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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 bla_{TEM} gene in only one (10%) *E. coli* isolate, while bla_{CTX-M} gene was prevalent in 70% *E. coli* isolates and 93.75% *Klebsiella* isolates. Conclusions: The present study showed predominance of bla_{CTX-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 bla_{TEM} and bla_{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-ATGAGT ATTCAACATTTCCG and R-TTAATCAGT GAGGCACCTAT for bla_{TEM} **(**Grimm *et al.,* 2004) and F-CGCTTTGCGATGTGCAG and R-ACCGCGATATCGTTGGT for bla_{CTX-M} (Paterson *et al.,* 2003) synthesized by Bangalore Genei (India) were used.

The PCR assay for bla_{TEM} was standardized as per the procedure described by Grimm *et al.,* (2004). The amplification of bla_{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 95^0C for 5 min followed by 35 cycles at 94^0C for 30 sec, annealing at 50^0C for 35 sec, elongation at 72^0 C for 40 sec and final extension at 72^0 C for 5 min. However, the amplification of bla_{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 *bla*$_{TEM}$ 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^{0}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°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 2x10^{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°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	MSHA positive
				No. (%)	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).

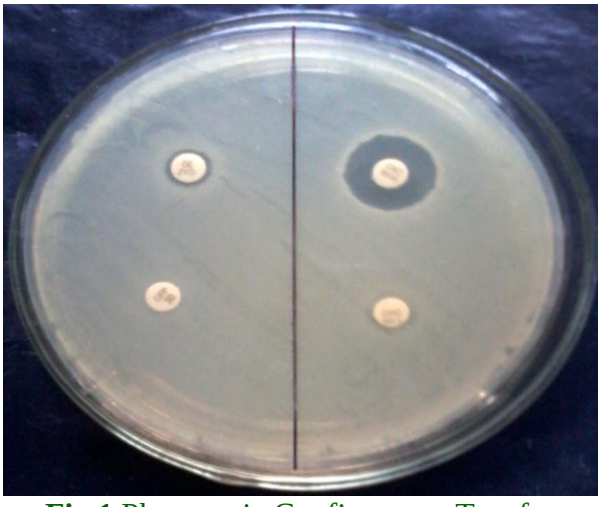

Fig.1 Phenotypic Confirmatory Test for ESBLs

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 bla_{TEM} gene (Fig 2). However, the prevalence of $bla_{CTX\text{-}M}$ 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 $bla_{CTX\text{-}M}$ 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 bla_{CTX-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.

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

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)

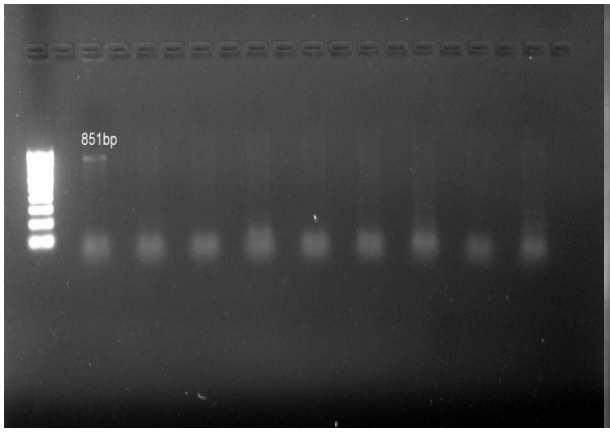

Fig.2 bla_{TEM}gene (851 bp)

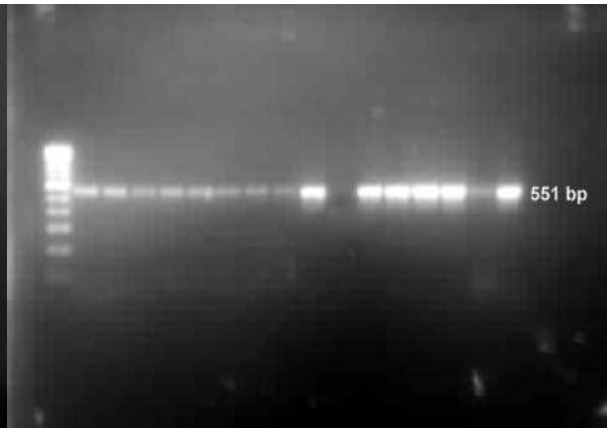

Fig.3 bla_{CTX-M} gene (551bp)

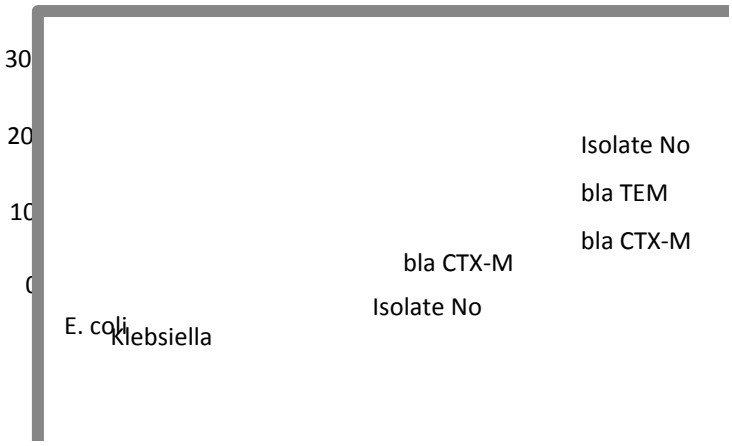

Fig.4 Distribution of ESBL genes in *E. coli* and *Klebsiella* isolates

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 fimbrae 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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