ORIGINAL ARTICLE

COMPARISON OF VARIOUS METHODS FOR THE DETECTION OF EXTENDED SPECTRUM BETA LACTAMASE IN KLEBSIELLA PNEUMONIAE ISOLATED FROM NEONATAL INTENSIVE CARE UNIT, AHMEDABAD

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ABSTRACT

Background & objectives: Several outbreaks of neonatal septicemia by extended spectrum beta lactamases (ESBL) producing isolates are not uncommon & are associated with increased mortality. Moreover, detection of ESBL in isolates that produce Amp C beta lactamases is problematic. So, clinical laboratories need to develop quick screening methods for detection of ESBL producing strains.

Materials & methods: Out of 600 samples of blood culture from neonatal intensive care unit (NICU) patients, 54 Klebsiella pneumoniae were tested for ESBL by screening & confirmatory tests recommended by clinical laboratory standard institute (CLSI). In addition, double disk synergy test (DDST), modified double disk synergy test (MDDST), direct modified three dimensional tests (DMTDT) & indirect modified three dimensional tests (IMTDT) were evaluated for optimum detection of ESBL in Amp C producing isolates.

Results: From 54 Klebsiella pneumoniae, 48 were screen positive for ESBL by CLSI criteria. ESBL detection was confirmed in 44 isolates. Phenotypic confirmatory disk diffusion method (PCDDT) by CLSI detected ESBL in 41/48 isolates. DDST using amoxicillin-clavulanic acid detected the same in 36/48 cases. MDDST using cefepime & piperacillin-tazobactam detected ESBL in 44/48 cases, including 3 isolates having Amp C enzyme which was confirmed by modified three dimensional tests for Amp C enzyme. DMTDT detected only 4 isolates with ESBL production. IMTDT detected all 44 ESBL producing isolates.

Conclusion: The prevalence of ESBL producing Klebsiella pneumoniae in our NICU was 81.48%. MDDST & DMTDT seem to be superior to PCDDT, DDST & IMTDT for detection of ESBL in Amp C producing isolates.

Key words: ESBL, Amp C, PCDDT, DDST, MDDST, DMTDT, IMTDT

INTRODUCTION

In spite of great advances in the antimicrobial therapy, neonatal life support measures and the early detection of risk factors, septicemia continues to be a major cause of mortality and morbidity among neonates around the world. [1] The most common organisms responsible for these infections are multidrug resistant gram negative bacilli, particularly members of the family Enterobacteriacae & non fermenting gram negative rods.[2] Several outbreaks of septicemia by gram negative isolates have been reported & phenomenon of isolation of extended spectrum beta lactamase producing isolates is not uncommon & is associated with increased mortality. Moreover, treatment of the infection caused by ESBL producers is complicated not only due to resistance to extended spectrum cephalosporins, but also because many ESBL genes are present on large plasmid which contain genes encoding resistance to many other antibiotics including aminoglycosides, chloramphenicol, sulphonamides and tetracyclines.[3,4]It is common practice to institute early empirical therapy with broad spectrum antibiotics in patients presenting with clinical features suggestive of septicemia or bacteremia.[5] Further, as report of blood culture isolation & susceptibility are usually available after 72 hours or more, any delay in the initiation of correct empirical therapy or improper choice of

antimicrobials cannot be justified.[3]Given the severity of septicaemia, such empirical therapy may be justified but the specific therapy based on the antibiogram of the isolate will definitely improve the therapeutic outcome.[5] Therefore, this study was designed to investigate the prevalence of ESBL producing Klebsiella pneumoniae in neonatal septicemia; to compare the reliability of various methods for the ESBL detection including those which can be used in the isolates possessing both ESBL and AmpC enzymes & to observe antimicrobial susceptibility pattern of ESBL & non ESBL producing isolates which would enable formulation of appropriate antimicrobial policy for such patients.

MATERIALS & METHODS

A total of 63 clinical isolates of Klebsiella spp. isolated from 600 samples of blood culture, from the suspected cases of the neonatal septicemia admitted in the neonatal intensive care unit at our teaching institute in Ahmedabad, Gujarat, West India during a period of February 2009 to July 2009 were included in this study.

