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RESEARCH ARTICLE

PREVALENCE AND MOLECULAR CHARACTERIZATION OF EXTENDED SPECTRUM BETA-LACTAMASES (ESBLs) PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

The aim of this study was to determine the prevalence of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* as well as genes encoding ESBLs. During this study, 1465 different clinical samples were tested in which 1255 (85.66%) samples showed growth of bacteria. Out of 1255 clinical samples, 1157 (92.19%) different strains of bacteria were isolated. *Escherichia coli* was the most prevalent 25.58% followed by *Klebsiella pneumoniae* (13.65%). Out of 296 *E. coli* isolates and 158 *K. pneumoniae* isolates, 247 (83.44%) and 128 (81.01%) isolates were ESBL producers, respectively. For testing with getotyping of isolates by Multiplex PCR detection using TEM, SHV, CTX-M genes, among the isolates harbouring single ESBL gene (61.53%), *bla*SHV, *bla*TEM and *bla*CTX-M were present in 42.30%, 19.23% and 11.53% strains of *E. coli* and *K. pneumoniae*, respectively. The 22 strains of *E. coli* and 16 strains of *K. Pneumoniae* had a single ESBL gene, although TEM and SHV types of ESBL were frequently found in *E. coli* (12/6) and *K. pneumoniae* (10/4), respectively. Two or more genes for ESBL were present in 14 (26.92%) of the 52 ESBL typeable isolates, *bla*TEM + *bla*SHV being the most common combination (9.61%), followed by *bla*TEM + *bla*CTX-M and bla SHV + blaCTX-M (5.76%). One strains of both *E. coli* and *K. pneumoniae* harbour 3 genes for ESBL. The majority of strains harboured two or more ESBL genes and the most common phenotypes were TEM, SHV and CTX-M. Identification of the genes is necessary for the surveillance of their transmission in hospitals.

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INTRODUCTION

β-Lactam antibiotics are commonly used to treat bacterial infections. The groups of antibiotics in this catagory include penicillins, cephalosporins, carbapenems & monobactams. Increased use of antibiotics, particularly the third generation of cephalosporins, has been associated with the emergence of β-Lactamases mediated bacterial resistance, which subsequently led to the development of ESBL producing bacteria. ESBLs are enzymes that mediate resistance to extended spectrum e.g., third generation cephalosporins as well as monobactams such as aztreonam (CLSI, 2010). These enzymes catalyze the hydrolysis of the β-lactam ring of antibiotic, thereby destroying the antimicrobial activity. ESBLs have been reported worldwide in many different genera of enterobactericeae and *Pseudomonas aeroginosa* (Friedman *et al*., 2005). 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.

The emergence of *Escherichia coli* and *Klebsiella pneumoniae* resistant to ceftazidime & other cephalosporins seriously compromised the efficacy of these life saving antibiotics (Perez *et al.,* 2007). The new bacterial beta-lactamases present in these common enteric bacilli (the parent TEM -1 and SHV-1 enzymes) demonstrated unique hydrolytic properties (Shobha *et al.,* 2007). ESBLs are inhibited in vitro by, β-lactamase inhibitors such as clavulanic acid and tazobactam. Some ESBLs are derived from earlier, broad-spectrum, β-lactamases (e.g., the TEM, SHV and OXA enzyme families) and differ from the parent enzyme by a few point mutations, which confer an extended spectrum of activity (Hawkey 2008). Point mutations in the SHV and TEM genes that resulted in single amino acid changes, (Gly 238 ñser, Glu 240-Lys arg 164-ser, arg164- His, Asp 179- Asn & Gul (Asp) 104- Lys) (Perez *et al.* 2007). More recently another family of ESBLs, the CTX-M types, has emerged and these ESBLs are becoming increasingly common (Hawkey, 2008). The TEM -1 enzyme was first reported from an *E.coli* isolate in 1965 and is now the commonest beta lactemase found in *Enterobactereceae* (Fonze *et al.,* 1995) and the older TEM is derived from Temoniera, a patient from whom the strain was first isolated in Greece (Turner 2005).

