Original Research Article

Isolation and Identification of Extended-Spectrum Beta-Lactamases Producing E. coli and Klebsiella from Human

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A B S T R A C T

The irrational and indiscriminate use of antibiotics has made antibiotic resistance as biggest health problem of India. Therefore, the present study was undertaken with the aim to isolate ESBL producing E. coli and Klebsiella from clinical human cases. A total of 100 urine samples of human were collected from pathology lab of Faizabad and Gorakhpur district and processed for isolation of E. coli and Klebsiella. All isolates were screened for ESBLs production by using Cefotaxim and Ceftazidime disk. The presumptive ESBL producers were further confirmed by combination disk test using ESBL kits and by targeting ESBL genes using PCR technique. The isolation rate of E. coli and Klebsiella was found to be 24% and 26% and all were found presumptive ESBL producers on preliminary screening. Further confirmation by combination disk test revealed 41.7% and 61.5% prevalence of ESBLs in E. coli and Klebsiella. The molecular study revealed blaTEM gene in only one (10%) E. coli isolate, while blaCTX-M gene was prevalent in 70% E. coli isolates and 93.75% Klebsiella isolates. Conclusions: The present study showed predominance of blaCTX-M gene in both E. coli and Klebsiella which indicates irrational use of antibiotics in human medicine posing a great risk to the health of both human and animal population.

Introduction

Antimicrobial resistance has become a most common problem in both human and veterinary medicine across the world. Currently, one of the most important resistance mechanisms in Enterobacteriaceae, which reduces the efficacy even of modern expanded-spectrum cephalosporins and monobactams, is mediated by extended-spectrum beta-lactamases (ESBLs) (Bonnet, 2004). ESBL are beta-lactamases capable of
conferring bacterial resistance to the penicillins; first-, second- and third-generation cephalosporins; and aztreonam (but not the cephamycins or carbapenems) and which are inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Paterson and Bonomo, 2005).

The incidence of ESBL producing strains among clinical isolates has been steadily increasing over the past years, resulting in limitation of therapeutic options. Therefore, present study was undertaken to know the ESBL status in human population, as no such study has been done previously in this area.

Materials and Methods

Sample Collection

A total of 100 urine samples of clinical human cases were collected from pathology labs of Faizabad and Gorakhpur districts of Uttar Pradesh in sterilized vials and brought to the laboratory under refrigerated condition.

Isolation and identification of E. coli and Klebsiella

The samples were processed for isolation of E. coli and Klebsiella using the method described by Cruickshank et al., (1975). The identification of E. coli and Klebsiella isolates was done on the basis of morphology, growth, biochemical characteristics as per the procedure of Edwards and Ewing (1972).

Identification of ESBL producers

The isolates identified as E. coli and Klebsiella were subjected to disk diffusion test for screening using cefotaxim and ceftazidime disks prescribed in CLSI guideline (2009). The test organisms were presumed as ESBL producers if the zone diameter for cefotaxim was ≤ 27 mm and for ceftazidime ≤ 22 mm. Phenotypic confirmation was done by double disks synergy test using ESBL kit 1 and kit 3 (Hi media).

The test organisms were considered as ESBL positive if a ≥ 5 mm increase in zone diameter was observed for two or more antimicrobial agents tested in combination with clavulanic acid versus its zone when tested alone.

Molecular characterization of ESBL producers

The ESBL genes $\text{bla}_{\text{TEM}}$ and $\text{bla}_{\text{CTX-M}}$ were targeted for molecular identification of ESBL. The DNA templates were prepared using snap-chill method as described by Franco et al., (2008). The primer sequence F-ATGAGATTCAACATTTCCG and R-TTAATCAGTGAGGCACCTAT for $\text{bla}_{\text{TEM}}$ (Grimm et al., 2004) and F-CGCTTTGCGATGTGCAG and R-ACCGCGATATCGTTGGT for $\text{bla}_{\text{CTX-M}}$ (Paterson et al., 2003) synthesized by Bangalore Genei (India) were used.

The PCR assay for $\text{bla}_{\text{TEM}}$ was standardized as per the procedure described by Grimm et al., (2004). The amplification of $\text{bla}_{\text{TEM}}$ was conducted in final volume of 20 µl containing 0.4µl of dNTP (50µM) mix, 1µl of forward and reverse primer (100pmol), 0.2µl of taq polymerase (1U), 2µl of MgCl$_2$ (2.5mM), 2µl of DNA template and 13.4 µl of nuclease free water.

