# PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES

**A Thesis**
**Submitted to the**
**Assam Agricultural University**

**In partial fulfillment of the requirements for the degree of**

## DOCTOR OF PHILOSOPHY

**IN**

## ANIMAL BIOTECHNOLOGY


**By**

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**July, 2019**

*Dedicated*
*To My*
*Beloved Husband*
*Biswajit Deka*

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This is to certify that the thesis entitled "**PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES**" submitted to the Faculty of Veterinary Science, Assam Agricultural University; in partial fulfillment for the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL BIOTECHNOLOGY** is a record of research work carried out by **DR. LEENA DAS** under my personal supervision and guidance.

All kinds of help received by her have been duly acknowledged.

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*Date :*

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# ABSTRACT OF THE THESIS

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# ABSTRACT

Extended-spectrum beta-lactamase producing Enterobacteriaceae has become a major threat to both animals and human health globally. The present study was undertaken to isolate and identify ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* from various sources, to study their resistant gene profile, to detect insertion sequences, to genogroup the isolates and to compare the efficacy of REP-PCR and PFGE to discriminate ESBL producing *E. coli* and *K. pneumoniae* isolates.

Out of 385 samples from various sources, 31 (8.05%) were positive for ESBL producing *E. coli*. Such isolates could be isolated from 10.05, 8.33, 15.63, 6.67 and 4.35 per cent of cattle milk, curd, chicken, pork and cattle faeces samples, respectively. However, no ESBL producing *E. coli* could be isolated from goat milk, goat faeces and beef samples. A total of 59 (15.32%) samples were positive for ESBL producing *K. pneumoniae*, which could be isolated from 14.35, 6.25, 21.43 and 34.78 per cent samples of cattle milk, chicken, beef and cattle faeces, respectively. No ESBL producing *K. pneumoniae* isolates could, however, be isolated from goat milk and faeces, curd and pork.

*In-vitro* drug susceptibility assay against 3$^{rd}$ and 4$^{th}$ generation cephalosporins showed resistance of all the 90 ESBL isolates to at least one antibiotic. In CDT, 93.55% of *E. coli* and 88.14% *K. pneumoniae* and in ESBL –E test, 96.77% *E. coli* and 88.14% *K. pneumoniae* showed positive results.

Antibiogram of the ESBL producing *E. coli* and *K. pneumoniae* showed resistance of 74.19% and 69.49%, respectively to ceftizoxime, 25.81% and 23.73% to both co-trimoxazole and tetracycline, 19.35% and 25.42% to ciprofloxacin, 9.68% and 16.95% to chloramphenicol, 3.23% and 5.08% to pipercillin-tazobactam, and 3.23% and 3.39% to gentamicin.

Resistance gene profiling showed *bla*CTX-M gene to be present in all the 90 (100%) ESBL isolates. The *bla*TEM gene was found in 54.84% and 55.93%, *bla*SHV gene in 90.32% and 77.97%, *Sul* 1 gene in 90.32% and 86.44% isolates. The *Int*1 gene was detected in 70.97% and 62.71% isolates, while *qnr*B gene was found in 3.23% and 10.17% of *E. coli* and K. *pneumoniae* isolates, respectively.

Out of the insertion sequences under study, *ISEcp*1 was found to be present in all the 90 (100%) ESBL producing isolates, followed by *IS*26 (100% and 90.32%) and *ISCR*1 (80.65% and 45.76%) in *E. coli* and *K. pneumoniae* isolates, respectively. All the 90 ESBL producing isolates were subjected to PCR for detection of *CTX*-M genogroups. All the 90 (100%) ESBL producing isolates were found to be positive for group 1 gene. A total of 80.65% and 55.93% *E. coli* and *K. pneumoniae* isolates, respectively showed presence of group 2 genes. The corresponding percentages for group 25 gene were

27.27% and 67.8%. However, group 9 gene could be detected in 5.08% of *K. pneumoniae* isolates only. None of the *E. coli* isolates were found be positive for group 8 and 9 genes, while no isolate of *K. pneumoniae* was found to be positive for group 8 gene.

The two molecular typing methods, REP-PCR and PFGE were found to show similar discriminatory power and could distinctly differentiate the ESBL producing E. *coli* and *K. pneumoniae* isolates. As both the methods were found equally competent, REP-PCR may be recommended as the preferred method of typing for epidemiological investigations owing to its advantages over PFGE in terms of rapidity, simplicity and ease of performance.

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| Abbreviation | Full form |
|---|---|
| °C | Degree Celsius |
| µg | Microgram |
| µl | Microlitre |
| bp | Base pair |
| DNA | Deoxy ribo nucleic acid |
| dNTP | Deoxynucleoside triphosphate |
| EDTA | Ethylene diamine tetra acetic acid |
| Fig. | Figure |
| g | Gram |
| hr | Hour (s) |
| µg | Microgram |
| mg | Milligram |
| min | Minute (s) |
| ml | Mllilitre |
| mM | Milimolar |
| NFW | Nuclease free water |
| ng | Nanogram |
| PCR | Polymerase Chain Reaction |
| pmol | Pico mol |
| rpm | Revolution per minute |
| sec | Second |
| TAE | Tris Acetate EDTA buffer |
| TBS | Tris buffer solution |
| UV | Ultra violet |
| PFGE | Pulsed-field gel electrophoresis |
| REP | Repetitive Extragenic Palindromic |
| REP-PCR | Repetitive element sequence based PCR |
| *XbaI* | *Xanthomonas badrii* |

# CHAPTER - I

# *Introduction*

**PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES**

## CHAPTER-I

# INTRODUCTION

Antibiotic resistance is a major problem of public health concern. Among the multi-drug resistant pathogens, the extended-spectrum beta-lactamase producing Enterobacteriaceae have emerged as a threat for human health worldwide (Pitout and Laupland, 2008). Extended spectrum β-lactamases (ESBLs) are enzymes carried by plasmids that show resistance to 3$^{rd}$ and 4$^{th}$ generation cephalosporins and also monobactam group of drugs. However, ESBLs are still sensitive to carbapenems and cephamycins. ESBLs are most commonly detected in Enterobacteriaceae like *Klebsiella pneumoniae* (KP) and *Escherichia coli*. These enzymes can be inhibited by β-lactamase inhibitors like clavulanic acid, sulbactam and tazobactam (Paterson and Bonomo, 2005). In the last few years, ESBL producing Enterobacteriaceae have been isolated from various sources like hospitals, domestic and wild animals, and also from different food sources, vegetables, healthy humans, and wastewater (Jouini *et al*., 2007; Poeta *et al*., 2009; Vinué *et al*., 2009; Ben Sallem *et al*., 2012; Blaak *et al*., 2014). Antibiotic resistance among pathogens is increasing due to occurrence of different variants of broad-spectrum β-lactamases (BSBL), known as extended spectrum β-lactamases (ESBL). These extended spectrum β-lactamases (ESBL) include TEM and SHV β-lactamase families, whereas other β-lactamases such as CTX-M, PER and KPC have also been described recently (Coque *et al*., 2008). ESBL enzymes belong to the Ambler class A and D β-lactamases. (Ambler *et al*., 1991). Majority of ESBLs in clinical isolates have been identified as SHV or TEM types, which have evolved from narrow-spectrum β-lactamases such as TEM-1, -2 and SHV-1.4. The most prevalent and commonly found beta-lactamase is TEM-1. It has been estimated that due to presence of TEM-1, more than 90% ampicillin resistance occurs in *E. coli* (Livermore, 1995). This enzyme can also hydrolyze penicillin and first generation cephalosporin group of drugs. TEM-2 is the first derivative of TEM-1 with a single replacement of amino acid (Du Bois *et al*., 1995). The CTX-M enzymes have originated from *Kluyvera* spp., and recently detected in Enterobacteriaceae from Europe, Africa, Asia, South America and North America (Bonnet, 2004).

ESBLs can be distinguished from other β-lactamases which are capable of hydrolyzing extended-spectrum cephalosporins like AmpC and carbapenemases. Carbapenemases may be further grouped as either metallo-β-lactamases (class B) and serine carbapenemases (classes A and D). Like ESBLs, AmpC β-lactamases hydrolyze third-generation cephalosporins, but unlike ESBLs, they are also active against cephamycins and can be inhibited by clavulanate or other β-lactamase inhibitors (Rupp *et al*., 2003; Jacoby *et al*., 2005). Carbapenemase-producing Enterobacteriaceae are currently relatively less in number, but there are concerns about the emergence and spread of these strains. Till now, at least 400 different types of β-lactamase enzymes originating from clinical isolates have been described and a website has been created to monitor the latest developments in newer types of β-lactamases (URL: http://www.lahey.org/ studies/webt.htm) (Jacoby *et al*., 2005).

The CTX-M β-lactamases can be divided into five clusters based on their amino acid sequences (Bonnet, 2004). The CTX-M-1 group includes CTX-M-1, -3, -10, -12, -15, -28, -30 and FEC-1; the CTX-M-2 group includes CTX-M-2, -4, -5, -6, -7, -20 and Toho-1; the CTX-M-8 group includes CTX-M-8; the CTX-M-9 group includes CTX-M-9, -13, -14 (also named CTX-M-18), -16, -17, -19, -21, -24, -27 and Toho-2; and the CTX-M-25 group includes CTX-M-25 and CTX-M-26. The CTX-M-2 group β-Lactamases are structurally related to the naturally produced β-lactamases of *Kluyvera ascorbata* (Humeniuk *et al*., 2002); CTXM- 8 is related to β-lactamase of *Kluyvera georgiana* (Poirel *et al*., 2002) and CTX -M- 1 group enzymes are related to the β lactamases *of Kluyvera cryocrescens* (Decousser *et al*., 2001). An enzyme identical to CTX-M-3 was isolated from a strain of *K. ascorbata* (Rodriguez *et al*., 2004) The CTX-M-9 group is related to enzymes from *Kluyvera* spp. isolated in Guyana, which were identical with CTX-M-14 ( Boyd *et al*., 2004).

The first CTX-M enzyme (previously known as MEN-1) was reported from France in 1991 (Bernard *et al*., 1992). The CTX-M-10 enzyme was found to be present in *E. coli*, *K. pneumoniae* and *Citrobacter freundii* isolated from outpatients in Spain (Valverde *et al*., 2004). The CTX-M enzymes generally have more activity against

cefotaxime than ceftazidime but some groups, such as CTX-M-15 and -19, also hydrolyse ceftazidime efficiently (Karim *et al*., 2001; Poirel *et al*., 2001).

For detection of ESBL in Enterobacteriaceae, particularly for *E. coli*, *Klebsiella* spp. and *Proteus* spp., the Clinical and Laboratory Standards Institute (CLSI; formerly known as the National Committee for Clinical Laboratory Standards) (CLSI, 2005) and the Health Protection Agency (HPA) in UK suggested some guidelines (British Society for Antimicrobial Chemotherapy, 2005). As per these guidelines, ESBL producing organisms can be detected by supplementation of either 8 mg/L (CLSI) or 1 mg/L (HPA) of cefpodoxime, 1 mg/L each of cefotaxime, ceftazidime, ceftriaxone, or aztreonam in media followed by phenotypical confirmation using both cefotaxime and ceftazidime in combination with clavulanic acid and by use of ESBL E-strips. In clinical laboratories of Europe, ESBL-producing organisms are detected by first line screening tests like double disc synergy test, combination of discs and microdilution tests by growing cultures in broth containing 1 mg/L of cephalosporin drug. Second confirmation of ESBL isolates is done by determination of MIC in broth containing cephlosporins with and without clavulanic acid or E-test.

Dissemination of CTX-M enzymes occurs due to involvement of different genetic mechanisms. Genes encoding enzymes of several CTX-M groups (CTX-M-1, CTX-M-2, CTX-M-9 and CTX-M-25) have been found downstream of an insertion sequence, ISEcp1 and it was experimentally proved that *ISE*cp1 is involved in mediating the capture of *bla*CTX-M genes from the chromosomes of *Kluyvera* spp. and facilitating their inter-replicon mobility inside *E. coli* hosts (Poirel *et al*., 2005; Lartigue *et al*., 2006). Genes encoding enzymes CTX-M-2 and CTX-M-9 have also been found to be associated with class 1 integrons within the CR1 region. *ISE*cp1 and the recombination system of CR1 regions have played a vital role in acquisition of *bla*CTX-M genes by conjugative plasmids and in inter-replicon dissemination. But association of CR1 region with Tn402-like backbones can provide further mechanism of mobility to the resistance genes. The horizontal gene transfer of *bla*CTX-M gene among strains of the same or different enterobacterial species is mostly mediated by plasmids (Bonnet, 2004).

Several genetic mechanisms have been found to involve in transmission of antimicrobial resistance genes. The term "mobilome" (Medini *et al*., 2005; Tettelin *et al*., 2008) is used for various mobile genetic elements (MGEs) including plasmids, transposons (Tn), insertion sequences (IS), integrons (intI) and introns. Horizontal gene transfer of MGEs occurs through conjugation, transformation and transduction (Norman *et al*., 2009; Woodford *et al*., 2011). Enzymes encoded by *blaCTX-M* genes are associated with transferable plasmids like transposons or cassettes in integrons.

Integrons are DNA fragments embedded in gene cassettes capable of disseminating antimicrobial resistance genes using mobile genetic elements (Stokes *et al*., 1989). These genes are usually composed of two conserved regions, *viz.* 50-conserved region (50-CS) and 30-conserved region (30-CS) separated by a variable region, which contains the gene cassettes. The 50-CS region is composed of the *int* gene that encodes for the enzyme integrase which belongs to tyrosine-recombinase family; a primary recombination site (*att*I); and a promoter (*Pc*), which ensures the transcription of the cassette genes. The 30-CS region is formed by a truncated quaternary ammonium compound resistant gene (*qacED1*); a sulfonamide resistance gene (*sul1*); and an unknown sequence (*orf5*) (Galani *et al*., 2006). Class 1 (*intI1*) and class 2 (*intI2*) integrons are most commonly involved in the antibiotic resistance mechanism (Fluit and Schmitz, 2004; Saenz *et al*., 2004; Machado *et al*., 2005; Odetoyin *et al*., 2017; Kaushik *et al*., 2018). The gene *intI3* has been found in Enterobacteriaceae and was first reported in a carbapenem-resistant *Serratia marcescens* (Arakawa *et al*., 1995) and has been reported in *Klebsiella pneumoniae* (Correia *et al*., 2003) and in other Enterobacteriaceae (Rizk and El-Mahdy, 2017). ESBL resistant genes have also been found to be associated with insertion sequences. Insertion sequences are the smallest transposable elements, <2.5 kb in size. These sequences are classified into families by different characteristics, with transposases (Mahillon and Chandler, 1998; Zhao *et al*., 2013). The antimicrobial resistance in ESBL has been found to be transferred mostly by *IS*26, *ISE*cp1, *ISCR*1 and *IS*903 in association with class 1 integrons (Arduino *et al*., 2002; Eckert *et al*., 2006; Diestra *et al*., 2008; Cullik *et al*., 2010; Cheng *et al*., 2016). Three subtypes of CTX-M genes (M-1, M-2 and M-9) are associated with insertion sequences, *ISEcp1* and hence are very widespread in dissemination. *ISE*cp1 is an insertion sequence that is weakly

related to other elements; they belong to the family of IS1380. *ISEcp1* elements and their remnants constitute an alternative promoter (Karim *et al*., 2001) which leads to expression of the blaCTX-M gene that expresses weakly in its natural reservoirs (Karim *et al*., 2001; Poirel *et al*., 2003).

Various molecular typing methods including repetitive extragenic palindromic polymerase chain reaction (REP-PCR) are used for molecular typing of gram-negative bacteria (Hulton **et al.,** ***1991; Versalovic*** **et al.,** ***1991).*** Repetitive sequence-based PCR (REP-PCR) is done based on amplification of the regions between non-coding repetitive DNA sequences interspersed throughout the genome (***Versalovic*** **et al.,** ***1991).*** The sequence sizes are specific to each type of bacteria, so the sizes of the fragments may vary among strains (***Versalovic*** **et al.,** ***1991***). The resulting band patterns can be used for DNA fingerprinting of a large variety of prokaryotic and eukaryotic organisms (Healy *et al.,* 2005*;* ***Versalovic*** **et al.,** ***1991***). The less time requirement for REP-PCR compared to PFGE, where it requires 2-4 days, made it most preferred method by many researchers. In addition, extensive training does not require for performing REP-PCR, while it is necessary in case of PFGE. This technique is low in cost and easy to use and can generate results in real time (Ross *et al.,* 2005)

Multiple DNA fragments of different sizes are generated through this PCR and the fragments produce a unique band pattern for each bacterial strain. Differences in the REP-PCR patterns can be used for typing of bacterial strains and to demonstrate strain variations (***Versalovic*** **et al.,** ***1991).*** Manual REP-PCR has been used in several studies for typing ESBL-producing *E. coli* (Edelstein *et al.,* 2003) and verocytotoxin-producing *E. coli* O157 (Hahm *et al*., 2003), although there may be difficulties in data interpretation and inter-laboratory comparisons due to lack of standardization.

A rapid, semi-automated, PCR-based typing system known as DiversiLab system is being used to perform repetitive element PCR (REP-PCR) (Healy *et al*., 2005). The semi-automated REP-PCR based DiversiLab system (bioMe´rieux, Basingstoke, UK) is based on the principle of microfluidic separation of PCR products (Healy *et al*., 2005). This automated REP-PCR has been used to differentiate among strains of different organisms such as *Acinetobacter* spp. (Carretto *et al*., 2008), methicillin-resistant

*Staphylococcus aureus* (Shutt *et al*., 2005; Ross *et al*., 2005), *Staphylococcus epidermidis* (Trevino *et al*., 2008), vancomycin resistant *Enterococcus* spp. (Sherer *et al*., 2005; Pounder *et al*., 2006), *Clostridium difficile* (Spigaglia *et al*., 2003), *Salmonella* (Wise *et al*., 2009), *Mycobacterium* spp. (Cangelosi *et al*., 2004) and fungi (Healy *et al*., 2004). These studies have revealed that the DiversiLab system based REP-PCR is a tool for rapid identification and typing of various infectious organisms (Healy *et al*., 2005).

The (GTG)5 repetitive sequence based REP-PCR has also been widely used for microbial typing of different strains of *Escherichia coli* (De Moura *et al.*, 2001; Jonas *et al.*, 2003; Brocchi *et al*., 2006; Mohapatra *et al*., 2007). This REP-PCR is used for amplification of DNA sequences flanked between the poly-trinucleotide (GTG) 5 repetitive sequences for discriminating *E. coli* strains (De Vuyst *et al*., 2008). The poly-trinucleotide (GTG) 5 motif represents a class of conserved repetitive sequences found in bacterial genomes (Versalovic *et al*., 1994). REP-PCR profiling using the (GTG)5 primer is found to be a promising and rapid molecular typing tool for lactobacilli (Gevers *et al*., 2001) and enterococci (Svec *et al*., 2005).

Pulsed-field gel electrophoresis (PFGE) is another technique to analyze large DNA fragments of the bacterial chromosome with high resolution, high repeatability, and good comparability, and is considered as the gold standard for bacterial typing (Helgason *et al*., 2000; Johnson *et al*., 2007). PFGE is considered as one of the best techniques for epidemiological analysis, differentiating bacterial strains and monitoring of the transmission of pathogens in the community. This technique has been applied for diagnosing diseases caused by a number of infectious pathogens including *Escherichia coli* O157:H7 (Bohm and Karch, 1992; Barrett *et al*., 1994), *Neisseria meningitidis* (Yakubu *et al*., 1994; Yakubu and Pennington, 1995), *Pseudomonas aeruginosa* (Kersulyte *et al*., 1995), *Listeria monocytogenes* (Brosch *et al*., 1991), *Bordetella pertussis* (de Moissac *et al*., 1994), *Legionella pneumophila* (Johnson *et al*., 1994), *Vibrio cholerae* (Cameron *et al*., 1994), *Staphylococcus aureus* (Schlichting *et al*., 1993) and enterococcal isolates (Murray *et al*., 1990). PFGE can also be successfully used to characterize those bacteria, which cannot be characterized by restriction enzyme analysis, phage typing, ribotyping, plasmid profiling and randomly amplified

polymorphic DNA analysis (Schoonmaker *et al*., 1992; Swaminathan and Matar, 1993; Saulnier *et al*., 1993; Barrett *et al*., 1994; Bannerman *et al*., 1995; Kersulyte *et al*., 1995). PFGE has become a standard technique for typing organisms due to its accuracy and reproducibility between different laboratories. However, there are some limitations like more time-consuming, tedious procedures for the purification of intact genomic DNA, lengthy restriction digestion and extended electrophoresis times (Maslow *et al*., 1993).

With the above facts in view, the present study was undertaken to characterize ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* with the following objectives:

1. To isolate and identify ESBL producing *E. coli* and *Klebsiella* from animal sources
2. To determine the type of ESBL resistance of the isolates phenotypically.
3. To characterize the isolates in terms of their resistance gene profiles by PCR.
4. To characterize the presence of CTX-M-genogroups and genotypes by molecular methods
5. To evaluate the clonal relationship among the isolates by genotyping using PFGE and REP-PCR

# CHAPTER - II

# *Review of Literature*

**PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES**

## CHAPTER-II

# REVIEW OF LITERATURE

### 2.1. HISTORICAL BACKGROUND:

**Extended Spectrum Beta Lactamases (ESBLs):**

**Extended Spectrum Beta Lactamases (ESBLs)** are some plasmid mediated enzymes which can hydrolyse oxyimino-cephalosporins and monobactam groups of drug but not cephamycins or carbapenems, and were first described in the year 1983 (Bradford, 2001). ESBLs have been described in a range of *Enterobacteriaceae* and *Pseudomonadaceae* from different parts of the world, but most commonly they are identified in *Klebsiella pneumoniae* and *Escherichia coli*. They belong to the class A and D of Ambler group β-lactamases (Ambler *et al*., 1991). The Class A enzyme activities can be inhibited by β-lactamase inhibitors like clavulanic acid, sulbactam and tazobactam, but these inhibitors are unable to inhibit the enzyme activities of class D enzymes. The most common ESBLs identified till now are SHV or TEM types, evolved from narrow-spectrum β-lactamases such as TEM-1, -2 and SHV-1 (Bradford, 2001). The CTX-M enzyme, one of the most common ESBLs, has originated from *Kluyvera* spp. and reports indicate that it is gaining prominence in *Enterobacteriaceae* from Europe, Africa, Asia, South America and North America (Bonnet, 2004). Some other types of ESBLs include GES/IBC, VEB and PER β lactamases, which are less prevalent worldwide (Girlich *et al*., 2001; Tzelepi *et al*., 2003; Weldhagen *et al*., 2003). The VEB enzymes are widely distributed among *Enterobacteriaceae* from South East Asia (Girlich *et al*., 2001) and *Acinetobacter* spp. from France (Poirel *et al*., 2003), whereas the GES/IBC enzymes have been reported from hospital settings of South Africa and France (Weldhagen *et al*., 2003). PER-1 β-lactamase has been reported from hospital borne infection of *Pseudomonas aeruginosa* and *Acinetobacter* spp. from Turkey (Weldhagen *et al*., 2003).

The ESBL-producing organisms are clinically important as they are found to be an important cause of treatment failure with β-lactam antibiotics (Bush, 2001; Bradford, 2001). The failure of treatment with β-lactam antibiotics in spite of the organism showing susceptibility to the drug in routine drug sensitivity test is one of the indicators

of production of ESBL by the pathogen. ESBLs are encoded by genes located on large plasmids and carry resistant genes against antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (Paterson, 2000). Co-transfer of the *qnr* gene by ESBL-producing organisms, which confer resistance to nalidixic acid with reduced susceptibility to fluoroquinolones has also been reported (Wang *et al*., 2004; Mammeri *et al*., 2005). The majority of ESBL-producing organisms have been isolated from hospital intensive care units (ICUs), but infections can occur in other areas of hospital (Livermore, 2003), long-term care facilities (Kassis-Chikhani *et al*., 2004) and nursing homes (Bradford *et al*., 1995).

*Escherichia coli* and *Klebsiella pneumoniae* are found to be the most common *Enterobacteriaceae* producing ESBL and causing blood stream infections (Laupland and Church, 2014). ESBL-producing *E. coli* and *K. pneumoniae* have been associated with increased rates of treatment failure, high mortality and high hospitalization cost (Tumbarello *et al*., 2010). There are several reports on the incidence of ESBL producing *E. coli* such as 7.4% in Spain (Rodriguez-Bano *et al*., 2010), 9.5% in North Korea (Kang *et al*., 2010) and 21.4% in Israel (Karfunkel *et al*., 2013). High prevalence of ESBL-producing *Enterobacteriaceae* in faecal samples of healthy individuals has also been reported from China with an incidence rate varying from 42.0% to 82.6% (Sun *et al*., 2014; Zhong *et al*., 2015; Zhang *et al*., 2015)

**2.2. *Klebsiella pneumoniae*:**

*Klebsiella pneumoniae* is a gram-negative, rod shaped organism under the family *Enterobacteriaceae* and is mostly associated with diseases like pneumonia, abscess, bacteraemia and urinary tract infections (Podschun and Ullmann, 1998; Ko *et al*., 2002)). Virulence factors of *K. pneumoniae*, including a capsule, mucoviscosity-associated gene (*magA*) and a regulator of mucoid phenotype (*rmpA*) gene, have been identified and found to play role in pathogenicity. The strains of K1 and K2 serotypes were found to be associated with the virulence mechanism in mouse model (Mizuta *et al*., 1983) and the *magA* and *rmpA* genes were found to play a role in causing hypermuco-viscosity (HV), with an antiphagocytic effect against macrophages and neutrophils (Fang *et al*., 2004; Yu *et al*., 2006; Siu *et al*., 2012). *Klebsiella pneumoniae* was found to be independently

involved as a risk factor for mortality due to community-acquired pneumonia (Paganin *et al*., 2004). It is also an important cause of community acquired diseases like necrotizing pneumonia, pyogenic liver abscesses and endogenous endophthalmitis (Podschun and Ullmann, 1998).

*Klebsiella pneumoniae* is found to be a major source of antibiotic resistance with its high prevalence rate. The rate of resistance of *K. pneumoniae* is increasing over the years which may vary from country to country. Countries like Eastern and South-Western Europe, as well as Mediterranean countries are endemic to MDR *K. pneumoniae* due to ESBL production, which causes non-susceptibility to third generation cephalosporins, fluoroquinolones and aminoglycosides that may exceed 50%–60%. Carbapenem-resistant *K. pneumoniae* (CRKP) has emerged in the year 2015 in some countries such as Romania, Italy and Greece with resistance rates of 40%–60%. Increase in prevalence of MDR and XDR *K. pneumoniae* worldwide reflects many factors that include (i) spread of High risk (HiR) global multi-resistant genetic lineages (Woodford *et al*., 2011); (ii) acquisition of successful multi-resistant plasmids and (iii) acquisition of resistance genes located on successful transposons.

Penicillin resistance in *K. pneumoniae* was reported early in the 1960s which led to the discovery of the first *β*-lactamase genes, *blaSHV*-1 and *bla*TEM-1. The *blaSHV*-2, first extended spectrum *β*-lactamase (ESBL) gene was identified in *K. pneumoniae* from a patient of hospital ICU in Germany (Kliebe *et al.*, 1985). The *blaSHV*-2 gene showed an extended-spectrum activity against *β*-lactam group of drugs, including third-generation cephalosporins and monobactams. Plasmid-mediated ESBL gene, *bla*TEM-3 was also first reported from *K. pneumoniae* in France (Sirot *et al.*, 1987). With the emergence of ESBLs in *K. pneumoniae* during the period from 1990 to 2000, it has become the major ESBL-producing *Enterobacteriaceae* associated with nosocomial infection. A prevalence rate of 40% ESBL producing *K. pneumoniae* among hospital isolates was reported from Israel and Spain (Navon-Venezia *et al.*, 2003; Canton *et al.*, 2008). *Klebsiella pneumoniae* reportedly harbored mainly TEM and SHV *β*-lactamases during that period (Chong *et al*., 2011), and resulted in spread of various alleles to many countries (Livermore, 2012). In the year 2000, hospital outbreaks occurred due to ESBLs producing *K. pneumoniae* because of acquisition of plasmids and transposons encoding

*bla*CTX-M-type ESBLs (Calbo and Garau, 2015). Other ESBL genes were transferred to *K. pneumoniae* by horizontal gene transfer (HGT) including *bla*OXA-type ESBLs (Evans and Amyes, 2014), *bla*GES and *bla*SFO (Bradford, 2001), or *bla*PER, *bla*TLA and *bla*VEB (Philippon *et al.*, 2016). *β*-lactamase inhibitor-resistant genes have emerged, which encode enzymes that are partially inhibited by clavulanic acid and tazobactam (Bush and Jacoby, 2010). According to the World Health Organization (WHO), the occurrence of ESBL-producing *K. pneumoniae* has reached an endemic rate of 50% worldwide and a resistance rate of 30% in the community demonstrating the widespread nature of this resistance (Antimicrobial Resistance, Global Report on Surveillance, WHO, 2014).

### 2.3 *Escherichia coli*

*Escherichia coli* was first identified by Theodor Escherich, a German paediatrician in the year 1885 and named it as *Bacterium coli commune* (Escherich, 1885). He identified the organism in the gut microflora of infants. Later on in the year 1919, Castellani and Chalmers defined the genus *Escherichia* and established the species *E. coli* (Castellani and Chalmers, 1919). Bacteria of the species *E. coli* are gram-negative, rod shaped, about 2.0–6.0 mm in length and 1.1–1.5 mm wide, non-spore forming and motile due to presence of peritrichous flagella. They are facultatively anaerobic and produce gas due to carbohydrate fermentation. The methyl red test is positive for *E. coli* which indicates mixed acid fermentation of glucose, but the Voges Proskauer test is negative. They produce indole but are unable to hydrolyze urea. They are unable to produce hydrogen sulphide when are grew on triple sugar iron (TSI) agar or Kligler's iron agar (KIA). *Escherichia coli* also cannot induce gelatin liquefaction through gelatinase activity. Most of the strains decarboxylate lysine and use sodium acetate but do not grow on Simmons' citrate agar, where citrate is the main carbon source. Most of the organisms are capsulated, the capsule being composed of acidic polysaccharides. Mucoid strains produce extracellular slime consisting of a polysaccharide K antigen or a common acid polysaccharide known as M antigen composed of colanic acid (Jimenez *et al.*, 2012). *Escherichia coli* exhibit fimbriae or pili of varying structure and antigenic specificity with hydrophobic nature. Generally *E. coli* is found in the digestive tract of warm-blooded animals, particularly humans. Detection

of the organism in potable water is used as an indicator of contamination with human or animal excreta, which is referred to as the coliform index (Leclerc *et al*., 2001).

There are mainly six diarrhoeagenic pathovars of *E. coli*, *viz*. enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enteroaggregative (EAEC) and diffuse adherent *E. coli* (DAEC). Each type is associated with diarrhoeal disease with different clinical symptoms. Enterohaemorrhagic (EHEC) *E. coli* O157:H7 is considered as the most pathogenic *E. coli* strain (Bavaro, 2012). Members of the 'O157' serogroup have the common somatic (cell surface) O antigen, while the flagellar H antigen is used to define the specific serotype.

Treatment of *E. coli* infection has become very difficult due to development of resistance against different antibiotics. There is an increased prevalence of extended-spectrum β-lactamase (ESBL) producing *E. coli* (Borjesson *et al*., 2013). ESBL producing *E. coli* confers resistance to most of the β-lactam antibiotics, including penicillins, monobactams and most of the cephalosporins. This is because of the presence of plasmid-mediated enzymes that hydrolyze the β-lactam antibiotics (Khalaf *et al*., 2009). There is an increased rate of hospital- as well as community-acquired infections and tends to be treated with carbapenems, as these are the last choice of drug against most of the ESBL- *E. coli*. Unfortunately, carbapenem resistance against *E. coli* is also emerging (Bush, 2010).