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The SHV-1 betalactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. Among the SHV type of ß-lactamases, SHV-5 was found to be responsible for outbreaks of nosocomial infection in several countries. Acquired resistance to beta-lactams is mainly mediated by extended-spectrum betalactamases (ESBLs) that confer bacterial resistance to all beta-lactams except carbapenems and cephamycins, which are inhibited by other beta lactamase inhibitors such as clavulanic acid. A shift in the distribution of different ESBLs has recently occurred in Europe, with a dramatic increase of CTX-M enzymes over TEM and SHV variants (Coque *et al.* 2008; Livermore and Canton 2007). The continuous pressure exerted by the use of newer expanded-spectrum beta-lactams promoted the development of new TEM and SHV derivates. There are so many types of ESBLs like TEM, SHV, CTX-M, OXA, AmpC but majority of the ESBLs are derivatives of TEM or SHV enzymes and these enzymes are most commonly found in *E. coli* and *K. pneumoniae* (Sharma *et al.,* 2010) and studies showed that new ones are being found every week. The rapid emergence of the ESBL-production among e*nterobacteriaceae* has already had serious clinical implications.

MATERIALS AND METHODS

Bacterial isolates

In this study, bacteria were isolated from different clinical samples such as urine, blood, sputum, pus/wound swab and cerebrospinal fluid (CSF). The clinical samples were collected from different pathology laboratories of Nagpur City. During this study, 1465 different clinical samples were collected for isolation of *E. coli* and *Klebsiella pneumoniae.* These samples were screened on different bacteriological media and identified on the basis of their morphological, cultural and biochemical characteristics.

Antimicrobial susceptibility testing

Susceptibility of all isolates was determined by the disk diffusion method on Mueller-Hinton agar following the zone size criteria as recommended by the Clinical and Laboratory Standards Institute (CLSI). The antibiotics that were tested included; Amikacin (30 µg/disc), Ampicillin (10 µg/disc), Penicillin (10 µg/disc), Cloxacillin (5 µg/disc), Erythromycin (15 µg/disc), Tetracycline (30 µg/disc), Gentamicin (10 µg/disc), Cotrimoxazole (25 µg/disc), Chloramphenicol (30 µg/disc), and some of the newer generation antibiotics including Cefixime (30 µg/disc), Cefuroxime (30 µg/disc), and Cefotaxime (30 µg/disc). The antibiotic susceptibility tests on the isolates were done according to the guideline set by the Clinical and Laboratory Standards Institute (CLSI, 2010).

E- strip method

Tested colonies from overnight culture were suspended with 0.85% of normal saline (NaCl) to a turbidity of 0.5 McFarland's. A sterile cotton swab was used to produce a uniform layer on a Mueller-Hinton agar plate and the excess moisture was allowed to be absorbed for about 15 min before the E-test strip was applied. The plate was incubated for 16 to 18 h at 37º C and the MIC end points were read where the inhibition ellipses intersected the strip (Bashir *et al.,* 2011).

Multiplex PCR for detecting TEM, SHV and CTX-M genes

DNA extraction from colony was done by alkaline lysis method

A single colony of each organism was inoculated from MacConkey agar into 5ml of Luria-Bertanii broth (LB) and incubated for 20 h at 37^{0}C. Cells from 1.5ml of the overnight culture was harvested by centrifugation at 12,000 rpm for 5 min. 1.5 ml from LB media containing cells was taken appendrof tube, than 100 µl TNE buffer was mixed. The mixture was centrifuged for 1 min at 10000 rpm and supernatant was discarded. Again 100 µl NaOH (50 mM) was added to pellet. After heating at 40^{0}C in water bath for 1 min, 60 µl of IM Tris HCl (PH 6.7) was added. Vortex, centrifuge at 10000 rpm for1 min was done. Then supernatant was used as template (1µl) (Medici *et al*. 2003).

