Original Article

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Microbiology Section

The Simultaneous Detection of the ESBL and the AmpC β-Lactamases in Gram Negative Bacilli

AVINASH LAGHAWE, NEELAM JAITLY, VILAS THOMBARE

ABSTRACT

Purpose: The purpose of this study was to detect the prevalence of the ESBLs and the AmpC β-lactamases in gram-negative bacilli.

Methods: The detection of ESBLs was done by using various third generation cephalosporins (3GC), along with imipenem, aztreonam, cefoxitin and ceftazidime + clavulanic acid. The criteria which were used for the identification of ESBL – were resistance to 3GC, sensitivity to cefoxitin and increase in the zone size by 5 mm or more of ceftazidime + clavulanic acid as compared to the ceftazidime zone size. The organisms which were resistant to cefoxitin were tested for the presence of AmpC by the AmpC disc test.

Results: A total of 432 isolates were isolated from 414 samples. Out of these 432 isolates, 85(19.67%) were (64 pure and 21 mixed) ESBL producers, 69(15.97%) were (48 pure and 21 mixed) AmpC β-lactamase producers and 299(69.22%) isolates didn't show any evidence of the production of β-lactamases. 21(4.86%) isolates were positive for both ESBLs and AmpC. Out of the 64 pure ESBL producers, 55 (85.94%) were from indoor patients and 9(14.06%) were from outdoor patients. Out of the 48 pure AmpC producers, 42(87.5%) were from indoor patients and 6(12.5%) were from outdoor patients. All (21) the mixed beta lactamase producers (ESBLs + AmpC) were from indoor patients.

Conclusion: Regular monitoring of the incidence of the β-lactamase production by the organisms is necessary. As the β-lactamase producing organisms are also present in the outdoor patients, they also should be screened for the production of β-lactamases. The detection of ESBLs and AmpC beta lactamases by this method is simple and any microbiology laboratory can do it along with the routine antibiotic susceptibility testing.

Key Words: ESBL, AmpC, Clavulanic acid, Cefoxitin, Third generation cephalosporins

INTRODUCTION

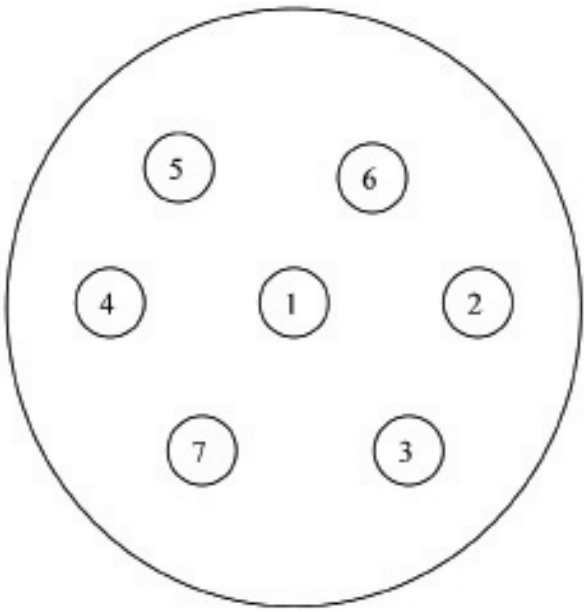
At least a quarter of all the illnesses for which patients consult their doctors are infective. Gram-negative bacteria may exhibit a reduced susceptibility to the β-lactam antibiotics by a number of mechanisms which include reduced outer membrane permeability, target site modification, and efflux of β-lactams out of the cell. However, by far, the most common mechanism of resistance is the enzymatic inactivation of the β-lactams by a β-lactamase [1]. The extensive use of the third generation cephalosporins (3GC) like cefotaxime, ceftriaxone and ceftazidime has led to the evolution of newer β-lactamases such as the Extended Spectrum Beta Lactamases (ESBLs). ESBLs are Plasmid-mediated enzymes that hydrolyze the oxyimino β-lactams (3GC) and the monobactams (aztreonam) but have no effect on the cephamycins (cefoxitin, cefotitan) and the carbapenems (Imipenem). Being plasmid mediated, they can be easily transferred from one organism to another. Based on their physical properties, more than 300 ESBLs have been identified and they have been found to be inhibited by clavulanic acid, sulbactam and tazobactam. This is the property which helps in their detection in the laboratory. ESBLs are more prevalent in the *Klebsiella species* and in *E. coli,* but they may also be found in other genera such as Citrobacter*, Serratia, Proteus, Salmonella, Enterobacter*, *Pseudomonas,* etc [2].