Extended-spectrum β-lactamase (ESBL)-producing *E. coli* (ESBLEC) has emerged as a significant cause of both community- and healthcare-associated infections worldwide (Pitout and Laupland, 2008). *Escherichia coli* also play a vital role in causing nosocomial infections (Rodriguez-Bano *et al*., 2006). The type of ESBL produced by *E. coli* has changed in recent years. The SHV and TEM types of ESBLs have been substituted by CTX-M β-lactamase (Canton and Coque, 2006). Previously, most of the ESBLEC strains were found to be clonally unrelated and the rapid emergence of ESBL was associated with dissemination of mobile genetic elements (Rodriguez-Bano *et al*., 2004). The spread of the O25b-ST131 clone producing CTX-M-15 and other β-lactamases has been described in the last decade (Coque *et al*., 2008; Nicolas-Chanoine *et al*., 2008). The first nationwide study of ESBLEC was developed in Spain in the year

2000 (Hernandez *et al*., 2005). The prevalence of ESBL producing *E. coli* isolates was found to be 0.5% with CTX-M-9, SHV-12, and CTX-M-14 being the most commonly found ESBLs. No CTX-M-15-producing *E. coli* strain could be found. Another nationwide study conducted in similar line in 2006 (Diaz *et al*., 2009)) because of perceived important changes in the epidemiology of ESBLEC. The prevalence of ESBLEC increased to 4.04% in the last 6 years (range, 0.4 to 20.3%) in Spain (Diaz *et al*., 2009). The prevalence rate of infection was determined as 32, 36, and 30 per cent, respectively for community-acquired, healthcare-associated, and nosocomial infection.

Although, ESBL production is mainly associated with hospital-borne infections caused by *Klebsiella pneumoniae*, frequently *E. coli* strains are found to be associated with community-acquired infections, mostly urinary tract infections (Paterson and Bonomo, 2005; Livermore *et al*., 2007). They also act as commensals that may be isolated from humans and food-producing animals (Mevius *et al*., 2012; Huijbers *et al*., 2013; Trott, 2013).

ESBL-producing *E. coli* is highly prevalent in poultry in the Netherlands. In the year 2009, ESBL or AmpC-producing *E. coli* were detected on 100% of Dutch broiler farms (Dierikx *et al*., 2013). Because of the high prevalence rate of ESBL- *E. coli* on Dutch retail chicken meat and the overlap between genotypes of chicken meat and clinical *E. coli* isolates (Leverstein-van Hall *et al*., 2011; Overdevest *et al*., 2011), chicken meat has been suggested as a potent source of ESBL-producing *E. coli* in the Netherlands.

## 2.4 CLASSIFICATION OF ESBLs

Beta-lactamase enzymes are classified by two general classification systems: the Ambler molecular classification and the Bush–Jacoby–Medeiros functional classification (Bush *et al*., 1995; Ambler, 1980). Beta-lactamase enzymes are classified into four classes as per Ambler scheme according to the protein homology of the enzymes. Beta-lactamases of class A, C and D are serine β-lactamases and the class B enzymes are under metallo-β-lactamases. The Bush–Jacoby–Medeiros functional classification is based on functional properties of the enzymes, *i.e.* the substrate and inhibitor profiles.

### 2.4.1 *SHV* Type β-lactamases

The SHV β-lactamase family was derived from *Klebsiella* spp. The *SHV*-1 sequence is the progenitor of the SHV class of enzymes found in *K. pneumoniae*. The gene encoding for SHV-1, or its precursor, LEN-1, resides within the chromosome of different strains of *K. pneumonia.* The *SHV*-1 β-lactamase gene evolved as a chromosomal gene in *Klebsiella* and was later incorporated into a plasmid that transmits to other enterobacteriaceae. The SHV-1 enzyme confers resistance to penicillins such as ampicillin, tigecycline and piperacillin but not to oxyimino substituted cephalosporins (Livermore, 1995). The SHV-1 β -lactamase is responsible for approximately 20% of the plasmid-mediated ampicillin resistance in *K. pneumoniae* (Tzouvelekis and Bonomo, 1999).

The SHV β- lactamase is more prevalent than the other types of ESBLs in clinical bacterial isolates (Jacoby, 1997). Most of the bacterial strains have *SHV* gene on their plasmids through the replacement of serine with glycine at position 238. It was also found that some strains change lysine with glutamate at position 240. The serine residue at position 238 is required for hydrolyzing ceftazidime, whether lysine residue is essential for hydrolyzing cefotaxime. Till date, more than 100 SHV varieties are known worldwide. The SHV-type of ESBLs is found in a wide range of *Enterobacteriaceae* (Harrif-Heraud *et al*., 1997), *P. aeruginosa* and *Acinetobacter* spp. (Huang *et al*., 2004; Poirel *et al*., 2004).

### 2.4.2 *TEM* Type β-lactamases

The TEM-1 β- lactamase was first reported from an *E. coli* isolate in the year 1965 and found to have substrate and inhibition profiles similar to that of SHV-1 (Datta and Kontomichalou, 1965). TEM-1 is capable of hydrolyzing penicillins and the first generation cephalosporins but unable to hydrolyze the oxyimino cephalosporin. TEM-3 is the first TEM variant with increased activity against extended spectrum cephalosporins (Sirot *et al*., 1987; Soughakoff *et al*., 1988). TEM-3 was first discovered in *K. pneumoniae* in France in 1984. Initially, TEM-3 was known as CTX-1, because of its activity against cefotaxime (Burn- Buisson *et al*., 1987). TEM-2 was the first derivative

of TEM-1 with a single amino acid substitution from the original β-lactamase (Barthelemy *et al*., 1985). This caused a shift in the isoelectric point from a pI of 5.4–5.6, but it did not change the substrate profile. TEM-3 is different from TEM-2, with replacement of two amino acids (Sougakoff *et al*., 1988). TEM-12 was found in a *Klebsiella oxytoca* isolate reported from Liverpool, England in 1982 with a ceftazidime resistant gene in the plasmid (Du Bois *et al*., 1995). The strain was isolated from a neonatal unit which had been stricken by an outbreak of *K. oxytoca* producing TEM-1 (Du Bois *et al*., 1995). Most commonly, TEM type ESBLs are found in *E. coli* and *K. pneumoniae* but it is also reported from other gram-negative organisms (Livermore, 1995) and in different genera of *Enterobacteriaceae* (*Enterobacter aerogenes, Enterobacter cloacae, Morganella morganii, Proteus mirabilis* and *Salmonella*) (Morosini *et al*., 1995; Marchandin *et al*., 1999). TEM type of β- lactamases have also been reported from non-*Enterobacteriaceae* like *P. aeruginosa* (Nordmann and Guibert, 1998).

### 2.4.3 *CTX – M* Type β-lactamases

The CTX-M β-lactamase was first described by Tzouvelekis in the year 2000 (Tzouvelekis *et al*., 2000). The term CTX-M beta-lactamase denotes its ability to hydrolyze cefotaxime (Bonnet, 2004). They can hydrolyze cephalothin better than benzyl-penicillin and cefotaxime better than ceftazidime (Bradford *et al*., 1998; Tzouvelekis *et al*., 2000). The enzyme has been found mostly in isolates of *Salmonella* Typhimurium, *E. coli* and other *Enterobacteriaceae* (Knothe *et al*., 1983; Gazouli *et al*., 1998). These enzymes are not very closely related to TEM or SHV β-lactamases (Tzouvelekis *et al*., 2000). In addition to the hydrolysis of cefotaxime, they can be better inhibited by the β-lactamase inhibitor tazobactam than by sulbactam and clavulanate (Bradford *et al*., 1998; Ma *et al*., 1998). CTX-M β-lactamases are classified under the functional group 2 (Bush and Jacoby, 2010) and found to originate from chromosomal ESBL genes found in *Kluyvera* spp. (Bush and Jacoby, 2012), an opportunistic pathogen under the family *Enterobacteriaceae*. The first CTX-M protein was discovered in the late 1980s and more than 100 variants have been sequenced till now (Bonnet, 2004). Based on the amino acid sequences, CTX-M enzymes can be divided into five sub-groups, CTX-M groups 1, 2, 8, 9, and 25 (Bonnet, 2004) found in different *Enterobacteriaceae*

including *Salmonella* (Bradford *et al*., 1998). The origin of the CTX-M β-lactamase is different from that of TEM and SHV type of ESBLs. The SHV and TEM enzymes were generated by amino acid substitutions of their parent enzyme, whereas, CTX-M enzymes were acquired by the horizontal gene transfer from other bacteria using conjugative plasmid or transposon. It has been found that the serine residue at position 237 present in all the CTX-M enzymes plays an important role in the extended-spectrum activity of the CTX-M-type b-lactamases (Tzouvelekis *et al*., 2000). The Arg-276 residue lies in a position equivalent to Arg-244 in TEM- or SHV-type ESBLs as suggested by molecular modeling and may also play a role in hydrolysis of oxyimino cephalosporins (Gazouli *et al*., 1998). The MIC test carried out for ceftazidime showed resistance by the CTX-M-type β-lactamases (Poirel *et al*., 2002). The CTX-M enzymes also hydrolyze the fourth generation cephalosporin, cefepime (Tzouvelekis *et al*., 2000). Unlike TEM and SHV β-lactamases, no point mutation has been detected in CTX-M enzyme.

### 2.4.4 OXA Type β-lactamases

The OXA-type β-lactamases have oxacillin-hydrolyzing abilities. These enzymes are characterized by hydrolysis rates for cloxacillin and oxacillin greater than 50% as that for benzyl penicillin (Bush *et al*., 1995). They are found to occur predominantly in *P. aeruginosa* (Weldhagen *et al*., 2003), but also have been detected in other gram-negative organisms. The most common OXA-type β-lactamase, OXA-1 has been reported in 1–10% of *E. coli* isolates (Livermore, 1995). The OXA ESBLs were discovered in *P. aeruginosa* isolated from a hospital in Ankara, Turkey. A derivative of OXA-10 (OXA-28) was found in a *P. aeruginosa* isolate in France (Poirel *et al*., 2001). OXA-18 and a derivative of the narrow spectrum OXA-13 β-lactamase, *i.e.* OXA-19, have also been from France in *P. aeruginosa* isolates (Philippon *et al*., 1997). There are very few epidemiological data on the geographical spread of OXA-type ESBLs (Philippon *et al*., 1997).

Plasmid-encoded β-lactamases could be divided into two groups, the TEM type of β-lactamase and a small group that can hydrolyze oxacillin, OXA type. Unlike the TEM

enzymes, OXA type β-lactamase has a heterogeneous substrate profile and is encoded by a much narrower range of plasmids. These enzymes have some properties similar to those found on a plasmid, R1818 (Datta and Kontomichalou, 1965), which was later renamed R46 (Meynell and Datta, 1966). They have a lower specific activity against penicillin than that of the TEM type of β-lactamases, but have much higher activity against oxacillin and methicillin. Emergence of these enzymes presumably coincided with the widespread introduction of flucloxacillin and methicillin for staphylococcal infection treatment. The OXA type of β-lactamases is less effective against the first generation cephalosporins and is poorly inhibited by β-lactamase inhibitors such as clavulanic acid.

### 2.4.5 *PER* Type β-lactamases

The PER (*Pseudomonas* extended resistance)-type β- lactamases share around 25–27% homology with TEM- and SHV-types of ESBLs (Bauernfeind *et al*., 1996). PER-1 β-lactamase can efficiently hydrolyze penicillins and cephalosporin group of drugs and is susceptible to β-lactmase inhibitors like clavulanic acid. PER-1 enzyme was first detected in *P. aeruginosa* (Neuhauser *et al*., 2003) and later in *S. enterica* serovar Typhimurium and *Acinetobacter* (Vahaboglu *et al*., 2001). In Turkey, 46% of nosocomial infections caused by *Acinetobacter* spp. and 11% of *P. aeruginosa* were found to produce PER-1 (Vahaboglu *et al*., 1997). PER-2 has a homology of 86% with PER-1, which was detected in *S. enterica* serovar Typhimurium, *E. coli*, *K. pneumoniae*, *Proteus mirabilis* and *Vibrio cholerae* O1 El Tor (Petroni *et al*., 2002). The PER-1 β-lactamase is found across Turkey in 60% of ceftazidime-resistant strains of *A. baumanii*, which represent 46% of the total isolates (Vahaboglu *et al*., 1997), while PER-2 has been found almost exclusively in South America.

### 2.4.6 *GES*, *VEB*-1, *BES*-1 and other ESBL type β-lactamases

The GES-1 ESBL was initially described in *K. pneumoniae* from a neonatal patient just transferred to France from French Guiana (Poirel *et al*., 2000). This enzyme has hydrolytic activity against penicillins and cephalosporins, but not against cephamycins or carbapenems. This type of β-lactamase is inhibited by β-lactamase

inhibitors. These properties of this enzyme resemble to other class A ESBLs; thus, GES-1 has been recognized as ESBLs. Other types of enzymes having ESBL activities are BES-1, CME-1, VEB-1, TLA-1 and SFO-1 (Bradford, 2001).

The GES-1 is not closely related to other plasmid-mediated ESBL enzymes and show 36% homology to a carbenicillinase from *Proteus mirabilis* (Poirel *et al*., 2000). VEB-1 was first reported in *E. coli* isolated from a patient from Vietnam and also subsequently reported from a patient from Thailand in a *P. aeruginosa* isolate (Naas *et al*., 1999). CME-1 β-lactamases was isolated from *Chryseobacterium meningosepticum* (Rossolini *et al*., 1999) and TLA-1 was detected in an *E. coli* isolate from a patient in Mexico (Silva *et al*., 2000). The PER-1, PER-2, VEB-1, CME-1, and TLA-1 β-lactamases show 40 to 50% homology. These ESBLs confer resistance to oxyimino-cephalosporins, particularly to ceftazidime and aztreonam. They show some homology to the chromosomal cephalosporinases in *Bacteroides* spp. and are thought to be originated from this species (Rossolini *et al*., 1999). SFO-1 is highly related to a class A ESBL of *Serratia fonticola*, which is transferable and can be induced to high-level production of β-lactamase by imipenem (Matsumoto and Inoue, 1999). The plasmid with gene encoding for SFO-1 β-lactamase also carries the *ampR* regulatory gene required for induction of class C β-lactamases. But unlike class C β-lactamases, SFO-1 cannot hydrolyze cephamycins and is inhibited by clavulanic acid (Matsumoto and Inoue, 1999).

## 2.5 EPIDEMIOLOGY AND PREVALENCE OF ESBLs

ESBLs have become the most common cause of nosocomial infection worldwide. The epidemiology of ESBLs is very complicated because of the involvement of certain factors like different geographical locations, countries, hospitals, the community and the host. Some other factors which involve in the epidemiology are different types of microorganisms (*E. coli* is more endemic and *K. pneumoniae* is more epidemic) and their mobile genetic elements, mostly plasmids. Moreover, there is involvement various reservoirs like the environment (*e.g.* soil and water), wild and farm animals and pets. Transmission of ESBL-producing organisms occurs from food and water and via direct or indirect contact (person to person) (Carattoli, 2008).

The first ESBL producer strain was identified from Germany (Knothe *et al*., 1983) and England (Du Bois *et al*., 1995). In France, the first ESBL was reported in the year 1986 and 54 patients of three intensive care units (ICUs) of a hospital were found as ESBL positive (Burn-Buisson *et al*., 1987). About 25 to 35% of nosocomial infections caused by *K. pneumoniae* in France were found positive for ESBL in 1990s (Marty and Jarlier, 1998). The rate of occurrence of *Enterobacteriaceae* producing ESBL in France was found to be less than 1%, but there was an increase in the rate of prevalence of CTX-M (Galas *et al*., 2008). Another study conducted in the year 2005 showed the reducing rate of prevalence of ESBL producers than in the previous years, like *P. mirabilis* (3.7% to1.3%), *Enterobacter aerogenes* (53.5% to 21.4%) and *K. pneumoniae* (9.4% to 3.71%). However, the interesting finding was an increased prevalence rate of ESBL producing *E. coli* from 0.2% to 2% (Arpin *et al*., 2007). There was an increase in ESBL producers in northern European countries such as Denmark, Norway and Sweden as per the national surveillance report. It was found that the prevalence rate of ESBL was more than 10% in Eastern European countries such as Hungary, Poland, Romania, Russia and Turkey. *Klebsiella pneumoniae* was found to be the dominant ESBL producers in most of the European countries. (Damjanova *et al*., 2007; Korten *et al*., 2007; Markovska *et al*., 2008). The most commonly involved β- lactamase enzymes were CTX-M-3, SHV-2 and SHV-5 in Eastern Europe countries.

As per the report of European Antimicrobial Resistance Surveillance System (EARSS), 2.6% *E. coli* and 1.7% *K. pneumoniae* strains were resistant to third-generation cephalosporins in Sweden in the year 2010 (EARSS, 2011). CTX-M-9 type of β –lactamase is most predominant in Spain and CTX-M-3 enzymes have been reported mostly from Eastern Europe and the most widespread ESBL is CTX-M-1 (including the CTX-M-15 type) throughout entire Europe (Coque *et al*., 2008a,b; Canton *et al*., 2008).

*Klebsiella pneumoniae* harbouring SHV-2 and SHV-5 were first reported during the period from 1988 to 1989 in Chile and Argentina (EARSS, 2011). The study showed a prevalence rate of 30 to 60% ESBLs producing *Klebsiella* spp. in Brazil, Colombia and Venezuela (Mendes *et al*., 2000). An increase in the rate of ESBL producers, *i.e.* 26% of

*E. coli* and 35% of *K. pneumoniae* were found in Latin American in the year 2008. Rate of ESBL producing *E. coli* was found the same (10%) in 2003 and 2004, while an increase of *K. pneumoniae* from 14% to 18% were found in 2003 and 2004 (Rossi *et al*., 2006).

In the United States, TEM-10 was reported as the first ESBL from a *K. pneumoniae* isolate in 1988 (Jacoby *et al*., 1988), which was followed by TEM-12 and TEM-26 (Bush, 2008). In 2001, 5.6% ESBL prevalence rate was reported from the United States (Winokur *et al*., 2001). A study conducted during 1998 to 1999 showed prevalence of 36.1% ESBL positive *K. pneumoniae* in a hospital in South Africa (Bell *et al*., 2002). The first study conducted in Tanzania in 2001–2002 for the presence of ESBL reported 25% ESBL positive *E. coli* and 17% of the *K. pneumoniae* harbouring mainly CTX-M-15 and TEM-63 (Blomberg *et al*., 2005). Another study conducted in a tertiary hospital in Mwanza, Tanzania showed a prevalence rate of 29% ESBLs in all gram-negative organisms. The study reported ESBL prevalence rate as 64% and 24% for *K. pneumoniae* and *E. coli*, respectively (Kariuki *et al*., 2007). ESBL prevalence rate of 63% in adults and 100% in children were found in a study performed at an orphanage in Mali (Tande *et al*., 2009). Herindrainy *et al.* (2011) observed that 10% of non-hospitalized patients carried ESBLs with CTX-M-15 enzyme.

The prevalence rate of ESBL is higher in Middle East than other parts of the world. A study conducted in Egypt for the period from 1999 to 2000 showed presence of 38% ESBLs producingstrains among *E. coli.* During 2007 to 2008, 45% of the *K. pneumoniae* isolates from urinary tract infections and 59.2% of *K. pneumoniae* isolated from respiratory infections were found as ESBL producers in Iran (Ghafourian *et al.*, 2011; Ghafourian *et al*., 2012). About 26% ESBL producing *K. pneumoniae* were detected from Saudi Arabia in the year 2008. The most predominant β-lactamases were SHV-12, CTX-M-15 and TEM-1 in ESBL producing organisms (Tawfik *et al*., 2011). In Lebanon, a study showed presence of β-lactamase producing bacteria in hospitalised patients (16%), healthcare workers (3%), and healthy subjects (2%) in 2003 (Moubareck *et al*., 2011) harbouring mostly CTX-M-15 enzyme (83%).

In Australia, the first ESBL positive strain was reported in *Klebsiella* spp. harboring the enzyme SHV (Mulgrave and Attwood, 1993). SHV-2 was the first β-lactamase detected in *K. pneumoniae* isolates from China in 1988 (Rossi *et al*., 2006). The SENTRY surveillance program reported an increased prevalence rate in *K. pneumoniae* (60%) and *E. coli* (13–35%) in different parts of China with CTX-M-14 and CTX-M-3 β-lactamase enzymes (Hirakata *et al*., 2005; Hawkey, 2008). In India, CTX-M-15 enzyme has been found to be most predominant β-lactamase, showing resistant to third generation antibiotics (66%) mostly in *K. pneumoniae* and *E. coli* isolates (Ensor *et al*., 2006). A rapid increase in the prevalence of NDM-1β-lactamase has been found from Varanasi, India (6.9%) and Rawalpindi, Pakistan (18.5%) (Perry *et al*., 2011). A study conducted in a tertiary hospital in Patiala, Punjab showed prevalence rate of ESBL producing strains as 44%, 48% and 50% among *K. pneumoniae, E. coli* and *P. aeruginosa* isolates, respectively (Rupinder *et al*., 2013). National Public Health Laboratory (NPHL), Kathmandu, Nepal reported prevalence of 31.57% of ESBL producing *E. coli* with co-resistance to different antibiotics (Thakur *et al*., 2013).

Duan *et al*. (2006) isolated ESBL-producing *Escherichia coli* from faecal samples and cloacal swabs from cattle, pigs, chicken, ducks, geese and pigeons in Hong Kong in 2002. Out of 734 faecal samples screened, six (2%) from pigs, three (3.1%) from cattle and one (3%) from pigeons showed positive for ESBL producing *E. coli*.

Mesa *et al*. (2006) screened a total of 9802 samples which included human infection samples from hospitals, faecal carriers, human sewage, pig, rabbit and poultry farm samples and different food samples and food-borne outbreak samples for the presence of ESBL producing *Enterobacteriaceae* in Spain. The study showed prevalence rate of 1.9% in human infections, 6.6% in faecal carriers, 0.4% in food samples, and 31.1% in food borne outbreaks and 20% in rabbit farms. Maximum prevalence was found in human sewage samples and samples of poultry farms (100%) followed by pig farm samples (80%).

A total of 334 faecal samples from different food-producing animals including pigs (59), cattle (124) and sheep (58) and 100 raw milk and 104 minced meat (beef and pork) samples from Switzerland were screened for presence of ESBL producing

microorganisms. The study revealed prevalence of ESBL in faecal samples as 15.3% from pig, 13.7% from cattle, 8.6% from sheep and 63.4% from chicken. Whereas, only one of the milk samples and none of the meat samples showed presence of ESBL. Of the total of 91 isolates, 89 were confirmed as *E. coli*, one of each *Citrobacter youngae* and *Enterobacter cloacae* (Geser *et al*., 2012).

A study carried out during 2007-2008, with swab samples from foods of animal origin mostly in Mediterranean countries showed presence of ESBL enzymes in 349 samples out of 419 swab samples (Tham *et al*., 2012).

Schmid *et al*. (2013) studied the prevalence of ESBL-producing *E. coli* in dairy cows and beef cattle in Bavaria, Germany, which included 30 mixed dairy and beef cattle farms and 15 beef cattle farms. A total of 598 samples were collected including faecal samples, boot swabs and dust samples. They found 196 (32.8%) positive samples for ESBL-producing *E. coli* from 39 farms (86.7%) out of 45. They also reported that samples of mixed farms yielded more ESBL producers than beef cattle farms.

## 2.6 PHENOTYPIC CONFIRMATION OF ESBLs

There are guidelines published by US Clinical and Laboratory Standards Institute (CLSI) and the UK Health Protection Agency (HPA) for detection of ESBLs in *Enterobacteriaceae* particularly for *E. coli*, *Klebsiella* spp., *Proteus* spp. and *Salmonella* (NCCLS, 2002; HPA, 2008). As per the guidelines, ESBLs can hydrolyze third-generation cephalosporins but they may be inhibited by β-lactamase inhibitors like clavulanate. Isolation of ESBL can be done with incorporation of antibiotics in the medium @ 8 mg/L (CLSI) or 1 mg/L (HPA) of cefpodoxime, 1 mg/L each of cefotaxime, ceftazidime, ceftriaxone, or aztreonam, followed by other tests (including the ESBL-E-test) with both cefotaxime and ceftazidime in combination with clavulanate with concentration of 4 µg/mL.

Jarlier *et al.* (1988) described double-disk test for detection of ESBL producing microorganisms. The test organism is to be swabbed onto a Mueller-Hinton agar plate

for that test and an antibiotic disk containing amoxicillin-clavulanic acid is placed in the center of the plate and disk containing β-lactam antibiotic alone is placed 30 mm away from the amoxicillin-clavulanic acid disk. Increase in the zone of inhibition of the β-lactam disk caused by the synergy of the clavulanic acid in the amoxicillin-clavulanic acid disk is considered as positive for ESBL (Jarlier *et al.*, 1988).

Thomson and Sanders reported a three-dimensional test for detection of ESBLs, where following inoculation of the organisms on a Mueller-Hinton agar plate, a slit is cut into the agar, in which a broth suspension of the test organism is introduced. Antibiotic disks are placed on the surface of the plate, 3 mm from the slit. Distortion or discontinuity in the expected circular zone of inhibition is considered as positive for ESBL (Thomson and Sanders, 1992).

Jacoby and Han (1996) described another test similar to double-disk test, in which 20 μg of sulbactam was added to disks containing one of the oxyimino-β-lactam antibiotics. An increase of 5 mm in the zone of inhibition of the combined antibiotic disk compared to the drug alone was considered as ESBL positive test.

Manufacturers have developed commercial disks containing both cephalosporin and clavulanic acid. A zone difference obtained with 10 μg disks containing cefpodoxime, ceftazidime or cefotaxime with or without the addition of 1 μg of clavulanic acid can detect the presence of ESBL (Carter, *et al.*, 2000; M'Zali *et al.*, 2000).

Another phenotypic confirmation test is used for determining MICs of either ceftazidime or cefotaxime with (@ 4 μg/ml) and without presence of clavulanic acid. A decrease in the MIC value of ≥3 two-fold dilutions in presence of clavulanic acid is considered as ESBL positive.

ESBL E-strips for detection of ESBL producers is one of the more sensitive tests that contain a gradient of ceftazidime on one side and ceftazidime with clavulanic acid on the other side. The test is considered positive, if it shows ≥3-dilution reduction in the MIC of ceftazidime in the presence of clavulanic acid (Linscott *et al.*, 2005)

## 2.7 ANTIBIOTIC SENSITIVITY ASSAY

Antimicrobial resistance is considered as one of the major threats emerging globally. The most common antibiotic resistance mechanism against β-lactam antibiotics occurs due to production of plasmid mediated enzymes β-lactamases. The production of extended-spectrum β-lactamases (ESBLs) leads to resistance of different gram-negative organisms against third-generation cephalosporins. Beta-lactamase enzymes are encoded by plasmid mediated genes, evolved from point mutations (Bradford, 2001).

A total of 116 (33.9%) *E. coli* isolates were detected from 342 urine samples collected from patients with urinary tract infection (UTI) from Khyber Teaching Hospital, Pakistan during the period from April 2005 and February 2006. The antibiotics used for the sensitivity assay were ampicillin (AMP), cephradine (CE), cefaclor (CEC), cefotaxime (CTX), ceftazidime (CAZ), doxycycline (DOX), ciprofloxacin (CIP), ofloxacin (OFL), enoxacin (ENX), nalidixic acid (NA), meropenem (MEM), imipenem (IPM), sulphamethoxazole + trimethoprim (SXT), gentamicin (CN) and kanamycin (K). Out of these, imipenem showed highest efficacy (98.3%) against the isolates, followed by meropenem (97.4%). Out of cephalosporins, cephradine showed 72% resistance, followed by cefaclor and ceftazidime (65% each), while 62% resistance was shown to cefotaxime. Maximum resistance (89%) was shown to ampicillin. Gentamicin resistance was found to be 51.7%, while 56.9% resistance was showed against kanamycin. All the three fluoroquinolones (ciprofloxacin, ofloxacin and enoxacin) were found to show similar resistance (62%). Resistance to combination of sulphamethoxazole and trimethoprim was recorded as 81%, while 79.3% resistance was recorded against doxycycline (Ullah *et al*., 2009).

A study conducted in a tertiary care teaching hospital for a period of 1 year from February 2008 and January2009 reported 213 ESBL isolates from pus, sputum, tracheal aspirate, cerebrospinal fluid, ascitic fluid, pleural fluid, blood and urine, which comprised of 132 *Escherichia coli*, 54 *Klebsiella pneumoniae* and 27 *Pseudomonas* spp. The *E. coli* isolates showed maximum sensitivity against imipenem (100%), followed by piperacillin-tazobactum (84%), amikacin (68%), gentamicin (9%), ciprofloxacin (9%) and amoxicillin-clavulanic acid (7%). The *K. pneumoniae* isolates were also found to be

highly sensitive to imipenem (98%), followed by piperacillin-tazobactum (68%), amikacin (40%), gentamicin (15%), ciprofloxacin (15%) and amoxicillin-clavulanic acid (5%). Approximately 87% ESBL producing *E. coli* and 88% *K. pneumoniae* isolates were respectively found to show multi-drug resistance to amoxicillin-clavulanic acid, gentamicin and ciprofloxacin (Umadevi *et al*., 2011).

Rajan and Prabavathy (2012) reported on 115 *E. coli* isolates from 562 urine samples of urinary tract infection from a hospital in Chennai during the period of March to April 2012, which were subjected to antibiotic sensitivity assay against antibiotics, *viz.* Ampicillin (30 µg), cephalexin (30 µg), ciprofloxacin (15 µg), nitrofurantoin (300 µg), gentamycin (10 µg), norfloxacin (10 µg), co-trimaxazole (25 µg), ofloxacin (5 µg), amoxycillin / clavulanic acid (30 µg), cefuroxime (30 µg) amikacin (10 µg), ceftazidime (30 µg) and cefpodoxime (10 µg). Out of these, ampicillin, cephalexin, ciprofloxacin, ceftazidime and cepodoxime showed 100% resistance followed by cefuroxime, norfloxacin and ofloxacin (98%). Co-trimoxazole showed a resistance rate of 72.5% followed by gentamicin (50%) and amoxyclav (25%). A very less resistance rate was shown against nitrofuranton (12%) and amikacin (3%).

Kumar *et al*. (2014) isolated100 ESBL producing *E. coli* from clinical samples including pus, urine, blood, cerebrospinal fluid (CSF), stool, sputum, ear swab and different body fluids during the period from September 2010 to March 2012 in Noida. Antimicrobial susceptibility test was performed by Kirby–Bauer disk diffusion method as per Clinical Laboratory Standards Institute (CLSI) guidelines using antibiotic discs, *viz.* ampicillin (10 µg), piperacillin (100 µg), piperacillin-tazobactam (100/10 µg), amoxicillin/clavulanic acid (20/10 µg), cefoperazone /sulbactam (75/10µg), ceftazidime/clavulanate (30/10 µg), cefoperazone (75 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (30 µg), ofloxacin (5 µg), norfloxacin (10 µg) and nitrofurantin (300 µg). The rate of susceptibility was found to be highest to imipenem (100%) followed by piperacillin/tazobactam (87.22%), cefoperazone/sulbactam (76.67%), amoxicillin/clavulanic acid (75.55%), and ceftazidime/ clavulanate (66.11%). The rate of susceptibility to different antibiotics were: cefotaxime (31.11%), ceftazidime (35.55%),

ceftriaxone (38.33%), cephamycin (31.11%), monobactam (31.11%), piperacillin (33.33%), cefoperazone (27.77%) and cefepime (35.55%).

Nwankwo *et al*. (2015) carried out a study during the period from January to July 2010 in Aminu Kano Teaching Hospital (AKTH), Kano, Nigeria with an aim to isolate different ESBL producing organisms from clinical samples. They found *E. coli* as the most predominant organism (30.5 %) followed by *Staphylococcus aureus* (21.3 %). ESBL producing organisms showed a high-level of resistance against the quinolones, aminoglycoside and cotrimoxazole but were found to be sensitive against carbapenems and levofloxaccin. *Escherichia coli* and *Klebsiella pneumoniae* showed a rate of 14 and 16 % sensitivity against amoxicillin/clavulanate and 43% and 21%, respectively against ceftazidime. But most bacterial pathogens showed more than 60 % sensitivity to ceftriaxone, gentamicin and levofloxacin.

Roshene *et al*. (2015) conducted a study in Saveetha Medical College, Chennai and isolated 20 ESBL producing *E. coli* from clinical samples like urine, stool, wound swab and pus. Antibiotic sensitivity assay was performed using antibiotics such as ampicillin, amoxicillin, amikacin, norfloxacin, ceftazidime, cefotaxime, ciprofloxacin and gentamicin as by Kirby Bauer disc diffusion method (CLSI, 2011). The study showed an increased (70%) sensitivity to amikacin followed by gentamicin (45%); 80- 90% of *E. coli* were found to show resistance to cephalosporin group of drugs.