DNA amplification in thermal cycler

PCR analysis for beta lactamase genes of the family TEM, SHV, CTX-M were carried out.

- **Preparation of reaction mixture**

For PCR amplification, 1 µl of template DNA was added to 50 µl of master mixture containing 4 µl of dNTPs mixture (2.5mM of each), 10X PCR buffer 5 µl (Ex Taq),0.5 µl of *Taq* polymerase (250 U), 1 µl of each primer stock solution (50pmol/µl), and remaining 38.5 µl volume was fulfilled by neuclease free water.

- **Amplification**

The prepared PCR tubes with master mixture were placed in the eppender off thermal cycler. Amplification was carried out according to the following thermal and cycling condition:

For TEM, SHV gene

Initial denaturation	at 94^{0}C	for 3 minute	
Denaturation	at 94^{0}C f	or 30 sec	35 cycles
Annealing	at 50^{0}C	for 30sec	
Extension	at 72^{0}C	for 2 min	
Final extension	at 72^{0}C	for 10 minutes	

For CTX-M gene

Initial denaturation	at 94^{0}C	for 7 minute	30 cycles
Denaturation	at 94^{0}C	for 50 sec	
Annealing	at 50^{0}C f	for 40sec	
Extension	at 72^{0}C	for 1 min	
Final extension	at 72^{0}C	for 5 minutes	

The PCR products were analyzed after electrophoresis in 1.0% agarose gel to detect specific amplified product by comparing with standard molecular weight marker. One percent agarose gel was prepared by melting 2.0 gm agarose in 200 ml of diluted TBE Buffer using a microwave woven. The melted agarose was allowed to cool to about 50ºC and 20 µl ethidium bromide was mixed and shacked and was poured into gel tray and combs were placed. After solidification of the gel, the comb was removed. During electrophoresis, the gel was placed in a Horizontal electrophoresis apparatus containing TBE buffer and ethidium bromide.

Loading and electrophoresis of the sample

Five µl of amplified PCR product was mixed with 2.0 µl of loading buffer. The mixture was slowly loaded into the well using disposable micropipette tips. Hundred bp molecular weight marker was loaded in one well to determine the size of the amplified PCR products. Electrophoresis was carried out at 100 volts for 35 minutes. The amplified products of the study samples were visualized by Gel-DOC system (Sharma *et al*., 2010).

RESULTS AND DISCUSSION

In this study, bacteria were isolated from different clinical samples such as urine, blood, sputum, pus/wound swab and cerebrospinal fluid (CSF). The clinical samples were collected from different pathology laboratories of Nagpur City. These samples were screened on different bacteriological media and identified on the basis of their morphological, cultural and biochemical characteristics. During this study, 1465 different clinical samples were tested in which 1255 (85.66%) samples showed growth of bacteria. Out of 1255 clinical samples, 1157 (92.19%) different strains of bacteria were isolated. Some clinical samples showed more than one bacterial colonies. Among the isolated organisms *Escherichia coli* was the most prevalent 25.58% followed by *Klebsiella pneumoniae* (13.65%). Out of 296 *E. coli* isolates and 158 *K. pneumoniae* isolates, 247 (83.44%) and 128 (81.01%) isolates were ESBL producers, respectively. In the present study, antibiotic susceptibility was tested against *E. coli* was found to be highly resistant to Gentamicin (97.29%) followed by Tetracyclin (95.94%), Amikacin (93.91%), Ampicillin (90.54%), Cefepime (87.83%), Ciprofloxacin (87.50%), and Co-trimaxazole (84.79%) (Wasnik *et al*., 2013). (Figure 1) Gentamicin and amikacin showed high resistant towards *E.coli,* the similar studies indicating relative efficacy of gentamicin and amikacin have been reported from Pakistan (Farooqi *et al.,* 2000). High resistance of *E. coli* to antimicrobial agents tested was observed in this study. Amikacin and ampicillin showed 93.91% and 90.54% resistant against *E. coli* respectively. This is similar to what was observed by Aibinu *et al.* (2004) who reported 100% resistance of their *E. coli* isolates to ampicillin and amikacin. The present studies also agree with other studies (Sasirekha *et al*., 2010).

