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Research Article

Detection of extended spectrum beta lactamase in *escherichia coli* and *Klebsiella pneumoniae* isolates from clinical samples

Shahitha S*, Kumaravel S, Poornima K

PG and Research Department of Microbiology, Muthayammal College of Arts and Science, Rasipuram, Tamil Nadu, India

ABSTRACT

The β -lactamases are the diverse family of enzymes that are capable of hydrolyzing a wide variety of β –lactam antibiotics. Extended spectrum beta lactamase (ESBL) is defined as β -lactamases capable of hydrolyzing oxyimino-cephalosporins that are inhibited by clavulanic acid and are placed into a functional group, the majority of strains belong to the family *Enterbacteriaceae.* The most common ESBL products are *Klebsiella pneumoniae* and *Escherichia coli.* The present study was carried out to find out the incidence of extended spectrum β lactamase in *Escherichia coli* and *Klebsiella pneumonia* isolated from various clinical samples.

Keywords: β –lactamases; *Klebsiella pneumoniae* and *Escherichia coli*

INTRODUCTION

β-lactam antibiotics are commonly used to treat bacterial infections. The groups of antibiotics in this category include penicillins, cephalosporins, carbapenems, and monobactams. Increased use of antibiotics, particularly the third generation cephalosporins has been associated with the emergence of β-lactamases a common mechanism of bacterial resistance. These enzymes that cause resistance to oxyimino-cephalosporins lead to the development of extended spectrum beta lactamase (ESBL) producing bacteria. The first report of ESBL *Klebsiella pneumoniae* appeared in Germany in 1983. (Sykes and Mathew, 1976) ESBLs have been reported from all parts of the world; however, prevalence various widely even in closely related regions. The true incident is difficult to determine because of the difficulty in detecting ESBL production and due to inconsistencies in testing and reporting.

The accelerated emergence of antibiotic resistance among the prevalent pathogens is the most serious threat to the management of infectious diseases. The production of β -lactamases is the main mechanism of bacterial resistance to these classes of antibiotics. The first β-lactamase was identified in *Escherichia coli* prior to the release of penicillin for use in medical practice. Many gram negative bacteria possess naturally occurring, chromosomally mediated β-lactamases (eg: Amp *C cephalosporinases* of *Enterobacteriaceae*). These enzymes may have some physiological role in peptidoglycan assembly or may arise to defend bacteria against β-lactams produced by environmental bacteria and fungi. The first plasmid mediated lactamases in gram negatives, TEM-1, was reported in 1965 from an *Escherichia coli* isolate belonging to a patient in Athens, Greece, named Temoniera (hence the designation TEM). The TEM-1 β-lactamase has spread worldwide and is now found among different species of members of *Enterbacteriaceae, Pseudomonas aeruginosa, Haemophilus influenzae* and *Neisseria gonorrhoeae.* Another common plasmid mediated β -lactamase found in *Klebsiella pneumoniae* and *Escherichia coli* is SHV-1 (named after the sulfhydril "variable" active site) (Bradford, 2001). TEM and SHV types are most often found in *Escherichia coli* and *Klebsiella pneumonia. However, they have also been found in Proteus sp, Providencia sp* (Jacoby, GA 1991).

* Corresponding Author
Email: shahi_sikkandar@yahoo.co.in
Contact: +91- 9443892846
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MATERIALS AND METHODS

Detection of extended spectrum β-lactamases using screening test (NCCLS, 2001)

Initially, screening test for ESBL production was done as part of routine susceptibility testing. Five antibiotic discs, namely ceftazidine (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefpodoxime (30 µg) and aztreonam (30 µg) were used for screening the ESBLs. Plates with Muller-Hindon agar (MHA) were prepared and inoculated with the test organism (turbidity corresponding to 0.5 McFarland's tube) to form a lawn culture. The above disks were applied on the surface of the agar. The plates were recorded by reading the zone diameter of the test organism.

Double disk approximation test (DDAT)

Muller-Hinton agar plates with a depth of 4 mm were prepared. With aseptic precautions 2 to 3 pure colonies of bacterial isolates were selected and inoculated into 2 ml of nutrient broth. This was incubated at 37°C for 2-3 hours to get a moderate turgidity equivalent to 0.5 Mcfarland standards. A sterile swab was dipped into a standardized inoculum and the soaked swab was rotated against the upper wall around the tube to express excess fluid. The entire surface of the muller-hinton agar was swabbed to form a lawn culture and the inoculum was allowed to dry for 15 minutes with lid in place. With sterile forceps, cefotaxime. This result was found to give the best result for detection of ESBL in our laboratory and was based on twice the radius of the inhibition zone produced by cefotxxime on its own. The plates were inverted and incubated at 37°C in an ambient air for 16-18 hours. Each plate was examined for enhancement of the zone of inhibition of cefotaxime disk at the slide facing augmentin disk. If the strain was an ESBL producer, then the zone around the cefotaxime disk was extended towards audementin disk. ATCC *Escherichia coli* (25922) was used as negative control and ATCC *Klebsiella pneumonia* (700603) was used as positive control. The above test was also done with augmentin and ceftazidime (30 µg), ceftriazone (30 µg), cefpodoxime (10 µg) and aztreonam (30 µg).