Koovapra *et al.* (2016) recovered 23 ESBL producing *Klebsiella pneumoniae* isolates from 340 bovine milk samples collected from healthy cows as well as cows with clinical and subclinical mastitis and subjected the isolates to antibiotic sensitivity test against 15 different antibiotics, *viz.* sulpha/trimethoprim (1.25/23.75 µg), chloramphenicol (30 µg), tetracycline (30 µg), gentamicin (10 µg), amikacin (30 µg), cefixime (5 µg), ceftizoxime (30 µg), cefoxitin (30 µg), gatifloxacin (5 µg), tobramycin (10 µg), ciprofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), pi-peracillin-tazobactam (100/10 µg) and cefepime-tazobactam (80/10 µg) as per the standard guidelines (CLSI, 2014). All the isolates were found to show 100% resistance against ceftriaxone, ceftazidime,

cefotaxime, aztreonam, cefpodoxime, ceftizoxime and cefexime followed by gentamicin (78%), tetracycline (74%), sulpha/trimethoprim combination (70%), cefepime (61%), ciprofloxacin and piperacillin/tazobactam (52%).

Onifade *et al.* (2018) reported isolation of ESBL producing bacteria from otitis media samples collected from hospitals in Nigeria. The most predominant bacteria isolated were *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Staphylococcus epidermidis* and *Streptococcus pyogenes*. Antibiotic sensitivity tests were performed using standardized agar disc diffusion test against the isolates as per CLSI, 2014. The antibiotic discs used in the study were *viz.* Cloxacillin (25 mg) (CXC), Cotrimoxazole (25 mg) (COT), Erythromycin (20 mg) (ERY), Gentamicin (10 mg) (GEN), Augmentin (30 mg) (AUG), Streptomycin (30 mg) (STR), Tetracycline (30 mg) (TET), Chloramphenicol (10 mg) (CHL), Amoxicillin (25 mg) (AMX), Nitrofurantoin (200 mg) (NIT), Nalidixic Acid (25 mg) (NAL), and Ofloxacin (5 mg) (OFL), respectively. Most of the isolates were found to be resistant against multiple antibiotics (MDR). *Streptococcus pneumoniae* was found to be resistant against CXC, AMX, AUG and TET while *S. pyogenes* was resistant to COT, CXC, AUG, AMX and TET respectively. *Staphylococcus epidermidis* was reported to show resistance against AMX, ERY, CHL and STR and *S. aureus* was resistant to AUG, AMX, COT and ERY. *Pseudomonas aeruginosa* was resistant to AMX, COT, ERY and TET, AUG and CHL, while *P. mirabilis* was found to be resistant against AMX, COT, ERY, TET, AUG and CXC. Another organism *K. pneumoniae* showed resistance to AMX, AUG, COT, NAL, and TET, but *E. coli* was found to show resistance against AMX, COT, ERY, TET, AUG and STR.

## 2.8 DETECTION OF AmpC B-LACTAMASE (ACBL)

Extended spectrum β-lactamases (ESBLs) and AmpC β-lactamases both are becoming global threat to animals as well as human beings for causing multidrug resistance to antibiotics resulting in treatment failure. These enzymes are produced by gram-negative bacteria mostly by *Klebsiella* spp. and *Escherichia coli.* AmpC β-lactamases are cephalosporinases which cannot be inhibited by clavulanic acid and can be differentiated from ESBLs because of their ability to hydrolyze cephamycins. ESBL genes are mostly plasmid-mediated and found in *K. pneumoniae, E. coli, Salmonella* and *Shigella* spp., while *Enterobacter* spp., *Providencia* spp., *Citrobacter freundii, Morganella morganii* and *Serratia marcescens* mostly harbour the AmpC β-lactamase genes that are either plasmid or chromosomally mediated. It has been reported that expression of chromosome-mediated AmpC genes may be constitutive or inducible. Initially, plasmid-mediated AmpC genes were thought to be non-inducible, but later on they were reported as inducible (Fortineau *et al*., 2001).

Coudron *et al*. (2000) and Ratna *et al*. (2003) screened AmpC producing *E. coli* isolates using cefoxitin (CX) discs. The isolates showing a zone diameter <18 mm were considered screen positive. Inhibitor based method (IBM) and modified three dimensional test (M3D) were used for both screen-positive and negative isolates for confirmation of AmpC enzyme. The ESBL producing isolates were screened by disc diffusion method as per CLSI guidelines and further confirmed by CLSI phenotypic confirmatory test (CLSI, 2004). Out of the 300 *E. coli* tested, 82.76% showed positive for ESBL production and 59% was AmpC screen positive. AmpC production was confirmed in 40% and 39% of *E. coli* isolates by IBM and M3D methods, respectively. Out of 39% AmpC producers, approximately 84.6% were both ESBL and AmpC producers, while only 15.38% isolates were AmpC producers alone.

Jennifer *et al.* (2005) reported 44 (31%) AmpC producers among 140 cefoxitin-resistant *Klebsiella* species, *Proteus mirabilis* and *Salmonella* species isolates (31%). Out of 44 positive isolates, 42 were confirmed plasmid-mediated AmpC β-lactamase producers by the isoelectric focusing overlay technique and multiplex PCR.

El-Hady and Adel (2015) detected 50 (33.8%) AmpC producers from 148 *Enterobacteriaceae* isolates from patients of Ain Shams University Hospital, Egypt. AmpC confirmation was done by three phenotypic tests, *viz.* AmpC E test, disk approximation test, and AmpC EDTA disc test. Molecular detection was done by multiplex PCR. All the 50 (100%) isolates were found to be positive by the three phenotypic tests and 46 (92%) isolates were reported to be positive by PCR.

Kaur *et al*. (2016) isolated 245 isolates from urine samples which included *Escherichia coli* (123), *Klebsiella pneumoniae* (87), *Proteus* spp. (20) *Enterobacter* spp. (9) and *Citrobacter* spp. (6). All the isolates were subjected to assay for AmpC β lactamase production by Modified three –dimensional test (MTDT) (Manchanda *et al*., 2003). Out of 143 positive AmpC isolates, screened using cephoxitin (30 µg) discs, 113 (46.1%) isolates were confirmed as AmpC producers. Isolates showing zone diameter of <18 mm in cephoxitin disc were considered as AmpC producers (Trivedi *et al*., 2013).

Koovapra *et al.* (2016) reported isolation of 23 ESBL producing *Klebsiella pneumoniae* from 340 bovine milk samples. Out of these isolates, seven were screened phenotypically as AmpC producers by cefoxitin-cloxacillin double disc synergy (CC-DDS) test (Polsfuss *et al*., 2011; Kar *et al.*, 2015), while the gene *bla*AmpC was detected in 20 (82.6%) ESBL isolates.

## 2.9 DETECTION OF METALLO-B-LACTAMASE

According to Ambler classification of β-lactamase, carbapenemase enzymes belong to classes A, B and D. The class B carbapenemases require one or two zinc ions for their increased catalytic activity and known as metallo-β-lactamases (MBLs) (Schlesinger *et al*., 2011). MBLs are considered as one of the most alarming β-lactamases because of their ability to hydrolyse all betalactam antibiotics (Noori *et al*., 2014). Till now no MBL inhibitors have been reported capable of producing these enzymes (King and Strynadka, 2011). Genes encoding MBL are disseminated from one bacterium to another through horizontal gene transfer (Noori *et al*., 2014).

A total of 84 MBL-producing gram-negative bacteria were isolated by Franklin *et al*. (2006). The isolates were subjected to phenotypic detection methods like double-disk synergy test, a combined-disk test and PCR was done to detect MBL genes. Both the phenotypic tests were reported to be positive for 66 (79%) isolates, whereas only 18(21%) isolates were found to be positive by combined-disk test. All the 84 (100%) isolates were found to carry genes encoding MBL.

Khosravi *et al*. (2012) isolated 90 imipenem resistant *P. aeruginosa* isolates from patients of University of Malaya Medical Centre, Malaysia during the period from October 2005 to March 2008. The imipenem resistant isolates were tested for MBL production by three phenotypic tests, *viz.* Combined Disk Test (CDT), Double Disk Synergy Test (DDST), imipenem/imipenem-inhibitor (MBL IP/IPI) E-Test. Genotyping was done by detection of MBL genes by PCR. All three phenotypic tests were found to show 100% sensitivity, while DDST was reported to be most specific of all the three (96.6%), followed by IP/IPI *E*- test (62.1%) and CDT (43.1%). The study showed presence of MBL genes in 32 isolates only.

EL-Mosallamy *et al*. (2015) isolated 100 *P. aeruginosa* from 220 random clinical samples. They obtained 25 imipenem resistant *P. aeruginosa* isolates, which were subjected to imipenem + EDTA combined disc test (IMP-EDTA CDT) for MBL production (Yong *et al*., 2002) and PCR to detect MBL genes. Phenotypically, 14 out of 25 (56%) were positive for MBL production and 15 (60%) isolates were found to carry MβL producing genes.

Anwar *et al*. (2016) isolated 112 *Acinetobacter baumannii* from urine, blood, sputum, pus, body fluids and tracheal secretions. Carbapenem resistance was found in 66 (58.9%) isolates by disc diffusion test. The carbapenem resistant isolates were further tested by Modified Hodge test (MHT), whereas Combined Disk Test (CDT) and Double Disk Synergy Test (DDST) tests were used phenotypically for MBL (Metallo β-lactamase) production. MHT showed presence of carbapenemase in 55 (83.3%) isolates. Out of 66 carbapenem resistant isolates, 63 were found to be positive for MBL production by CDT and 51 were found positive by DDST.

Koovapra *et al*. (2016) reported 23 ESBL producing *Klebsiella pneumoniae* from milk samples of three different states of India, West Bengal, Jharkhand and Mizoram. Out of 23 isolates, five were found to be resistant against imipenem. However, when tested with imipenem-EDTA disc synergy test and modified hodge test (MHT), none of the isolates were found to be positive by any of these tests or by PCR.

## 2.10 DETECTION OF RESISTANCE GENES BY SIMPLEX PCR

A study carried out by Chen *et al.* (2010) revealed 342 cefotaxime resistant isolates among 1914 thermo tolerant coliform isolates. A total of 319/1915 (16.7%) isolates were confirmed as ESBL by disc diffusion test. PCR was preformed to detect *bla*TEM, *bla*CTX-M, *bla*SHV and *bla*OXA genes. Single *bla* gene was found in 47.3% (151 strains) including 22.6% CTX-M (72strains) 16.3% TEM (52 strains) and 8.5% SHV (27strains). A total of 49.5% isolates were found to carry more than one gene. The prevalence of combined *bla*TEM and *bla*CTX-M was found to be 30.4%, followed by 9.1 % for both *bla*SHV and *bla*CTX-M genes, 6% for both *bla*TEM and *bla*SHV genes and 4.1% for all three *bla*TEM, *bla*SHV and *bla*CTX-M genes. None of the isolates was found to harbor the *bla*OXA gene.

Cabral *et al.* (2012) characterized 24 *K. pneumoniae* isolates obtained from hospital patients in Brazil. DDST (double-disc synergy test) identified only 9 (37.5%) isolates as ESBL producers. PCR was carried out for detection of β-lactamase genes, *viz.* *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*KPC, *bla*VIM, *bla*IMP and *bla*SPM. The *bla*SHV gene was found to be the most predominant (100%) of all the genes, followed by *bla*CTX-M-2 (62.5%), *bla*KPC (41.7%) and *bla*TEM (29%). None of the isolates was found to carry *bla*VIM, *bla*IMP and *bla*SPM genes. Eleven isolates were found to carry at least three β-lactamase genes and two isolates were found to harbor *bla*SHV, *bla*TEM, *bla*CTX-M-2 and *bla*KPC genes.

Ibrahim *et al.* (2016) isolated 126 *E. coli* strains from slurry and faecal samples collected from a dairy unit. Out of 126 isolates, 53 (42.06%) were found to show resistance against cefotaxime and/or ceftazdime. Phenotypically, 25/53 (47.1%) isolates were confirmed as ESBL producers. All the 25 ESBL isolates were subjected to PCR for

resistant gene profiling with *bla*CTX-M, *bla*TEM, *bla*OXA1 and OXA2 and *bla*SHV genes. None of the isolates were found to harbor *bla*SHV gene. The rate of presence of other genes was 22.6% for *bla*CTX-M, 7.5% for *bla*TEM and 3.7% for *bla*OXA-1.

Koovapra *et al*. (2016) isolated 23 *K. pneumoniae* ESBL producing isolates from bovine milk samples, all of which were subjected to PCR to detect *bla*AmpC, *bla*SHV, *bla*TEM, *bla*CTX-M, *bla*NDM, *bla*VIM, *bla*IMP, *bla*OXA-48, *bla*KPC, *int*1, *int*2, *qnr*S, *qnr*B, *qnr*A, *qep, aac* and *sul*-1 genes. Out of all the resistant genes screened, *bla*CTX-M was found to be the most predominant, *i.e.* 82.6%, followed by *bla*TEM (34.8%) and *bla*SHV (13%). Eight isolates were found to carry more than one gene like six isolates harboured *bla*CTX-M and *bla*TEM, and one isolate each harboured *bla*SHV, *bla*CTX-M and *bla*SHV, *bla*TEM genes.

Salah *et al.* (2016) carried out a study during the period from May 2013 to July 2015 at the National Institute of Hygiene, Lome in Togo and isolated 91 *E. coli* strains resistant to different third generation antibiotics. They could detect ESBL genes, *viz. blaTEM, blaSHV, bla*CTX-M-G1 (CTX-M-1, 3 or 15), *bla*CTX-M-G2 and *bla*CTX-M-G9 (CTX-M-9 and CTX-M-14) among the isolates. The study revealed presence of a low rate of ESBL genes alone, *i.e.* 19/91 (20.88%) in comparison to multiple genes together like 72/91 (79.21%) *E. coli* isolates showing presence of more than one ESBL resistance gene. Only one (1.1%) strain was found to harbor the *bla*TEM gene and 18/91 (19.78%) isolates were found to have *blaCTX-M*-1 gene, while none of the isolates were found to have *bla*SHV, *bla*CTX-M-2 and *bla*CTX-M-G-9 genes. The rate of occurrence of TEM/SHV and TEM/CTX-M1 genes were 1.10% and 57.14%, respectively and for TEM/SHV/CTX-M1 gene, it was 20.88%.

Kpoda *et al.* (2018) reported prevalence of 187 ESBL producing *Enterobacteriaceae* isolates from clinical samples of hospitals in Ouagadougou Burkina Faso., *viz. Klebsiella* spp. and *Escherichia coli*. The ESBL isolates were subjected to PCR for detection of *bla*TEM, *bla*CTX-M, *bla*SHV genes. The rate of occurrence of *bla*CTX-M, *bla*TEM and *bla*SHV genes in *E. coli* was 39.6, 24.6 and3.7%, respectively. While in *Klebsiella* spp., the corresponding rates were 5.9% for *bla*CTX-M , 2.7% for *bla*TEM and 1.6% for *bla*SHV gene.

A total of 211 ESBL-producing *E. coli* isolates were obtained from different clinical samples and were identified by MALDI-TOF by Yasir *et al.* (2018). They carried out PCR for detection of *bla*TEM, *bla*SHV, *bla*CTX-M and *bla*OXA genes. The prevalence of *bla*CTX-M and *bla*TEM was found to be 95.3% and 83.9%, respectively, whereas, *bla*OXA and *bla*SHV were detected in 6.6% and 5.2% isolates, respectively. Approximately, 82.5% of the isolates carried more than one resistant gene. The combination of *bla*CTX-M and *bla*TEM was found to be 79.1%, while all the three genes, *viz. bla*CTX-M, *bla*TEM and *bla*SHV was reported in only 2.4% isolates. The prevalence of *bla*CTX-M-1 *bla*CTX-M-9, *bla*CTX-M-8/25 and *bla*CTX-M-2 was found to be 74.6%, 20.4%, 2.5 and 1.5%, respectively.

## 2.11 CTX-M GENOGROUPING USING PCR

CTX-M-is a type of plasmid-encoded β-lactamases, capable of hydrolyzing cephalosporins and are also inhibited by clavulanic acid and tazobactam. These enzymes come under group 2be of the Bush, Jacoby and Medeiros classification, which also contains the extended spectrum derivatives of TEM-1 & 2 and SHV-1 (Bush *et al*., 1995). Based on the difference in amino-acid sequence, CTX-M enzyme is classified into the five major phylogenetic groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25.

Xu *et al*. (2005) genogrouped 62 *bla*CTX-M-positive isolates and standardized a multiplex PCR for detection of all the five groups of CTX-M. They found presence group 1 (40 CTX-M-15 and 3 CTX-M-3) in 43 isolates, followed by 16 isolates of group 9 (10 CTX-M-14 and six CTX-M-9) and 3 isolates of group 25/26 (CTXM- 26). None of the isolates was found to possess group 2 and group 8 genes. The multiplex PCR revealed 100% similar results with simplex PCR for CTX-M genogrouping.

Tofteland *et al*. (2007) reported 52 (60%) *E. coli* isolates out of 87 and 21 (84%) *K. pneumoniae* out of 25 isolates as ESBL producers. All the isolates were subjected to PCR for detection of resistant genes. The *bla*CTX-M gene was detected in 45 (90%) ESBL *E. coli* isolates including CTX-M-1 group in 29 isolates, CTX-M-9 group in15 isolates and CTX-M-2 group only in one isolate. Out of 19 *K. pneumoniae* isolates, only

three isolates were found to harbor *bla*CTX-M gene, including 2 of group 1 and one of group 9. No isolate was found to carry groups 8 and 25 genes.

Mirzaee *et al*. (2009) screened 250 *E. coli* isolates from three hospitals of Tehran, Iran. Phenotypic test confirmed 140 (56%) isolates as ESBL producers. The ESBL isolates were further subjected to multiplex PCR for detection of *bla*CTX-M genogroups. Out of 140 isolates, 50 (35.7%) showed presence of group 1, followed by 5 (3.5%) isolates of group 9 and 1 (0.7%) of group 25/26. No isolate was found to belong to group 2 and group 8.

Geser *et al*. (2012) confirmed a total of 91 isolates comprising of 89 *E. coli*, one each of *Citrobacter youngae* and *Enterobacter cloacae* from raw milk, bulk tank milk and minced beef and pork. ESBL producing isolates were screened by PCR for presence of CTX-M genogroups. A total of 78 isolates (85.7%) were found to show presence of CTX-M group 1, while six isolates (6.6%) produced CTX-M group 9 enzymes.

Hu *et al*. (2013) confirmed a total of 139 ESBL *E. coli* isolates from water (26), pig faecal sample (31), healthy humans (46), and hospitalized patients (36) and carried out PCR for detection of CTX-M subgroups. A total of 33 (23.7%) isolates were reported to carry CTX-M- group 1, while 99 (71.2%) isolates were found to carry CTX-M group 9 and 7 (5%) isolates were found to carry both group 1 and group 9. The *bla*CTX-M-14 gene was found as the most prevalent CTX-M group 9 gene, and *bla*CTX-M-55 and *bla*CTX-M-15 were the most prevalent CTX-M group 1 genes.

Shahid *et al.* (2014) carried out both monoplex and multiplex PCR assays using 80 ESBL producing Enterobacteriaceae comprising of 75 *E. coli* and five *Klebsiella pneumoniae.* A total of 93.8% (75/80) isolates showed amplifcation of CTX-*M* genes by multiplex PCR. By monoplex-PCR, 93.3% (70/75) were found to harbour CTX-M genogroup-1 and 6.7% (5/75) harboured genogroup-9. Out of 70 CTX-M genogroup-1 isolates, 65 were *E. coli* and five were *K. pneumoniae*, whereas genogroup-9 isolates were reported as *E. coli*.

Woodford *et al*. (2005) designed a multiplex PCR for performing CTX-M genogrouping in 633 Enterobacteriaceae isolates. The study revealed presence of group 1 CTX-M enzymes in 547 (86.4%) of the 633 isolates, which included 429 *E. coli*, 114 *Klebsiella spp*., three *Enterobacter spp.* and one *Morganella* sp. CTX-M group 9 enzymes were found to be present in 81 (12.8%) isolates, including 74 *E. coli*, six *Klebsiella* spp. and one *Enterobacter* spp. The remaining three *E. coli* isolates were found to harbor group 2 CTX-M enzymes, while group 8 CTX-M 8 was found in only one *E. coli* isolate. One *E. coli* isolate was reported to harbor both group 1 and group 9 enzymes. No isolate was found to belong to CTX-M group 25.

## 2.12 DETECTION OF INSERTION SEQUENCE USING PCR

The insertion sequences are the smallest transposable elements less than 2.5 kb and classified into families on the basis of their characteristics with transposases (Mahillon and Chandler, 1998; Zhao and Hu, 2013). ESBL resistant genes have been found to be associated with insertion sequences. It has been reported that *IS*26, *ISE*cp1, *ISCR*1 and *IS*903 along with class 1 integron are the most frequently found insertion elements with ESBL resistance (Arduino *et al*., 2002; Eckert *et al*., 2006; Diestra *et al*., 2008; Cullik *et al*., 2010; Cheng *et al*., 2016). Many insertion sequences have been identified upstream of the *bla*CTX-M genes, like *ISEcp1*, *ISCR*1, *IS*10 and *IS*26. Of all these insertion sequences, *ISE*cp*1* is the most commonly found IS upstream of different *bla*CTX-M gene and was first reported in 1999 adjacent to CTX-M 15. *ISEcp1* was found to be associated with all groups of CTX-M except group 8. *ISCR1* has been reported to be associated with CTX-M groups 2 and 9, while *IS*10 was related to CTX-M group 8.

Raji *et al.* (2015) screened a total of 73 Enterobacteriaceae isolates, of which 38 (52.1 %) were confirmed as ESBL producers comprising of 21 (55.3 %) *E. coli*, 12 (31.6 %) *K. pneumoniae*, 3 (7.9 %) *Proteus* spp., and one (2.6 %) each of *M. morganii* and *Citrobacter freundii.* All the ESBL isolates were screened for the presence of *ISE*cp1 element by PCR and 30 (79%) isolates were found positive, which also showed presence of *bla*CTX-M gene.

Ali *et al.* (2016) screened 1252 mastitic milk samples and isolated 36 (23.53%) ESBL producing *E. coli.* All the ESBL isolates were screened for the presence of insertion sequence *ISCR*1 and was found in 22 (66.11%) ESBL producing *E. coli* isolates. *ISCR*1 was also found to be associated with *bla*CTX-M, *bla*TEM and *bla*SHV genes in 16, 3, and 4 isolates, respectively.

Roshani *et al.* (2018) recovered 56 *E. coli* and 24 *K. pneumoniae* isolates from different samples. All the 80 isolates were subjected to PCR for detection of three insertion sequences, *viz. ISE*cp1, *IS*26 and *IS*903. About 69.64% (39/56) and 96.42% (54/56) of *E. coli* isolates were found to carry *IS*903 and *ISE*cp1 genes, respectively, while *ISE*cp1 was found in 21( 87.5%) and *IS*903 was found in 18 (75%) *K. pneumoniae* isolates, while all the 80 (100%) isolates were found to be positive for presence of *IS*26 element.

Etayo *et al.* (2018) isolated 150 ESBL-producing *Escherichia coli* of food products (48), farms and feeds (20), rivers and wastewater treatment plants (33) and human origins, including healthy volunteers (13) and hospital inpatients (36). PCR was performed to detect *IS*Ecp1, *ISCR*1, *IS*26 and *IS*903 associated with resistant genes. Out of all the insertion elements, *IS*26 was found to be the most prevalent (99.4%), followed by *IS*Ecp1 (68%), *IS*903 (65.3%) and *ISCR*1 (12.6%). All the insertion elements were found in all different samples except *ISCR*1, which could not be found in farm and feed samples.

## 2.13 MOLECULAR DIVERSITY SCREENING METHODS

### 2.13.1 Repetitive Extragenic Palindromic Sequence Based PCR (REP-PCR)

Identification of an epidemic strain is often critical to the success of epidemiological investigations aimed at preventing the spread of infection and eradicating its source (Threlfall and Frost, 1990). The repetitive extragenic palindromic polymerase chain reaction (REP-PCR) has been applied for typing different gram-negative organisms (***Hulton*** *et al.*,1991; ***Versalovic*** **et al., *1991).*** Research has shown that REP-PCR has the ability to fingerprint strains of *Escherichia coli* and *K. pneumoniae* and

other gram-negative bacteria (***Ishii and Sadowsky, 2009; Hernandez* et al.*, 2005; Zowawi* et al.*, 2014; Naas* et al.*, 2016).***

The poly-trinucleotide (GTG)5 motif is a class of conserved repetitive sequences present in most of the bacterial genomes (Versalovic *et al*., 1994). The (GTG)5-PCR fingerprinting has been used by many researchers now a days for molecular typing of many organisms, *viz. Acinetobacter baumanii* (Huys *et al*., 2005), *Salmonella enterica* (Rasschaert *et al*., 2005), *Campylobacter concisus* (Matsheka *et al*., 2006), *Enterococcus faecium* (Svec *et al*., 2005; Jurkovic *et al*., 2007), *Escherichia coli* (Mohapatra *et al*., 2007: Mohapatra *et al*., 2008), *Streptococcus mutans* (Svec *et al*., 2008) and for some lactic acid bacteria isolated from human blood cultures (Svec *et al*., 2007).

Lim *et al*. (2009) carried out comparative genotyping of 51 antibiotic resistant *K. pneumoniae* using RAPD, ERIC-PCR, REP-PCR and PFGE. RAPD using the OPAB11 primer revealed 49 unique profiles for all the 51 isolates. ERIC- and REP- PCR resulted in 46 and 50 different genotypes, respectively, while PFGE using *Xba*I revealed 47 distinct pulse field patterns. Genotyping of the *K. pneumoniae* isolates using ERIC-PCR, RAPD, REP-PCR and PFGE showed genetically diverse and heterogeneous profiles.

Lim *et al*. (2009) detected 47 multi-drug resistant ESBL producing *E. coli* from hospitals of Malayasia. The isolates were further genotyped by four different fingerprinting techniques, *viz.* RAPD, ERIC-PCR, REP-PCR and PFGE. RAPD was performed using OPAB04 and OPB17 primers, ERIC-PCR by ERIC-1 primer and REP primer was used for REP-PCR. PFGE was performed using XbaI enzyme. RAPD generated 44 and 43 profiles by use of OPAB04 and OPB17 primers, respectively, while both REP-PCR and ERIC-PCR produced 45 different patterns. Pulse-field gel electrophoresis generated 44 distinct pulso-types consisting of 12–26 restriction fragments.

Diaz *et al*. (2010) screened 1008 samples for isolation of ESBL producing organisms and confirmed 254 isolates as ESBL producers. The clonal relationship was studied by REP-PCR using (GTG)5 primer. The REP-PCR findings revealed presence of 214 different clones in all the isolates, seven profiles included two isolates each, and one profile included three isolates.

Valenza *et al*., (2015) identified 31 ESBL producing Enterobacteriaceae from 156 fecal samples. Molecular typing of the *E. coli* (23) isolates was done by the semi-automated repetitive sequence- based (REP-PCR) DiversiLab System This study revealed 97% similarity in 15 (65.2%) of 23 isolates.

Ghasemian *et al.* (2017) isolated 200 *K. pneumoniae* isolates from various sources from Tehran, Iran, of which 87 (43.5%) were confirmed as ESBL. The prevalence of *bla*TEM gene was reported to be as 87.3%. All the *bla*TEM producing isolates were subjected to REP-PCR using REP 1 primer. The rep- PCR pattern revealed six clusters for all different isolates and they showed 90% similarity among 50-80% isolates which suggested a polyclonal spread of TEM type of β-lactamase among the isolates.

Ghasemian *et al.* (2018) conducted a study in 80 ESBL-producing *K. pneumoniae* isolates and found presence of *bla*CTX-M gene in 74 (92.5%) isolates. REP-PCR was performed in all the CTX-M1 producing isolates to check genetic relatedness of the isolates. Among these 74 isolates, six different genotype clusters were found. Mostly, they showed 40 to 50% similarity, while six isolates showed >70% similarity. In this study**,** REP-PCR typing showed a wide diversity with no genetic relation.

Sekhar *et al.* (2018) isolated 122 (81.3%) *E. coli* isolates from 150 faecal samples of chicken, ducks, quails, turkey and fancy birds and 12(80%) isolates from 15 human stool samples. A total of 12 *E. coli* isolates out of 134 were found as ESBL producers. ESBL producing isolates were genotyped by (GTG)5 REP-PCR and found 4-21 bands per isolate, which ranged from >100 bp to 2500 bp in size. The Rep- PCR profiles revealed 11 patterns among the ESBL isolates. The study showed four major clusters with 70% similarity. Two *E. coli* isolates showed identical REP fingerprints, which were also found to belong to the same serotype (O)

### 2.13.2 Pulse-Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a tool for epidemiological studies, which helps in analyzing large bacterial DNA fragments with high resolution, repeatability and good comparability. It is considered as the gold standard for bacterial typing (Helgason *et al*., 2000; Johnson *et al*., 2007). This technique has high

reproducibility and can differentiate unrelated strains and can also establish a relationship among the isolates of same origin. PFGE is being used as a tool for diversity studies in different organisms including *E. coli* (Kiratisin *et al*., 2008; Mendonça *et al*., 2009) and *K. pneumoniae* (Arlet *et al*., 1994; Li *et al*., 2009).

Nemoy *et al*. (2005) carried out a study from 2001-2002 and isolated 40 ESBL producing *E. coli* from ICU patients. The ESBL isolates were evaluated by PFGE using *Xba*I enzyme and MLST for molecular typing. PFGE generated 19 pulse-field patterns among the ESBL isolates, while MLST produced 22 different sequence types. Simpson's indices of diversity for PFGE and MLST were found to be 0.895 and 0.956, respectively.

Feizabadi *et al*. (2010) screened 75 ESBL producing *K. pneumoniae* from hospitals of Tehran. A total of 71 isolates were genotyped by PFGE as the other four isolates did not yield good quality DNA. These 71 isolates generated 62 different genotypes.

Barguigua *et al*. (2011) screened 767 *E. coli* and 36 *K. pneumoniae* isolates and detected only 10 *E. coli* (1.3%) and two *K. pneumoniae* (5.6%) as ESBL producers. All the *E. coli* isolates were genotyped by PFGE. The study revealed seven different pulsed-field profiles (PFPs) among the *E. coli* isolates with >80% similarity.

Chandel *et al.* (2011) collected 1738 blood samples from infants from four different hospitals of India and found 155 isolates as ESBL producers out of 252 gram negative bacilli. All the ESBL producing isolates were found to show multiple genetic profiles (total of 23) in PFGE. More than 40% of all ESBL- isolates formed three pulsotypes, *viz.*PFP I, II and III, with PFP-II being the largest cluster.

Mohajeri *et al*. (2014) isolated 200 uropathogenic *E. coli* from hospitalized patients in Iran, of which 49 (24.5%) isolates were confirmed as ESBL. These isolates were analysed by PFGE by digesting with *Xba*I. PFGE revealed a total of 10 different pulsotypes with nine common clones and one single clone. It also showed a high similarity, *i.e.* 96–99.5% between isolates, which indicated prevalence of these isolates among the community.

Kao *et al*. (2016) identified 56 ESBL isolates from hospitalized patients. Of these, 46 were identified as *E. coli*, seven were *K. pneumoniae* and three were *K. oxytoca*. PFGE was performed with all the ESBL producing isolates, which showed >80% similarity among the isolates. The study revealed genetic heterogeneity in CTX-M

producing isolates, while eight *E. coli* and three *K. pneumoniae* isolates were found to be genetically related.

A study carried out in a hospital by Popova *et al*. (2018) revealed 155 Enterobacteriaceae from respiratory tract infection samples, of which 121 (78.1%) were confirmed as *E. coli* and 96 (79.3%) of these isolates were ESBL producers. PFGE was performed using *Xba*I enzyme for all the isolates which revealed 12 different pulsotypes on the basis of their band patterns with 100% similarity within the pulsotype.