Table 1. Primers used for detection and sequencing of resistant genes

Target	Primer name	Primer sequence (5`→3`)	Product size (bp)	References
BlaTEM	TEM-F	TCGCCGCATACACTATTCTCAGAATG	445	Monstein *et al*. (2005)
	TEM-R	TTGGTCTGACAGTTACCAATGC		
BlaSHV	SHV-F	TGCTTTGTTCGGGCCAA	747	Monstein *et al*. (2005)
	SHV-R	ATGCGTTATATTCGCCTGTG		
BlaCTX-M	CTX-F	TGGGTAATAGTACCAGAACAGCG	593	Monstein *et al*. (2005)
	CTX-R	ATGTGCAGACCAGTAAGTATGGC		

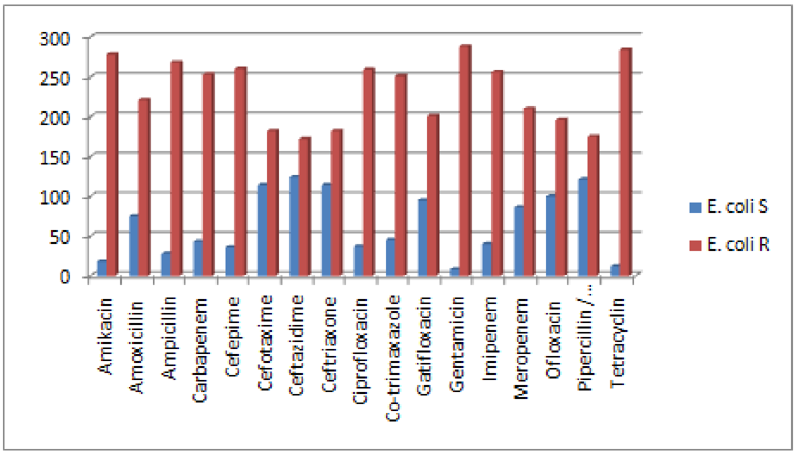

Figure 1. Antibiotic susceptibility testing of *E.coli*

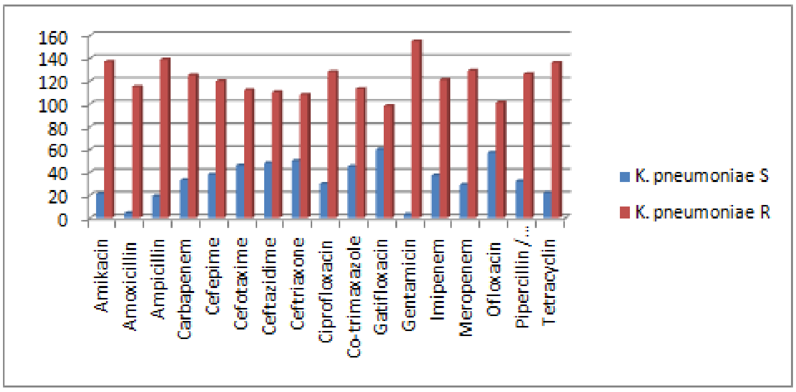

Figure 2. Antibiotic susceptibility testing of *K. pneumoniae*

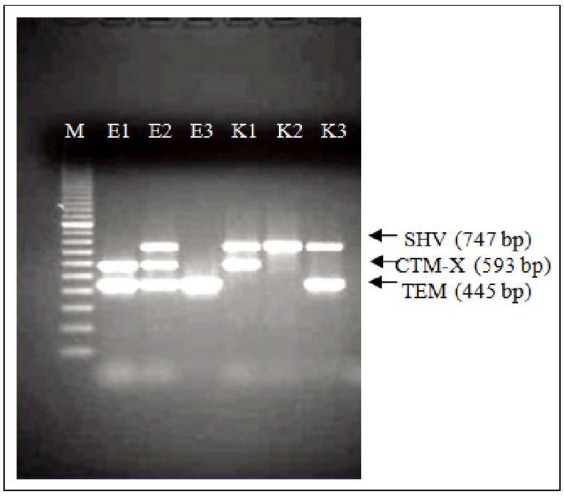

Figure 3. Multiplex PCR detection of TEM, SHV, CTX-M in Gram negative bacteria. M : Marker; Lane 1 – 3 : E1 – E3 (*E.coli* Isolate); Lane 4 -6 : K1 – K3 (*K. peumoniae* isolate)

Table 2. Extended spectrum β-lactamase (ESBL) genotypes in *E. coli* and *K. pneumoniae* strains

Positive by PCR for ESBL genes	Number amplified		
	E. coli (n = 30)	*K. pneumoniae* (n = 22)	Total N = 52 (%)
A. Single ESBL gene	22	16	38 (61.53)
blaTEM only	12	10	22 (42.30)
blaSHV only	06	04	10 (19.23)
blaCTX-M only	04	02	06 (11.53)
B. Two or more ESBL genes	08	06	14 (26.92)
blaTEM + blaSHV	03	02	05 (9.61)
blaTEM + blaCTX-M	01	02	03 (5.76)
blaSHV + blaCTX-M	02	01	03 (5.76)
blaTEM + blaSHV + blaCTX-M	01	01	02 (3.84)

