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Research Article

Phenotype Prevalence of ESBLs and Genotype Prevalence of CTX-M in bacterial isolates from lower respiratory tract specimens in tertiary care centre in central Kerala

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Abstract

Background and Objectives: *Extended spectrum beta lactamases (ESBLs) are responsible for resistance to third generation Cephalopsorins and Monobactums which form the mainstay of therapy in majority of clinical conditions. This study was performed to generate data on ESBLs and their CTX-M genotypes as prevalent in tertiary care centre at Kottayam, Kerala, India. The prevalence of ESBLs among Gram negative rods in specimens from lower respiratory tract in tertiary care centre at Kottayam was detected using standard methods and their CTX-M genotypes were detected using RT-PCR. The risk factors associated with the emergence of ESBLs and the spread of CTX-M enzymes were discussed.*

Objectives: *To determine the phenotype prevalence of ESBLs and genotype prevalence of CTX-M among Gram negative rods in specimens from respiratory tract at Government Medical College Hospital, Kottayam using standard methods and RT-PCR respectively.*

Materials and Methods: *1216 specimens from lower respiratory tract namely sputum, broncholaveolar lavage and tracheal aspirate were collected during June 2017 to November 2017. Culture yielded 290 isolates of which 40 were multidrug resistant (MDR) strains. Phenotypic testing of ESBLs was done using Clinical Laboratory Standards Institute (CLSI) guidelines- disc diffusion testing. Genotypic testing of CTX-M Beta-lactamase was done using RT-PCR using primers from Gen Bank for Group I- CTX-M1, Group II- TOHO1, Group III- CTX-M825 and Group IV- CTX-M914.*

Results: *Phenotypic testing showed that out of 40 MDR strains, all 40 tested positive, of which 31/220 (14%) were Klebsiella pneumoniae and 9/70 (12.8%) were E coli. Genotypic testing showed that of the 40 MDR strains, 15% of Klebsiella pneumoniae and 11% of E coli tested positive for Group III CTX-M while all tested negative for Group IV CTX-M. 10-14% of E coli and Klebsiella pneumoniae isolates tested positive for Groups I and II of CTX-M.*

Conclusion: *Even though CTX-M ESBLs which originated in Kluyvera species were first reported from Japan in 1980s, over the last decades, these genes have dispersed globally among Gram negative rods creating chaos in chemotherapy. This study of lower respiratory tract specimens from tertiary care centre reveals prevalence of ESBLs among multidrug resistant(MDR) strains at 72.7% in phenotype testing and 0-100% in the four gene clusters of CTX-M in genotype testing. Since the clinical microbiology laboratory is the first line of defence in the detection and control of ESBLs and failure to detect ESBLs implies treatment failure, this study stresses the importance of routine ESBL testing of atleast MDR strains and the need for clinicians to practise antibiotic stewardship earnestly.*

Keywords: *Extended spectrum beta lactamases (ESBLs), CTX-M gene, Multidrug resistant (MDR).*

Introduction

Even though Alexander Fleming only humbly predicted that bacteria would eventually develop resistance towards penicillin, today antibiotic resistance has become a huge public health concern. Over the last half century, third generation Cephalosporins form the most commonly used antibiotic class and today there is global dissemination of Extended spectrum beta lactamases (ESBLs), the most common of which is CTX-M[1,2,4,5,6]. They hydrolyse third and fourth generation Cephalosporins and Monobactums but not Cephamycins and Carbapenems. Although the first report of CTX-M was by Bauernfeind et al in 1989, in the following years, Gram negative rods evolved in such a way that resulted in a CTX-M pandemic with reports pouring in from all over the world as documented by Coque in 2006, Canton in 2008 and Bush in 2010 and many others [2,3,5,6,8,9]. This study attempts to trace CTX-M ESBLs in respiratory tract samples in tertiary care centre at Kottayam in central Kerala, India.

CTX-M ESBLs belong to class A of Ambler classification and 2be of Bush-Jacoby-Medeiros classification[1,3]. They are located in plasmids generally but their origin points to chromosomal *bla* genes present in *Kluyvera* species from where they spread to Gram negative rods through mobile genetic elements. A review of global CTX-M research reveals that CTX-M have replaced TEM, SHV and other ESBL variants through their horizontal transfer by insertion sequences and transposons as concluded in the review article by Rafael canton et al in 2012[4,5,6].

In this study, the clinical scenario here points to increased usage of third generation Cephalosporins. Cefotaxime used to be the most common antibiotic used for most clinical situations deserving second line antibiotics but the use has come down owing to reports of CTX-M ESBLs. Presently, Ceftriaxone and Ceftazidime are used for situations in which bacteria belonging to the family Enterobactericeae are isolated. The phenotype testing for these controls and clinical strains included screening tests for Cefpodoxime, Ceftazidime and Cefotaxime and confirmatory tests with Ceftazidime-Clavulanic acid and Cefotaxime-Clavulanic acid, all by disc diffusion[7].

