



Detection of *bla*_{TEM} and *bla*_{CTX-M} genes by multiplex polymerase chain reaction amongst uropathogenic *Escherichia coli* strains isolated from hospitalized patients in Kolkata, India

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Received: 24 October 2011

Revised: 04 November 2011

Accepted: 05 November 2011

Key words: UPEC, ESBL, Multiplex PCR.

Abstract

Production of extended-spectrum β -lactamase (ESBL) is one of the most important resistance mechanisms that hamper the antimicrobial treatment of infections caused by uropathogenic *Escherichia coli* (UPEC). The objective of the study was to determine the *bla*_{TEM} and *bla*_{CTX-M} producers simultaneously in a single tube reaction by multiplex PCR technique using gene specific primer sets. A total of 100 *E. coli* strains, isolated from hospitalized patients were phenotypically screened for ESBL production using drug-inhibitor combination disks, namely, ceftazidime-clavulanate (30+10 μ g) and cefotaxime-clavulanate (30+10 μ g) following CLSI guidelines. Out of the phenotypically screened 85 ESBL producers, multiplex PCR carried out on plasmid DNA alone indicated 50.5 % and 55.3% positivity for *bla*_{CTX-M} and *bla*_{TEM} gene respectively, whereas PCR on both plasmid and genomic DNA showed 81.1 % positivity for *bla*_{CTX-M} and 83.5% positivity for *bla*_{TEM} genes. A high percentage (52.9%) of the isolates was found to harbor both genes simultaneously. Moreover amplification of both plasmid and genomic DNA from the non-ESBL producers indicated that 6 of the isolates harbored *bla*_{TEM} gene. Amplification of whole genomic DNA increased the positivity of detection, compared to amplification of plasmid DNA alone, suggesting β -lactamase expression was controlled by both chromosomal and plasmid DNA. This study for the first time reports the prevalence of the two most common ESBL responsive genes using multiplex PCR in hospitalized patients from Kolkata, India. This technique provided an efficient and rapid differentiation of ESBLs and could be used as a rapid tool for epidemiological studies.

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Introduction

Urinary tract infection (UTI) is the second most common infectious presentation in community practice. Worldwide, about 150 million people are diagnosed with UTI each year, costing the global economy in excess of 6 billion US dollars (Moura *et al.*, 2009). *Escherichia coli* are one of the most common causative agents of UTI. UTIs are often treated with different broad-spectrum antibiotics. The extensive uses of antimicrobial agents have invariably resulted in the development of antibiotic resistance, which, in recent years, has become a major problem worldwide (Kumar *et al.*, 2006).

The development of extended-spectrum cephalosporins in the early 1980s was regarded as a major addition to our therapeutic armamentarium in the fight against beta-lactamase-mediated bacterial resistance (Bush, 2002). Regrettably, the emergence of *Escherichia coli* resistant to ceftazidime, cefotaxime and other cephalosporins seriously compromised the efficacy of these life-saving antibiotics. These extended spectrum beta-lactamases (ESBLs) were mutant, plasmid-mediated beta-lactamases derived from older, broad-spectrum beta-lactamases (*e.g.*, TEM-1, TEM-2, SHV-1), have an extended substrate profile which allows hydrolysis of all cephalosporins, penicillins, and aztreonam (Perez *et al.*, 2007, Harada *et al.*, 2008). A recent development after the year 2000 was the identification of ESBLs of the CTX-M type that were also becoming a major threat for patients in the hospital, long-term care facilities and in the community dwellers. Failure to detect ESBL production by routine disk-diffusion tests has been well documented (Paterson and Yu, 1999).

Moreover, many clinical laboratories are not fully aware of the importance of ESBLs and their detection procedures. ESBLs infected patients are at risk of having fatal outcome. It is also very important to determine the type of ESBL producers in order to assess the spread of antimicrobial resistance and also

for the administration of appropriate antibacterial agents to combat the disease. The antibiotic resistance of uropathogens has been changing over the past years, both in community and nosocomial infection (Kahan *et al.*, 2006, Manges *et al.*, 2006). There is not much information available on resistance pattern of hospital and community acquired UTIs in the eastern region of India.