All isolates were highly resistant to ampicillin, which is in agreement to the findings of (Nagoba *et al.,* 2008). Densenclos *et al.* (1998) reported 53% of their *E. coli* isolates were resistant cotrimoxazole and 67% to tetracycline. Their finding is in harmony with the report of this study, showing 84.79% and 95.94% resistance to co-trimoxazole and tetracycline respectively. Similarly, 90% isolates were resistant to tetracycline by Maynard *et al*. (2004) also reported an increase in the tetracycline resistance in human isolates. The antibiotic susceptibility testing showed that Ceftriaxone, Tetracyclin and Ciprofloxacin had the highest resistant of 61.48% 95.94% & 87.5% respectively (Figure 1). This results completely agree with a study done by P. L. Winokur *et. al.* (2001) which reported that ESBL strains show high level of co-resistance to ciprofloxacin, aminoglycosides, tetracycline, and suphanethoxazole/Trimethoprim.

It was also similar in Farooqui *et al.* (2001) who reported a relatively high resistance of 25% to quinolones and Khan and Ahmed (2001) who reported 46% resistance in Pakistan. In this study, out of 296 *E. coli* isolates, 247 isolates or 83.44% exhibited multiple drug resistance. The similar result was found in many studies multi drug resistance has serious implications for the empiric therapy of infections caused by *E. coli* and for the possible co-selection of antimicrobial resistance mediated by multi drug resistance plasmids (Sherley *et al.,* 2004). The multidrug resistant *E. coli, i*.e. isolates resistant to fifteen antibiotics, were observed to be very common in this study. The growing antimicrobial resistance may be due to unrational use of antibiotics and the transfer of resistance genes by transport means including antibiotic resistant plasmids, bacterio-phages, transposons and integrons.