In 2004, J D D Pitout with A Hossain and N D Hanson published the first classification of CTX-M Beta lactamases with four groups[17,18]. According to this, Group I CTX-M included 1, 3 CTX-M enzymes; Group II included TOHO enzyme, group III included 8 CTX-M enzyme and group IV included 9 CTX-M enzyme. In this study, the primers were used accordingly, and the genotypes among MDR strains of bacteria of the family Enterobactericeae were determined.

Materials and Methods

Bacterial Isolates: In this study, from 1216 samples from respiratory tract during June to December 2017, a total of 290 isolates of Gram negative rods were obtained in culture and were selected for processing. All isolates were plated on Blood agar, Chocolate agar and MacConkey agar and identification was based on biochemical reactions.

Screening for ESBLs in routine Antimicrobial susceptibility testing: 43 MDR strains were identified by routine antimicrobial testing done as per Clinical Laboratory Standards Institute (CLSI) guidelines. The isolates were identified as susceptible, intermediate or resistant by disc diffusion. The drugs tested include Ampicillin, Cephalexin, Cefotaxime, Gentamicin, Amikacin, Ciprofloxacin, Cefoperazone-Sulbactum, Piperacillin-Tazobactum and Meropenem. The control strain E coli ATCC 25922 was used.

Phenotypic Confirmatory tests for ESBLs: 40 multidrug resistant strains (resistant to more than three classes of antibiotics) were selected. The control strains used were *Klebsiella pneumoniae* 700603 and *E coli* ATCC 25922. Apart from these, three control strains from patients in a reference laboratory that contained CTX-M gene group I, II and III but not CTX-M group IV were tested. The testing was disc diffusion method as per CLSI guidelines- for *K pneumoniae* and *E*

coli, Cefpodoxime 10 µg, Ceftazidime 30µg, Aztreonam 30µg, Cefotaxime 30µg and Ceftriaxone 30µg. The criteria for performance of ESBL test was- in case of *Klebsiella pneumoniae* and *E coli*, Cefpodoxime zone diameter <= 17mm, Ceftazidime <=22mm, Aztreonam <=27mm, Cefotaxime <=27mm and Ceftriaxone <=25mm.

The β-lactam combination agents used were Ceftazidime-Clavulanate 30/10µg and Ceftazidime-Clavulanate 30/10µg. In case of *Klebsiella pneumoniae*, increase in zone diameter of 5mm with Ceftazidime-Clavulanate and 3mm with Cefotaxime-Clavulanate and in case of *E coli*, increase in 2mm diameter with β-lactam combination agents were taken as positive for presence of ESBLs.

CTX-M gene identification: As per the classification of CTX-M genes by J D D Pitout et al, there are four groups identified by four primers namely Group I CTX-M gene by CTX-M1, Group II CTX-M gene by TOHO1, Group III CTX-M gene by CTX-M825 and Group IV CTX-M gene by CTX-M914. The testing was done by RT-PCR as per CDC guidelines. DNA template preparation and PCR amplification were carried out on thermal cycler of Applied Biosystems 7500 Fast Real-Time PCR system. The 40 MDR isolates were tested and along with them, three isolates from a reference laboratory which were used as positive control.

Results

The specimens collected from lower respiratory tract infections include sputum, bronchoalveolar lavage and tracheal aspirate as in Table 1. The most common specimen was sputum accounting for 76% of specimens followed by bronchoa-lveolar lavage and tracheal aspirate.

28% of specimens were from Pulmonology unit, 22% from Cardiothoracic unit and 20% from critical care unit- both medical and surgical. The remaining 30% of specimens were from Medicine, Nephrology and Neurosurgery units. 77% of specimens were from males and 23% were from females. 8% of specimens were positive for Acid fast bacilli either in smear or culture. 10% of patients were started on ATT, of which 2% was empirical ATT. 1.5% of specimens were positive for Candida albicans. They were treated with either Fluconazole or Amphotericin B.

Table 2 shows the prevalence of ESBLs among 31 isolates of *Klebsiella pneumoniae* and 9 of *E coli* of Enterobactericeae. Table 3 shows the prevalence of CTX-M Beta-lactamases- Groups I, II, III and IV among Gram negative rods as present in *Klebsiella pneumoniae* and *E coli* and three control strains from a reference laboratory. The risk factors for ESBL producing strains include prolonged antibiotic usage with third generation Cephalosporins, ICU stay with lines/ catheter and previous and multiple invasive procedures.