Identification of the all isolates was done by standard methods and the antibiotic susceptibility was determined by Kirby-Bauer disk diffusion method as per CLSI recommendations. [3] Following tests are used for determination of ESBL activity.

Screening test and phenotypic confirmatory disc diffusion test (PCDDT)

These were applied as per CLSI guidelines by using control strains of Escherichia coli ATCC 25922 (Beta – Lactamase negative) and Klebsiella pneumoniae ATCC 700603 (ESBL positive). [3]

Double disk synergy test (disk approximation test)

This test was done by using a disc of augmentin (20µg amoxicillin + 10µg clavulanic acid) and discs of cefpodoxime (30µg), ceftazidime (30µg) and cefotaxime (30µg); which were placed around augmentin disc keeping the distance of 16 to 20 mm from it. (centre to centre). The organisms were considered to be producing ESBL when the zone of inhibition around any of these cephalosporin discs showed a clear-cut increase towards the augmentin disc.[6]

Modified double disk synergy test (MDDST)

The original double disk synergy test was modified for detecting ESBL in Amp C producing clinical isolates. A disc of augmentin was placed in the centre; then discs of cefpodoxime (30µg), ceftazidime (30µg), cefotaxime (30µg), aztreonam(30ug) and cefepime (30µg) were kept around it at distance ranging between 16 and 20 mm from the augmentin disc (centre to centre), and a disc of piperacillin-tazobactam (100/10µg) was placed at a distance ranging between 22 and 25 mm from the cefepime disc. The organisms were considered to be producing ESBL when the zone of inhibition around cefepime or any of the extended-spectrum cephalosporin discs showed a clear-cut increase towards the piperacillin-tazobactam disc or augmentin disc. [6]

Modified three dimensional tests (MTDT)

(i) Direct modified three dimensional test (DMTDT)

MHA plates were inoculated with test strains matching 0.5 McFarland turbidity standards as described for disk diffusion test and a disc of ceftazidime, ceftriaxone, cefotaxime or aztreonam was placed in the center of the plate. A well of 4 mm (diameter) was punched at a distance of 2 mm from the antibiotic disc. The inoculum (30µL) of the test strain preadjusted to 5.0 McFarland standards was seeded into the well. Heart shaped distortion of zone of inhibition with growth of test organism appearing behind the well and reaching the well was indicative of an ESBL production.[7]

(ii) Indirect modified three dimensional test (IMTDT)

It is same as DMTDT except, MHA plates were seeded with the inoculum of a standard sensitive strain (E. coli ATCC 25922) adjusted to 0.5 McFarland standard instead of test strain. Heart shaped distortion of zone of inhibition around the β -lactam disc was indicative of an ESBL production. [7]

Modified three-dimensional testwas used for detection of AmpC β-lactamase in ESBL positive isolates with reduced susceptibility to cefoxitin (≤18mm).[6]

RESULTS & OBSERVATIONS

A total of 600 blood culture samples from NICU patients suspected of having septicemia were processed for the detection of Klebsiella pneumoniae organism with ESBL production. Out of 600 cases, growth was obtained in 266 blood culture samples. (Gram positive cocci-86, Gram negative bacilli-170 & candida species-10). Among these 266 samples, Klebsiella species were isolated in 63 samples from which Klebsiella pneumoniae were isolated in 54 cases, and remaining 9 were other Klebsiella species (6 were Klebsiella oxytoca and 3 were Klebsiella ornithinolytica). Table 1 shows resistance pattern of Klebsiella pneumoniae.

Table 1: Bacteriological profile of neonatal septicemia

Organism isolated	Positive isolates (n=266) (%)
Staphylococcus coagulase negative	73 (27.44)
Klebsiella species	63 (23.68)
Pseudomonas aeruginosa	42 (15.78)
Acinetobacter species	37 (13.90)
E. coli	26 (9.77)
Candida species	10 (3.75)
Staphylococcus aureus	08 (3.00)
Streptococcus species	03 (1.12)
Proteus vulgaris	01 (0.37)
Morganella species	01 (0.37)
Enterococus species	01 (0.37)