Akya *et al*. (2019) confirmed 40 isolates as ESBL producers out of 100 *K. pneumoniae* isolates obtained from clinical samples from hospitals. Of these, 30 were reported to harbor CTX-M gene. The 30 CTX-M producing isolates were subjected to PFGE for genotyping with use of *Salmonella enterica* serovar Braenderup H9812 as marker. PFGE of *bla*CTX‑M positive *K. pneumoniae* isolates generated 19 clusters different genotypic patterns

# CHAPTER - III

# *Materials and Methods*

**PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES**

## CHAPTER-III

# MATERIALS AND METHODS

## 3. SAMPLING

### 3.1. STUDY AREA

For the present study, milk and faecal samples were collected from apparently healthy animals and poultry from Khanapara, Jorabat, Chandrapur, Hajo and Sualkuchi areas of Kamrup district, Assam, and different areas of Nagaon, Morigaon, Barpeta and Bongaigaon districts.

### 3.2. DURATION OF STUDY

The present study was conducted during the period from May 2017 to June 2018 to isolate and identify ESBL producing *Klebsiella pneumoniae* and *Escherichia coli* from animal sources.

### 3.3. NATURE OF SAMPLES

Samples collected for the present investigation included milk samples from healthy cows and goats, meat samples from chicken, pig, goat and cow, and faecal samples from cows and goats. Samples of curd prepared from cow milk were also collected for the study. The samples were collected from animals which were under antibiotic treatment or having previous history of treatment with antibiotics.

### 3.4. BUFFERS, REAGENTS AND MEDIA

Detailed composition of all buffers, reagents and media used in this study are mentioned in the Appendix.

### 3.5 EXAMINATION OF SAMPLES

#### 3.5.1. Isolation of *Klebsiella* spp. and *Escherichia coli*

The following media were used for isolation of Extended Spectrum β-Lactamase (ESBL) producing *Klebsiella* spp. and *Escherichia coli*

(a) **Media used for primary isolation**:

- Luria Burtoni broth supplemented with cefotaxime @ 1 mg/L was used for primary isolation of both the organisms.
- Mac Conkey's Lactose Agar (MLA) supplemented with cefotaxime @ 1 mg/L was used as a selective medium for isolation of ESBL producing *Klebsiella* spp. and *Escherichia coli.*

(b) **Media used for purification of colonies**: Mac Conkey's Lactose Agar (MLA) and Eosine Methylene Blue Agar (EMBA) both supplemented with cefotaxime @ 1 mg/L were used for purification of suspected colonies of *Klebsiella* spp. and *Escherichia coli*, respectively.

(c) **Media for maintenance of culture**: Nutrient agar (with 1.5% Agar agar) stabs and 16% glycerol were used for preservation of the isolates.

**3.5.2. Inoculation of samples:**

For primary isolation, the samples were inoculated in test tubes containing 5 ml of Luria Burtoni broth supplemented with cefotaxime @ 1 mg/L and the tubes were incubated aerobically at 37° C for 24 hours. A loopful of broth culture was then streaked onto the Mac Conkey's Lactose Agar (MLA) plates supplemented with cefotaxime @ 1 mg/L and incubated for a period of 24 hours at 37° C. Subsequently, the suspected colonies of ESBL producing *Klebsiella* spp. were subcultured on MLA plate and ESBL producing *E. coli* colonies were subcultured on Eosin Methylene Blue Agar (EMBA) plates for purification. The plates were then incubated aerobically at 37° C for 24 hours. Single colonies from MLA and EMBA plates were smeared and stained by Gram's staining method and examined microscopically.

**3.5.3. Maintenance of cultures:**

After purification, the suspected colonies of different isolates of both ESBL producing *Klebsiella* spp. and *E. coli* were preserved in nutrient agar stabs and as glycerol stocks with 16% glycerol. Tryptone soy agar stabs were kept at 4°C and the glycerol stocks were maintained at -80°C ultra-freezer.

### 3.5.4. Characterization and identification of the isolates:

Characterization and preliminary identification of the suspected isolates of *Klebsiella* spp. and *E. coli* were done on the basis of morphology, colony characteristics and biochemical tests following the methods described by Patel *et al*. (2017) for *Klebsiella* and Koneman *et al*. (1992) and Baron *et al*. (1994) for *E. coli*.

**Morphology:**

Morphological characteristics of the isolated cultures were studied by Gram's staining depending on its shape, size and arrangement along with staining reaction.

**Colony characteristics:**

The colony characteristics of the isolates, *viz.* colour, size, shape and appearance of the colonies on culture media were recorded.

**Biochemical tests:**

Biochemical tests required for identification of *Klebsiella pneumoniae* were performed as per the methods described by Foster and Bragg (1962) and Darrell and Hurdle (1964). For this, the isolates were subjected to the following biochemical tests:

(a) Sugar fermentation tests: Peptone water medium with 1% sugar was inoculated with the test culture and incubated at 37°C for 48 hours. Fermentation of glucose, lactose, sucrose, mannitol and dulcitol was tested.

(b) Citrate utilization test: Simmon's citrate agar slants were inoculated with the test culture and incubated at 37°C for overnight.

(c) Urease production test: Christensen's urea agar slants were inoculated with the test culture and incubated at 37°C for 48 hours.

(d) $H_2S$ production Test: Lead acetate papers were inserted over the peptone water culture and incubated for 24-48 hours at 37°C.

(e) Methyl red and Voges-Proskauer Tests: Organisms were grown in buffered glucose broth (Glucose phosphate peptone water broth) at 37°C for 48 hours and subsequently added with MR and VP reagents, respectively for reading the results as per standard methods.

(f) Indole test: Production of indole was tested by a colorimetric reaction using Kovac's reagent in cultures grown in Peptone water broth for 24 hours at 37°C.

Biochemical tests required for identification of *Escherichia coli* were performed as per the methods described by Smith and Holdeman (1968), Willis (1977) and Koneman *et al*. (1992). The isolates were subjected to the following biochemical tests as per the methods described above:

(a) Citrate utilization test

(b) Urease production test

(c) Methyl red and Voges-Proskauer tests

(d) Indole test

## 3.6. MOLECULAR DETECTION OF *Escherichia coli* BY SIMPLEX PCR

### 3.6.1. Reference strains

Standard reference strains of *Escherichia coli* and *Klebsiella pneumoniae* used in this study were obtained from American Type Culture Collection (ATCC), U.S.A.

**TABLE 3.1: REFERENCE CULTURES OBTAINED FROM AMERICAN TYPE CULTURE COLLCETION (MTCC)**

| Sl. No. | Strain No. | Name |
|---|---|---|
| 1. | ATCC 25922 | *Escherichia coli* |
| 2. | ATCC 700603 | *Klebsiella pneumoniae* |

### 3.6.2 DNA Extraction

Genomic DNA of *Escherichia coli* was extracted using a commercial kit (Geneaid DNA isolation kit**)** following the protocol prescribed by the manufacturer. The concentration of the purified DNA was determined using a Pico Drop spectrophotometer.

## 3.7 CONFIRMATION OF THE *Escherichia coli* ISOLATES BY PCR AMPLIFICATION OF *uid*A GENE:

The identity of *Escherichia coli* isolates was confirmed by amplification of the *uid*A gene by polymerase chain reaction (PCR). The *uid*A gene encodes the enzyme β -D-glucoronidase. Amplification of this conserved gene sequence by PCR was used for the identification of *E. coli*.

**TABLE 3.2. PRIMER SEQUENCES FOR AMPLIFICATION OF *uid*A GENE BY SIMPLEX PCR**

| Primer | Primer sequence (5′ - 3′) | Amplicon size | Reference |
|---|---|---|---|
| Forward | TATGGAATTTCGCCGATTTT | 166 bp | Heijnen and Medema (2006) |
| Reverse | TGTTTGCCTCCCTGCTGCGG | | |

### 3.7.1. PCR amplification of *uid*A gene

Amplification of the *uid*A gene was done in a thermocycler (Eppendorf, Germany) using the components of the reaction mixture and the cycling conditions as shown below:

**TABLE 3.3: PCR REACTION MIXTURE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermoscientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.4. PCR CONDITIONS USED FOR AMPLIFICATION *uid*A GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 54 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

## 3.8 CONFIRMATION OF *Klebsiella pneumoniae* ISOLATES BY PCR AMPLIFICATION OF *rpo*B GENE:

The identity of *Klebsiella pneumoniae* isolates was confirmed by amplification of the *rpo*B gene by polymerase chain reaction. The *rpo*B gene encodes the enzyme RNA polymerase β subunit. Amplification of this conserved gene sequence by PCR was used for the identification of *K. pneumoniae* isolates.

**TABLE 3.5. PRIMER SEQUENCES FOR AMPLIFICATION OF *rpo*B GENE BY SIMPLEX PCR**

| Primer | Primer sequence (5′ - 3′) | Amplicon size | Reference |
|---|---|---|---|
| Forward | CAA CGG TGT GGT TAC TGA CG | 108 bp | Chander *et al.* (2011) |
| Reverse | TCT ACG AAG TGG CCG TTT TC | | |

**TABLE 3.6: PCR REACTION MIXTURE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermoscientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.7. PCR CONDITIONS USED FOR AMPLIFICATION *rpo*B GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 51 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

## 3.9. CONFIRMATION AND VISUALISATION OF PCR AMPLICONS

The PCR amplified products were detected by gel electrophoresis using 1.5% agarose gel. For this, the gel casting tray was set up by placing a comb in the slot containing 16 wells. A volume of 100 ml of 1.5% agarose gel was prepared by adding 1.5 g of agarose in 100 ml of 1 X TAE buffer. The mixture was heated for melting in a

microwave oven for 2 minutes. The molten agarose was cooled to 56°C and ethidium bromide @ 0.5 µg/ml was added as a stain to visualise the products later. After proper mixing, it was poured on the gel casting tray and allowed to solidify without disturbing the tray. After solidification, the comb was removed carefully and 1X TAE buffer was poured in the gel casting tray until the gel was submerged. Each well was loaded with 3.0 µl of amplified product and 1 µl of 6X loading dye. Along with the PCR amplicons, a 100 bp DNA ladder, a template-negative control and a positive control were also loaded. Electrophoresis was carried out at 90 volt for 1 hour at room temperature. The gel was then visualised using a Gel documentation system (Bio-Rad, USA) and the result was recorded.

## 3.10 CONFIRMATION OF ESBL PRODUCING ISOLATES PHENOTYPICALLY

### 3.10.1 Screening of the isolates for confirming as ESBL by drug sensitivity assay against 3$^{rd}$ and 4$^{th}$ generation cephalosporins

Drug sensitivity assay was performed on Mueller-Hinton agar (Himedia, India) using the agar diffusion method with antimicrobial disks (Himedia, India) according to the Clinical and Laboratory Standards Institute (CLSI, 2014) guidelines.

**Table 3.8. LIST OF ANTIMICROBIAL AGENTS USED**

| Serial no. | Antimicrobial agents | Concentration (mcg) |
|---|---|---|
| 1. | Cefotaxime (CTX) | 30 |
| 2. | Ceftriaxone (CTR) | 30 |
| 3. | Cefpodoxime (CPD) | 10 |
| 4. | Ceftazidime (CAZ) | 30 |
| 5. | Cefepime (CPM) | 30 |
| 6. | Aztreonam (AT) | 30 |

**Test procedure:**

The test was performed by inoculating the suspected pure cultures on Luria Burtoni (HiMedia, India) broth tubes and incubating at 37° C aerobically for 6 to 8

hours. About 1 ml of the culture from the broth was uniformly spread on the surface of Mueller-Hinton agar plates (HiMedia, India) and kept undisturbed for minimum 10 minutes. Excess of the inoculated broth was discarded from the plates and the antimicrobial discs were placed on the surface by gently pressing down with a pair of sterile forceps at an adequate distance. The plates were incubated at 37°C for 24 hours in an inverted manner. The results were interpreted according to zone size interpretative table provided by the manufacturer of the discs.

### 3.10.2 Screening of isolates for confirming as ESBL by Combination of Disc Diffusion Tests (CDT)

The CDT test was performed on Mueller-Hinton agar (Himedia, India) using cefotaxime and cefotaxime+ clavulanic acid combined antimicrobial disks (Himedia, India) as per CLSI, 2014

**TABLE 3.9. LIST OF ANTIMICROBIAL AGENTS USED**

| Serial no. | Antimicrobial agent | Concentration (mcg) |
|---|---|---|
| 1. | Cefotaxime (CTX) | 30 |
| 2. | Cefotaxime/Clavulanic acid | 30/10 |

**Test Procedure**

The test was performed by inoculating the suspected pure colonies on Trypton Soya Broth (HiMedia, India) broth tubes and incubating at 37° C aerobically for 6 to 8 hours. A sterile non-toxic cotton swab was dipped into the standardized inoculum and the soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid. The culture was streaked on to the Mueller hinton agar (Himedia, India) plate with the swab three times, turning the plate at 60° angle between each streaking and allowed the inoculum to dry for 5-15 minutes. The antimicrobial discs were placed by keeping a distance of 24 mm on the surface by gently pressing down with a pair of sterile forceps at an adequate distance. The plates were incubated at 37°C for 16-18 hours in an inverted manner. The results were interpreted according to zone size interpretative table provided by the manufacturer of the discs.

**Interpretation**

A zone diameter difference of ≥ 5 mm between Cefotaxime 30 mcg disc and Cefotaxime/Clavulanic acid 30/10 mcg disc was interpreted as ESBL positive positive.

### 3.10.3 Screening of isolates for confirming as ESBL by ESBL-E TEST

The ESBL-E test test was performed on Mueller-Hinton agar (Himedia, India) using Triple ESBL-E strip (Himedia, India) as per CLSI, 2014.

**TABLE 3.10. LIST OF ANTIMICROBIAL AGENTS USED**

| Sl. no. | Composition of MIC strip | Concentration (mcg/ml) |
|---|---|---|
| 1. | Ceftazidime, Cefotaxime & Cefepime Mix | 0.125-16 |
| 2. | Ceftazidime, Cefotaxime & Cefepime Mix + Clavulanic acid | 0.032- 4 |

**Test Procedure**

The test was performed by inoculating the suspected pure colonies on Trypton Soya Broth (HiMedia, India) broth tubes and incubating at 37° C aerobically for 6 to 8 hours. A sterile non-toxic cotton swab was dipped into the standardized inoculum and the soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid. The culture was streaked on to the Mueller hinton agar (Himedia, India) plate with the swab three times, turning the plate at 60° angle between each streaking and allowed the inoculum to dry for 5-15 minutes. With the help of the sterile applicator from the self sealing bag, MIC strip was lifted and placed on agar plate swabbed with test culture. The plates were incubated at 37°C for 16-18 hours in an inverted manner. The results were interpreted as per the interpretive criteria provided by the manufacturer of the strip.

**Interpretation**

**ESBL positive strain:**

Ratio of the value obtained for MIX: the value of MIX in combination with Clavulanic acid (MIX+) is more than or equal to 8 (MIX/ MIX+ = ≥ 8)

**ESBL positive strain:**

Ratio of the value obtained for MIX: the value of MIX in combination with Clavulanic acid (MIX+) is less than 8 (MIX/ MIX+ = < 8)

**ESBL (nonconclusive):**

No zone of inhibition is obtained on either side

### 3.11. ANTIMICROBIAL SENSITIVITY TESTING

Antimicrobial sensitivity tests were performed on Mueller-Hinton agar (Himedia, India) using the agar diffusion method with antimicrobial disks (Himedia, India) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

**TABLE 3.11. LIST OF ANTIMICROBIAL AGENTS USED**

| Serial no. | Antimicrobial agents | Concentration (mcg) |
|---|---|---|
| 1. | Co-trimoxazole (COT) | 25 |
| 2. | Chloramphenicol (C) | 30 |
| 3. | Tetracycline (TE) | 30 |
| 4. | Gentamicin (GEN) | 30 |
| 5. | Ceftizoxime (CZX) | 30 |
| 6. | Ciprofloxacin (CIP) | 5 |
| 7. | Imipenem (IPM) | 10 |
| 8. | Meropenem (MRP) | 10 |
| 9. | Pipercillin-tazobactam (PIT) | 100/10 |

**Test procedure:**

The test was performed by inoculating the suspected pure cultures on Luria Burtoni (HiMedia, India) broth tubes and incubating at 37° C aerobically for 6 to 8 hours. About 1 ml of the culture from the broth was uniformly spread on the surface of Mueller-Hinton agar plates (HiMedia, India) and kept undisturbed for minimum 10 minutes. Excess of the inoculated broth was discarded from the plates and the antimicrobial discs were placed on the surface by gently pressing down with a pair of sterile forceps at an adequate distance. The plates were incubated at 37°C for 24 hours in an inverted manner. The results were interpreted according to zone size interpretative table provided by the manufacturer of the discs.

### 3.12 DETECTION OF AmpC B-LACTAMASE:

#### 3.12.1 Cefoxitin-cloxacillin double disc synergy (CC-DDS) test

The CC-DDS test was performed on Mueller-Hinton agar (Himedia, India) using cefoxitin and cefoxitin-cloxacillin combined antimicrobial disks (Himedia, India) as described by Polsfuss *et al*. (2011).

**TABLE 3.12. LIST OF ANTIMICROBIAL AGENTS USED**

| Serial no. | Antimicrobial agent | Concentration (mcg) |
|---|---|---|
| 1. | Cefoxitin (CX) | 30 |
| 2. | Cefoxitin-cloxacillin (CXX) | 200 |

**Test Procedure**

The test was performed by inoculating the suspected pure colonies on Luria Burtoni (HiMedia, India) broth tubes and incubating at 37° C aerobically for 6 to 8 hours. A sterile non-toxic cotton swab was dipped into the standardized inoculum and the soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid. The culture was streaked on to the Mueller hinton agar (Himedia, India) plate with the swab three times, turning the plate at 60° angle between each streaking and allowed the inoculum to dry for 5-15 minutes. The antimicrobial discs were placed by

keeping a distance of 24 mm on the surface by gently pressing down with a pair of sterile forceps at an adequate distance. The plates were incubated at 37°C for 16-18 hours in an inverted manner. The results were interpreted according to zone size interpretative table provided by the manufacturer of the discs.

**Interpretation**

A zone diameter difference of ≥ 4 mm between Cefoxitin 30 mcg disc and Cefoxitin-Cloxacillin 30-200 mcg disc was interpreted as AmpC positive.

## 3.13 DETECTION OF METALLO β-LACTAMASE

### 3.13.1 Modified-Hodge Test

Modified-Hodge test was performed as described by Birgy *et al*. (2012) by preparing 0.5 McFarland dilution of the *E. coli* ATCC 25922 in 5 ml of Mueller-Hinton broth (Himedia, India). The culture was diluted into 1:10 by adding 4.5 ml of Mueller-Hinton broth into 0.5 ml of broth culture comparable to the the 0.5 McFarland standard. The diluted (1:10) culture was streaked on to Mueller-Hinton agar plate and allowed to dry for 3-5 min. An ertapenem (10 mcg) antibiotic disc (Himedia, India) was placed at the centre of the plate and the test isolates were streaked in a straight line from the edge of the disc to the edge of the plate. The plate was incubated at 37°C for16-18 hours.

**Interpretation**

A clover leaf-type indentation at the intersection of the test organism and *E. coli* 25922 growth on the agar plate was considered positive

### 3.13.2. Imipenem-EDTA Disc Synergy test

Imipenem-EDTA Disc Synergy test was performed using MIC strips (Himedia, India) containing imipenem (4- 256 mcg/ml) in one side and imipenem and EDTA (1-64 mcg/ml) in combination on the other side as described by Birgy *et al*. (2012).

**Test Procedure**

Mueller-Hinton agar (Himedia, India) plates were prepared for the test and the culture was inoculated in Luria Burtoni broth (HiMedia, India) and incubated at 37°C for 6-8 hours. A sterile cotton swab was dipped into the inoculum and the soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid. The test culture was inoculated on the agar plate for three times, turning the plate at 60° angle between each streaking. With the help of applicator, the strip was placed at a desired position on the agar plate swabbed with test culture.

**Interpretation**

When the ratio of the value for Imipenem (IPM): the value of Imipenem + EDTA (IPM+EDTA) was more than to 8 or if zone was observed on the side coated with Imipenem+EDTA and no zone was observed on the opposite the side coated with Imipenem, culture was interpreted as MBL positive.

When the ratio of the value for Imipenem (IPM): the value of Imipenem + EDTA (IPM+EDTA) was less than or equal to 8, it was considered as MBL negative.

## 3.14 SCREENING FOR PRESENCE OF ESBL RESISTANCE GENES BY PCR

The standard strains and field isolates of ESBL-producing *E. coli* and *K. pneumoniae* were screened for the presence of six important resistant genes, *viz. bla*TEM, *bla*SHV**,** *bla*CTX-M, *Sul* 1, *qnr*B, *Int*1 by PCR using the primers shown in Table 3.10. Reported primer sequences were used for all the six genes for the present study.

**TABLE 3.13. PRIMER SEQUENCES FOR DETECTION OF RESISTANCE GENE BY SIMPLEX PCR**

| Gene | Primer | Sequence (5′-3′) | Product size | Reference |
|---|---|---|---|---|
| *bla*TEM | Forward | ATA AAA TTC TTG AAG ACG AAA | 1080 bp | Weill *et al.* (2004) |
| | Reverse | GAC AGT TAC CAA TGC TTA ATC A | | |
| *bla*SHV | Forward | AGG ATT GAC TGC CTT TTT G | 393 bp | Kar *et al*. (2015) |
| | Reverse | ATT TGC TGA TTT CGC TCG | | |
| *bla*CTX-M | Forward | TTT GCG ATG TGC AGT ACC AGT AA | 544 bp | Edelstein *et al*.(2003) |
| | Reverse | CGA TAT CGT TGG TGG TGC CAT A | | |
| *Sul* 1 | Forward | CGG CGT GGG CTA CCT GAACG | 433 bp | Kar *et al*. (2015) |
| | Reverse | GCC GAT CGC GTG AAG TTC CG | | |
| *qnr*B | Forward | GAT CGT GAA AGC CAG AAA GG | 476 bp | Kar *et al*. (2015) |
| | Reverse | ATG AGC AAC GAT GCC TGG TA | | |
| *Int*1 | Forward | ACGAGCGCAAGGTTTCGGT | 564 bp | Li *et al*. (2014) |
| | Reverse | GAAAGGTCTGGTCATACATG | | |

**TABLE 3.14: PCR REACTION MIXTURE FOR *bla*TEM GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.15. PCR CONDITIONS USED FOR AMPLIFICATION *bla*TEM GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 43 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.16: PCR REACTION MIXTURE FOR *bla*SHVGENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.17. PCR CONDITIONS USED FOR AMPLIFICATION *bla*SHV GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 30 |
| Annealing | 45 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.18: PCR REACTION MIXTURE FOR *bla*CTX-M GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.19. PCR CONDITIONS USED FOR AMPLIFICATION *bla*CTX-M GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 30 |
| Annealing | 53 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.20: PCR REACTION MIXTURE FOR *sul*1 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.21. PCR CONDITIONS USED FOR AMPLIFICATION *sul*1GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 58 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.22: PCR REACTION MIXTURE FOR *Int*1 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermoscientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer,<br>4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 10.0 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 13.0 µl |
| Total | 25.0 µl |

**TABLE 3.23. PCR CONDITIONS USED FOR AMPLIFICATION *Int*1 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 60 | 45 sec | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.24: PCR REACTION MIXTURE FOR *qnr*B GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 10.0 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 13.0 µl |
| Total | 25.0 µl |

**TABLE 3.25. PCR CONDITIONS USED FOR AMPLIFICATION *qnr*B GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 57 | 45 sec | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

## 3.15. CONFIRMATION AND VISUALISATION OF PCR AMPLICONS

The PCR amplified products were detected by gel electrophoresis using 1.5% agarose gel. For this, the gel casting tray was set up by placing a comb in the slot containing 16 wells. A volume of 100 ml of 1.5% agarose gel was prepared by adding 1.5 g of agarose in 100 ml of 1 X TAE buffer. The mixture was heated for melting in a microwave oven for 2 minutes. The molten agarose was cooled to 56°C and ethidium bromide @ 0.5 µg/ml was added as a stain to visualise the products later. After proper mixing, it was poured on the gel casting tray and allowed to solidify without disturbing the tray. After solidification, the comb was removed carefully and 1X TAE buffer was poured in the gel casting tray until the gel was submerged. Each well was loaded with 3.0 µl of amplified product and 1 µl of 6X loading dye. Along with the PCR amplicons, a 100 bp DNA ladder, a template-negative control and a positive control were also loaded. Electrophoresis was carried out at 90 volt for 1 hour at room temperature. The gel was then visualised using a Gel documentation system (Bio-Rad, USA) and the result was recorded.

### 3.16 SCREENING FOR PRESENCE OF INSERTION SEQUENCES BY PCR

The standard strains and the field isolates of *Escherichia coli* and *Klebsiella pneumoniae* were screened by amplification of partial gene fragments of three different insertion sequences, *viz*. *ISE*cp1, *ISCR*1 and *IS*26 by PCR using the specific primers shown in Table 3.23. For the present study, specific primers for *IS*26 were designed against the corresponding gene sequences downloaded from NCBI (AccessionNo KT725789; Primer sequence position: Forward: 69713-69732; Reverse: 70208-70227) using Primer 3 software, while for *ISE*cp1 and *ISCR*1 reported primers were used.

**TABLE 3.26. PRIMER SEQUENCES FOR DETECTION OF INSERTION SEQUENCES BY SIMPLEX PCR**

| Gene | Primer | Sequence (5'-3') | Product size | Reference |
|---|---|---|---|---|
| *ISE*cp1 | Forward | GCAGGTCTTTTTCTGCTCC | 527 bp | Karim *et al*. (2001) |
| | Reverse | ATTTCCGCAGCACCGTTTGC | | |
| *ISCR*1 | Forward | CGC CCA CTC AAA CAA ACG | 469 bp | Kiiru *et al*. (2013) |
| | Reverse | GAG GCT TTG GTG TAA CCG | | |
| *IS*26 | Forward | AACGCGGAGTGAATGTCGAT | 515 bp | Self-designed |
| | Reverse | TAGTGCACGCATCACCTCAA | | |

**TABLE 3.27. PCR REACTION MIXTURE FOR *ISE*cp1 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.28. PCR CONDITIONS USED FOR AMPLIFICATION *ISE*cp1 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 54 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.29. PCR REACTION MIXTURE FOR *ISCR*1 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.30. PCR CONDITIONS USED FOR AMPLIFICATION *ISCR*1 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 51 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.31. PCR REACTION MIXTURE FOR *IS*26 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.32. PCR CONDITIONS USED FOR AMPLIFICATION *IS*26 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 54 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

## 3.17. CONFIRMATION AND VISUALISATION OF PCR AMPLICONS OF INSERTION SEQUENCES

The PCR amplified products were detected by gel electrophoresis using 1.5% agarose gel. For this, the gel casting tray was set up by placing a comb in the slot containing 16 wells. A volume of 100 ml of 1.5% agarose gel was prepared by adding 1.5 g of agarose in 100 ml of 1 X TAE buffer. The mixture was heated for melting in a microwave oven for 2 minutes. The molten agarose was cooled to 56°C and ethidium bromide @ 0.5 µg/ml was added as a stain to visualise the products later. After proper mixing, it was poured on the gel casting tray and allowed to solidify without disturbing the tray. After solidification, the comb was removed carefully and 1X TAE buffer was poured in the gel casting tray until the gel was submerged. Each well was loaded with 3.0 µl of amplified product and 1 µl of 6X loading dye. Along with the PCR amplicons, a 100 bp DNA ladder, a template-negative control and a positive control were also loaded. Electrophoresis was carried out at 90 volt for 1 hour at room temperature. The gel was then visualised using a Gel documentation system (Bio-Rad, USA) and the result was recorded.

### 3.18. CONFIRMATION OF INSERTION SEQUENCES BY SEQUENCING

The amplified products of all the three insertion sequences were purified by Nucleospin PCR clean up kit and sent for sequencing to 1$^{st}$ base sequencing system, Malaysia. Sequencing was done using both forward and reverse primers. The results were analyzed by BioEdit software and alignment using NCBI-BLAST server to identify the sequence specificity.

### 3.19. SCREENING OF CTX-M GENOGROUPS BY PCR

On the basis of the amino acid sequences, CTX-M enzyme was classified into five different subgroups, *viz.* CTX-M Group 1, CTX-M Group 2, CTX-M Group 8, CTX-M Group 9 and CTX-M Group 25 (Rasmussen & Hoiby, 2014). The standard strains and the field isolates of *Escherichia coli* and *Klebsiella pneumoniae* were screened by amplification of partial gene fragments of all the five CTX-M subgroups by PCR using the primers shown in Table 3.30. Reported primer sequences were used for all the five genes in the present study.

**TABLE 3.33. PRIMER SEQUENCES FOR DETECTION OF CTX-M GENOGROUPS BY SIMPLEX PCR**

| Gene | Primer | Sequence (5'-3') | Product size | Reference |
|---|---|---|---|---|
| CTX-M Group 1 | Forward | AAAAATCACTGCGCCAGTTC | 415 bp | Woodford *et.al.* (2006) |
| | Reverse | AGCTTATTCATCGCCACGTT | | |
| CTX-M Group 2 | Forward | TGATACCACCACGCCGCTC | 341 bp | Xu *et al.* (2005) |
| | Reverse | TATTGCATCAGAAACCGTGGG | | |
| CTX-M Group 8 | Forward | TCGCGTTAAGCGGATGATGC | 688 bp | Woodford *et.al.* (2006) |
| | Reverse | AACCCACGATGTGGGTAGC | | |
| CTX-M Group 9 | Forward | CAAAGAGAGTGCAACGGATG | 205 bp | Woodford *et.al.*. (2006) |
| | Reverse | ATTGGAAAGCGTTCATCACC | | |
| CTX-M Group 25 | Forward | CAATCTGACGTTGGGCAATG | 292 bp | Mirzaee *et.al.* (2009) |
| | Reverse | ATAACCGTCGGTGACAATT | | |

**TABLE 3.34. PCR REACTION MIXTURE FOR CTX-M Group 1 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.35. PCR CONDITIONS USED FOR AMPLIFICATION CTX-M Group 1 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 52 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.36. PCR REACTION MIXTURE FOR CTX-M Group 2 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.37. PCR CONDITIONS USED FOR AMPLIFICATION CTX-M Group 2 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 55 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.38. PCR REACTION MIXTURE FOR CTX-M Group 8 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.39. PCR CONDITIONS USED FOR AMPLIFICATION CTX-M Group 8 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 53 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.40. PCR REACTION MIXTURE FOR CTX-M Group 9 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.41. PCR CONDITIONS USED FOR AMPLIFICATION CTX-M Group 9 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 49 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

**TABLE 3.42. PCR REACTION MIXTURE FOR CTX-M Group 25 GENE**

| Components | Volume |
|---|---|
| 2X Dream Taq Master Mix (Thermo Scientific)<br>Composition: 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). | 12.5 µl |
| Template DNA (100 ng/µl) | 1 µl |
| Forward primer (0.01 nanomole/µl) | 0.5 µl |
| Reverse primer (0.01 nanomole/µl) | 0.5 µl |
| Nuclease free water | 10.5 µl |
| Total | 25.0 µl |

**TABLE 3.43. PCR CONDITIONS USED FOR AMPLIFICATION CTX-M Group 25 GENE**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 48 | 1 min | |
| Extension | 72 | 30 sec | |
| Final Extension | 72 | 5 min | 1 |
| Final Hold | 4 | Infinite | |

## 3.20. CONFIRMATION AND VISUALISATION OF PCR AMPLICONS OF CTX-M GENOGROUPING

The PCR amplified products were detected by gel electrophoresis using 1.5% agarose gel. For this, the gel casting tray was set up by placing a comb in the slot containing 16 wells. A volume of 100 ml of 1.5% agarose gel was prepared by adding 1.5 g of agarose in 100 ml of 1 X TAE buffer. The mixture was heated for melting in a microwave oven for 2 minutes. The molten agarose was cooled to 56°C and ethidium bromide @ 0.5 µg/ml was added as a stain to visualise the products later. After proper mixing, it was poured on the gel casting tray and allowed to solidify without disturbing the tray. After solidification, the comb was removed carefully and 1X TAE buffer was poured in the gel casting tray until the gel was submerged. Each well was loaded with 3.0 µl of amplified product and 1 µl of 6X loading dye. Along with the PCR amplicons, a 100 bp DNA ladder, a template-negative control and a positive control were also loaded. Electrophoresis was carried out at 90 volt for 1 hour at room temperature. The gel was then visualised using a Gel documentation system (Bio-Rad, USA) and the result was recorded.