Since a plasmid or transposon can carry several resistance indexes, simultaneous resistance to multiple antimicrobial agents may be developed and the result would be MDR organisms. For example, resistance to co-trimaxazole is usually accompanied by resistance to Ampicillin, cephalothin and Tetracycline. Chloramphenicol, Cephotaxime, amikacin and imipenem can be still prescribed for UTI as their resistance rate still under control. The high level of antibiotic resistance among *E. coli* isolates may be due to self prescription policy, comparatively cheaper antibiotics intake, lack of dependency on laboratory guidance and in adequate doses of antibiotics intake. In many areas of India antibiotics can be easily available over the counter without prescription of registered medical practitioner. This is the main cause of misuse of antibiotics responsible for the increasing emergences of MDR. Antibiotic resistance is a major clinical problem in treating infections caused by microorganisms. The resistance to the antimicrobials has increased over the years. Resistance rates vary from country to country (Kahan *et al.,* 2006). In Pattukkottai, there is an evidence for increase in antibiotic resistance. In this study, higher percentage of resistance was noted against Gentamicin (98.10%) followed by Ampicillin (87.97%), Amikacin (86.70%), Tetracyclin (86.07%), Ciprofloxacin (81.01%), Meropenem (81.64%), Amoxicillin (72.78%) and Co-trimaxazole (71.51%). (Figure No. 2) The comparative similar result was previously reported in other hospital as well as in other institutions in the various country (Daoud *et al.,* 2006). Amoxicillin resistance (72.78%) observed in the present study was lower than the reports of Sonavane *et al.* (2008) was found (97.7%). The resistance of *Klebsiella* spp. to cotrimaxazole (71.51%) was high, compared to most Indian studies (Biswas *et al.,* 2006).

Only Arjunan *et al.* (2010) from Tirunelveli reported cotrimaxazole resistance, (14.3%) which was lower than what has been observed in this study. Resistance to Ciprofloxacin is high in *Klebsiella pneumoniae*. The present study recorded 81.01% *K. pneumoniae* were resistant to Ciprofloxacin and 86.07% *K. pneumoniae* resistant to Tetracycline this is in agreement with other study done by Aktas *et al.* (2002). Cephalosporins, particularly second and third generation cephalosporins have been used for *Klebsiella* infections (Jett *et al.,* 1995). In this study, 59.45% and 70.88% of *K. pneumoniae* isolates were resistant to the third generation cephalosporin (Ceftazidime, Cefotaxime respectively). The study conducted in Pakistan have recorded a total of 54.3% isolates were resistant to the third generation cephalosporins (ceftazidime and cefotaxime) (Ullah *et al.,* 2009). Study from India has recorded 84% resistance to cefotaxime (Singh *et al.,* 2003). The study conducted in US 95% of the *K. pneumoniae* isolates showed resistance to at least one of the three third generation cephalosporin (3GC (ceftazidime, cefotaxime, ceftriaxone) used for the study. 87% of the *K. pneumoniae* isolates showed resistance to all the three third generation cephalosporin antibiotics and this resistance to all the three 3GC was found to coexist with resistance to other antibiotics.

Aminoglycosides have good activity against clinically important gram negative bacilli. Amikacin and Gentamicin showed 86.70% & 98.10% resistance towards *K. pneumoniae* isolates respectively. This result is higher than the study conducted in Doha Qatar. The study done in Doha Qatar showed that all gram negative bacilli were sensitive to amikacin and resistance of GNB to gentamicin was 20% (Ahmed *et al.,* 2013). Similarly, the study from India has reported 39.10% activity of amikacin and 16.7% activity of gentamicin in *Klebsiella* (Revathi G, Puri 1998). This may be due to increased use of amikacin and gentamicin in India and as compared to Nepal. Pattern of resistance to aminoglycosides is affected by selective pressure in different regions (Miller *et al.,* 1997). The observed resistance in *Klebsiella pneumoniae* to Ofloxacin was 63.92%. This is lower than studies conducted in India, (Gonzalez *et al.,* 1998 & Ahmed *et al*., 2013) and higher than those of USA (Miller *et al.,* 1997).