CCLS phenotypic confirmatory test

Performed by disk diffusion method as prescribed by NCCLS (2000) using β lactum and β lactamase inhibitor disk. Muller's Hinton agar plates were swapped with the test organism having the turbidity equivalent to 0.5 McFarland's standard. The was allowed to dry for 15 minutes with lid in place. Aseptically antibiotic discs of ceftazidime (30 µg) and ceftazidime / clavulanic acid (30/10 µg) were placed on the surface of the agar. The plates were incubated at 35°C for 16-18 hours and the diameters of the zone of inhibition produced were recorded.

Minimum inhibitory concentration (MIC) determination by e-test

ESBL E-test was used for MIC determination of cefotaxime and ceftazidime and confirmation of ESBL status. Sterile, non toxic swab (not too tightly spun) was dipped into the inoculum suspension. Excess fluid was removed by pressing the swab against the inside wall of the test tube and the entire agar surface was swabbed three times, rotating the plate approximately 90 degrees each time to ensure an even distribution of inoculum. Keep the plate at room temperature for about 15 minutes so that the surface is completed dry before applying E test ESBL strips.

With a pair of forceps, hold the strip tightly at areas labeled TZ(ceftazidime), TZL (ceftazidime with clavulanic acid) or ESBL and placed them on a dry clean surface. With an E-test applicator or forceps, apply both strips to the inoculated agar plate. Make sure the whole length of the strips is in complete contact with the agar surface. If necessary, remove air pockets by pressing gently on the strip with forceps, always moving from the minimum concentration upwards. Small bubbles under the strip will not affect results. Once applied, the strip cannot be moved because of the instantaneous release of antibiotic into the agar. The plates were incubated at 35°C for 16-18 hours in an ambient atmosphere. The results were interpreted as positive or negative according to the ESBL ratio obtained after calculation.

Ratio of ceftazidime / clavulanic acid MIC equals to or greater than (>) 8 indicates the presence of ESBL. Isolates, which were found to be resistant to ceftazidime / clavulanic acid were further tested for Amp C production.

Detection of Amp C β- lactamases

Modified three dimensional test

The organism was inoculated in to trypticase soy broth and incubated at 37°C for overnight. Then the overnight inoculum was pelleted by centrifugation at 3000 rpm for 15 minutes. Crude enzyme extract was prepared by repeated freeze thawing of the bacterial pellet (approximately 10 cycles). Lawn culture of *Eschrichia coli* ATCC 25922 was prepared on a Muller Hinton agar plate, and cefoxitin (30 µg) disc was placed in the center of the plate. Linear slits (3 cm) were cut using sterile surgical blade, 3mm away from cefoxitin disc, 30 µl of the enzyme extract was loaded in the slit and the plate was kept upright for 5 to 10 minutes until the liquid dried and was then incubated at 37°C for 24 hours. Enhanced growth of the surface organism at the point where the slit inserted the zone of inhibition of cefoxitin was considered as three dimensional tests and was interpreted as evidence for the presence of Amp C –lactamases.

RESULTS

There were 206 numbers of *Escherichia coli* and 84 number of *Klebsiella pneumoniae* isolated from various samples like urine, pus, blood, sputum, etc.,

NCCLS Confirmatory Test

Results of the screen test done by oxyimino-cephalosporins (ceftazidime, cefotaxime, ceftriaxone, efpodoxime and aztreonam). According to National Committee for Clinical Laboratory Standards (NCCLS) criteria for the screen test all the 290 isolates were considered as suspicious for ESBL production. Among the cephalosporins tested 56% of the isolates shown by the zone of inhibition.