## 3.21. MOLECULAR TYPING OF *Escherchia coli* and *Klebsiella pneumoniae*

### 3.21.1 Repetitive-Extragenic Palindromic Sequence Based PCR (REP-PCR)

REP-PCR was performed using (GTG) 5 primer by modified protocol of Mohapatra *et al.* (2007).

**TABLE 3.44. PRIMER SEQUENCE FOR REP-PCR**

| **Primer** | **Primer sequence (5′ - 3′)** | **Reference** |
|---|---|---|
| (GTG)5 | GTGGTGGTGGTGGTG | Mohapatra *et al.* (2007) |

**TABLE 3.45. REACTION COMPONENT STANDARDIZED FOR REP-PCR**

| Components | Volume |
|---|---|
| DNA (100 ng/ µl) | 0.5 µl |
| Primer (0.01 nanomole/µl) | 0.75µl |
| 10 mM dNTP | 0.5 µl |
| 10 X Taq buffer | 2.5 µl |
| Taq (5 U/µl) | 0.2 µl |
| Nuclease-free water | 20.55 µl |
| Total | 25.0 µl |

**TABLE 3.46. CYCLING CONDITION STANDARDIZED FOR REP-PCR**

| Steps | Temperature (°C) | Duration | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denaturation | 94 | 30 sec | 30 |
| Annealing | 51 | 1 min | |
| Extension | 65 | 8 min | |
| Final Extension | 65 | 8 min | 1 |
| Final Hold | 4 | Infinite | |

### 3.21.2. Visualization of REP-PCR products

The amplified products were subjected to gel electrophoresis on 1.2% agarose gel with ethidium bromide @ 0.5 µg/ml as stain at 80 volt for one and half hour. The visualization of the gel was done by using a Gel documentation system (Bio-Rad, USA) and the result was recorded.

### 3.21.3 Pulse-Field Gel Electrophoresis (PFGE)

PFGE was done as per the modified protocol described by Hussain *et al.* (2015). Briefly, each isolate was inoculated into tryptone soy agar plate and incubated

aerobically for 16 hours at 37 °C. The isolates were then inoculated on LB broth and were kept at incubator cum shaker at 300-350 rpm for 11-12 hours. The OD was adjusted to 0.6 to 0.8 at 600 nm using a spectrophotometer (Specord 50 plus, Analytik Jena, Germany). A volume of 1.5 ml of culture from broth was taken in a micro-centrifuge tube and centrifuged at 1000 G for five minutes and supernatant was discarded. The pellet was resuspended in 500 µl cell suspension buffer (CSB) (10 mM Tris-HCl, 20 mM NaCl, 50 mM EDTA). Simultaneously, 2% low melting agarose (Sigma) in autoclaved water was melted and equilibrated to 50 °C in a digital heat block (Eppendorf, Germany). Then thiourea was added to agarose solution @ 200 µM final concentration. The bacterial suspension was added to heated agarose solution and was mixed thoroughly avoiding bubble formation. The mixture was then immediately transferred to reusable plug moulds (BioRad, USA) and was allowed to solidify at 4 °C for 15 - 20 minutes. The solidified plugs were then removed with a spatula and placed into a sterile 2 ml micro-centrifuge tube containing one ml lysozyme solution (10 mM TRIS-HCl, 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine) with lysozyme (2 mg/ml). The tubes were then incubated in a shaking water bath (350 rpm) at 37° C overnight (16 hours). The lysozyme solution was removed carefully from the tubes next day and the plugs were washed seven to eight times with autoclaved water for 30 minutes eachto remove the lysozyme completely. After washing, the plugs were immersed in one ml of proteinase K solution (1 mg/ml) reaction buffer buffer (100 mM EDTA, 0.2% sodium deoxycholate, 1% sodium-lauryl sarcosine) and incubated at 50 °C for 16 hours in the shaking water bath (350 rpm). Next day, the plugs were washed four times in washing buffer (10 mM Tris-HCl, 50 mM EDTA) for 1 hour at room temperature in a thermomixer (350 rpm) (Eppendorf, Germany). The fourth wash contained 1 mM PMSF to inactivate the residual proteinase K. Then the plugs were washed for four times for half an hour each in one ml 0.1 X wash buffer in thermomixer at 350 rpm and cut into two pieces. One piece from plug was transferred to a fresh 2 ml micro-centrifuge tube containing 250 µl reaction buffer (Tango buffer, Thermo Scientific) and incubated for one hour at room temperature without shaking. Then the buffer was carefully removed and fresh 250 µl reaction buffer was added, along with 50 units of *Xba*1 (Thermo Scientific) and the tube was kept overnight at 37°C for restriction

digestion. *Salmonella branderup* strain was also grown on tryptone soy agar plates aerobically to use it as a pulse marker. The plugs were also made for *E. coli* and *K. pneumoniae* isolates and digested with 50 U of *Xba*1 restriction enzyme for overnight at 37°C. After restriction digestion, the plugs were equilibrated in gel running buffer (0.5X TBE) for half an hour. The gel casting tray was made ready by adjusting the tip of the comb 1-1.5 mm above the surface. The plugs were then loaded on the margin of the teeth of the combs and allowed to dry for about 10 -15 minutes. Simultaneously, one gram pulse field certified (Sigma) agarose was melted in 100 ml of 0.5 X TBE containing 200 μM thiourea and allowed to cool to about 50°C. Then the agarose was poured slowly and carefully so that the plugs were not dislodged from the teeth of the comb and the gel was allowed to get solidified for 30 minutes. After proper solidification, the comb was carefully removed and the gel with the base plate was put into the electrophoresis chamber of the CHEF Mapper (BioRad, USA) and 2.2 litres of 0.5 X TBE (with 200 μM thiourea) was poured into the chamber. The CHEF Mapper was allowed to run. Electrophoresis was done using auto-algorithm mode at 6 volt/cm having initial switch time of 4 second and final switch time of 40 seconds at 120° angle for 18 hours. After electrophoresis, the gel was transferred to a staining tray and stained with ethidium bromide solution in distilled water (0.5 μg/ml) for 30 minutes and washed with distilled water for 30 minutes to 1 hour. The DNA fragments were visualised and photographed with the help of Gel Documentation System (BioRad, USA).

## 3.22. DETERMINATION OF DISCRIMINATORY POWER OF THE MOLECULAR TYPING METHODS

The discriminatory power is the average probability that a typing system will assign a different type to two unrelated strains randomly sampled from a population. This was measured by calculating the Simpson's index of diversity (D) (Hunter and Gaston, 1988).

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} Xj(Xj-1),$$

Where S is the number of type isolates described, Xj is the number of isolates belonging to $j^{th}$ group and N is the total number of isolates in the sample.

## 3.23. PHYLOGENETIC ANALYSIS BASED ON RESULTS OF REP-PCR AND PFGE

The DNA fingerprints of the isolates revealed by both the molecular typing techniques, *viz*. REP-PCR and PFGE were used for construction of phylogenetic tree to study the evolutionary relationship among the isolates. The dendrograms were constructed by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method using Jaccard's coefficient using the software Gel Compar

# CHAPTER - IV

# *Results and Discussion*

**PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES**

## CHAPTER-IV

# RESULTS AND DISCUSSION

The extended-spectrum β-lactamases (ESBLs) producing Enterobacteriaceae carry certain plasmid-encoded enzymes, which can efficiently hydrolyze and confer resistance to β-lactam group of antibiotics. ESBLs are predominantly found in *Escherichia coli* and *Klebsiella* spp., but may also occur in other Enterobacteriaceae (ECDPC, 2014). The emergence of ESBL has become public health concern because of increased morbidity, mortality and healthcare costs (Pitout Laupland, 2008). The most commonly found ESBLs are SHV or TEM types, evolved from narrow-spectrum β-lactamases such as TEM-1, -2 and SHV-1 (Bradford, 2001). Another most common type of ESBL is CTX-M, originated from *Kluyvera* spp. and it is gaining prominence in *Enterobacteriaceae* from Europe, Africa, Asia, South America and North America (Bonnet, 2004). The ESBL-producing organisms are clinically important because of treatment failure with β-lactam antibiotics (Bush, 2001; Bradford, 2001). The treatment failure with β-lactam antibiotics, in spite of the organism showing susceptibility to the drug in routine drug sensitivity test, is one of the indicators of production of ESBL by the pathogen. *Escherichia coli* and *Klebsiella pneumoniae* are reported to be the most common Enterobacteriaceae producing ESBLs and causing blood stream infections (Laupland and Church, 2014). ESBL-producing *E. coli* and *K. pneumoniae* have been found to be associated with increased rates of treatment failure, high mortality and high hospitalization cost (Tumbarello *et al*., 2010).

Several mechanisms have been involved in acquisition and transfer of antimicrobial resistant genes known as “mobilome” (Medini *et al.*,2005; Tettelin *et al*., 2008), which is composed of mobile genetic elements (MGEs), *viz.* plasmids, transposons (Tn), insertion sequences (IS), integrons (IntI), and introns. Conjugation, transformation and transduction are the main mechanisms for the horizontal transfer of MGEs (Norman *et al*., 2009; Woodford *et al*., 2011).

The insertion sequences are the smallest transposable elements less than 2.5 kb and are classified on the basis of their characteristics with transposases (Mahillon and Chandler, 1998; Zhao and Hu, 2013). It has been reported that *IS*26, *ISE*cp1, *ISCR*1, and *IS*903, in association with class1 integrons, are mostly involved in antimicrobial

resistance (Arduino *et al*., 2002; Eckert *et al*., 2006; Diestra *et al*., 2008; Cullik *et al*., 2010; Cheng *et al*., 2016).

CTX-M enzymes are found to be very rapidly disseminated among communities and are reported from various countries (Bauernfeind *et al*., 1996; Radice *et al*., 2002; Quinteros *et al*., 2003; Kim *et al*., 2005; Livermore *et al*., 2005). The genogroups of CTX-M extended-spectrum β-lactamases have been circulating for more than two decades since its first appearance in 1990 (Shahid *et al*., 2011).

Pulsed-field gel electrophoresis (PFGE) and repetitive extragenic palindromic sequence based PCR (REP-PCR) are most commonly used techniques to differentiate related strains of bacteria and for epidemiological studies during outbreaks (Wirth *et al*., 2006; Hawser *et al*., 2013; Horner *et al*., 2013; Wang *et al*., 2013). PFGE is considered as the gold standard among all the molecular typing methods, because of high discriminatory power. However, it is laborious and time-consuming, and requires a skilled person (Brolund *et al*., 2010). If DNA degradation occurs during plug preparation, no banding pattern may be observed. Whereas compared to PFGE, REP-PCR is less time-consuming and easy to perform without the need of a skilled person. Both these techniques have been found to show comparable discriminatory power (Pitout *et al*., 2009).

## 4.1 ISOLATION OF ESBL PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* FROM VARIOUS SOURCES

In the present investigation, a total of 385 samples collected from different sources were examined for the presence of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*. The samples included 209 cattle milk, 23 goat milk, 12 curd samples (indigenously prepared), 32 chicken meat, 15 pork, 14 beef, and faecal samples of cattle (69) and goat (11). The samples were collected from animals which were under antibiotic treatment or having previous history of treatment with antibiotics. Out of these 385 samples, 31 (8.05%) were found to be positive for ESBL producing *E. coli* and 59 (15.32%) were for ESBL producing *Klebsiella pneumoniae* (Table 4.1).

**TABLE 4.1: ISOLATION OF ESBL PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* FROM VARIOUS SOURCES**

| Nature of sample | Source | Number of samples tested | Number positive for ESBL isolates | |
|---|---|---|---|---|
| | | | ***E. coli*** | ***K. pneumoniae*** |
| Raw milk | Cattle | 209 | 21 (10.05) | 30 (14.35) |
| | Goat | 23 | - | - |
| Curd (Indigenous) | Cattle | 12 | 1 (8.33) | - |
| Meat | Chicken | 32 | 5 (15.63) | 2 (6.25) |
| | Pork | 15 | 1 (6.67) | - |
| | Beef | 14 | - | 3 (21.43) |
| Faeces | Cattle | 69 | 3 (4.35) | 24 (34.78) |
| | Goat | 11 | - | - |
| **Total** | | **385** | **31 (8.05)** | **59 (15.32)** |

Figures in parenthesis indicate percentages

In the present study, 21 (10.05%) and 30 (14.35%) out of 209 cattle raw milk samples yielded ESBL producing *E. coli* and *K. pneumoniae*, respectively, while none of the 23 goat raw milk samples yielded ESBL producing *E. coli* and *K. pneumonia* (Table: 4.1). None of the 12 curd samples tested in the present study yielded ESBL producing *K. pneumonia*, while only 1 (8.33%) yielded *E. coli*. A total of 5 (15.63%) and 2 (6.25%) among 32 chicken samples examined were found positive for ESBL producing *E. coli* and *K. pneumoniae*, respectively. None of the 15 pork samples yielded presence of *K. pneumoniae* isolates, while only 1 (6.67%) yielded *E. coli*. No ESBL producing *E. coli* isolate could be obtained from the 14 beef samples examined and only 3 (21.43%) of these samples yielded *K. pneumoniae*. While 3 (4.35%) and 24 (34.78%) of 69 cattle faecal samples yielded ESBL producing *E. coli* and *K. pneumoniae* isolates, respectively,

none of the 11 goat faecal samples were found to be positive for ESBL producing *E. coli* and *K. pneumoniae.*

Isolation of ESBL producing *K. pneumoniae* from 6.76 per cent of 340 bovine milk samples was reported by Koovapra *et al*. (2016). The percentage of recovery of ESBL isolates in the present study was higher from raw milk samples of cattle, which might be due to smaller sample size.

Isolation of ESBL-producing *E. coli* from faecal samples and cloacal swabs from cattle, pigs, chicken, ducks, geese and pigeons was reported by Duan *et al*. (2006) in Hong Kong. Out of 734 faecal samples screened, 6 (2%) from pig, 3 (3.1%) from cattle and 1 (3%) from pigeon yielded ESBL producing *E. coli* which was in agreement with the present investigation. Another study conducted by Brower *et al*. (2017) in Punjab, India reported isolation of 305(59.8%) *E. coli*, 13(2.55%) *K. pneumoniae*, 8(1.56%) *Escherichia fergusonii*, 3(0.59%) *Proteus mirabilis*, and 1 (0.19%) *Escherichia hermannii* from 510 cloacal samples of poultry, which was higher for *E. coli*, but lower for *K. pneumoniae* compared to that of the present study.

Gundogan *et al*. (2011) reported a higher isolation rate of 35% (21/60) ESBL producing *K. pneumoniae* compared to the present study from beef and chicken samples in Turkey, which might be due to geographical and climatic variations or influence of managemental and host factors on acquiring infection from the environment.

## 4.2 BIOCHEMICAL CHARACTERIZATION OF ESBL producing *E. coli* AND *K. pneumoniae* ISOLATES:

All the 31 ESBL producing *E. coli* isolates recovered from different sources showed important biochemical characteristics typical of the species. They were positive for methyl red test, indole production, but were negative for Voges-Proskauer (VP) test and urease production and citrate utilization.

Similarly, all the 59 *K. pneumoniae* isolates showed important biochemical characteristics typical of the species and fermented glucose, lactose, sucrose, mannitol and dulcitol. All the isolates were positive for Voges-Proskauer (VP) test and urease

production and citrate utilization, but were negative for methyl red test, indole production.

## 4.3. CONFIRMATION OF *Escherichia coli* ISOLATES BY AMPLIFICATION OF *uid*A GENE

All the 31 suspected *E. coli* isolates obtained in the present study were subjected to PCR targeting amplification of species-specific *uid*A gene. The *uid*A gene was detected in all the 31 (100%) isolates (Fig. 4.1) and hence all the isolates were confirmed as *E. coli*. Martins *et al*. (1993) confirmed 97.7% out of 435 *E. coli* isolates by amplification of *uid*A gene.

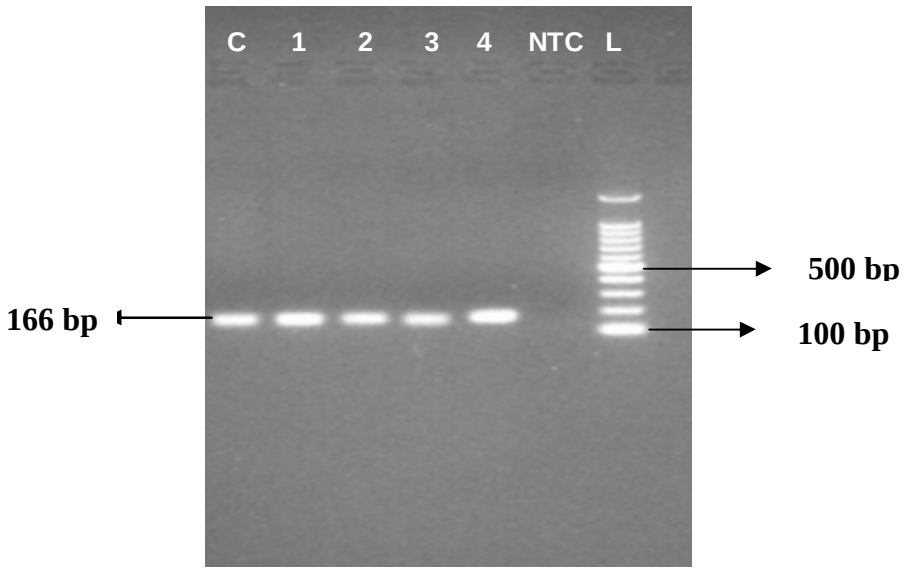


**FIG. 4.1. AMPLIFIED PRODUCTS OF *uid*A GENE IN 1.5% AGAROSE GEL**

Lane 1: Positive control,
Lane 2 to 5: Amplified products,
Lane 6: No-template control,
Lane 7: 100 bp DNA ladder

## 4.4. CONFIRMATION OF *K. pneumoniae* ISOLATES BY AMPLIFICATION OF *rpo*B GENE

All the 59 suspected *K. pneumoniae* isolates obtained in the present study were subjected to PCR targeting amplification of species-specific *rpo*B gene. The gene was detected in all the 59 (100%) suspected *K. pneumoniae* isolates (Fig.4.2). Garza-Ramos *et al*. (2018) found 36 (92.4%) of 39 suspected *K. pneumoniae* isolates from Mexico to be positive for *rpo*B gene in PCR.

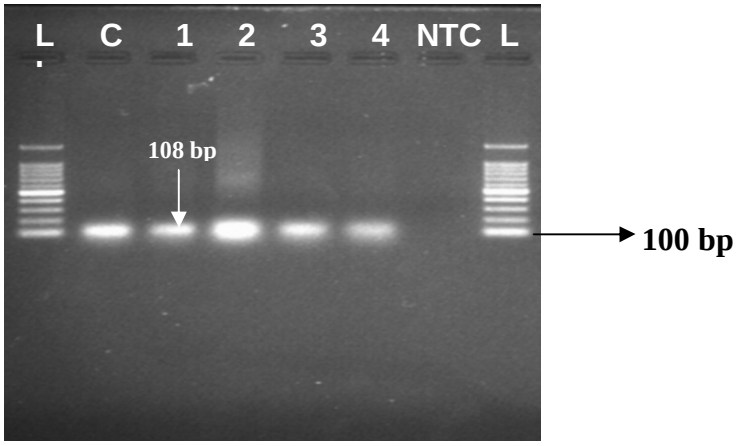


**FIG 4.2. AMPLIFIED PRODUCTS OF *rpo* B GENE IN 1.5% AGAROSE GEL**

Lane 1 and 8: 100 bp DNA ladder
Lane 2: Positive control,
Lane 3 to 6: Amplified products,
Lane 7: No-template control,

## 4.5. CONFIRMATION OF ESBL PRODUCING ISOLATES PHENOTYPICALLY

### 4.5.1. Confirmation of the ESBL producing isolates by drug sensitivity assay against 3rd and 4th generation cephalosporins

All the 31 ESBL producing *E. coli* isolates were subjected to drug sensitivity assay as one of the methods of phenotypic confirmation of ESBL production in the present study. Out of all the six antibiotics used, 100% resistance was shown to ceftriaxone and cefpodoxime (Table 4.4), followed by cefotaxime (96.77%). As high as 83.87% *E. coli* isolates showed resistance to Aztreonam, while 77.42% of the isolates were resistant to cefepime and 58.06% to ceftazidime.

**TABLE 4.2. CONFIRMATION OF ESBL ISOLATES BY DRUG SENSITIVITY ASSAY AGAINST 3rd and 4th GENERATION CEPHALOSPORINS**

| Antibiotic used | *Escherichia coli* (n = 31) | *Klebsiella pneumoniae* (n = 59) |
|---|---|---|
| | No of resistant isolates | No. of resistant isolates |
| Cefotaxime (30 mcg) | 30 (96.77) | 57 (96.61) |
| Ceftriaxone (30 mcg) | 31 (100) | 55 (93.22) |
| Ceftazidime (30 mcg) | 18 (58.06) | 46 (77.97) |
| Cefpodoxime (10 mcg) | 31 (100) | 59 (100) |
| Cefepime (30mcg) | 24 (77.42) | 30 (50.85) |
| Aztreonam (30 mcg) | 26 (83.87) | 53 (89.83) |

Figures in parenthesis indicate percentages

In the present investigation, all the 59 ESBL producing *K. pneumoniae* isolates were subjected to drug sensitivity assay. All (100%) isolates showed resistance to cefpodoxime, followed by cefotaxime (96.61%) and ceftriaxone (93.22%), aztreonam (89.83%), ceftazidime (77.97%) and cefepime (50.85%).

Koovapra *et al*. (2016) reported 100% resistance of *K. pneumoniae* isolates from bovine milk to each of ceftriaxone, ceftazidime, cefotaxime, aztreonam, cefpodoxime, which was in partial agreement with the findings of the present study.

Kumar *et al*. (2014) also reported high level of resistance (64.45% to 68.89%) to ceftazidime, cefotaxime, ceftriaxone and aztreonam respectively among 180 ESBL *E. coli* isolates. The finding was also in partial agreement with that of the present study.

Mobile genetic elements have been found to play an important role in the dissemination of antibiotic resistant organisms among human, animals and environmental sources. These genetic elements are commonly known as “mobilome” (Medini *et al*., 2005; Tettelin *et al*., 2008). They are composed of a variety of mobile genetic elements (MGEs), including plasmids, transposons (Tn), insertion sequences

(IS), integrons (intI), and introns. Conjugation, transformation, and transduction are mainly found to involve in the mechanisms of the horizontal transfer of MGEs (Norman *et al*., 2009; Woodford *et al*., 2011). Integrons are DNA elements capable of capturing antimicrobial gene cassettes and disseminating through an MGE (Stokes *et al*., 1989). The *bla*ESBL genes have been found to be associated with insertion sequences. The insertion sequences are the smallest transposable elements, less than 2.5 kb in size and are classified on the basis of their characteristics with transposases (Mahillon and Chandler, 1998). It has been reported that IS26, ISEcp1, ISCR1, and IS903, in combination with class1 integrons, are the most involved elements in the antimicrobial resistance (Arduino *et al*., 2002; Cheng *et al*., 2016).

**TABLE 4.3. NUMBER OF ISOLATES SHOWING MULTIPLE DRUG RESISTANCE**

| No. of antibiotics against which resistance shown | No. of isolates showing resistance | |
|---|---|---|
| | ***Escherichia coli*** **(n = 31)** | ***Klebsiella pneumoniae*** **(n = 59)** |
| 6 | 15 (48.39) | 30 (50.85) |
| 5 | 9 (29.03) | 16 (27.12) |
| 4 | 6 (19.35) | 8 (13.56) |
| 3 | - | 2 (3.39) |
| 2 | 1 (3.23) | 3 (5.08) |

Figures in parenthesis indicate percentages

Drug sensitivity assay of the *E. coli* (31) and *K. pneumoniae* (59) isolates against the six antibiotics showed multiple drug resistance in all the isolates. Out of the 31 *E. coli* isolates, 48.39%, 29.03%, 19.35% and 3.23% showed resistance to six, five, four and two antibiotics, respectively. On the other hand, a total of 50.85%, 27.12%, 13.56%, 3.39% and 5.08% ESBL producing *K. pneumoniae* isolates showed resistance to six, five, four, three and two antibiotics, respectively. Multiple drug resistance exhibited by the isolates might be due to acquiring multiple resistance genes through mobile elements

like plasmids, transposons and insertion sequences, which generally regulate ESBL-related resistance among *E. coli* and *K. pneumoniae.*

Stanley *et al*. (2018) reported detection of high level of antibiotic resistance to cotrimoxazole (74%), ampicillin (67%), amoxicillin/clavulanate (37%) and ciprofloxacin (31%) among *E. coli* isolates. In *K. pneumoniae*, cotrimoxazole (68%) and amoxicillin/clavulanate (46%) resistance was found to be the most frequent. A total of 57% and 82% of *E. coli* and *K. pneumoniae* isolates, respectively were found to be resistant to at least three classes of antibiotics. Carbapenem resistance was not detected among *K. pneumoniae*, while only 0.6% of the *E. coli* were resistant to carbapenems. Isolates producing ESBLs comprised 12% and 23% of *E. coli* and *K. pneumoniae*, respectively. These findings were in close agreement with that of the present study.

### 4.5.2 Confirmation of ESBL producing isolates by Combination of Disc Diffusion Tests (CDT)

All the suspected ESBL producing *E. coli* (31) and *K. pneumoniae* (59) isolates were also subjected to combination of disc diffusion tests (CDT) for phenotypic reconfirmation of ESBL production. In all, 93.55% and 88.14 % of *E. coli* and *K. pneumoniae*, respectively showed positive results in CDT (Table 4.6).

**TABLE 4.4. CONFIRMATION OF ESBL ISOLATES BY COMBINATION OF DISC DIFFUSION TESTS (CDT)**

| ***Escherichia coli*** | | ***Klebsiella pneumoniae*** | |
|---|---|---|---|
| **No. of isolates tested** | **No. positive** | **No. of isolates tested** | **No. positive** |
| 31 | 29 (93.55) | 59 | 52 (88.14) |

Figures in parenthesis indicate percentages

Koovapra *et al*. (2016) reported 100% positivity in CDT among 23 ESBL producing *K. pneumoniae*, which was in close agreement to the findings of the present

study. Kumar *et al*. (2014) reported confirmation of ESBL production in 55.55% of 180 *E. coli* isolates by CDT.

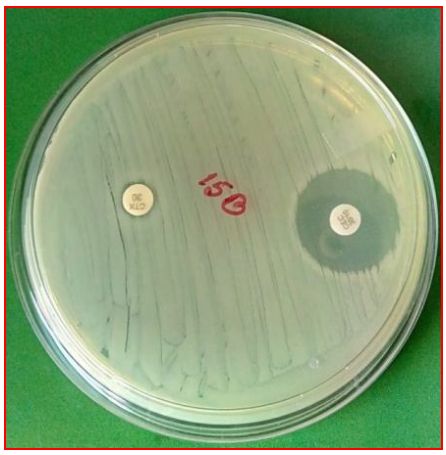


**FIG 4.3. DIFFERENCE IN THE ZONE OF INHIBITION SHOWED BY CEFOTAXIME AND CEFOTAXIME + CLAVULANIC ACID IN COMBINATION OF DISC DIFFUSION TESTS**

### 4.5.3. Confirmation of ESBL producing isolates by ESBL-E TEST

Out of the 31 *E. coli* isolates, 30 (96.77%) were positive for ESBL production by ESBL-E test, while 52 (88.14%) out of 59 isolates of *K. pneumoniae* were positive in ESBL E-test (Table 4.7).

**TABLE 4.5. CONFIRMATION OF ESBL ISOLATES BY ESBL-E TEST**

| ***Escherichia coli* (n = 31)** | | | ***Klebsiella pneumoniae* (n = 59)** | | |
|---|---|---|---|---|---|
| **Positive** | **Negative** | **Non-conclusive** | **Positive** | **Negative** | **Non-conclusive** |
| 30 (96.77) | 1 (3.23) | - | 52 (88.14) | 2 (3.39) | 5 (8.47) |

Figures in parenthesis indicate percentages

Koovapra *et al*. (2016) reported 100% positivity among 23 ESBL producing *K. pneumoniae* isolates in ESBL-E test, which was in close agreement to the findings of the present study. On the other hand, Prabha *et al*. (2016) reported 35% out of 100

Enterobacteriaceae isolates to be positive by ESBL E-test. This was, however, not in conformity with the present study.

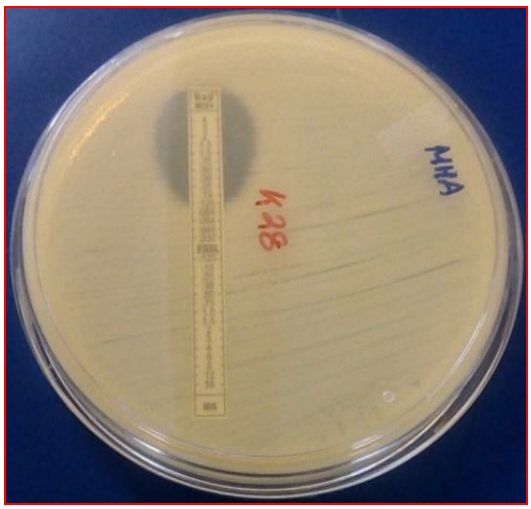


**FIG 4.4. ELLIPSE INTERSECTING THE STRIP IN A POSITIVE ESBL-E TEST**

## 4.6. ANTIBIOGRAM OF THE ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES FROM DIFFERENT SOURCES

All the ESBL producing isolates (31 *E. coli* and 59 *K. pneumoniae*) were subjected disc diffusion test to determine their susceptibility to nine different antimicrobial agents. The *E. coli* isolates showed highest resistance to ceftizoxime (74.19%), followed co-trimoxazole and tetracycline (25.81% each) and ciprofloxacin (19.35%). Resistance to chloramphenicol was 9.68%, while only 3.23% of the *E. coli* isolated showed resistance to gentamicin and pipercillin-tazobactam. None of the isolates, however, showed resistance to merpenem and imipenem.

**TABLE 4.6. ANTIBIOGRAM OF ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES FROM DIFFERENT SOURCES**

| Antimicrobial agents tested | *Escherichia coli* (n = 31) | *Klebsiella pneumoniae* (n = 59) |
|---|---|---|
| | No. of resistant isolates | No. of resistant isolates |
| Ceftizoxime (30 mcg) | 23 (74.19) | 41 (69.49) |
| Chloramphenicol (30 mcg) | 3 (9.68) | 10 (16.95) |
| Ciprofloxacin (5 mcg) | 6 (19.35) | 15 (25.42) |
| Co-trimoxazole (25 mcg) | 8 (25.81) | 14 (23.73) |
| Gentamicin (30 mcg) | 1 (3.23) | 2 (3.39) |
| Imipenem (10 mcg) | - | - |
| Meropenem (10 mcg) | - | - |
| Pipercillin-tazobactam (100/10 mcg) | 1 (3.23) | 3 (5.08) |
| Tetracycline (30 mcg) | 8 (25.81) | 14 (23.73) |

Figures in parenthesis indicate percentages

The ESBL producing *K. pneumoniae* isolates showed highest level of resistance to ceftizoxime (69.49%), followed by ciprofloxacin (25.42%), and co-trimoxazole and tetracycline (23.73% each). Comparatively lower level of resistance was shown by the isolates to chloramphenicol (16.95%), pipercillin-tazobactam (5.08%) and gentamicin (3.39%). None of the isolates showed resistance to merpenem and imipenem.

The present findings were in conformity with that of Umadevi *et al*. (2011), who reported 100% sensitivity of ESBL *E. coli* isolates to imipenem followed by piperacillin-tazobactum (84%), amikacin (68%), gentamicin (9%), ciprofloxacin (9%) and amoxicillin-clavulanic acid (7%). They also trecorded high sensitivity of ESBL producing *K. pneumoniae* to imipenem (98%), followed by piperacillin-tazobactum (68%), amikacin (40%), gentamicin (15%), ciprofloxacin (15%) and amoxicillin-clavulanic acid (5%).