The high sensitivity was found against Ceftazidime, Ofloxacin and Gatifloxacin can be prescribed for the treatment in *Klebsiella pneumoniae* infection. The proportion of isolates of *K. pneumoniae* exhibiting the ESBLs phenotype has increased progressively. The data generally reflect the seriousness of the antimicrobial resistance among bacterial pathogens in Nepal. Globally increasing resistance trends to multiple antibiotics in *K. pneumoniae* have complicated the management of these infections. The result of present study also noted an increased resistance to the commonly used antibiotics against *K. pneumoniae* infections. More concerning was the emergence and increase in the isolation rates of ESBLs producing *K. pneumoniae* over the years. Studies from both developed and developing countries have reported an increasing trend in the isolation of ESBLs positive *K. pneumoniae* (Coque *et al.,* 2008) A study done at Kasturba Medical College Mangalore, (Shah *et al*., 2010) in their studies sixteen ESBLs producing *Klebsiella* isolates (26.66%) were detected, which was lower than the present study. In this study the total 158 *K.pneumoniae* were isolated from different clinical samples. Out of 158 *K. pneumoniae*, 128 (81.01%) strains were found to be ESBL MDR. Overall ESBLs prevalence elsewhere in Asia, the percentage of ESBL production in *K. pneumoniae* varies like 8.5% in Taiwan (Yan *et al.,* 2001) and 12% in Hong Kong (Bradford, 2001). Similarly, at the University Hospital of the West Indies, a tertiary care hospital in Jamaica, 18.2% of the total *K. pneumoniae* was found to be ESBLs producer. A study done in a general hospital of Saudi Arabia showed that 6% of all isolates were MDR and 4.8% were positive for ESBLs (Kader *et al.,* 2004). As emphasized by various authors, prevalence of ESBLs positive strains in a particular region or even hospital is variable and is associated with frequency of treatment of bacterial infections with β-lactam antibiotics as well as with colonization of patients hospitalized for over 10 days by ESBLs positive strains (Agata *et al.,* 1998).

Characterization of Multiple Drug Resistant (MDR) *s*trains using molecular method: (Genotyping Resistance Testing)

The characterization of presence of multiple drug resistant (MDR) strains using molecular method can be done by selecting the bacteria on the basis of their highest resistant pattern against the antibiotic tested. Those bacteria having high resistant pattern were selected for the presence of resistant gene. *bla*TEM, *bla*SHV and *bla*CTX-M among the total *Enterobacteriaceae* in this study were recorded while compare with earlier studies done for the presence of *bla*TEM, *bla*SHV and *bla*CTX-M (Ahmed *et al*., 2009). The rate of co-existence of two different ß-lactamase encoding genes among *Enterobacteriaceae* in this study was also reported by Ahmed *et al*. (2009). The dominance of *bla*CTX-M gene either alone or in association with *bla*TEM among *E. coli* isolates was reported previously in Egyptian clinical setting (Fam and El-Damarawy, 2008). The previous findings demonstrate the increasing tendency of *K. pneumoniae* and *E. coli* to accumulate ß-lactamase resistance determinants which is higher among *K. pneumoniae* isolates than *E. coli* isolates. The previous finding ascertain also the predilection of *E. coli* to aggregate *bla*CTX-M and *bla*TEM genes together as well as the predilection of *K. pneumoniae* to associate either *bla*CTX-M together with *bla*TEM or *bla*TEM together with *bla*SHV. Existence of *bla*SHV among *E. coli* isolates is seldom encountered. (Figure No. 3)