Therefore, we investigated the presence of *bla*_{TEM} and *bla*_{CTX-M} in the ESBL-producing UPEC by multiplex polymerase chain reaction using gene specific primers at our hospital from the eastern region of India. By using this method, ESBL-producing *E. coli* collected from urine specimen was characterized at the molecular level.

Materials and methods

Bacterial isolates

100 non-duplicate *E. coli* isolates were selected for the molecular study. All these isolates were obtained from consecutively collected urine samples in the Department of Microbiology at Calcutta School of Tropical Medicine, Kolkata, India from patients admitted to The Carmichael Hospital for Tropical Diseases, Kolkata. The *E. coli* isolates were biochemically identified based on the colony morphology on blood agar and MacConkey agar plates and was speciated by standard biochemical tests (Myer and Koshi, 2001). The study protocol was approved by the institutional ethical committee.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing of the uropathogenic isolates were performed by the disk diffusion method as described by Clinical Laboratory Standard Institute (former National Committee for Clinical Laboratory Standards, NCCLS) (CLSI, 2006) using cefotaxime (30µg), ceftazidime (30µg), nalidixic acid, ciprofloxacin, meropenem and nitrofurantoin (200µg). Phenotypic confirmatory test for ESBL producers were determined using ceftazidime-

clavulanate (30+10µg) and cefotaxime-clavulanate (30+10µg) combination disks. Antimicrobial agents (disks) tested were obtained from Hi-Media labs, Mumbai, India. *E. coli* ATCC 25922 was used as a negative control strain. All the strains were further screened for ESBL producing genotypes.

Preparation of plasmid DNA and genomic DNA

A single colony of each organism was inoculated from MacConkey agar into 5ml of Luria-Bertanii broth (Hi-media diagnostic, Mumbai) and incubated for 20 h at 37° C. Cells from the overnight culture were harvested by centrifugation at 12,000 rpm for 5 min. Plasmid DNA were isolated by alkaline lysis method (Sambrook *et al.*, 2001). Genomic DNA was purified by phenol extraction and ethanol precipitation method (Sambrook *et al.*, 2001). The DNA samples were stored at -20 °C until use.

Multiplex PCR amplification of bla_{TEM} and bla_{CTX-M} genes

Multiplex PCR was standardized to identify two different DNA targets (*bla_{TEM}* ; 861bp and *bla_{CTX-M}* ; 546 bp) within the bacterial isolates simultaneously. The extracted plasmid DNA and genomic DNA was used as the template in the PCR reaction. Primers used to detect *bla_{TEM}* specific gene were 5'-ATGAGTATTCAACATTTCCGTG-3' (forward primer) and 5'-TTACCAATGCTTAATCA GTGAG- 3' (reverse primer) (Hosoglu *et al.*, 2007). To detect *bla_{CTX-M}* gene, a degenerate primer set comprising of forward primer: 5' ATGYGCAGYACCAGTAAG 3'(Y represents T/C) and reverse primer: 5' ATATCRTTGGTGGTGCCRT 3'(R represents A/G) (Jemima and Varghese, 2008) were used. The chosen primers were able to recognize all the known *bla_{TEM}* and *bla_{CTX-M}* variants of UPEC strains. They were procured from IDT, USA. Final reaction volume of 20 µl was prepared with H₂O (Mili-Q grade), 50 pmol and 20 pmole of *bla_{CTX-M}* and *bla_{TEM}* gene specific primers respectively, 250 µM of each dNTP, 1.5 unit of Taq polymerase (Sibenzyme), 2.0 µl of 10X PCR buffer containing 1.5 mM MgCl₂ and 100 ng DNA

template. Amplification reactions were carried out in a thermocycler (ABI, Veriti, USA) under the following conditions: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 52 °C for 30 sec and elongation at 72°C for 1 min. The final elongation step was extended to 10 min at 72°C. The amplified products were separated in 1.5 percent agarose gel containing ethidium bromide (0.5 mg/ml). A 100 bp DNA ladder (Genei, Bangalore, India) was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were visualized and documented using a gel documentation system (BioRad, USA).