Kumar *et al*. (2014) reported highest susceptibility of ESBL producing *Escherichia coli* to imipenem (100%), followed by piperacillin/tazobactam (87.22%), cefoperazone/sulbactam (76.67%), amoxicillin/clavulanic acid (75.55%), and ceftazidime/ clavulanate (66.11%), ceftriaxone (38.33%), ceftazidime (35.55%),

cefepime (35.55%), cephamycin (31.11%), monobactam (31.11%), piperacillin (33.33%), cefotaxime (31.11%) and cefoperazone (27.77%). These findings were in close conformity with the present investigation.

**TABLE 4.7. NUMBER OF ISOLATES SHOWING MULTIPLE DRUG RESISTANCE**

| No. of agents against which showed resistance | No. of isolates showing resistance | |
|---|---|---|
| | ***Escherichia coli* (n=31)** | ***Klebsiella pneumoniae* (n=59)** |
| 5 | 2 (6.45) | - |
| 4 | 3 (9.68) | 3 (5.08) |
| 3 | 1 (3.23) | 12 (20.34) |
| 2 | 3 (9.68) | 15 (25.42) |
| Total no. of multi-drug resistant isolates | 9 (29.32%) | 30 (50.85) |

Figures in parenthesis indicate percentages

Out of 31 ESBL producing *E. coli* isolates, 6.45%, 9.68%, 3.23% and 9.68% showed resistance to five, four, three and two antibiotics, respectively. On the other hand, 5.08%, 20.34% and 25.42% of the ESBL producing *K. pneumoniae* (59) isolates showed resistance to four, three and two different antibiotics, respectively. Overall, 29.32% of *E. coli* and 50.85% of *K. pneumonia* isolates showed multiple drug resistance. This indicated that the resistance acquired by the isolated was mainly plasmid-mediated.

## 4.7. DETECTION OF AmpC TYPE β-LACTAMASE (ACBL)

All the 90 ESBL producing isolates (31 *E. coli* and 59 *K. pneumoniae*) were subjected to cefoxitin-cloxacillin double-disc synergy test for detection of ampC type β-lactamase. None of the *E. coli* and *K. pneumoniae* isolates was found to produce ampC β-lactamase.

Kaur *et al*. (2016) reported isolation of 143 ESBL producing isolates including *Escherichia coli, Klebsiella* spp., *Proteus* spp., *Enterobacter* spp. and *Citrobacter* spp. after screening using cephoxitin (30 µg) discs and confirmed 113 (46.1%) of them as

AmpC producers by modified three-dimensional test. Koovapra *et al.* (2016) also found 7 (30.43%) out of 23 ESBL *K. pneumoniae* isolates to be ampC producers by cefoxitin-cloxacillin double disc synergy test. These findings were, however, contradictory to that of the present study.

Extended spectrum β-lactamases (ESBLs) are plasmid-mediated enzymes which can hydrolyze penicillins and third generation cephalosporins and aztreonam (Pfaller and Segreti, 2006). They are not active against cephamycins (cephoxitin and cefotetan) and can be inhibited by β-lactamase inhibitors (clavulanic acid). AmpC co-production with ESBL has made them phenotypically more complex. AmpC β-lactamases are cephalosporinases that can hydrolyze cephamycins and are poorly inhibited by clavulanic acid. Genes for ESBLs are located mainly on plasmids, while AmpC β-lactamases genes are either plasmid or chromosomally encoded. Chromosomally encoded AmpC are found in *Enterobacter* spp., *Providencia* spp., *Citrobacter freundii*, *Morganella morganii*, and *Serratia marcescens.* Chromosomal AmpC genes expression of may be constitutive or inducible. Plasmid mediated AmpC are found in *K. pneumoniae*, *E. coli*, *Salmonella* spp. and *Shigella* spp. Plasmid mediated AmpC genes were initially thought to be non-inducible, but later on inducible Amp C genes on plasmids have been reported (Fortineau *et al*., 2001).

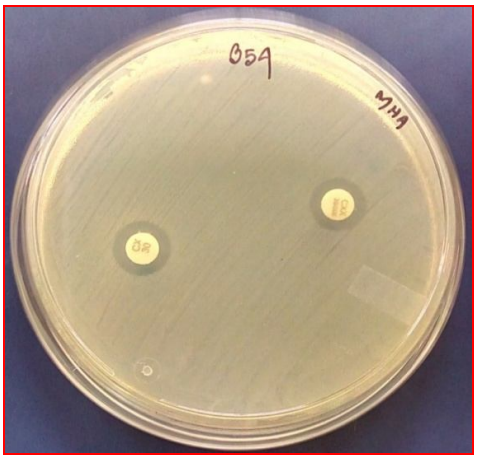


**FIG 4.5. DIFFERENCE IN ZONE OF INHIBITION BETWEEN CEFOXITIN AND CEFOXITIN-CLOXACILLIN DISCS AS REVEALED BY CEFOXITIN-CLOXACILLIN DOUBLE-DISC SYNERGY TEST**

## 4.8. DETECTION OF METALLO –β LACTAMASE PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES

All the 90 ESBL producing isolates (31 *E. coli* and 59 *K. pneumoniae*) were subjected to MHT and Imipenem-EDTA MIC test. None of the isolates was found to produce metallo- β lactamase by any of the two tests. The present investigation was in agreement with Koovapra *et al*. (2016), who reported none of 23 ESBL *K. pneumoniae* isolates to produce metallo β lactamase as detected by MHT and Imipenem-EDTA MIC tests.

Carbapenemase enzymes belong to classes A, B, and D according to molecular Ambler classification. The class B carbapenemases require one or two zinc ions for their full catalytic activity and these enzymes are therefore called metallo β lactamase (MBLs) (Schlesinger *et al*., 2011). MBLs are considered as one of the most important carbapenemases because of their ability to hydrolyse all betalactam antibiotics (Noori *et al*., 2014). There are no clinically approved MBL inhibitors till now, making these enzymes a serious threat to human health (King and Strynadka, 2011). MBL encoding genes can be easily disseminated from one organism to another through mechanism of horizontal gene transfer (Noori *et al*., 2014).

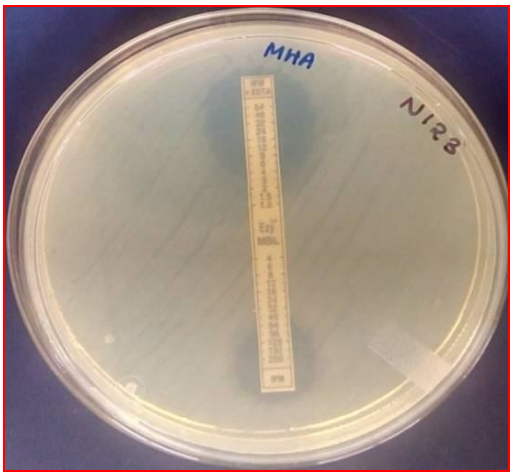


**FIG. 4.6. ELLIPSE INTERSECTING THE STRIP IN IMIPENEM-EDTA STRIP**

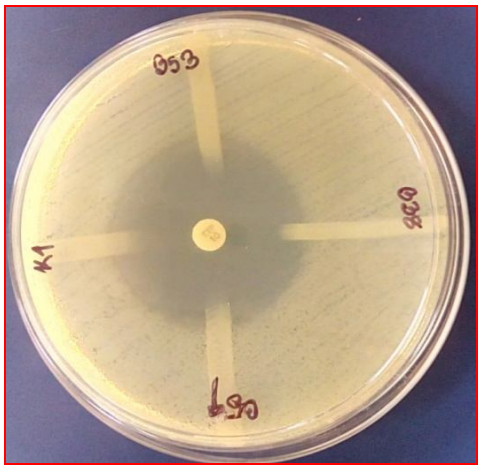

**FIG. 4.7. NO CLOVER LEAF FORMATION IN MODIFIED HODGE TEST**

## 4.9. SCREENING OF ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES FOR PRESENCE OF RESISTANCE GENES

All the confirmed ESBL isolates (31 *E. coli* and 59 *K. pneumoniae*) were screened by amplification of partial gene fragments of six antibiotic resistance genes, *viz.* *bla*TEM, *bla*SHV, *bla*CTX-M, *qnr*B, *Sul* 1 and *Int*1 by PCR. Of these, *bla*CTX-M was found to be predominant (100%), followed by *bla*SHV and *Sul* 1 (90.32% each) and *Int*1 (70.97%). The rate of occurrence of *bla*TEM was 54.84%, while only 3.23% of the isolates were found to harbor the *qnr*B gene (Table 4.12).

**TABLE 4.8. RESISTANCE GENE PROFILING OF ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES BY POLYMERASE CHAIN REACTION**

| Resistance gene | No. of isolates showing presence of the gene | |
|---|---|---|
| | ***E. coli* (n = 31)** | ***K. pneumoniae* (n = 59)** |
| *bla*TEM | 17 (54.84) | 33 (55.93) |
| *bla*SHV | 28 (90.32) | 46 (77.97) |
| *bla*CTX-M | 31 (100) | 59 (100) |
| *qnr*B | 1 (3.23) | 6 (10.17) |
| *Sul* 1 | 28 (90.32) | 51 (86.44) |
| *Int*1 | 22 (70.97) | 37 (62.71) |

Figures in parenthesis indicate percentages

Likewise, all the 59 (100%) *K. pneumoniae* isolates showed presence of *bla*CTX-M gene, followed by *Sul* 1(86.44%), *bla*SHV (77.97%), *Int*1 (62.71%), *bla*TEM (55.93%) and *qnr*B (10.17%).

In this aspect, the present study was in close agreement with Yasir *et al.* (2018), who reported occurrence of *bla*CTX-M and *bla*TEM genes in 95.3% and 83.9% of 211 ESBL producing *E. coli* isolates, whereas, *bla*OXA and *bla*SHV were detected only in 6.6% and 5.2% isolates respectively, which was not in agreement with the present study.

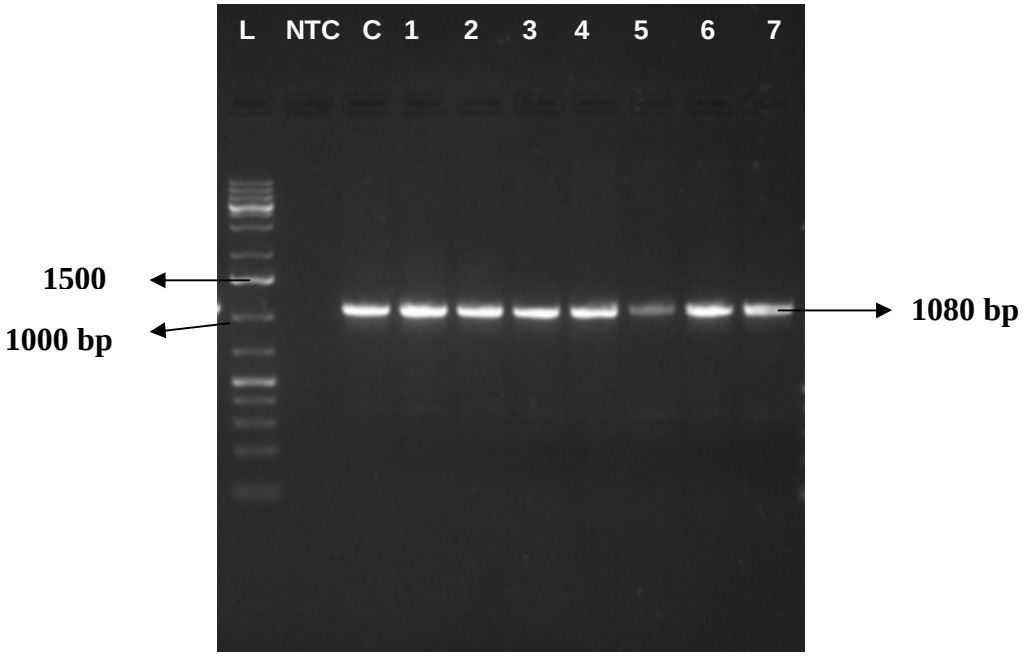


**FIG. 4.8. AMPLIFIED PRODUCTS OF *bla*TEM GENE IN 1.5% AGAROSE GEL**

Lane 1: 1Kb DNA ladder,
Lane 2: No-template control,
Lane 3: Positive control
Lane 4 to 6: Amplified products of *E. coli*,
Lane 7 to 10: Amplified products of *K. pneumoniae*

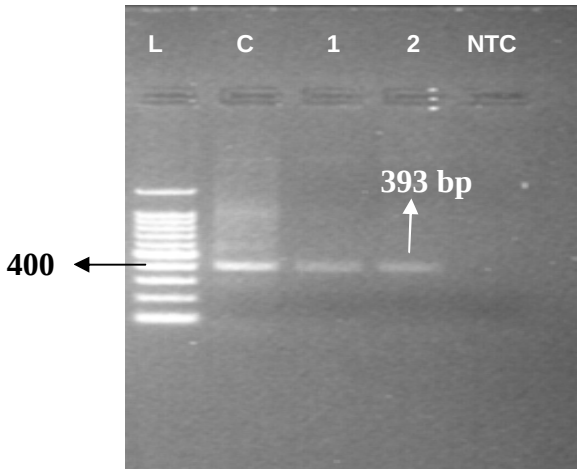


**FIG. 4.9. AMPLIFIED PRODUCTS OF *bla*SHV GENE IN 1.5% AGAROSE GEL**

Lane 1: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3: Amplified product of *E. coli*
Lane 4: Amplified products of *K. pneumoniae*
Lane 5: No-template control

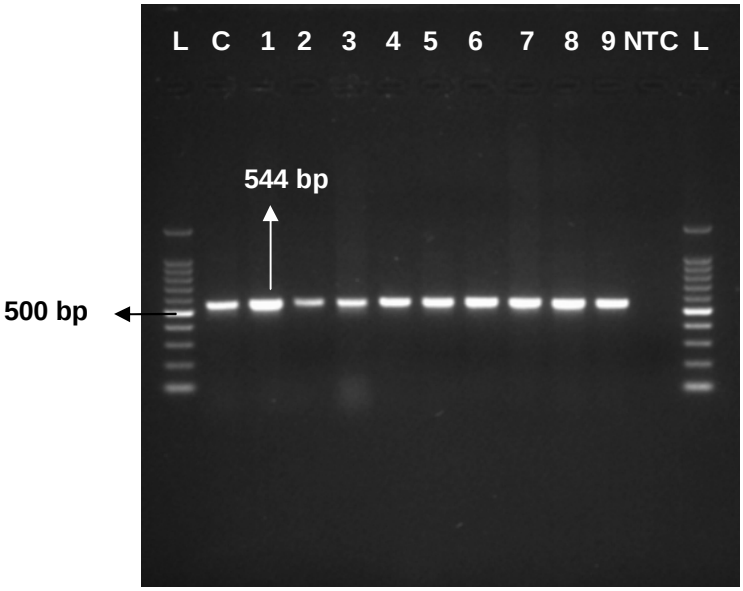


**FIG. 4.10. AMPLIFIED PRODUCTS OF *bla*CTX-M GENE IN 1.5% AGAROSE GEL**

Lane 1 and 13: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 to 6: Amplified products of *E. coli*,
Lane 7 to11: Amplified products of *K. pneumoniae*,
Lane 12: No-template control

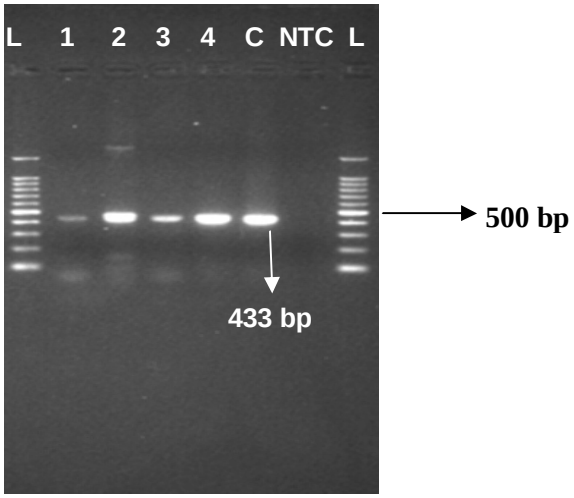


**FIG 4.11. AMPLIFIED PRODUCTS OF *Sul*1 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 8: 100 bp DNA ladder,
Lane 2 and 3: Amplified products of *E. coli*,
Lane 4 and 5: Amplified products of *K. pneumoniae*
Lane 6: Positive control;
Lane 7: No-template control

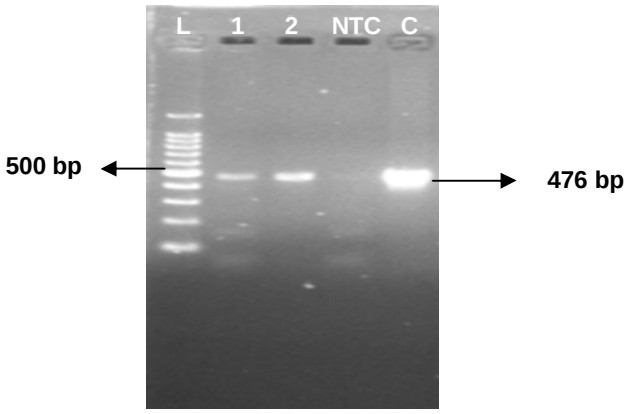


**FIG. 4.12. AMPLIFIED PRODUCTS OF *qnr*B GENE IN 1.5% AGAROSE GEL**

Lane 1: 100 bp DNA ladder,
Lane 2: Amplified product of *E. coli;*
Lane 3: Amplified product *K. pneumoniae*,
Lane 4: No-template control,
Lane 5: Positive control

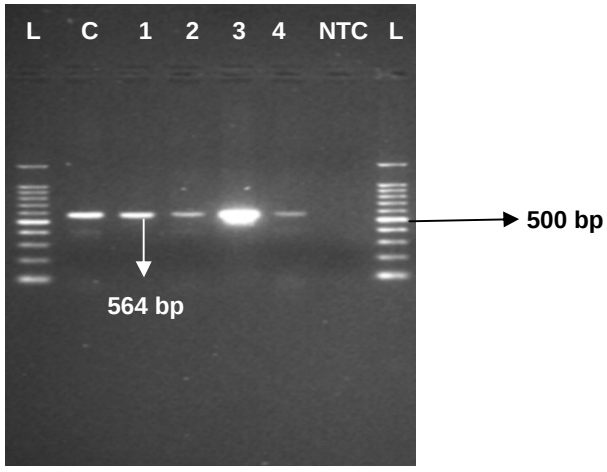


**FIG. 4.13. AMPLIFIED PRODUCTS OF *Int* 1 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 8: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 and 4: Amplified products of *E. coli*,
Lane 5 and 6: Amplified products of *K. pneumoniae*,
Lane 7: No-template control

Koovapra *et al*. (2016) reported *bla*CTX-M gene as the most predominant (82.6%) resistance gene among ESBL producing *E. coli* isolates, followed by *bla*TEM

(34.8%) and *bla*SHV (13%). While 52.2% isolates showed presence of *Int*1 gene and 47.84% isolates were positive for *Sul*1, only 4.35% isolates were positive for *qnr*B gene. This was in close agreement with the present study. Ibrahim *et al*. (2016) reported presence of *bla*CTX-M, *bla*TEM and *bla*OXA-1 genes in 22.6%, 7.5% and 3.7% of 25 ESBL producing *E. coli* isolates, respectively. They also did not observed presence of *bla*SHV gene in any of the isolates. This was also in partial agreement with the present findings.

## 4.10. DETECTION OF INSERTION SEQUENCES (IS) IN ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES BY PCR

The ESBL producing *E. coli* (31) and *K. pneumoniae* (59) isolates were subjected to PCR for detection of insertion sequences by amplification of partial gene fragments of the genes, *viz. ISEcp*1, *IS* 26 and *ISCR*1. *ISEcp*1 was found to be present in all the 90 (100%) ESBL isolates, followed by *IS* 26 (100% in *K. pneumoniae* and 90.32% in *E. coli* isolates (Table 4.13). The insertion sequence *ISCR*1 was found to be present in 80.65% *E. coli* isolates and 45.76% *K. pneumoniae* isolates.

**TABLE 4.9. SCREENING OF INSERTION SEQUENCES (IS) IN ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES BY PCR**

| IS type | No. of isolates showing presence of Insertion Sequence | |
|---|---|---|
| | ***Escherichia coli*** **(n = 31)** | ***Klebsiella pneumoniae*** **(n = 59)** |
| *ISEcp*1 | 31 (100) | 59 (100) |
| *IS* 26 | 28 (90.32) | 59 (100) |
| *ISCR*1 | 25 (80.65) | 27 (45.76) |

Figures in parenthesis indicate percentages

The present study was in agreement with the report of Roshani *et al.* (2018), who observed presence of *IS*903 and *ISE*cp1 in 69.64% and 96.42% of ESBL producing *E. coli* isolates, while 87.5% and 75% of ESBL producing *K. pneumoniae* isolates, respectively showed presence of *ISEcp1* and *IS903*. Another insertion sequence *IS*26 was

found in all the *E. coli* and *K. pneumoniae* isolates, which was also in close agreement with the present study.

Etayo *et al*. (2018) reported presence of *IS*26 in 99.4%, ISEcp1 in 68%, *IS*903 in 65.3% and *ISCR*1 in 12.6% of ESBL producing *E. coli* isolates. This was also in partial agreement with the present study.

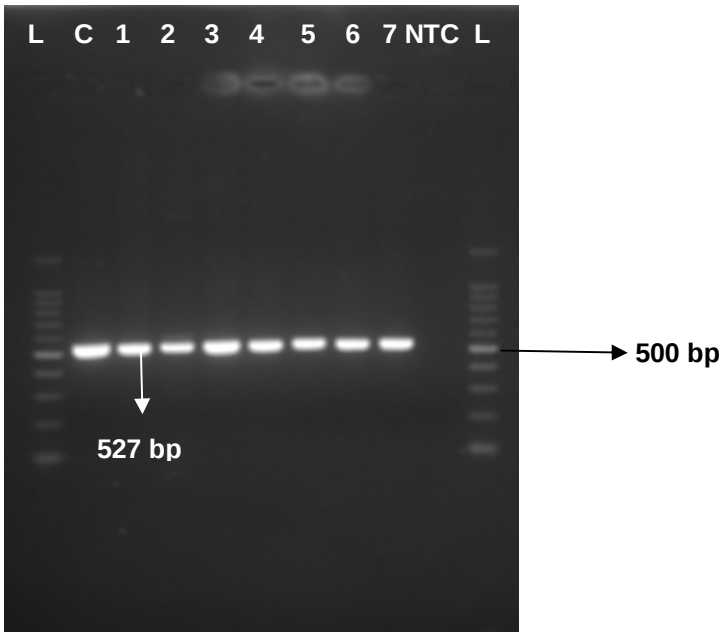


**FIG. 4.14. AMPLIFIED PRODUCTS OF *ISE*cp1 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 11: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 to 6: Amplified products of *E. coli*,
Lane 7 to 9: Amplified products of *K. pneumoniae*,
Lane 10: No-template control

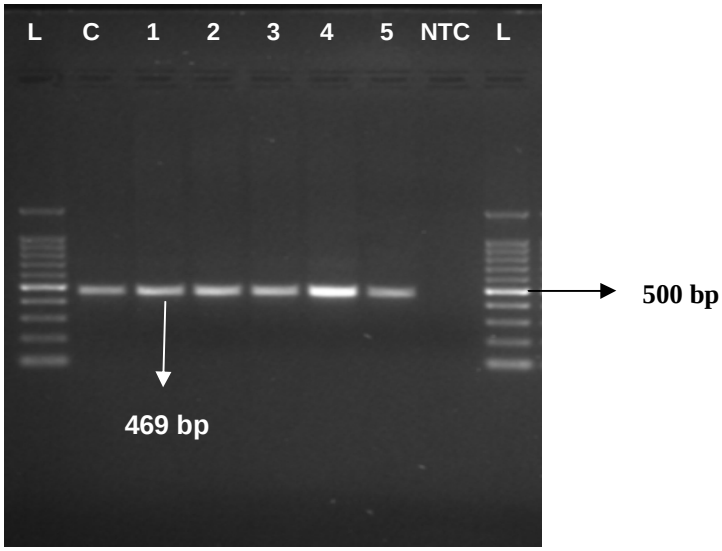


**FIG. 4.15. AMPLIFIED PRODUCTS OF *ISCR*1 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 9: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 to 5: Amplified products of *E. coli*,
Lane 6 and 7: Amplified products of *K. pneumoniae*,
Lane 8: No-template control

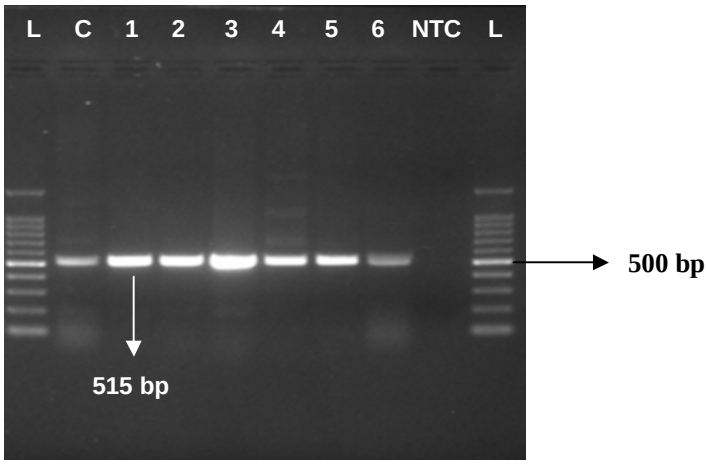


**FIG. 4.16. AMPLIFIED PRODUCTS OF *IS*26 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 10: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 to 5: Amplified products of *E. coli*,
Lane 6 to 8: Amplified products of *K. pneumoniae*,
Lane 9: No-template control

### 4.10.1. Confirmation of insertion sequences by sequencing

PCR amplified products of *ISEcp*1, *IS* 26 and *ISCR*1 were sequenced by outsourcing at 1st Base DNA sequencing, Malaysia. Representative figures of each insertion sequences and the BLAST results are given in Figures 4.17 and 4.18 (*ISE*cp1) and 4.19 and 4.20 (*ISCR*1), 4.21 and 4.22 (*IS*26).

5′-AACAAATAAAATCAAGATGAATCATATAAAGACCATGCTCTGCGGTCACTT
CATTGGCATTGATAAGTTAGAACGTCTAAAGCTACTTCAAAATGATCCCCTCGT
CAACGAGTTTGATATTTCCGTAAAAGAACCTGAAACAGTGTCACGGTTTCTAGG
AAACTTCAACTTCAAGACAACCCAAATGTTTAGAGACATTAATTTTAAAGTCTTT
AAAAAACTGCTCACTAAAAGTAAATTGACATCCATTACGATTGATATTGATAGT
AGTGTAATTAACGTAGAAGGTCATCAAGAAGGTGCGTCAAAAGGATATAATCCT
AAGAAACTGGGAAACCGATGCTACAATATCCAATTTGC-3′

**FIG. 4.17. CONSENSUS SEQUENCE OF *ISE*cp1 GENE OF *K. pneumoniae***

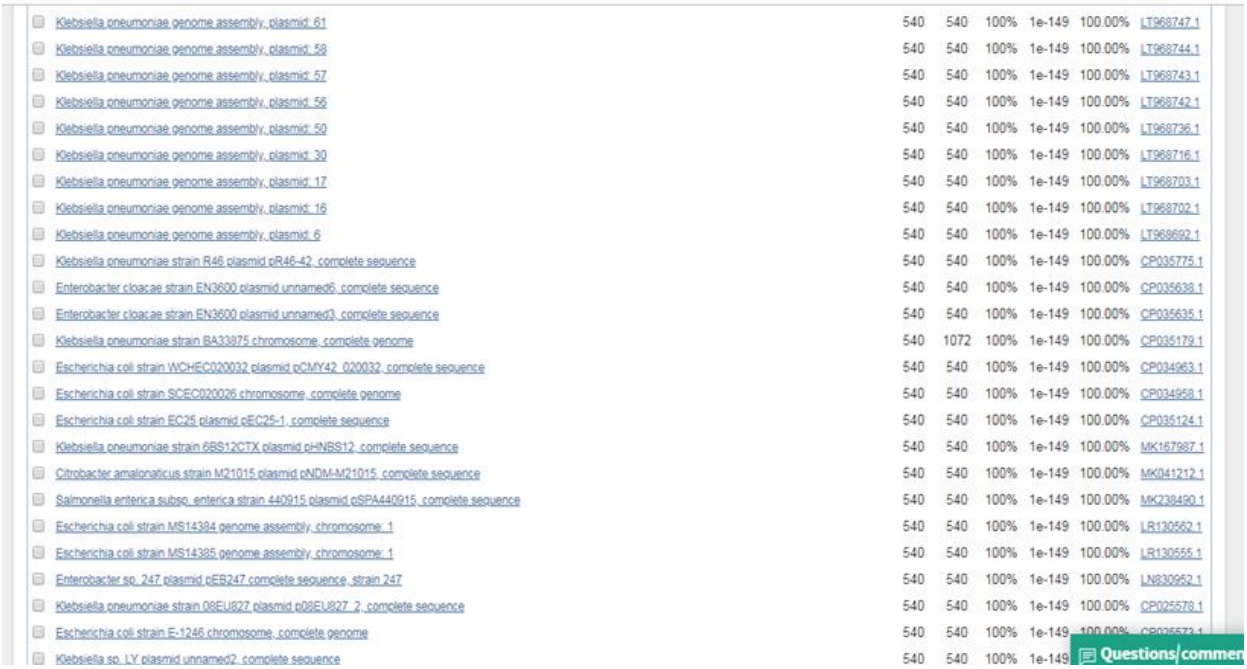


**FIG. 4.18. SEQUENCE SIMILARITY OF *ISE*cp1 GENE REVEALED BY BLAST ANALYSIS**

5’-CAAACGGTTACGAACGCCACCAACCCGACCAGACGCTGCTCTACCAGCTGGTTGA
GCAGCACTACCCA GCCTTCAAAGCCTCACTCGAAGCCCAAGGTCAACACCTGCCTC
GCTACATCCAACAAGAATTCAACGACCTCCTCCAATGTGGCCGTCTGGAGTATGGTT
TCATGCGGGTTCGCTGCGAGGATTGTCATCACGAGCGTCTGGTCGCCTTCAGCTGTAA
ACGACGCGGCTTTTGCCCTAGCTGCGGTGCCCGCCGGATGGCCGAGAGTGCGGCGCT
GCTGATAGACGAAGTCTTCCCCAAGGAGCCCATTCGCCAGTGGGTGCTCAGCTTTCCT
TTCCAGCTACGCTTTTTGCTGGCTCGCCATCCCCAGCTGATGGGCCAGGTCTTGAGTAT
CGTCTATCGTACACTCTCAACTCATCTGA-3’