Table 1: Study Format

1216 samples from Respiratory tract- Sputum, FOB wash and Tracheal aspirate
290 isolates- *Klebsiella pneumoniae* and *E coli*
ESBL phenotyping of 43 isolates by CLSI method
Genotyping of 43 isolates for CTX-M genes-four groups- by RT-PCR

Table 2: Prevalence of ESBLs among Gram negative rods- Phenotype testing

Nature of Isolate	Number of Isolates	Number of ESBL Positive Isolates
Klebsiella pneumoniae	220 (82%)	31 (14%)
E coli	70 (18%)	9 (12.8%)
Total	290 (100%)	40 (13.7%)

Table 3: Prevalence of CTX-M Beta-lactamases among Gram negative rods- Genotype testing

CTX-M group and Primer	*Klebsiella pneumoniae* (220isolates)	*E coli* (70 isolates)
Group I- CTX-M1	27 (12%)	7 (10%)
Group II- TOHO1	30 (14%)	8(11%)
Group III- CTX-M825	32 (15%)	8 (11%)
Group IV- CTX-M914	0	0

Discussion

In this study, the phenotypic testing of ESBLs was done as per CLSI guidelines of 2017 which includes disc diffusion with Cefpodoxime, Cefotaxime, Ceftriaxone, Ceftazidime, Aztreonam and the combination discs of Cefotaxime-Clavulanate and Ceftazidime-Clavulanate. It is realized that the phenotypic testing performed is but a cumulative demonstration of different mechanisms of antibiotic resistance that includes decreased drug entry, efflux pumps, release of enzymes such as inducible AmpC, ESBLs, alteration of target proteins and development of alternative pathways by bacteria and that it is not specific for CTX-M ESBLs. This study documents 14% of both *Klebsiella pneumoniae* and 12.8% *E coli* of Enterobactericeae as positive for ESBLs phenotyping[10,11,12,13,14,15,16].

Based on the phylogenetic tree analysis of all CTX-M Beta lactamases so far reported, the CTX-M lineage was initially differentiated into clusters as derived from chromosomal bla gene KluC in *Kluyvera cryocrescens*, KluA in *K ascorbata*, and KluG and KluY in *K georgiana* which were considered as the progenitors of CTX-M-1, CTX-M-2 and CTX-M-9 clusters respectively[19,20]. In this study, the genotype testing was done by RT-PCR with primers for the four groups of CTX-M as given in the J D D Pitout et al classification. In case of Group III CTX-M, this gene was present in 15% of isolates of Klebsiella pneumoniae and 11% of E coli. In case of Group I and Group II CTX-M, varying range from 10 to 14% were positive for the Gram negative rods. All isolates tested negative for Group IV CTX-M[21,22,23].

This study highlighted two case histories. In case of one male patient, a tailor, aged 35 years from central Kerala, who was admitted in cardiology ICU one month and was in and out of ventilator most of the time, one sputum sample and one tracheal aspirate were received for culture and sensitivity and from both these samples, culture yielded multidrug resistant *Klebsiella pneumoniae* that tested positive for ESBLs both phenotypically and for CTX-M genotypically. Inspite of earnest efforts, the patient succumbed after one month in the ICU.

In case of one female patient, a housewife, aged 23 years from central Kerala, she was referred from adjacent local government hospital as a case of post operative sepsis with multiorgan dysfunction/ HELPP/ acute fatty liver of pregnancy following Caesarean section. Her baby was normal but she presented with oozing from the wound site, anemia, thrombocytopenia and oliguria. She was in critical care ICU for three months and in the dialysis ward for one month. It was an eventful period in which she was started on dialysis and was on and off the ventilator. During this period, two sputum samples and five tracheal aspirates were received for culture and sensitivity. Three out five tracheal aspirates yielded in culture- multidrug resistant *Klebsiella pneumoniae* which tested positive for presence of ESBLs both phenotypically and CTX-M Groups I, II, III genotypically. Therapy was administered as per existing antibiotic policy and she survived on injection Piperacillin-Tazobactum, Meropenem and Colistin during various but separate periods.

In this study, the risk factors contributing to the prevalence of ESBLs include prolonged antibiotic usage with third generation antibiotics, prolonged ICU stay with indwelling lines and catheters and previous/ multiple invasive procedures/ surgeries. In these cases, the patient almost behaves like an immunocompromised subject with decreased immunity and is highly susceptible to otherwise minor pathogens. The presence of ESBLs warrants the use of higher antibiotics such as Meropenem and Colistin which are not without side effects and complications.

Conclusion

The presence of ESBLs in Gram negative rods makes them resistant to third generation Cephalosporins and Monobactums. Even though, initially guidelines for phenotypic testing of ESBLs were put forth for routine testing by CLSI, it is presently recommended only for epidemiological and infection control purposes in

the current manual. Nevertheless, the clinical microbiology laboratory is the first line of defence in the detection and control of ESBLs and failure to detect ESBLs implies treatment failure. This study of lower respiratory tract specimens from tertiary care centre in Kerala demonstrates a prevalence of 14% for *Klebsiella pneumoniae* and 12.8% for *E coli* of ESBLs among multidrug resistant isolates of Enterobactericeae when tested phenotypically.

Even though CTX-M ESBLs which originated in Kluyvera species were first reported from Japan in 1980s, over the last decades, these genes have dispersed globally among Gram negative rods creating chaos in chemotherapy. This study demonstrates a range of prevalence of 0 to 19% of the four groups of CTX-M ESBLs in *Klebsiella pneumoniae* and *E coli* when tested genotypically.

Therefore this study in central Kerala stresses the importance of routine ESBL testing of atleast MDR strains and also points to an urgent need among clinicians and surgeons to adhere to a uniform hospital antibiotic policy and practise antibiotic stewardship earnestly.

Conflict of Interest: Nil

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