Results and discussion

ESBLs have different levels of activity against the oxyimino beta lactams. In this study two third generation cephalosporin (ceftazidime and cefotaxime) drugs were used to initially screen the probable ESBL producers. An overall drug sensitivity pattern was also investigated using nalidixic acid, ciprofloxacin, nitrofurantoin and meropenem on 100 non-repetitive *E. coli* strains isolated from urine samples of the hospitalized patients. Antibiotic susceptibility results following CLSI guidelines indicated that the overall resistance of the *E. coli* strains to the different antibiotics tested were as follows: ceftazidime(CAZ) 81.25 % %, cefotaxime(CTX) 85 %, nalidixic acid (NA) 98.75 % and ciprofloxacin(CIP) 86.25 % (Fig.1). Moreover all the isolates were found susceptible to nitrofurantoin and meropenem that indicated the above drugs as potential agents to treat the patient population. Furthermore phenotypic screening test using ceftazidime-clavulanate and cefotaxime-clavulanate combinations indicated that out of 100 isolates, 85 showed positivity for ESBL production by increase in zone diameter to ≥5mm for either antimicrobial agent: ceftazidime (30µg) or cefotaxime (30µg) tested in presence of clavulanate (10µg) than alone. Amongst the 85 ESBL producing urinary isolates, 60 of them were obtained from patients with

diabetes, anaemia, or other hepatobiliary diseases and were found to possess UTI on routine urine culture. Moreover 11 were obtained from the patients showing signs of fever and were sent for routine urine culture sensitivity to exclude UTI.

Table 1. Extended spectrum β -lactamase genotypes in uropathogenic *E. coli* isolates (n =85).

ESBL genes	Number of strains n (%)	
	Plasmid DNA	Plasmid + genomic DNA
<i>bla_{CTX-M}</i>	43 (50.5)	69 (81.1)
<i>bla_{TEM}</i>	47 (55.3)	71 (83.5)

A study by Navon-Vanenzia *et al.*, (2003) indicated that phenotypic tests for ESBL detection needed to be evaluated periodically, as their performance may change with the introduction of new enzymes. Most investigators in India have used phenotypic methods, and have reported prevalence ranging from 6.6 to 88.8 per cent. A study by Grover *et al.*, (2006) on phenotypic and genotypic methods of ESBL detection also concluded PCR to be a reliable method of ESBL detection. The techniques were necessary for the task of identifying the ESBL producers and designating the exact ESBL subtypes that remained undetected by phenotypic analysis (Sharma *et al.*, 2010). ESBLs are mainly coded by three important genes, namely *bla_{TEM}*, *bla_{SHV}* and *bla_{CTX-M}*. It was also reported that sometimes multiple genes are responsible for production of ESBLs in a single isolate. In the present study, two sets of primers were used to amplify *bla_{TEM}* and *bla_{CTX-M}* genes by multiplex PCR on both plasmid and chromosomal DNA of the *E. coli* strains. PCR products were analyzed by agarose gel electrophoresis. Fig. 2, represents distinct electrophoretic banding patterns of the amplified products of the two genes (*bla_{TEM}* ; 861bp and *bla_{CTX-M}* ; 546 bp). PCR results indicated that out of the 85 ESBL positive *E. coli* isolates, 69 harbored *bla_{CTX-M}* gene and 71 harbored *bla_{TEM}* gene. Moreover a high percentage (52.9%) of the isolates was found to harbor both genes

simultaneously. PCR carried out on plasmid DNA alone indicated 50.5 % and 55.3% positivity for *bla_{CTX-M}* and *bla_{TEM}* gene respectively (Table 1), whereas PCR on both plasmid and chromosomal DNA showed 81.1 % positivity for *bla_{CTX-M}* and 83.5% positivity for *bla_{TEM}* genes.