**FIG 4.19. CONSENSUS SEQUENCE OF *ISCR*1 GENE OF *Escherichia coli***

| | | | | | | |
|---|---|---|---|---|---|---|
| Escherichia coli strain A1_180 plasmid unnamed1, complete sequence | 793 | 1586 | 100% | 0.0 | 100.00% | CP040382.1 |
| Escherichia coli NIPH17_0020 plasmid pNIPH17_0020_1 DNA, complete sequence | 793 | 793 | 100% | 0.0 | 100.00% | LC483178.1 |
| Acinetobacter baumannii strain P7774 plasmid unnamed1, complete sequence | 793 | 1579 | 100% | 0.0 | 100.00% | CP040260.1 |
| Acinetobacter baumannii NU-60 DNA, complete genome | 793 | 793 | 100% | 0.0 | 100.00% | AP019685.1 |
| Escherichia coli strain J53 plasmid pMG252A, complete sequence | 793 | 4759 | 100% | 0.0 | 100.00% | MK733575.1 |
| Pseudomonas aeruginosa strain PA298 plasmid pBM908, complete sequence | 793 | 793 | 100% | 0.0 | 100.00% | CP040126.1 |
| Acinetobacter baumannii strain VB35575 chromosome, complete genome | 793 | 1586 | 100% | 0.0 | 100.00% | CP040087.1 |
| Acinetobacter baumannii strain VB33071 chromosome, complete genome | 793 | 793 | 100% | 0.0 | 100.00% | CP040084.1 |
| Acinetobacter baumannii strain VB16141 plasmid unnamed1, complete sequence | 793 | 1581 | 100% | 0.0 | 100.00% | CP040051.1 |
| Acinetobacter baumannii strain VB35435 plasmid unnamed1, complete sequence | 793 | 793 | 100% | 0.0 | 100.00% | CP040057.1 |
| Acinetobacter baumannii strain VB35179 plasmid unnamed1, complete sequence | 793 | 1577 | 100% | 0.0 | 100.00% | CP040054.1 |
| Pseudomonas aeruginosa strain 1160 plasmid p1160-VIM, complete sequence | 793 | 1586 | 100% | 0.0 | 100.00% | MF144194.2 |
| Acinetobacter baumannii strain TG22182 chromosome, complete genome | 793 | 793 | 100% | 0.0 | 100.00% | CP039993.1 |
| Salmonella enterica subsp. enterica strain CFSA664 plasmid pCFSA664-1, complete sequence | 793 | 793 | 100% | 0.0 | 100.00% | CP033353.2 |
| Raoultella ornithinolytica strain WLK218 plasmid pWLK-238550, complete sequence | 793 | 1586 | 100% | 0.0 | 100.00% | CP038277.1 |
| Providencia heimbachae strain 99101 plasmid unnamed, complete sequence | 793 | 793 | 100% | 0.0 | 100.00% | CP028385.1 |
| Escherichia coli strain EC-129 plasmid pEC129_2, complete sequence | 793 | 793 | 100% | 0.0 | 100.00% | CP038455.1 |
| Providencia rettgeri strain Pr-15-2-50 chromosome, complete genome | 793 | 2379 | 100% | 0.0 | 100.00% | CP039844.1 |
| Acinetobacter baumannii strain TG22627 chromosome, complete genome | 793 | 793 | 100% | 0.0 | 100.00% | CP039520.1 |
| Acinetobacter baumannii strain TG22653 chromosome, complete genome | 793 | 793 | 100% | 0.0 | 100.00% | CP039518.1 |
| Escherichia coli J53 plasmid pMG252, complete sequence | 793 | 1586 | 100% | 0.0 | 100.00% | MK638972.1 |
| Raoultella ornithinolytica B1645-1 plasmid pCYNDM01, complete sequence | 793 | 1584 | 100% | 0.0 | 100.00% | MK510953.1 |
| Salmonella enterica subsp. enterica serovar Agona strain 44 plasmid, partial sequence | 793 | 793 | 100% | 0.0 | | |
| Salmonella enterica subsp. enterica serovar 4 [5],12:i:- strain 77 plasmid, partial sequence | 793 | 1586 | 100% | 0.0 | | |



**FIG. 4.20. SEQUENCE SIMILARITY OF *ISCR*1 GENE REVEALED BY BLAST ANALYSIS**

5’-TGTCGATCACTCCACGATTTACCGCTGGGTTCAGCGTTATGCGCCTGAAATGGA
AAAACGGCTGCGCTGGTACTGGCGTAACCCTTCCGATCTTTGCCCGTGGCACATGG
ATGAAACCTACGTGAAGGTCAATGGCCGCTGGGCGTATCTGTACCGGGCCGTCGAC
AGCCGGGGCCGCACTGTCGATTTTTATCTCTCCTCCCGTCGTAACAGCAAAGCTGCA
TACCGGTTTCTGGGTAAAATCCTCAACAACGTGAAGAAGTGGCAGATCCCGCGATT
CATCAACACGGATAAAGCGCCCGCCTATGGTCGCGCGCTTGCTCTGCTCAAACGCGA
AGGCCGGTGCCCGTCTGACGTTGAACACCGACAGATTAAGTACCGGAACAACGTGA
TTGAATGCGATCATGGCAAACTGAAACGGATAATCGGCGCCACGCTGGGATTTAAAT
CCATGAAGACGGCTTA-3’

**FIG. 4.21. CONSENSUS SEQUENCE OF *IS*26 GENE OF *K. pneumoniae***

| | | | | | | |
|---|---|---|---|---|---|---|
| Klebsiella pneumoniae strain CR-HvKP4 plasmid pCR-HvKP4-p3, complete sequence | 859 | 2579 | 100% | 0.0 | 100.00% | CP040542.1 |
| Klebsiella pneumoniae strain CR-HvKP4 plasmid pCR-HvKP4-pKPC, complete sequence | 859 | 10317 | 100% | 0.0 | 100.00% | CP040541.1 |
| Klebsiella pneumoniae strain CR-HvKP4 chromosome, complete genome | 859 | 1719 | 100% | 0.0 | 100.00% | CP040539.1 |
| Klebsiella pneumoniae strain CR-HvKP5 plasmid pCR-HvKP5-p3, complete sequence | 859 | 2579 | 100% | 0.0 | 100.00% | CP040548.1 |
| Klebsiella pneumoniae strain CR-HvKP5 plasmid pCR-HvKP5-KPC, complete sequence | 859 | 10317 | 100% | 0.0 | 100.00% | CP040547.1 |
| Klebsiella pneumoniae strain CR-HvKP5 chromosome, complete genome | 859 | 1719 | 100% | 0.0 | 100.00% | CP040545.1 |
| Klebsiella pneumoniae strain CR-HvKP1 plasmid pCR-HvKP1-p3, complete sequence | 859 | 2579 | 100% | 0.0 | 100.00% | CP040536.1 |
| Klebsiella pneumoniae strain CR-HvKP1 plasmid pCR-HvKP1-KPC, complete sequence | 859 | 10317 | 100% | 0.0 | 100.00% | CP040535.1 |
| Klebsiella pneumoniae strain CR-HvKP1 chromosome, complete genome | 859 | 2579 | 100% | 0.0 | 100.00% | CP040533.1 |
| Salmonella enterica subsp. enterica serovar Typhimurium strain TJWQ005 chromosome, complete genome | 859 | 3942 | 100% | 0.0 | 100.00% | CP040458.1 |
| Salmonella enterica subsp. enterica serovar Typhimurium strain TJWQ005 plasmid unnamed1, complete sequence | 859 | 8551 | 100% | 0.0 | 100.00% | CP040457.1 |
| Salmonella enterica subsp. enterica serovar California 2,4-dienoyl-CoA reductase [NADPH] (fadH), Putrescine aminotransferase (yjiG), and Hypothetical protein genes, | 859 | 10153 | 100% | 0.0 | 100.00% | MH079550.1 |
| Escherichia coli strain M63c plasmid pMB2, complete sequence | 859 | 4299 | 100% | 0.0 | 100.00% | MK492688.1 |
| Raoultella ornithinolytica B1645-1 plasmid pCYNDM01, complete sequence | 859 | 2892 | 100% | 0.0 | 100.00% | MK510953.1 |
| Enterobacter cloacae strain Effluent_2 plasmid unnamed3, complete sequence | 859 | 11029 | 100% | 0.0 | 100.00% | CP039321.1 |
| Enterobacter cloacae strain Effluent_2 plasmid unnamed2, complete sequence | 859 | 2579 | 100% | 0.0 | 100.00% | CP039320.1 |
| Enterobacter cloacae strain Effluent_2 plasmid unnamed1, complete sequence | 859 | 1719 | 100% | 0.0 | 100.00% | CP039319.1 |
| Enterobacter cloacae strain Effluent_4 plasmid unnamed | 859 | 6730 | 100% | 0.0 | 100.00% | CP039305.1 |
| Enterobacter cloacae strain Effluent_4 plasmid unnamed1, complete sequence | 859 | 4287 | 100% | 0.0 | 100.00% | CP039304.1 |
| Enterobacter cloacae strain Effluent_3 plasmid unnamed2, complete sequence | 859 | 10169 | 100% | 0.0 | 100.00% | CP039313.1 |
| Enterobacter cloacae strain Effluent_3 plasmid unnamed1, complete sequence | 859 | 2579 | 100% | 0.0 | 100.00% | CP039312.1 |
| Citrobacter portucalensis strain Effluent_1 plasmid unnamed2, complete sequence | 859 | 6013 | 100% | 0.0 | 100.00% | CP039329.1 |
| Citrobacter portucalensis strain Effluent_1 plasmid unnamed1, complete sequence | 859 | 859 | 100% | 0.0 | | |



**FIG. 4.22. SEQUENCE SIMILARITY OF *IS*26 GENE REVEALED BY BLAST ANALYSIS**

## 4.11. SCREENING OF ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES FOR PRESENCE OF *CTX*-M GENOGROUPS

All the 31 ESBL *E. coli* isolates were subjected to PCR for detection of *CTX*-M genogroup-specific genes. All the 31 (100%) isolates were found to be positive for group 1 gene, followed by group 2 (80.65%) and group 25 (27.27%). None of the isolates were found be positive for group 8 and 9 genes.

Group 1 gene was also found to be present in all the 59 (100%) *K. pneumoniae* isolates. While group 25 specific gene was found in 67.8%, group 2 in 55.93% and group 9 in 5.08% isolates only. None of the isolates was found to be positive for group 8 specific gene.

**TABLE 4.10. SCREENING OF ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES FOR PRESENCE OF *CTX*-M GENOGROUP-SPECIFIC GENES BY PCR**

| CTX-M Gene Subgroups | No. of isolates belonging to the group | |
|---|---|---|
| | ***E. coli* (n = 31)** | ***K. pneumoniae* (n = 59)** |
| CTX-M GROUP 1 | 31 (100) | 59 (100) |
| CTX-M GROUP 2 | 25 (80.65) | 33 (55.93) |
| CTX-M GROUP 8 | - | - |
| CTX-M GROUP 9 | - | 3 (5.08%) |
| CTX-M GROUP 25 | 9 (27.27) | 40 (67.8) |

Figures in parenthesis indicate percentages

Woodford *et al.* (2005) reported presence of group 1 specific gene in 86.4% isolates out of 633 ESBL producing *E. coli* isolates, followed by group 9 (12.8%) gene and group 2 (0.47%) genes. While group 8 gene was found only in 1(0.16%) isolate, none of the isolates were found show presence of group 25 gene. This was in partial agreement with the present study.

Shahid *et al.* (2014) reported presence of group1 gene in 93.3% isolates, out of 80 ESBL producing *E. coli* (75) and *K. pneumoniae* (5), followed by group 9 (6.7%). None of the isolates were found to be positive for group 2, group 8 and group 25 genes. This was also in partial agreement with the present study.

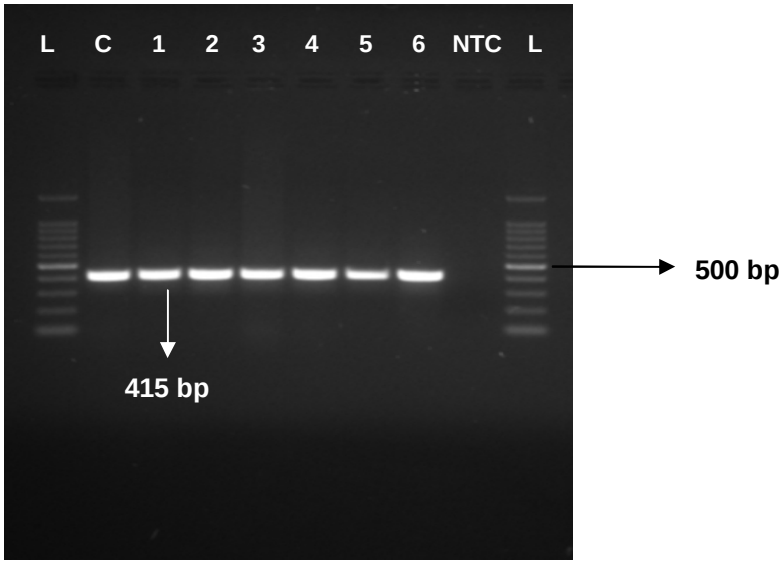


**FIG. 4.23. AMPLIFIED PRODUCTS OF *CTX*-M GROUP1 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 10: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 and 4: Amplified products of *E. coli*,
Lane 5 to 8: Amplified products of *K. pneumoniae*
Lane 9: No-template control

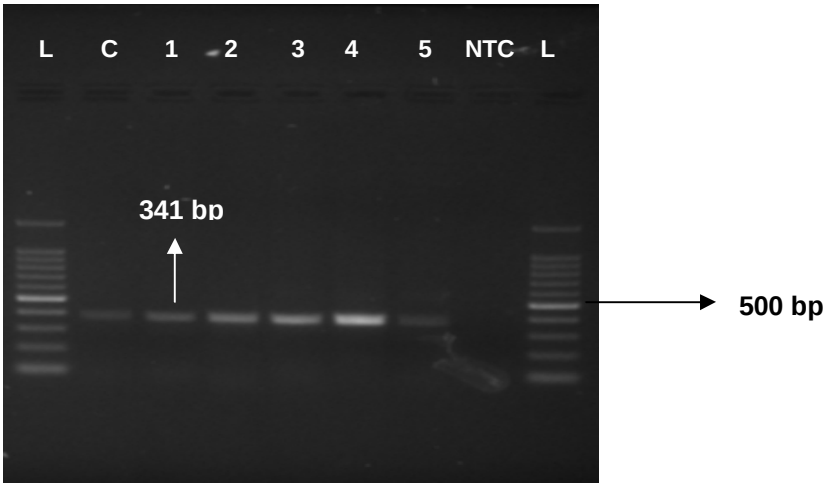


**FIG. 4.24. AMPLIFIED PRODUCTS OF *CTX*-M GROUP2 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 9: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 and 4: Amplified products of *E. coli*,
Lane 5 to7: Amplified products of *K. pneumoniae*,
Lane 8: No-template control

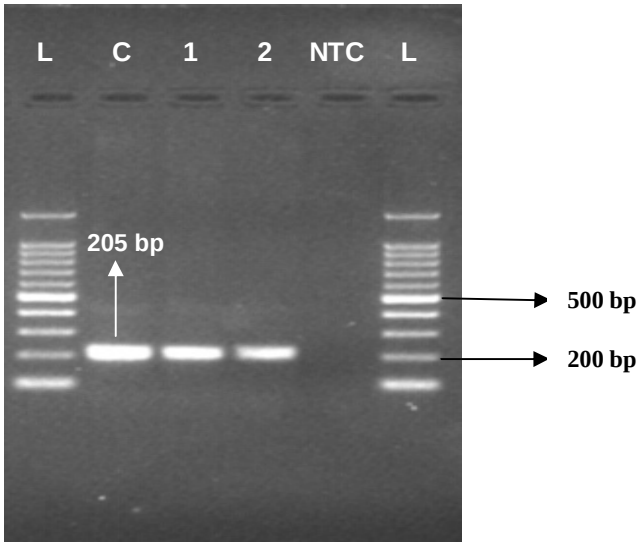


**FIG. 4.25. AMPLIFIED PRODUCTS OF *CTX*-M GROUP 9 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 6: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 and 4: Amplified products of *K. pneumoniae*,
Lane 5: No-template control

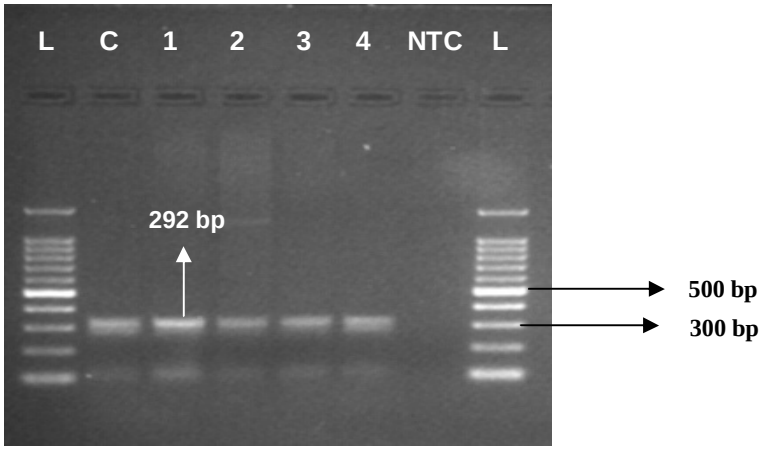


**FIG. 4.26. AMPLIFIED PRODUCTS OF *CTX*-M GROUP 25 GENE IN 1.5% AGAROSE GEL**

Lane 1 and 8: 100 bp DNA ladder,
Lane 2: Positive control,
Lane 3 and 4: Amplified products of *E. coli*,
Lane 5 and 6: Amplified products of *K. pneumoniae*,
Lane 7: No-template control

## 4.12. MOLECULAR TYPING OF ESBL PRODUCING *E. coli* AND *K. pneumoniae* ISOLATES

### 4.12.1. REP-PCR analysis

In the present investigation, a total of 90 ESBL producing (31 *E. coli* and 59 *K.* pneumoniae) isolates were subjected to REP-PCR using (GTG) 5 primer. Based on the banding patterns, dendrograms were drawn for different species using Gel Compar software.

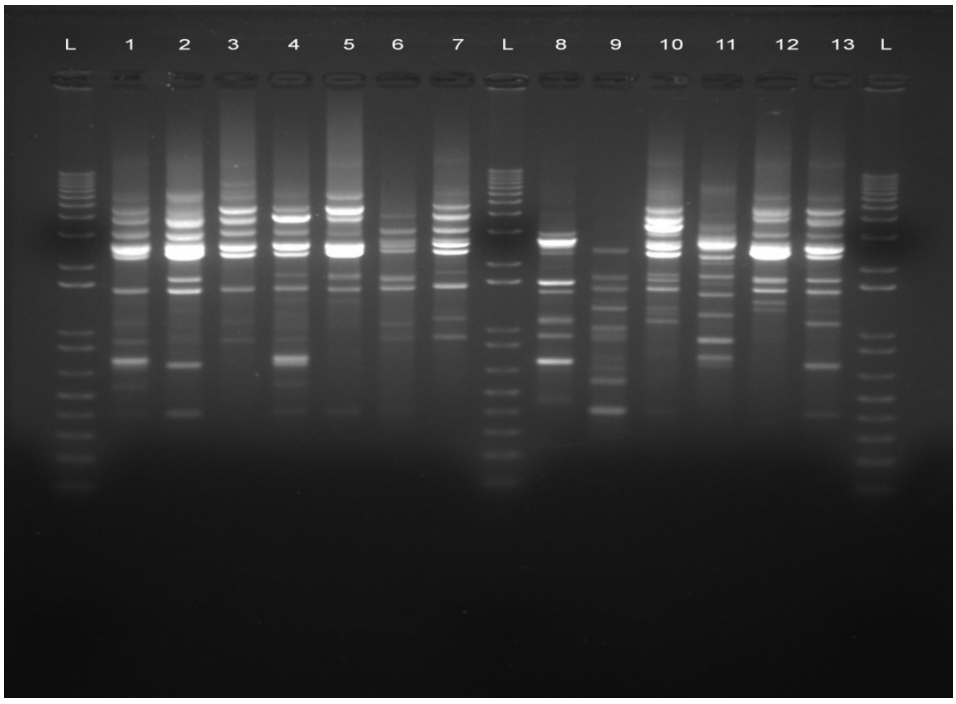


**FIG. 4.27. VISUALIZATION OF REP-PCR BANDING PATTERNS OF ESBL-PRODUCING *K. pneumoniae* ISOLATES IN 1.2% AGAROSE GEL**

Lane 1, 9 and 16: 1 Kb DNA ladder,
Lane 2 to 8 and 10 to 15: ESBL producing *K. pneumoniae* isolates showing variable banding patterns

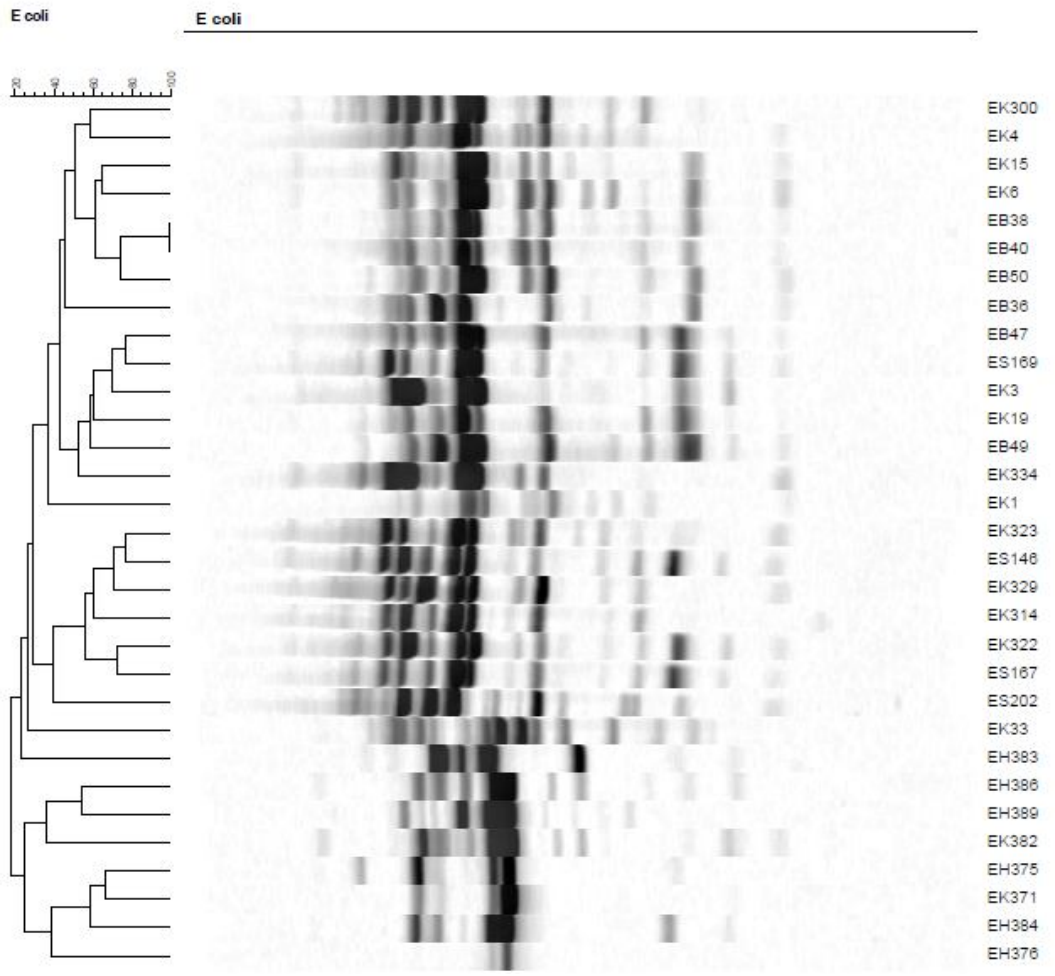


**FIG. 4.28. DENDROGRAM BASED ON REP-PCR BANDING PATTERNS OF ESBL PRODUCING *E. coli* ISOLATES**

**Interpretation**

The isolates were divided into 13 distinct clusters and the D value was calculated to be 0.92. Except for two isolates from milk of cattle (Bongaigaon), all other isolates showed variable banding patterns with 20% to 80% similarity among them. It was interesting to observe that except for one isolate from faeces of cattle, all other isolates (six from cattle and one from poultry from Hajo area of Kamrup district) formed a single cluster and it was the root cluster for all other isolates. However, they were divided into two sub-clusters. No distinct relation could be observed between clustering pattern and species, nature of sample or place of origin, except for the isolates from Hajo area as described above.

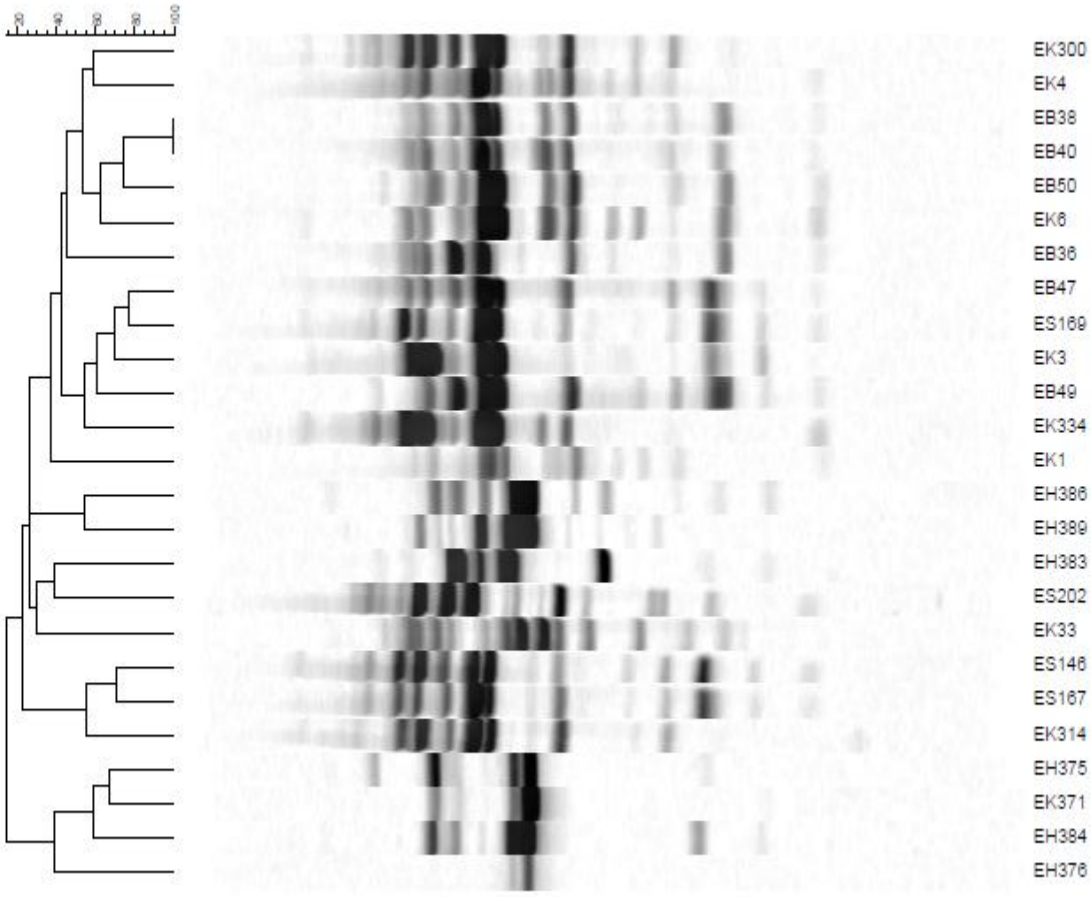


**FIG. 4.29. DENDROGRAM BASED ON REP-PCR BANDING PATTERNS OF ESBL PRODUCING *E. coli* ISOLATES FROM CATTLE**

**Interpretation**

The isolates from cattle were divided into 10 distinct clusters and the D value was calculated to be 0.91. Each of the 25 isolates showed variable band patterns and the percentage of similarity varied from 20% to 78%, except for two isolates (EB 38 and EB 40) from cattle milk from Bongaigaon, which showed 100% similarity. Four isolates of cattle (three from milk and one from faeces) from Hajo area formed the root cluster for all other isolates.

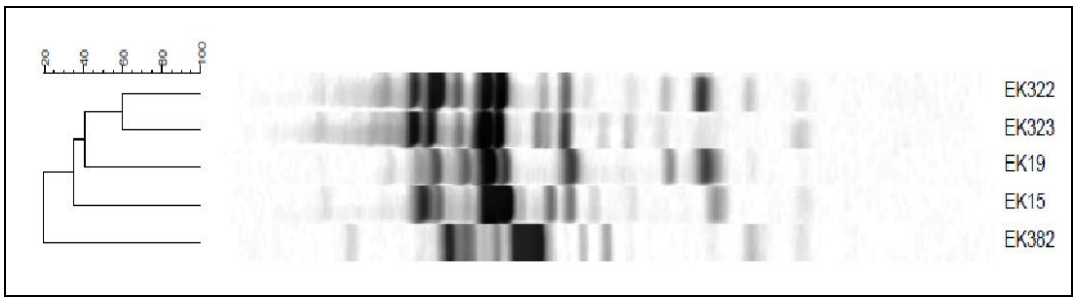


**FIG. 4.30. DENDROGRAM BASED ON REP-PCR BANDING PATTERNS OF ESBL PRODUCING *E. coli* ISOLATES FROM POULTRY**

**Interpretation**

The isolates were divided into four distinct clusters and the D value was calculated to be 0.90. All the five isolates showed variable band patterns with 20% to 60% similarity among them.

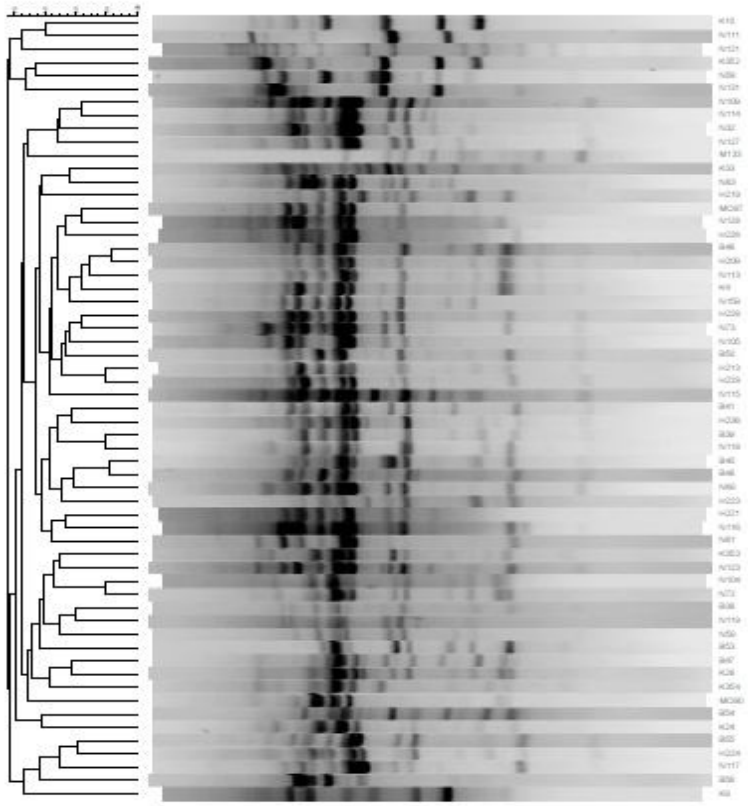

**FIG. 4.31. DENDROGRAM BASED ON REP-PCR BANDING PATTERNS OF ESBL PRODUCING *K. pneumoniae* ISOLATES**

**Interpretation**

Each of the 59 *K. pneumoniae* isolates showed variable banding patterns and the percentage of similarity among the isolates varied from 15% to 90%. The isolates were divided into 23 distinct clusters and six isolates from cattle (two from Kamrup and four from Nagaon) were separated in a root cluster. The D value was calculated to be 0.96. No definite relation could be observed between the clustering pattern and the source of the isolates in terms of either species, type of sample or place of sample collection.