The PCR cycling condition included initial denaturation at 95°C for 5 min followed by 35 cycles at 94°C for 30 sec, annealing at 50°C for 35 sec, elongation at 72°C for 40 sec and final extension at 72°C for 5 min. However, the amplification of $\text{bla}_{\text{CTX-M}}$ gene was conducted as per the procedure described by Paterson et al., (2003) with slight
modification. The PCR mix composition and cycling conditions were same as for $\texttt{bla}_{\text{TEM}}$ except the primer concentration and annealing temperature.

The primer concentration was 48pmol for forward and 54pmol for reverse primer, volume 2µl each while annealing temperature was $55^\circ$C. The amplified PCR products were run in 1.5% agarose gel and visualized and analyzed under gel documentation system (Uvi tech, UK).

**Haemagglutination Assay**

ESBL positive isolates were also screened for mannose resistant haemagglutination (MRHA) and mannose sensitive haemagglutination (MSHA) property by using the method of Green and Thomas (1981) with slight modification. The isolates sub-cultured in nutrient broth for 24 hrs at $37^\circ$C were centrifuged at 10,000 rpm for 10 min.

The pellet so obtained was washed in phosphate buffer saline and cell concentration was adjusted to approximately $2\times10^{10}$ CFU/ml by the McFarland turbidimetric method. The 2% (W/V) D (+) mannose solution was then mixed with equal amount of 5% sheep RBCs suspension and kept for few min at $4^\circ$C before its use to demonstrate mannose sensitive haemagglutination.

Fifty microliter of bacterial suspension was emulsified in equal amount of PBS at two spots on a microscopic slide. Then, fifty microliter of 5% RBC suspension with and without mannose was added on these spots. The contents were mixed thoroughly by rotating the slide gently in circular manner.

The isolates showing clumping of RBCs without mannose were considered as HA positive. However, clumping of RBCs mixed with mannose was regarded as mannose resistant haemagglutination (MRHA) and absence of clumping at the same spot was considered as mannose sensitive haemagglutination (MSHA). The suspension of RBCs with and without mannose in PBS was taken as negative control.

**Results and Discussion**

In India, there is irrational and indiscriminate use of antibiotics in human and veterinary practices. Therefore, extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* has become widespread in hospitals and is spreading increasingly in community settings where they cause a variety of infections (Maina et al., 2013).

Keeping in view above, the present study was undertaken with the aim to know status of ESBL producing *E. coli* and *Klebsiella* spp in this area. Overall 95 isolates were recovered out of 100 urine sample collected from clinical human cases but based on growth, staining and biochemical characteristics, merely 24 and 26 isolates could be identified as *E. coli* and *Klebsiella* (Table1).

**Table 1** Occurrence of ESBL, MRHA and MSHA among *E. coli* and *Klebsiella* isolates

| S. No. | Name of Organism | No. of isolates | ESBL positive isolates | MRHA positive No. (%) | MSHA positive No. (%) |
|-------|------------------|----------------|-----------------------|-----------------------|-----------------------|
| 1.    | *E. coli*        | 24             | 10                    | 3(12.5)               | 7(87.5)               |
| 2.    | *Klebsiella*     | 26             | 16                    | 6(23.07)              | 10(76.93)             |
The isolates grew luxuriantly and selectively on MLA showing rose pink color, a characteristic of lactose fermenter although Klebsiella colonies were light pink and mucoid in appearance. The tiny metallic sheen colonies on EMB were considered as E. coli, while purple dark centered colonies with mucoid rim were regarded as Klebsiella.

Comparatively low isolation rate in this study may be ascribed to the source of samples that were from the patients having undergone prolong antibiotic treatment. Similar finding were reported by Rajan and Prabavathy (2012) from Chennai as they found only 20.46 percent cultures positive for E. coli out of 562 urine samples suspicious of UTI.

Likewise, Ravichitra et al., (2014) reported low (26 percent) isolation rate of Klebsiella spp. from urine sample, which was similar to our finding. Manjula et al., (2014) could also isolate only 19.9 percent Klebsiella from urine sample of pregnant women from Karnataka (India).