Table 2: Antibiotic resistance pattern of ESBL producing Klebsiella pneumoniae

Name of Antibiotic	Susceptible isolates	Resistance (in %)
Cefaclor	00	100
Ceftazidime	00	100
Cefotaxime	00	100
Ceftriaxone	00	100
Cefpodoxime	00	100
Cefipime	03	93.18
Aztreonam	00	100
Cefoxitin	41	06.81
Amoxicillin-clavulanic acid	41	06.81
Piperacillin/tazobactam	32	27.27
Imipenem	44	00
Moxifloxacin	39	11.36
Amikacin	29	34.09
Cotrimoxazole	09	79.54
Tetracycline	02	95.45

Prevalence of ESBL producing Klebsiella pneumoniae was detected by following tests - screening and phenotypic confirmatory tests as per CLSI guidelines, double disk synergy test, modified double disk synergy test and direct and indirect three dimensional tests.[3,6,7] Our study showed prevalence of ESBL in Klebsiella pneumoniae was 81.48% (44/54). Table 2 shows percentage of ESBL producing Klebsiella pneumoniae by different methods. Table 3 shows comparison of prevalence of ESBL in Klebsiella pneumoniae in our study and with other studies.

Table 3: Comparision of various methods of ESBL detection

ESBL detection methods	Positive (n=44) (%)
PCDDT(Phenotypic confirmatory disk diffusion test)	41 (93.18)
DDST(Double disk synergy test)	36 (81.81)
MDDST(Modified double disk synergy test)	44 (100)
DMTDT(Direct modified three dimensional test)	04 (9.09)
IMTDT(Indirect modified three dimensional test)	44 (100)

Figure.1: Positive phenotypic confirmatory disk diffusion test by CLSI for ESBL showing ≥ 5 mm increase in zone diameter of ceftazidime (CA) in the prescence of inhibitor clavulanic acid (CAC), synergism is also seen between these two antibiotics.

Detection of AmpC β-lactamases in ESBL positive strain by Modified three-dimensional test showed, there were 4 isolates that produced AmpC enzymes.

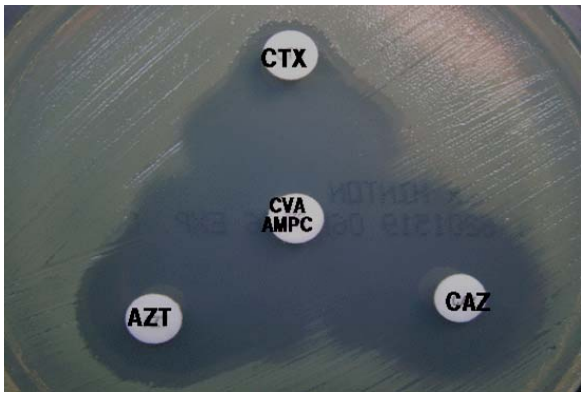

Figure.2: Positive double disk synergy test for ESBL showing synergy between amoxicillin-clavulanic acid (CVA/AMPC) & indicator antibiotics like cefotaxime (CTX), Ceftazidime (CAZ), aztreonam (AZT)

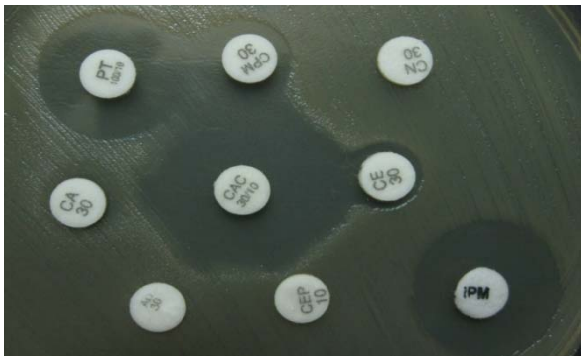

Figure.3: Positive modified double disk synergy test for ESBL showing synergy between cefipime (CPM) &piperacillin-tazobactam (PT).Amoxicillin-clavulanic acid (AC) is also showing synergy against cefipime (CPM), cefotaxime (CE), ceftazidime (CA).The organism also exhibits resistance to Aztreonam (AO), cefpodoxime (CEP) & cefoxitin (CN) and sensitivity to imipenem (IPM)

Figure.4: Positive modified double disk synergy test for ESBL showing synergy between cefepime (CPM) &piperacillin-tazobactam (PT).Here amoxicillin-clavulanic acid (AC) is not able to detect synergy against cefotaxime (CE) & ceftazidime (CZ).