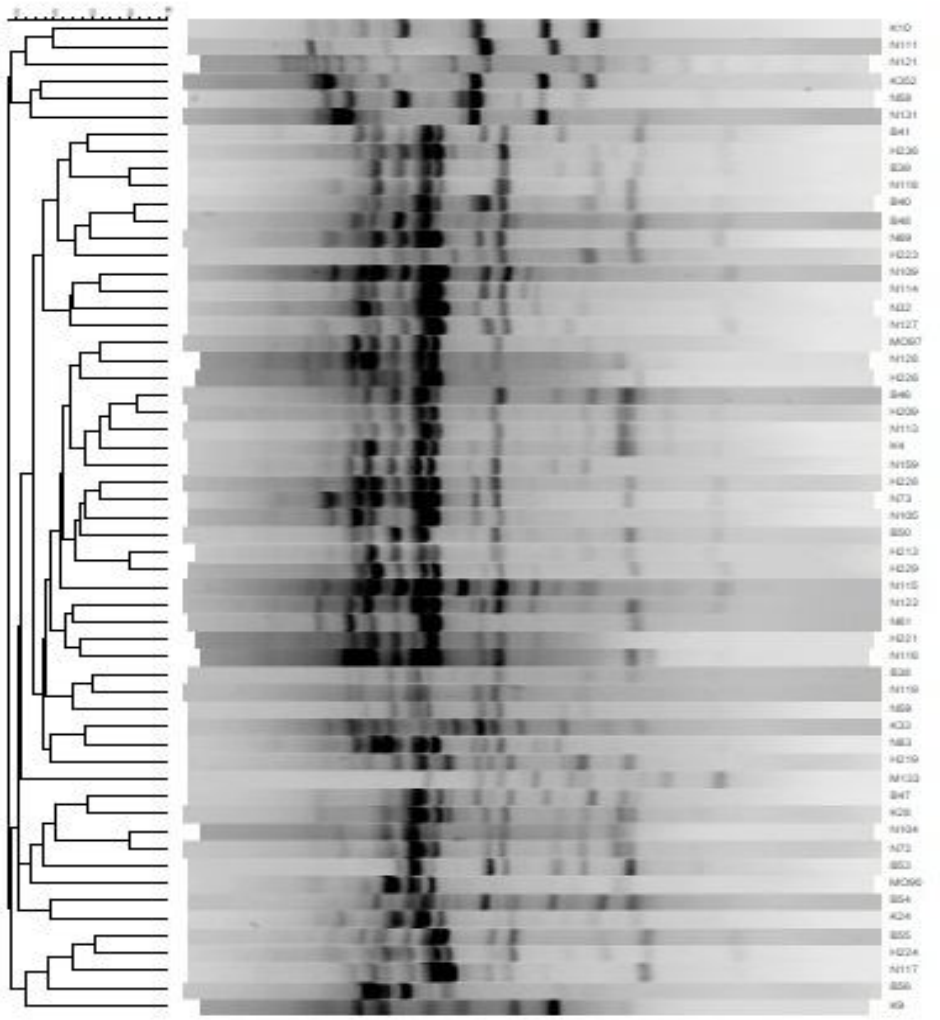

**FIG. 4.32. DENDROGRAM BASED ON REP-PCR BANDING PATTERNS OF ESBL PRODUCING *K. pneumoniae* ISOLATES FROM CATTLE**

**Interpretation**

Each of the 57 isolates showed variable banding patterns and the percentage of similarity among the isolates varied from 18% to 88%. The isolates were divided into 22 distinct clusters and six isolates from cattle (two from Kamrup and four from Nagaon) were separated in a root cluster. The D value was calculated to be 0.96. No distinct relation could be observed between clustering pattern and species, nature of sample or place of origin.

Deplano *et al.* (2011) reported DiversiLab based REP-PCR as a technique with excellent performance and a reliable typing tool for investigation of outbreaks and epidemiological study caused by ESBL producing Enterobacteriaceae isolates. Chandramohan and Revell (2012) reported REP-PCR as one of the best molecular tools for typing of ESBL producing *E. coli* isolates and the method was highly reproducible and could establish good clonal relation among the isolates.

Sekhar *et al*. (2017) reported (GTG)5 REP-PCR as an effective genotyping tool for rapid and reliable discrimination (D value > 0.9) of ESBL *E. coli* strains of diverse origin based on their genotype. These findings were in close agreement with that of the present study and based on these findings, the REP-PCR could be confirmed as a reliable typing method for ESBl producing Enterobacteriacae, which is effective, rapid and easy to perform.

**4.12.2. PFGE Analysis**

In the present study, a total of 90 ESBL producing (31 *E. coli* and 59 *K. pneumoniae*) isolates were subjected to PFGE using *Xba*I enzyme @ 50 units per plug. Based on the banding patterns exhibited in PFGE, dendrograms were drawn for the two different species using Gel Compar software.

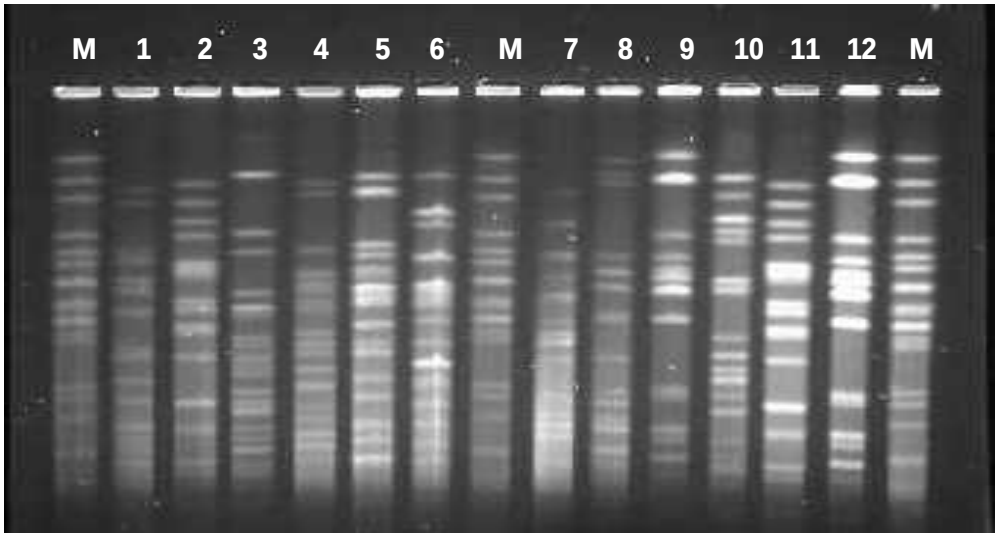


**FIG. 4.33. VISUALIZATION OF PFGE BANDING PATTERNS OF ESBL PRODUCING *K. pneumoniae* IN 1% PFC AGAROSE GEL**

Lane 1, 8 and 15: *Salmonella* Braenderup H9812 Strain, Lane 2 to 7 and 9 to 14: ESBL producing *K.pneumoniae* isolates showing variable banding patterns in PFGE

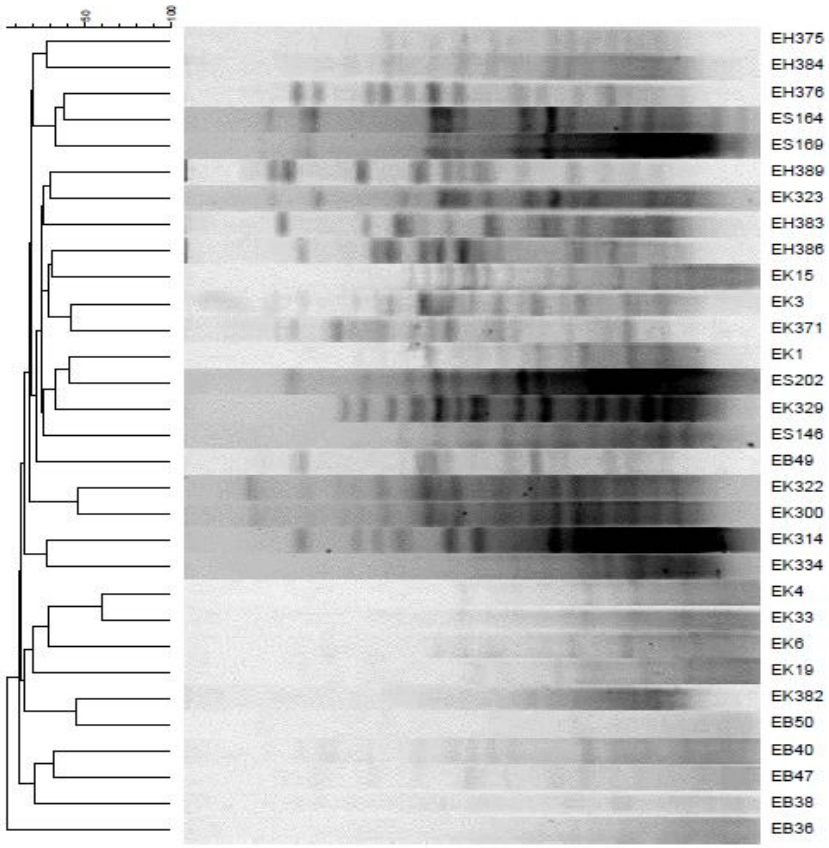


**FIG. 4.34. DENDROGRAM BASED ON PFGE BANDING PATTERNS OF ESBL PRODUCING *E. coli* ISOLATES**

**Interpretation**

The isolates were divided into 12 distinct clusters and the D value was calculated as 0.93. All the 31 *E. coli* isolates showed variable banding patterns with 10%-70% similarity among them. One isolate of cattle milk from Bongaigaon (EB 36) formed the root cluster. No distinct relation could be observed between clustering pattern and species, nature of sample or place of origin, except for isolate from Bongaigaon as described above.

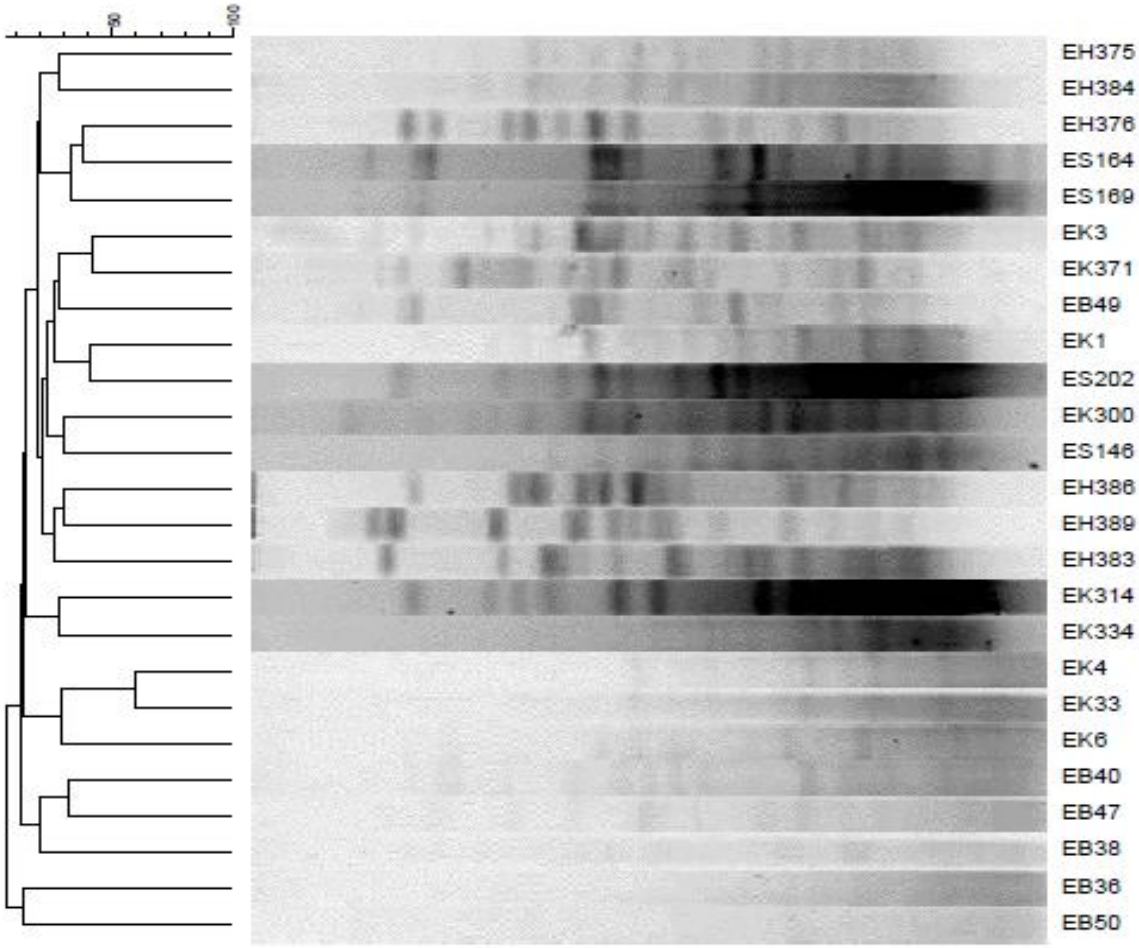


**FIG 4.35. DENDROGRAM BASED ON PFGE BANDING PATTERNS OF ESBL PRODUCING *E. coli* ISOLATES FROM CATTLE**

**Interpretation**

The isolates were divided into 10 distinct clusters and the D value was calculated to be 0.93. All the 25 isolates showed variable banding patterns with 12% to 65% similarity among them. Two isolates of cattle milk from Bongaigaon (EB 36 and EB 50) formed the root cluster for all other isolates**.**

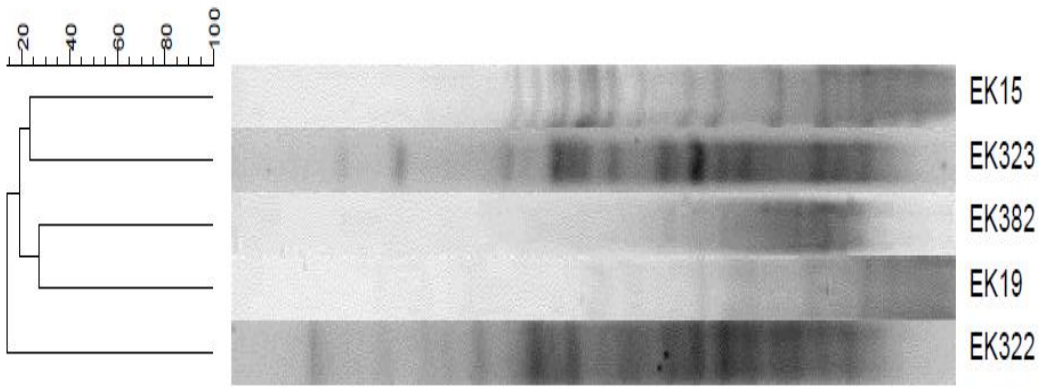


**FIG. 4.36. DENDROGRAM BASED ON PFGE BANDING PATTERNS FOR THE ESBL PRODUCING *E. coli* ISOLATES FROM POULTRY**

**Interpretation**

The isolates were divided into three distinct clusters and the D value was calculated to be 0.80. All the five isolates showed variable band patterns with 15% to 32% similarity among them. One isolate from meat (chicken) collected from Kamrup district (EK 322) formed the root cluster**.**

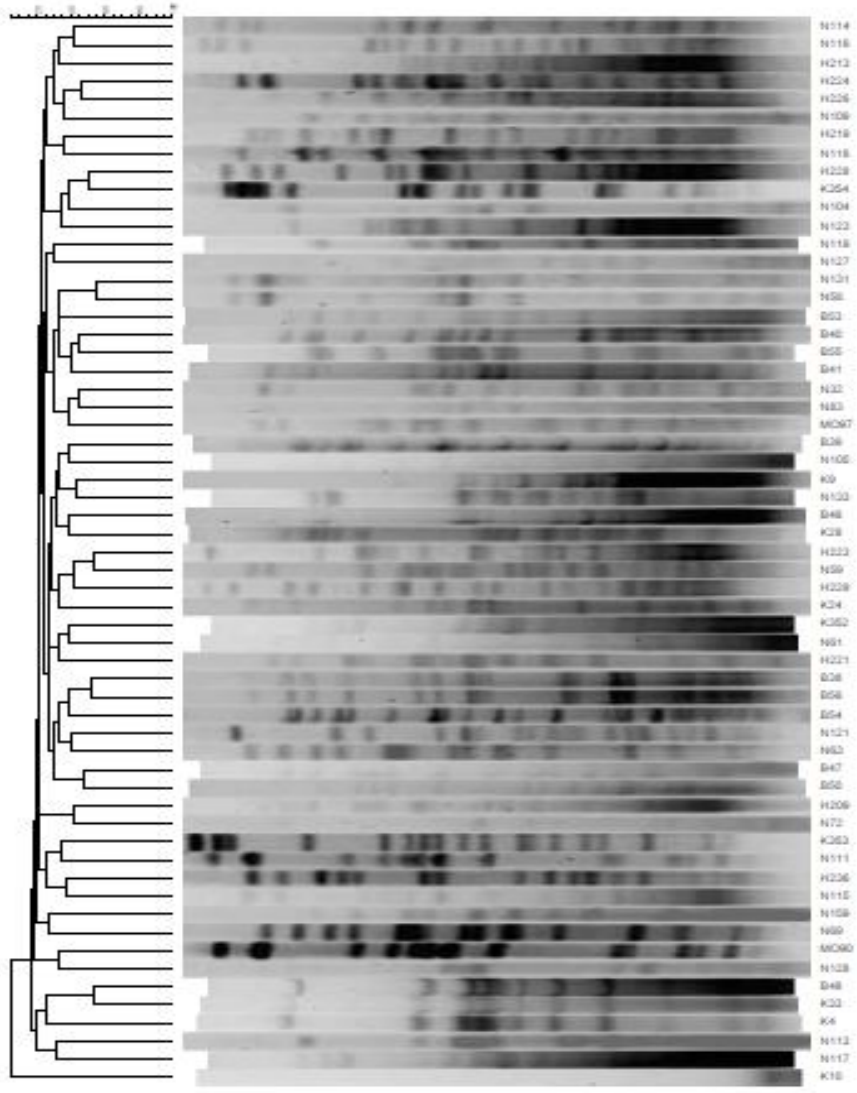

**FIG. 4.37. DENDROGRAM BASED ON PFGE BANDING PATTERNS OF ESBL PRODUCING *K. pneumoniae* ISOLATES**

**Interpretation**

Each of the 59 *K. pneumoniae* isolates showed variable banding patterns and the percentage of similarity among the isolates varied from 10% to 55%. The isolates were divided into 22 distinct clusters and one isolate (K10) from cattle milk collected from Kamrup district formed the root cluster. The D value was calculated to be 0.96. No definite relation could be observed between the clustering pattern and the source of the isolates in terms of either species, type of sample or place of sample collection.

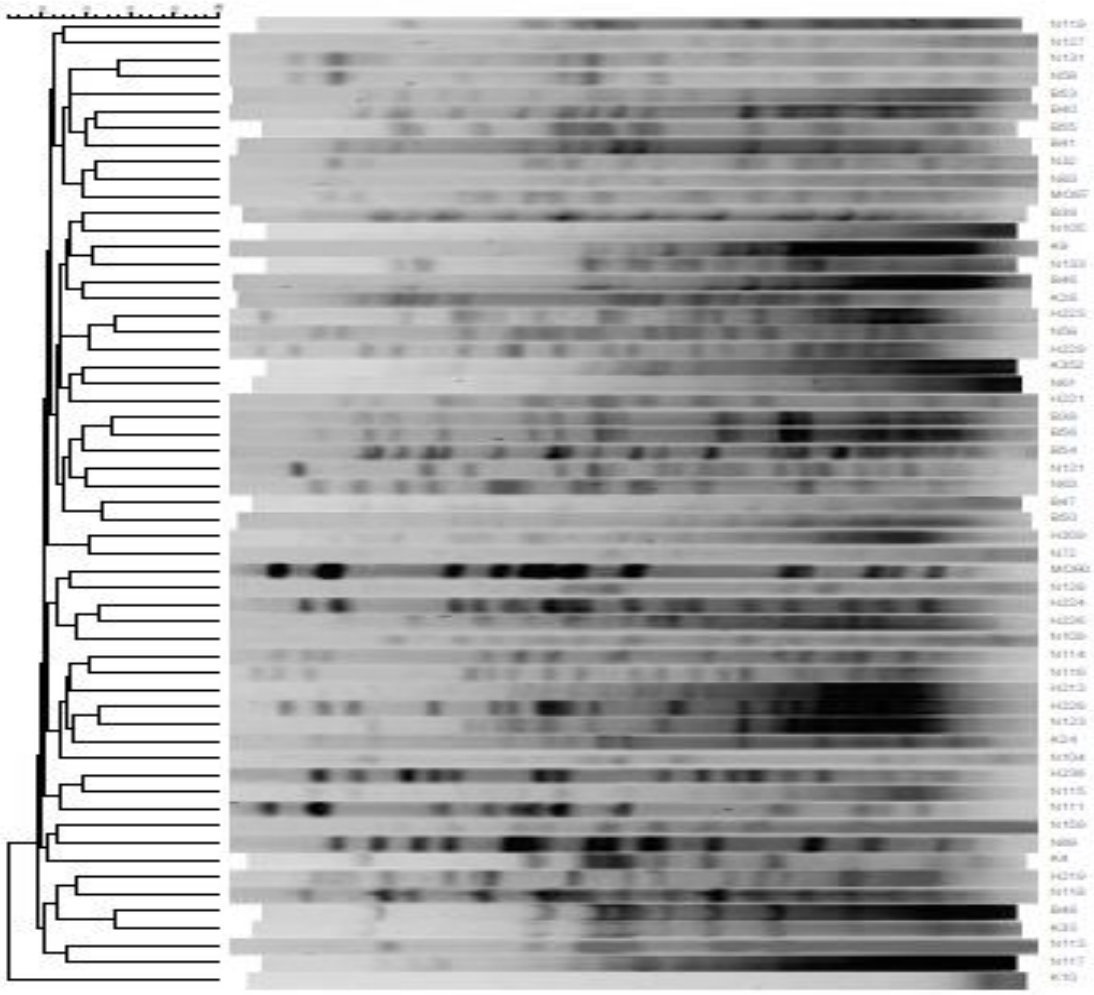

**FIG. 4.38. DENDROGRAM BASED ON PFGE BANDING PATTERNS OF ESBL PRODUCING *K. pneumoniae* ISOLATES FROM CATTLE**

**Interpretation**

Each of the 57 *K. pneumoniae* isolates showed variable banding patterns and the percentage of similarity among the isolates varied from 10% to 55%. The isolates were divided into 23 distinct clusters and one isolate from cattle milk collected from Kamrup district (K10) formed the root cluster. The D value was calculated to be 0.97. No distinct relation could be observed between clustering pattern and species, nature of sample or place of origin.

Bae *et al.* (2014) reported PFGE as the best tool for typing ESBL producing *E. coli* isolates with a higher discriminative power compared to REP-PCR. PFGE could group *E. coli* isolates belonging to different sequence types in a better way, whereas REP-PCR accurately grouped *E. coli* isolates belonging to the same sequence type compared to PFGE. This study showed that the appropriate molecular typing method for an epidemiological study should be chosen based on the particular characteristics of that study. These findings were, however, not in agreement with that of the present study.

Pitout *et al.* (2009), on the other hand, reported DiversiLab based automated REP-PCR as a better tool for molecular typing of ESBL isolates compared to PFGE. REP-PCR could identify *E. coli* clone ST131 producing CTX-M-15 in a better way than PFGE.

In the present investigation, both PFGE and REP-PCR showed almost the same D (Simpson's Index of diversity) value suggesting comparable discriminatory power of these methods with almost similar clustering patterns demonstrated among the ESBL-producing *E. coli* and *K. pneumoniae* isolates. Moreover, REP-PCR could distinguish *E. coli* isolates of different places of origin in a better way compared to PFGE. However, no distinct relation could be observed between the clustering pattern of REP-PCR and PFGE, and *CTX*-M genogrouping and insertion sequences. As both the typing methods were found equally competent, REP-PCR may be preferred as a typing method for epidemiological investigations, as it has several advantages over PFGE like less time-consuming, cost-effective, no need of highly skilled worker and easy to perform.

# CHAPTER - V

# *Summary and Conclusion*

**PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES**

## CHAPTER-V

# SUMMARY AND CONCLUSION

The present study was carried out with a view to isolate and identify ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* from milk, meat and faecal samples of animals, to study resistance profiles of the isolates, to genotype on the basis of CTX-M genogroups, to detect insertion sequences related to antibiotic resistance and to study the molecular diversity among the isolates by REP-PCR and PFGE.

A total of 385 samples were collected including raw milk (209) and faeces (69) of cattle, milk (23) and faeces (11) of goat, curd (12), chicken (32), beef (14) and pork (15). Among the 385 samples examined, 31 (8.05%) were positive for ESBL producing *E. coli* (Table 4.1). A total of 21 (10.05%) *E. coli* isolates were obtained from cattle milk samples, 1 (8.33%) from curd, 5 (15.63%) from chicken, 1 (6.67%) from pork and 3 (4.35%) from cattle faeces. Whereas, ESBL producing *E. coli* could not be isolated from goat milk, goat faeces and from beef samples.

Of the 385 samples, 59 (15.32%) were positive for ESBL producing *K. pneumoniae.* A total of 30 (14.35%) *K. pneumoniae* isolates were obtained from cattle milk, 2 (6.25%) from chicken, 3 (21.43%) from beef and 24 (34.78%) from cattle faeces. ESBL producing *K. pneumoniae* could not be isolated from goat milk, goat faeces, curd and pork.

All the 31 (100%) suspected *E. coli* isolates were confirmed by amplification of species-specific *uid*A gene by PCR. All the 59 (100%) suspected *K. pneumoniae* isolates were similarly confirmed by amplification of species-specific *rpo*B gene by PCR.

Phenotypic confirmation of the ESBL production by the isolates (90) was done in three steps, *viz.* subjecting the isolates against 3$^{rd}$ and 4$^{th}$ generation antibiotics, by combination of disc diffusion tests (CDT) and by ESBL-E test. In drug susceptibility test, all the *E. coli* isolates (100%) showed resistance to ceftriaxone and cefpodoxime, followed by 96.77% to cefotaxime, 83.87% to aztreonam, 77.42% to cefepime and 58.06% to ceftazidime.

All the *K. pneumoniae* isolates (100%) also showed resistance to cefpodoxime, followed by cefotaxime (96.61%) and ceftriaxone (93.22%), aztreonam (89.83%), ceftazidime (77.97%) and cefepime (50.85%)

In CDT, 93.55% of *E. coli* and 88.14% *K. pneumoniae* isolates showed positive results. Whereas, in ESBL-E test, 96.77% *E. coli* and 88.14% *K. pneumoniae* isolates were found positive.

Antibiogram of the ESBL producing *E. coli* (31) isolates revealed highest resistance to ceftizoxime (74.19%), followed by co-trimoxazole and tetracycline (25.81% each), ciprofloxacin (19.35%), chloramphenicol (9.68%), and gentamicin and pipercillin-tazobactam (3.23% each). The *K. pneumoniae* (59) isolates showed highest resistance to ceftizoxime (69.49%), followed by ciprofloxacin (25.42%), co-trimoxazole and tetracycline (23.73% each), chloramphenicol (16.95%), pipercillin-tazobactam (5.08%) and gentamicin (3.39%). All the ESBL producing *E. coli* and *K. pneumoniae* isolates were found to be sensitive to imipenem and meropenem.

Out of the 31 ESBL producing *E. coli* isolates, 6.45%, 9.68%, 3.23% and 9.68% isolates showed resistance to five, four, three and two antibiotics, respectively. On the other hand, 5.08%, 20.34% and 25.42% of the ESBL producing *K. pneumoniae* (59) isolates showed resistance to four, three and two different antibiotics, respectively. Overall, a total of 29.32% of *E. coli* and 50.85% of *K. pneumoniae* isolates showed multiple drug resistance. None of the 90 ESBL isolates were found to be positive for AmpC and metallo β-lactamase production.

All the 90 ESBL producing isolates (31 *E. coli* and 59 *K. pneumoniae*) were subjected to PCR for detection of six different resistant genes, *viz. bla*TEM, *bla*SHV, *bla*CTX-M, *qnr*B, *Sul* 1 and *Int*1. The *bla*CTX-M gene was found to be predominant (100%) in case of both *E. coli* and *K. pneumoniae* isolates. The *bla*TEM gene was found in 54.84% *E. coli* and 55.93% *K. pneumoniae* isolates. The *bla*SHV gene was found in 90.32% *E. coli* and 77.97% *K. pneumoniae* isolates, while 90.32% of *E. coli* and 86.44% *K. pneumoniae* showed presence of *Sul*1 gene. The *Int*1 gene was found in 70.97% *E.*

*coli* and 62.71% *K. pneumoniae* isolates, while *qnr*B gene was found in 3.23% *E. coli* and 10.17% *K. pneumoniae* isolates.

Out of the three insertion sequences under study, *viz. ISEcp*1, *IS* 26 and *ISCR*1, *ISEcp*1 was found to be present in all the 90 (100%) ESBL producing isolates, followed by *IS* 26 in 100% *K. pneumoniae* and 90.32% *E. coli* isolates. On the other hand, *ISCR*1 was found to be present in 80.65% *E. coli* and 45.76% *K. pneumoniae* isolates.

All the 90 ESBL producing isolates were subjected to PCR for detection of *CTX*-M genogroups. All the 31(100%) *E. coli* isolates were found to be positive for group 1 gene, followed by group 2 (80.65%) and group 25 (27.27%). None of the isolates were found be positive for group 8 and 9 genes. All the 59 (100%) *K. pneumoniae* isolates were also found to be positive for Group 1 gene, while group 25 gene was found in 67.8%, group 2 in 55.93% and group 9 in 5.08% isolates only. None of the isolate was found to be positive for group 8 gene.

REP-PCR created 13 different clusters among the 31 ESBL producing *E. coli* isolates. The cattle *E. coli* (25) and the poultry *E. coli* (5) isolates were divided into 10 and 4 clusters, respectively. Among the 59 *K. pneumoniae* isolates, 23 different clusters were recorded, while cattle (57) isolates were divided into 22 clusters. The D value for REP-PCR ranged from 0.90 to 0.96.

Pulsed field gel electrophoresis divided the 31 ESBL producing *E. coli* isolates into 12 disctinct clusters. The cattle (25) and the poultry (5) *E. coli* isolates were divided into 10 and 3 clusters, respectively. Similarly, the ESBL producing *K. pneumoniae* (59) isolates were divided into 22 distinct clusters. The D value of PFGE ranged from 0.8 to 0.97.

To summarize the present findings, the following salient points could be highlighted:

1. The overall rates of occurrence of ESBL-producing *E. coli* and *K. pneumoniae* were found to be 8.05% and 15.32%, respectively.

2. No ESBL producing *K. pneumoniae* could be isolated from goat milk and faeces, curd and pork samples. Similarly, no ESBL-producing *E. coli* could be isolated from goat milk and faeces, and beef samples.

3. Antibiotic sensitivity assay for phenotypic confirmation showed all *K. pneumoniae* and *E. coli* isolates to be resistant to cefpodoxime.

4. In CDT, 93.55% and 88.14% *E. coli* and *K. pneumoniae* isolates, respectively were found positive. The corresponding percentages by ESBL-E test were found to be 96.77% and 88.14%.

5. Antibiogram revealed none of isolates to be resistant to imipenem or meropenem.

6. None of the isolate was found to produce AmpC type and metallo β-lactamase.

7. All the 90 (100%) isolates (31 *E.* coli and 59 *K. pneumoniae*) were found to possess *bla*CTX-M gene by PCR.

8. Among the insertion sequences, *ISE*cp1 was found to be predominant (100%) in both *K. pneumoniae* and *E. coli*, while IS-26 was also found to be present in all (100%) the isolates of *K. pneumoniae.*

9. CTX-M genogrouping showed presence of group 1 gene in all the 90 (100%) isolates. While none of the isolates showed presence of group 8 gene, only 3 *K. pneumoniae* isolates were found to show presence of group 9 gene.

10. Both PFGE and REP-PCR showed closely comparable D value (Discriminatory power) with almost similar clustering patterns among the ESBL-producing *E. coli* and *K. pneumoniae* isolates. However, compared to PFGE, REP-PCR could distinguish *E. coli* isolates of different places of origin better.

11. No distinct relation could be observed between the clustering pattern revealed by either REP-PCR or PFGE, and *CTX*-M genogrouping or insertion sequences.

12. As both the molecular typing methods were found equally competent, REP-PCR may be preferred for epidemiological investigations considering its advantages like rapidity, simplicity and ease of performance

# Bibliography

**PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES**

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# *Appendix*

## PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATES FROM ANIMAL SOURCES

# APPENDIX

**1. PREPARATION OF 10X TAE BUFFER:**

| | |
|---|---|
| Tris-Acetate | 0.04 M |
| EDTA | 0.001M |
| pH adjusted to 8.0 | |

**2. TE BUFFER:**

10 mM Tris HCL (pH - 8)
1 mM EDTA
(Stored at 4° C)

3. **PREPARATION OF 10X TBE BUFFER**:

| | |
|---|---|
| Tris-Borate | 0.045 M |
| EDTA | 0.001M |
| pH adjusted to 8.0 | |

**4. ETHIDIUM BROMIDE SOLUTION:**

The solution was prepared by adding 250 ml of distilled water to 125 µl of stock ethidium solution (10 mg of ethidium bromide to 1 ml of distilled water) for staining the gel.

**5. GEL LOADING DYE:**

| | |
|---|---|
| Bromophenol blue | 0.25% |
| Sucrose | 40 % |
| Distilled water | 100 ml |
| Stored at 4°C | |