Phenotypic Confirmation Test

Out of 290 isolates tested for ESBL detection, by phenotypic confirmation test using ceftazidime and cefta-

Table 1: ESBL producing strains of E.coli by phenotypic confirmation test

S.No	Sample	*E.coli* (ESBL)	*E.coli* (NON ESBL)
1	Urine	84	66
2	Pus	16	7
3	Blood	7	5
4	Wound swap	5	1
5	Tracheal aspirate	4	-
6	Sputum	3	4
7	Catheter tip	1	1
8	Bal	-	2
	Total	120	86

Table 2: ESBL producing strains of Klebsiella pneumoniae by phenotypic confirmation test

S.No	Sample	*Klebsiella pneumonia* (ESBL)	*Klebsiella pneumonia* (NON ESBL)
1	Urine	84	66
2	Pus	16	7
3	Blood	7	5
4	Wound swap	5	1
5	Tracheal aspirate	4	-
6	Sputum	3	4
7	Catheter tip	1	1
8	Bal	-	2
	Total	120	86

Table 3: Double disk approximation test (DDAT)

S.No	Methods	*E.coli*		*Klebsiella pneumonia*	
1.	Double disk approximation test (DDAT)	ESBL	NON ESBL	ESBL	NON ESBL
		118	-	40	-

zidime/clavulanic acid: 162 isolates were found to be ESBL producers (55.86%). (table-1 and 2)

Double Disk Approximation Test (DDAT)

Escherichia coli (120 ESBL producers and 86 non ESBL producers) and *Klebsiella pneumoniae* (42 ESBL producers and 42 Non ESBL producers) were tested for ESBL production by DDAT. Double disk approximation test detected only 118 and 40 of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae*. It was negative in all ESBL negative bacterial isolates.(table-3).

E-Test

E – test is a modified MIC; the E-strip has two sides, on one side ceftazidime (TZ) in graded concentration and on the other side ceftazidime with clavulanic acid (TZL) in graded concentrations is present. ESBL producers show the elliptical zone of inhibition to ceftazidime (TZ) and ceftazidime clavulanic(TZL) acid. Threefold reduction in MICs will be taken has been positive for ESBL production in that isolate 100 out of 120 ESBLs producing *Escherichia coli* and showed in E-test method (49 %). The remaining isolates to give false negative results. Probably, because they also were found to produce Amp C beta lactamase enzyme. 35 out of 42 (83 %) ESBL producing *Klebsiella pneumoniae* Showed ESBL productions by E – test, other isolates to give false negative results.

Amp C Detection Method

A total of 120 *Escherichia coli* and 42 *Klebsiella pneumoniae* which are ESBL producers were screened for Amp C beta lactamase production by cefoxitin (30 µg) disc diffusion method and confirmed by inhibitor an inhibitor method using boronic acid. Amp C production was observed in 94% of ESBL producing *Escherichia coli* and 90% of ESBL producing *Klebsiella pneumoniae* in Amp C detection method.

DISCUSSION

Extended spectrum β – lactamases are rapidly evolving group of β – lactamases which share the ability to hydrolyze 3rd generation cephalosporines and aztreonam yet are inhibited by clavulanic acid. Incidence of ESBL producing gram negative bacteria are increasing alarmingly world over. The extended spectrum β – lactams became widely used for the treatment of serious infections due to gram negative bacteria's in the 1980s. ESBLs are now a problem in hospitalized patient's worldwide. The ESBL phenomenon began in western Europe, most likely because extended spectrum β – lactam antibiotics were first used there clinically. In united states occurrence of ESBL production in *Enterobactericeae* ranges from 0-25 % depending upon the institution, with the national average being around 3%. In the netherlands, a survey of 11 hospital laboratories

showed that < 1 % of *Escherichia coli* and *Klebsiella pneumoniae* strains possessed an ESBL. However, in France, as many as 40 % of *Klebsiella pneumoniae* isolates were found to be ceftazidine resistant. Across Europe, the incidence of ceftazidime resistance among strains was 20 % for non –ICU isolates and 42 % for isolates from patients in the ICU. In Japan, the percentage of β – lactam resistance due to ESBL production in *Escherichia coli* and *Klebsiella pneumonia* remains very low.(Joumana et al., 2003). Double disk approximation test is a useful detection method but needs vigourous standardization and proper placement of discs. Thomson and Senders reported 79% sensitivity while Cormican et al ., Sukhla et al., and Abiugail et al., observed sensitivity of 87%, 90.6%and 93.3% respectively. A successful approach for the control over the spread of ESBL producing organisms involved switching to different classes of board spectrum antibiotics for the treatment of serious infections. The two most successful replacement antibiotics have been imipenem and piperacillin / tazobactem. (Bush et al., 1995).In this study, A total of 290 isolates of which 206 *Escherichia coli* and 84 *Klebsiella pneumoniae. ESBL* production was found to be present in 58.25 % of *Escherichia coli* and 50 % of *Klebsiella pneumoniae.* Double disk approximation test has an agreement of only 52.44% with the phenotypic confirmatory test. E- test detects only 49 % of ESBL producing *Escherichia coli* and 83% of ESBL producing *Klebsiella pneumoniae.* False negative E-test results were due to co-production of Amp C *–β* lactamase enzyme by these isolates. Amp C production was observed in 94 % of ESBL producing *Escherichia coli* and 90 % of ESBL producing *Klebsiella pneumoniae.*

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