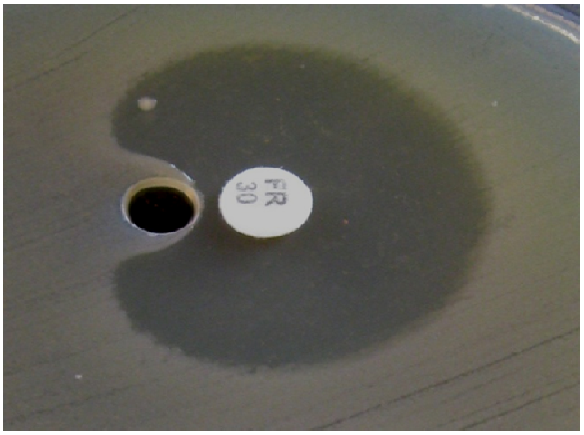

Figure.5: Positive modified indirect three dimentional test for ESBL showing heart shaped distortion of the zone of inhibition around cetazidime disc near well inoculated with heavy suspension of test organism.

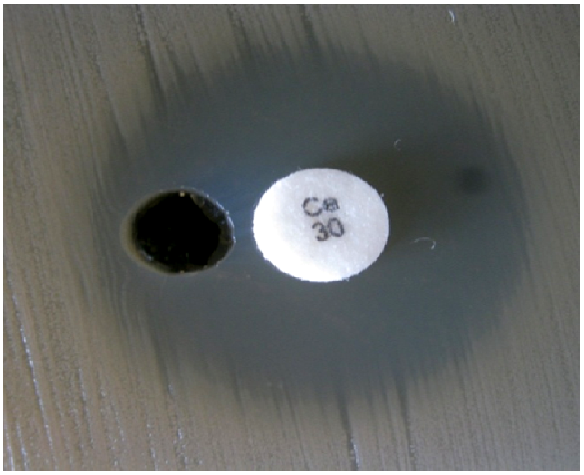

Figure.6: Negative indirect modified three dimentional test for ESBL showing clear zone of inhibition around cetazidime disc.

Figure.7: Negative modified direct three dimentional test for ESBL showing no zone of inhibition around ceftazidime disc thus masking test interpretation

DISCUSSION

Neonatal septicemia is the single most important cause of neonatal deaths in the community accounting for over half of them.[9].It is a life-threatening emergency, and rapid treatment with antibiotics is essential for favorable outcome. [10] For effective management of neonatal septicemia cases, the study of bacteriological profile with their antibiotic sensitivity pattern plays a significant role. [11]

In this study blood culture positivity rate in neonatal septicemia cases was 44.33% (266/600). A study done by Roy I et al reported positivity rate 47.5% which was well correlated with our study[12].Gram-negative isolates constituted major group (63.90 %), than Gram positive isolates (32.33%) and remaining were the candida spp. which is comparable with the study of Nalini et al (58.5 % and 48.5% respectively).[2]

Of all the bacterial isolates the most frequent gram negative offender was Klebsiella spp. (24.6%) and Klebsiella pneumoniae was isolated in 21.09% of cases. Roy I et al also found the Klebsiella spp. as the most prevalent Gram negative isolates, and our study is well co-related with his study.[12]

Table 4: Comparison of our study with other studies for prevalence of ESBL producing Klebsiella pneumoniae

Authors	Prevalance of ESBL in Klebsiella pneumoniae
Amita Jain & Rajesh Mondal[16]	58.00%
Bhattacharjee A et al[14]	62.50%
Brendan D Crowley[17]	25.00%
Jain et al[20]	86.60%

ESBLs are now a problem in hospitalized patients throughout the world. The prevalence of ESBLs among the clinical isolates varies greatly worldwide and in geographic areas and are rapidly changing over time.[13] Among the all the ESBL detection methods indirect modified three dimensional test and modified double disc synergy tests were the most sensitive methods as shown in table 3.

