Microbiological method gave positive results in 209 resistant isolates and 01 sensitive isolate. Bacterial isolates which were resistant to ampicillin but failed to give a positive result for β-lactamase test indicates that the resistance mechanism may be different from β-lactamase production.

says rather than determinations of ampicillin MICs, because the latter may indicate false susceptibility.

This study implies that though there is a good correlation between penicillin and ampicillin susceptibility and β-lactamase production, it is necessary to screen the isolates showing sensitive and intermediate susceptibility to penicillin and ampicillin for β-lactamase tests be-

Table - 6. Correlation of ampicillin susceptibility and β-lactamase production.

Ampicillin	Iodometric	Acidometric	Microbiological
*Sensitive = 2	02	00	01
#Resistant = 238	205	208	209
Total = 240	207	208	210
Percentage	86.25	86.66	87.5

* for GNB – MIC ≤ 8 μg/ml, for *Staphylococci* MIC ≤ 0.25 μg/ml.
\# for GNB – MIC ≥ 32 μg/ml, for *Staphylococci* MIC ≥ 0.5 μg/ml.

Similar kind of study was conducted by other authors. Lee WS *et al*. [18] reported 13 (24%) ampicillin resistant *H.influenzae* (n=54) and 31 (51%) penicillin resistant *S.aureus* (n=60) isolates positive for β-lactamase production by iodometric method.

Doern GV *et al*. [4] reported 58 (78%) apparently sensitive (ampicillin MIC 0.008-4μg/ml) isolates of *B.catarrhalis* (n=74) to be β-lactamase producers by acidometric and iodometric methods. Such strains would be considered susceptible to ampicillin if the judgment was made solely on the basis of *in-vitro* determinations. There have been several reports, however, of therapeutic failures when ampicillin has been used to treat infections caused by β-lactamase positive strains. Because of this, it appears that therapeutic decisions are best predicted on the results of β-lactamase as-

cause; though they may appear susceptible by disc diffusion method they may be potent β-lactamase producers.

Our data indicate that there is a satisfactory correlation between MIC values of penicillin and ampicillin and β-lactamase production. From the results a conclusion can be drawn that isolates showing penicillin MIC ≤0.125μg/ml and ampicillin MIC ≤8μg/ml should be checked for β-lactamase production, because there are several reports of therapeutic failures when ampicillin has been used in the treatment; furthermore β-lactamase testing of isolates with MIC more than the above values would not be necessary.

In the clinical microbiology laboratory apart from the epidemiological importance the main significance of the β-lactamase production is directly related to the degree of urgency for

the institution of specific antimicrobial therapy in a given patient. Standard antimicrobial sensitivity tests by diffusion methods require overnight incubation while automated procedures take several hours. A rapid reliable β-lactamase test may make possible early implementation of appropriate therapy. Present study recommends that in view of the noted bacterial resistance to drugs, tests for β-lactamase should be carried out on the routine basis to meaningfully correlate the finding of Kirby-Bauer disk diffusion method.

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