The expression of the AmpC β-Lactamases can be generated by chromosomal or plasmid genes. The plasmid mediated AmpC β-lactamases are thought to have originated from the chromosomes of several Enterobacteriaceae species and they are rarely inducible. Unlike ESBLs, the plasmid encoded cephalosporinases are active against the cephamycins and they are not inhibited by clavulanic acid. The plasmid mediated AmpC β-lactamases were first reported in 1988. The number of infections which are caused by the AmpC producing organisms is increasing. Distinguishing between the AmpC and the ESBL producing organisms has epidemiological significance and it may have a therapeutic importance as well [3]. The AmpC producing organisms can act as hidden reservoirs for ESBLs. Enterobacteriaceae which produce both AmpC and ESBLs have been increasingly reported worldwide. Also, the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs [4].

With this background, the current study was undertaken to detect the prevalence of ESBLs and AmpC β-lactamases in gram-negative bacilli, as not many such studies have been done on this topic, especially in central India.

MATERIALS AND METHODS

All the gram-negative bacilli which were isolated from various clinical samples from hospitalized patients as well as from patients who attended the out patients departments were collected and identified by standard biochemical reactions. Anti-microbial susceptibility testing was performed by the Kirby Bauer disc diffusion method for cefotaxime, ceftriaxone, ceftazidime, ceftazidime + clavulanic acid, imipenem, cefoxitin and aztreonam. The disc placement was designed in such a fashion so as to assess the ESBLs and the Amp C enzymes [Table/Fig-1]



[Table/Fig-1]: The disc placement was designed in such a fashion to assess ESBL and Amp C enzymes
1-Imipenem, 2-Cefotaxime, 3-Cefoxitin, 4-Ceftazidime, 5-Ceftazidime + Clavulanic acid, 6-Aztreonam, 7-Ceftriaxone

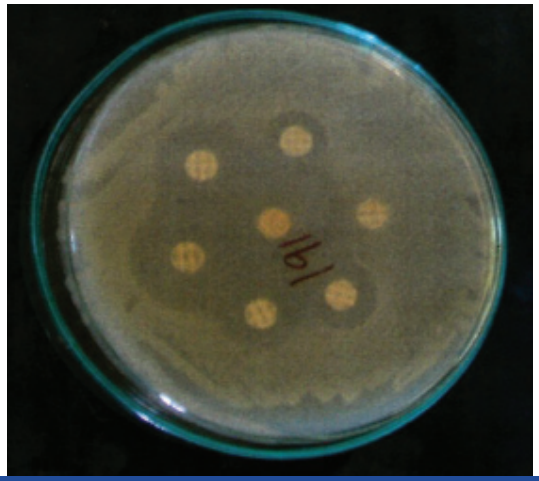
Imipenem, an inducer, was placed in the centre and on either side of it, at a 15 mm distance, ceftazidime and cefotaxime (indicators of induction) were placed. The ceftazidime + clavulanic acid disc was placed 15-20 mm apart from the ceftazidime disc. In addition, another inducer, cefoxitin, was placed 15 mm apart from cefotaxime (indicator).

The cefoxitin disc was placed opposite to that of ceftazidime + clavulanic acid to avoid any effect of the inducible β-lactamase on the zone of inhibition of ceftazidime + clavulanic acid. Aztreonam was placed in between ceftazidime + clavulanic acid and cefotaxime, while ceftriaxone was placed in between cefoxitin and ceftazidime. All the discs were placed at a distance of 15-20 mm from each other [5].