ESBL screening method recommended by CLSI showed that cefpodoxime had highest sensitivity than any other cephalosporin and identified that 48 out of 54 isolates of Klebsiella pneumoniae showed decreased susceptibility to at least any one indicator antibiotic for ESBL detection. Out of 48 screening positive Klebsiella pneumoniae, ESBL production was confirmed in 44 isolates. So the prevalence of ESBL producing Klebsiella pneumoniae in our Neonatal ICU was 81.48%. A study done by Amita Jain et al also found cefpodoxime to be the most efficient antimicrobial agent as screening test.[16]

Various methods had been used for optimum detection of ESBL. PCDDT was identified as most sensitive & specific method for the detection of ESBL in Klebsiella species and was capable of detecting ESBL in 41(93.18%) isolates out of 44 ESBL producing strains,

likewise, DDST, using amoxiclav as inhibitor of ESBL, showed positive result in only 38 isolates (81.81%). So among these two methods, PCDDT was more sensitive than DDST. This was also shown by the study done by MKR Khan et al at New Delhi in 2008.[6] Here, decreased sensitivity of both the tests can be explained by the presence of strains which produce both ESBL & inducible AmpC enzymes. Clavulanic acid which was used in the standard tests for ESBL detection (PCDDT & DDST) act as inducers of high level AmpC production and it led to resistance to 3rd generation cephalosporins as well 3rd generation cephalosporins+clavulanic acid. So even if ESBL was present, it would not be detected and resulted in false negative test. [6]

In MDDST, we had used piperacillin- tazobactam as ESBL inhibitor. Another modification in the original double disc synergy test was use of 4th generation cephalosporins & optimum spacing of drugs for detection of synergy. If Amp C was present, cefepime would be sensitive so synergy could be seen with 4th generation cephalosporins & inhibitor antibiotic. [6] Tazobactam & sulbactam were less likely to induce AmpC B-lactamase.So, simultaneous Amp C induction did not occur. So piperacillin- tazobactam or ampicillin-Sulbactam remained sensitive in susceptibility testing & synergy could be seen with 3rd generation cephalosporins or 4th generation cephalosporins & piperacillin- tazobactam or ampicillin- sulbactam. [6] This MDDST had identified all 44 (100%) ESBL positive isolates. It could also identified 3 other isolates, which were not detected by standard tests (PCDDT & DDST).Thus it had 100% sensitivity & specificity for detection of ESBL. A study of MKR Khan et al had similar findings, in which DDST was positive in 25/40 isolates, MDDST was positive in 40/40 isolates, PCDDT was positive in 39/40 isolates. [6]

The presence of inducible AmpC enzyme in 3 isolates were confirmed by the MTDT by using cefoxitin disc.[6] DMTDT could identify only 4 (9.09%) ESBL positive isolates while IMTDT identified all 44 ESBL positive isolates. Thus it was most sensitive (100 %) among all other tests. It was superior in detecting ESBL than PCDDT & DDST in our study as well as in other studies also. A study carried out by T Menon et al at Chennai in 2003 had shown that out of total 70 isolates, 14 were ESBL positive. DDST had identified 2/14 (14.2%) & MTDT had identified 12/14 (85.7%) which was well corelated with our study. [7] Thus, PCDDT& DDST should be used in the isolates which produce only ESBL but are not useful for detection of ESBL in isolates producing AmpC enzyme like Enterobactor, Serratia, Citrobacter and now with Klebsiella species also.

Prevalence of ESBL in Klebsiella pneumoniae in NICU in our study was 81.48%. Susceptibility pattern of ESBL positive isolates showed that all 2nd generation cephalosporins, 3rd generation cephalosporins and aztreonam were resistant. The cefepime was resistant in 3 isolates. The imipenem was sensitive in all ESBL producing isolates. Amoxiclav was sensitive in 41 cases while piperacillin- tazobactam was sensitive in 32 cases. Co-resistance to other group of drugs was noted in ESBL producing organisms. Tetracycline showed 95.45% of resistance while cotrimoxazole showed 79.54% of resistance. In addition to imipenem, good activity against ESBL producing Klebsiella pneumoniae was noted by amikacin & moxifloxacin. Amikacin was sensitive in 29 cases while moxifloxacin was sensitive in 39 cases. So depending on the sensitivity pattern, these drugs can be given to the serious neonatal septicemic patient. Amikacin and higher quinoloners are good alternatives and they will also provide some economic relief to the patients. Imipenem drug should be reserved for the future resource. Continued monitoring of the susceptibility pattern of the organisms is necessary in clinical settings to detect true burden of the antibiotic resistance for proper disease management.

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