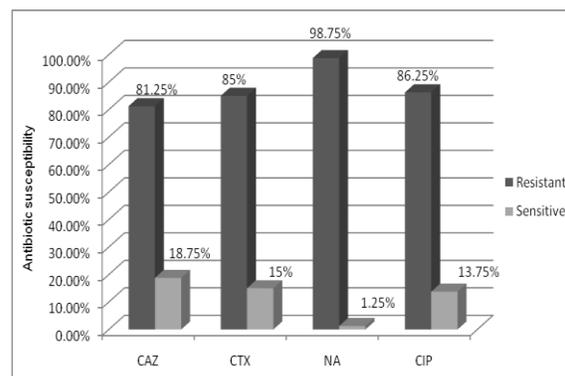


Fig. 1. Antibiotic resistance/sensitivity pattern of the *Escherichia coli* isolates (n=100) recovered from UTI patients. CAZ- ceftazidime, CTX- cefotaxime, NA- nalidixic acid, CIP-ciprofloxacin. All assays were done in triplicate with each clinical isolate.

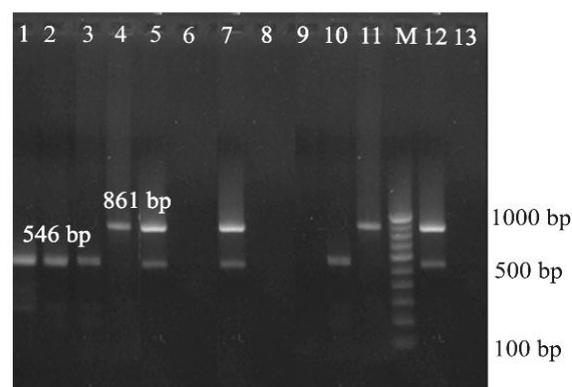


Fig. 2. Electrophoretic banding pattern on 1.5 % agarose gel showing products of multiplex PCR amplification with *bla_{TEM}* (861 bp) and *bla_{CTX-M}* (546 bp)-specific primers. Lanes 1-11; 11 uropathogenic *E. coli* isolates, Lane 6, 8, 9 were non-ESBL producers. Lane 12- in-house positive control for *bla_{CTX-M}* and *bla_{TEM}*, containing both the genes. Lane 13- negative control, (PCR product without the DNA template). M represents; 100 bp DNA ladder used as the molecular size standard, the different sizes of the markers are shown on the right side of the gel picture.

Some ESBLs may fail to reach a level to be detectable by disk diffusion tests but result in treatment failure in the infected patient. PCR amplification of *bla_{CTX-M}* and *bla_{TEM}* gene on plasmid and chromosomal DNA of the phenotypically screened non-ESBL producers indicated that out of the 15 isolates 6 isolates harbored *bla_{TEM}* genes. Therefore PCR could detect 91 % ESBL producing isolates in *E. coli* with TEM and CTX-M primers.

In this study we targeted the amplification for *bla_{TEM}* and *bla_{CTX-M}* genes. Amplification of whole genomic DNA increased the positivity of detection, compared to amplification of plasmid DNA alone, suggesting β -lactamase expression controlled by both chromosomal and plasmid DNA.

Conclusion

In conclusion this study for the first time reports the prevalence of *bla_{TEM}* and *bla_{CTX-M}* genes in uropathogenic *E. coli* strains isolated from hospitalized patients in Kolkata, an eastern region in India. ESBL producing isolates were screened by CLSI method. Multiplex PCR detected ESBL genes in 91 % of the isolates. The alarming spread of the most common ESBL responsive genes (*bla_{CTX-M}* and *bla_{TEM}*) can be successfully detected by this technique that will facilitate the monitoring process and will provide a valuable clinical and research tool. Therefore routine phenotypic tests for ESBL detection accompanied with genotype analysis by multiplex PCR will help to prevent the efficacy of the life-saving antibiotics. Moreover, detection of *bla_{CTX-M}* and *bla_{TEM}* both on plasmid and chromosome gave better understanding of ESBL production.

Acknowledgements

This work was carried out at the Department of Biochemistry and Medical Biotechnology, Calcutta School of Tropical Medicine, Kolkata, West Bengal, India. The authors would like to thank Professor Krishnangshu Ray, Director of the Institute, Professor

Indranil Chakraborty, Head of the Department of Biochemistry and Medical Biotechnology and Professor K.K. Halder, Head of the Department of Microbiology for their kind cooperation.

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