INTERPRETATION [5]

An isolate was suspected to be an ESBL producer if it had the zone sizes for the cephalosporins like cefotaxime (30μg) ≤ 27 mm, ceftazidime (30μg) ≤ 22 mm, ceftriaxone (30μg) ≤ 25 mm and aztreonam (30μg) ≤ 27 mm.

The criteria which were used for deciding whether an organism was an ESBL producer [Table/Fig 2]:



[Table/Fig-2]: Extended spectrum beta lactamase production

1) Zone diameter for various 3GCs as has been mentioned above.
2) Susceptibility to cefoxitin
3) Increase in the zone size with the addition of an inhibitor, by ≥ 5 mm.

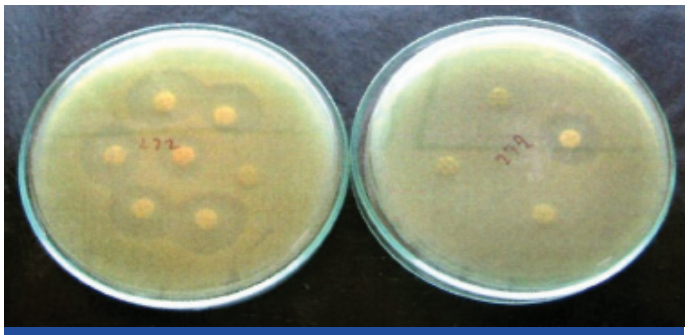
The criteria which were used for suspecting whether an organism was an AmpC producer [Tabe/Fig-3]:

1) Zone diameter for various 3GCs as has mentioned above.
2) Resistance to cefoxitin
3) No increase in the zone size with the addition of an inhibitor, by ≥ 5 mm

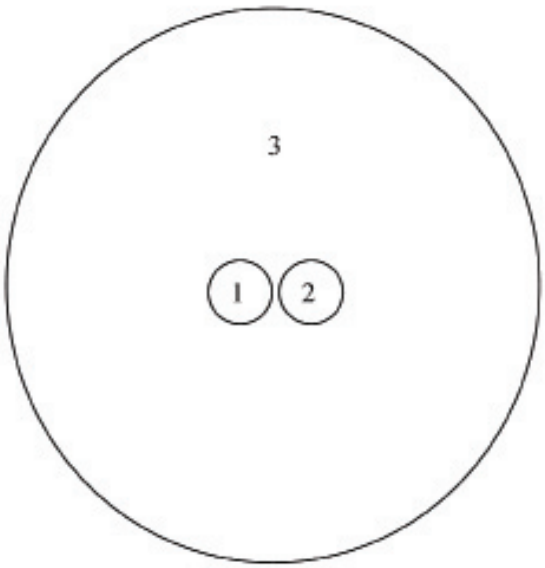
CONFERMATION OF THE AMPC β-LACTAMASES [TABLE/FIG-4] [6]

The isolates which showed blunting of the ceftazidime or the cefotaxime zone of inhibition which was adjacent to the inducer (imipenem or cefoxitin) or those which showed a reduced susceptibility to either of the above test drugs (ceftazidime and cefotaxime) and cefoxitin were considered as "screen positive" and they were selected for the confirmation of the AmpC β-lactamases.

A lawn culture of *E.coli* ATCC 25922 was prepared on a Mueller-Hinton agar plate. A sterile disk (6mm) was moistened with sterile saline (20μl) and it was inoculated with several colonies of the test organism. The inoculated disk was then placed besides a cefoxitin disk (almost touching) on the inoculated plate. The plate was incubated overnight at 35^{0}C. A positive test appeared as a flattening or an indentation of the cefoxitin inhibition zone in the vicinity of the test disk. A negative test produced a undistorted zone.