Genomic DNA isolated from 52 phenotypic confirmed ESBL-producing organisms (*E. coli*-30 and *K. pneumoniae*-22) were subjected to PCR using pairs of primers. All isolates was found to be ESBL positive and these isolates could be typed for one or more genes. Among the isolates harbouring single ESBL gene (61.53%), *bla*SHV, *bla*TEM and *bla*CTX-M were present in 42.30%, 19.23% and 11.53% strains of *E. coli* and *K. pneumoniae*, respectively. The 22 strains of *E. coli* and 16 strains of *K. Pneumoniae* had a single ESBL gene, although TEM and SHV types of ESBL were frequently found in *E. coli* (12/6) and *K. pneumoniae* (10/4), respectively. Two or more genes for ESBL were present in 14 (26.92%) of the 52 ESBL typeable isolates, *bla*TEM + *bla*SHV being the most common combination (9.61%), followed by *bla*TEM + *bla*CTX-M and blaSHV + blaCTX-M (5.76%). One strains of *E. coli* and one strains of *K. pneumoniae* harbour 3 genes for ESBL. The most common combination was *bla*TEM + blaSHV + *bla*CTX-M (3.84%). There is a difference between strains harbouring a single ESBL gene (61.53%) compared to those with two or more genes (26.92%) (Table 2). ESBLs have been reported from all parts of the world. However, prevalence varies widely even in closely related regions. The true incidence is difficult to determine because of the difficulty in detecting ESBL production & due to inconsistencies in testing & reporting

(Yushaíu *et al.,* 2010). Prevelance of ESBL in many parts of the world was (10-40%) among *E. coli* and *Klebsiella pneumoniae* (Rupp and Paul 2003). The prevalence of ESBLs in Europe is higher than in the USA but lower than in Asia and South America (Girlich *et al.,* 2004). In 2007 in Asia pacific region was found to harbour plasmid borne ESBLs 62% and 75% in *E. coli* and *Klebsiella* spp. respectively (Bell *et al.* 2007). ESBL production rate was 43%, 73.8%, 96% and 70% in *E. coli* and 60% in *Klebsiella* spp. in Pakistan, Iraq, Iran and India respectively in 2009, 2011 and last two were in 2010 (Ali 2009; Al- Charrakh *et al.* 2011; Sharma *et al.,* 2010). There were a limited number of studies on prevalence of ESBL showing a high rate in Bangladesh, where *E. coli* and *K. pneumoniae* were 43.2% and 39.5% respectively in 2004 (Rahman *et al.* 2004) and at Rajshahi in Bangladesh it was 57.89% in *Klebsiella* spp followed by *Proteus* spp. 50.0%, *E. coli* 47.83% and *Pseudomonas* spp 31.35% in 2010 (Haque and Salam 2010). In France CTX-M-1, CTX-M-3 and CTX-M 14, lactamases from Enterobacteriaceae was isolated in 2002 (Dutour *et al.,* 2002). Again in Iran SHV, CTX-M, TEM, PER were 26%, 24.5%, 18%, 7.5% respectively in 2010. In India in 2006 CTX-M-15 was 73% in *E. coli* and 72% in *Klebsiella* spp. and in 2010 TEM and SHV were 30% and 38% respectively (Ensor *et al*., 2006; Sharma *et al*., 2010).

Extended spectrum β-lactamase (ESBL) genotypes in *E. coli* and *K. pneumoniae* strains

To conclude, phenotypic methods are only screening methods for detection of ESBLs in a routine laboratory. The prevalence of ESBL-producing *E. coli* and *K. pneumoniae* had means of 61.53% and 26.92%, respectively. The prevalence of ESBL-producing *E. coli* and *K. pneumoniae* in surgical clinics was higher compared to that in clinics of internal medicine. The genotypic methods help us to confirm the genes responsible for ESBL production. The majority of our strains harboured two or more ESBL genes and the most common phenotypes were TEM, SHV and CTX-M. In this study used a multiplex PCR for the detection of *bla*TEM, *bla*SHV and *bla*CTX-M genes in ESBL-producing *E. coli* and *K. pneumoniae*. This method provided an efficient, rapid differentiation of ESBLs. Appropriate antibiotic policy and infection control measures in hospital settings are crucial to overcome the problems associated with infections by ESBL-producing strains.

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