Identification of ESBL producers among E. coli and Klebsiella isolates was done by screening test followed by phenotypic confirmatory test (Fig1).

On preliminary screening, all isolates of E. coli (100%) and Klebsiella (100%) were presumed to be ESBL producers as per CLSI recommendations (Wayne, 2009).

Since the affinity of ESBLs for different substrates is variable, the use of more than one antimicrobial agent for screening improved the sensitivity of detection (Wayne, 2009).

By phenotypic confirmation method, the potential ESBL producers in E. coli and Klebsiella spp. were found to be 41.67% and 61.54%, respectively (Table 1).

Similar findings have earlier been reported from Karnataka (Rao, et al., 2014), Tamilnadu (Thenmozhi and Sureshkumar, 2013) and Sikkim (Tsering, et al., 2009) and other parts of India.

Over the last two decades, the incidence of infection caused by multidrug resistance Klebsiella strain has increased (Morgan et al., 1984). In a study from Delhi, 68% of Gram negative bacteria were found to be ESBL producers, with 80% of Klebsiella being ESBL producers (Mathur et al., 2002).

In this study too, the frequency of ESBL producer was higher in Klebsiella isolates rather than E. coli isolates. Thus in most of the studies, Klebsiella pneumoniae is more often reported as major ESBL producer (Gupta et al., 2002; Gales et al., 2002; Akata et al., 2003).

The molecular study of all ESBL producing E. coli isolate (10%) revealed only one blatTEM gene (Fig 2). However, the prevalence of blatCTX-M gene in this study was found to be 70% and 93.75% in E. coli and Klebsiella isolates, respectively (Table 2). Almost, all the Klebsiella isolates harboured blatCTX-M gene (Fig 3).
Our finding coincided with previous studies, because since 2000, the CTX-M enzymes have formed a rapidly growing family of u cephalosporins especially ceftriaxone and cefotaxime or may be associated with high mobilization of the encoding genes (Barguigua et al., 2011).

Barlow et al., (2008) also reported that the blaCTX-M gene have been mobilized to plasmid almost 10 times more frequently than other class A beta-lactamases.

The present study also indicated the predominance of CTX-M gene in this region of UP (Fig 4). A phenotypic assay was also conducted to determine the virulence of ESBL positive isolates. The ability to cause agglutination of erythrocytes is an indirect evidence of presence of fimbriae and can be used as a simple indirect method of virulence testing (Tabasi et al., 2015).

In present study, out of 24 E. coli, 3 (12.5 percent) isolates showed haemagglutination with sheep erythrocytes of which all were MRHA positive. In case of Klebsiella isolates, 09 out of 26(34.61 percent) showed hemagglutination of which 6 (23.08 percent) isolates were MRHA positive and 3 (11.54 percent) isolates were MSHA positive.

| S. No. | Bla gene          | E. coli (%) | Klebsiella (%) |
|--------|-------------------|-------------|----------------|
| 1      | TEM              | 1(10)       | None           |
| 2      | CTX-m            | 7(70)       | 15(93.75)      |
| 3      | TEM and CTX-m    | 1(10)       | None           |
| 4      | Non TEM and non CTX-m | 1(10) | 1(6.25)       |

Table 2 ESBL gene distribution among E. coli and Klebsiella isolates

Fig. 2 blaTEM gene (851 bp)  
Fig. 3 blaCTX-M gene (551 bp)
Rest isolates were negative for haemagglutination with sheep erythrocytes (Table-1). The findings were in agreement with observation of Tabasi et al., (2015) although degree of haemagglutination has been reported to differ with erythrocytes of different origin.

The studies have shown that P fimbriae encoded by pap (pyelonephritis-associated pilus) operon are the most important mannose-resistant adhesions, although they are expressed by only a limited number of *E. coli* serotypes as evident from present study (Jadhav, 2011).

The isolates showing MSHA property might be having type 1 fimbriae that are Mannose sensitive adhesions present in many strain of *E. coli* including non-pathogenic one (Jadhav, 2011). Strains with MRHA property may be regarded as uropathogenic as they have been reported to attach in higher number to human urinary tract epithelial cells (Hagberg *et al.*, 1981).

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