[Table/Fig-3]: AmpC β-lactamase production



[Table/Fig-4]: AMP C Disk Test
1- Cefoxitin disk, 2- Disk smeared with test organisms, 3- Lawn culture of E.coli ATCC25922

RESULTS

This study was conducted from February 2005 to July 2007 in the Department of Microbiology, NKP Salve Institute of Medical Sciences and Research Center and Lata Mangeshkar Hospital, Digdoh, Nagpur, India. A total of 432 isolates were isolated from 414 samples. Out of the 414 samples, 392 were mono microbial and 22 were poly microbial. The maximum number [154(35.65%)] of isolates were of *E.coli,* followed by those of *Pseudomonas aeruginosa* [113(26.16%)] and *Klebsiella pneumoniae* [111(25.69%)]. Other isolates were those of *Enterobacter cloacae* [19(4.4%)], *Proteus mirabilis* [14(3.24%)], non-fermenters [9(2.08%)], *Citrobacter freundii* and *Serratia marcescens* [4(0.93%)]. Of the 432 isolates, 137 were found to be resistant to cefoxitin and to one or more of the third generation cephalosporins. When the AmpC disk test was applied, 69 isolates were found to be positive for the AmpC β-lactamases.

Out of the 432 isolates, [Table/Fig-5] 85(19.67%) were ESBL producers, 69(15.97%) were AmpC β-lactamase producers and 299(69.22%) isolates didn't show any evidence of the production of β-lactamases. 21(4.86%) isolates were positive for both ESBLs and AmpC.

organisms	ESBL (%)	Ampc (%)	No β-Lactamase (%)	No. of Isolates
E. coli	36 (23.38)	11 (7.14)	111 (72.08)	154
Pseudomonas aeruginosa	11 (11.5)	22 (19.47)	82 (72.57)	113
Klebsiella pneumoniae	22 (19.82)	13 (11.71)	82 (73.87)	111
Enterobacter cloacae	4 (21.05)	10 (52.63)	7 (36.84)	19
Proteus mirabilis	5 (35.71)	3 (21.43)	8 (57.14)	14
Non-fermenters	0 (0)	4 (44.44)	5 (55.56)	9
Citrobacter freundii	5 (62.5)	5 (62.5)	1 (12.5)	8
Serratia marcescens	0 (0)	1 (25)	3 (75)	4
Total	85 (19.67)	69 (15.97)	299 (69.12)	432

Table/Fig-5: β-Lactamase Production In Various Organisms

ESBL production was observed in 23.38% of isolated *E.coli*, 19.82% of the Klebsiella *pneumoniae* isolates and in 62.5% of the *Citrobacter freundii* isolates. Out of the 23.38% ESBL producing *E. coli*, 20.78% produced pure ESBLs and 2.6% produced mixed ESBLs i.e. ESBLs + AmpC. Out of the 19.82% ESBL producing *Klebsiella pneumoniae*, 14.41% produced pure ESBLs and 5.41% produced mixed ESBLs i.e. ESBLs + AmpC. The AmpC production was predominant in *Citrobacter freundii* (62.5%), followed by *Enterobacter cloacae* (52.63%) and the non-fermenters (44.44%).* A mixed β-lactamase (ESBL and AmpC) production was observed in 4 - *E.coli*, 4 - *Ps. aeruginosa, 6 - Klebsiella pneumoniae, 2 - Enterobacter cloacae, 2 - Proteus mirabilis, and 3-Citrobacter freundii* isolates.

All the β-lactamase producing organisms were susceptible to Imipenem.

Out of the 64 pure ESBL producers, 55 (85.94%) were from indoor patients and 9(14.06%) were from outdoor patients. Out of the 48 pure AmpC producers, 42(87.5%) were from indoor patients and 6(12.5%) were from outdoor patients. All (21) the mixed beta lactamase (ESBL + AmpC) producers were from indoor patients.

DISCUSSION

The knowledge on the extent of the ESBL mediated resistance appears to be limited due to the inability of the standardized methods of susceptibility testing or the commercially available systems to detect this resistance [7]. The emergence and the spread of the ESBL producing strains have led to questions regarding the optimal therapy for infections which are caused by the ESBL producing strains [8].

The confirmation of the ESBL production by clavulanic acid inhibition can be difficult in some strains, not only because the activity of the β-lactamase varies with different substrates, but also because the organism may contain additional resistance mechanisms that can mask the presence of the ESBL activity [9].

The AmpC β-lactamases are cephalosporinases which belong to the molecular class C, as was classified by Ambler and group I under a classification scheme of Bush and Jacoby et al. These are clinically significant as they may confer resistance to a wide variety of β-lactam drugs which include α-methoxy-β-lactams, narrow, expanded and broad-spectrum cephalosporins, aztreonam and most significantly, the β-lactam plus the β-lactamase inhibitor combinations [10].

The AmpC producing organisms can act as a hidden reservoir for the ESBLs. Enterobacteriaceae which produce both AmpC and ESBLs have been increasingly reported worldwide. Also, the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs [4].

In our study, 19.67% were ESBL producers as against Emery et al's [11] and Spanu et al's [12] findings which reported ESBL prevalence in 1.2% and 6.3% isolates respectively. Stratchounski et al [13] and Sanguinetti et al [14] had reported in their studies that 66.7% and 62.55% were ESBL producers. The studies which were carried out by Cormican et al [7], D'Agata E. et al [15], Navon et al [16], Tankhiwale et al [17] and Wang et al [18] reported isolation rates of 17.33%, 13.1%, 19%, 19.35% and 16.67% respectively. This also proved that the prevalence of ESBLs among the clinical isolates varied from country to country and institution to institution within the country.

In our study, AmpC production was seen in 15.97% isolates as against Ratna AK et al's [19] findings (3.3%) and Rodrigues et al's [5] findings (66.43%). Moland et al [20] and Sanguinetti et al [14] found the prevalence of the AmpC production to be 10.67% and 15.1% respectively.

Though the presence of the beta lactamases in the indoor patients was significantly high as compared to that in the outdoor patients, beta lactamases are also found in community acquired infections.

CONCLUSION

Regular monitoring of the incidence of the β-lactamase production by the organisms is necessary. Along with the detection of ESBL, it is necessary to detect AmpC β-lactamases, as they can act as hidden reservoirs for the ESBLs. Enterobacteriaceae which produce both AmpC and ESBLs have been increasingly reported worldwide. Also, the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs.

To prevent the spread of the β-lactamase producing strains, hospitals must have functional hospital infection control committee with an appropriate hospital antibiotic policy, with regular updates.

As the β-lactamase producing organisms are also present in the outdoor patients, they also should be screened for the presence of β-lactamases. Also, attempts should be made to prevent the dissemination of the β-lactamases in the community.

The carbapenems should be kept as reserve drugs and they should be used only in patients who have infections which are caused by multi-drug resistant strains, especially the strains which produce extended-spectrum and AmpC β-lactamases.

The detection of ESBLs and AmpC beta lactamases by this method is simple and any microbiology laboratory can do it along with the routine antibiotic susceptibility testing.

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AUTHOR(S):

1. Dr. Avinash Laghawe
2. Dr. (Ms.) Neelam Jaitly
3. Dr. Vilas Thombare

PARTICULARS OF CONTRIBUTORS:

1. M.D.(Microbiology) Assist. Professor, Dept. of Microbiology, Sri Aurobindo Inst. Of Medical Sciences, Indore, M.P. , India.
2. M.D.(Microbiology) Professor, Dept. of Microbiology, NKP Salve Inst. of Medical Sciences and Research Centre, Nagpur, MH, India.
3. M.D. (Microbiology) Professor and HOD, Dept. of Microbiology, NKP Salve Inst. of Medical Sciences and Research Centre, Nagpur, MH, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Avinash Laghawe
Asst. Professor, Dept. of Microbiology,
SAIMS Medical College, Sanwer Road, Indore,
Madhya Pradesh, India - 453111
Phone: 09713357133
E-mail: lavinash@rediffmail.com; dravinash73@gmail.com

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