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Phenotypic and genotypic characterization of extended-spectrum beta-lactamase producing *Escherichia coli* from raw milk in Manipur

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ABSTRACT

This study aimed to determine the prevalence of Extended Spectrum Beta-Lactamase (ESBL) producing *Escherichia coli* in raw milk collected from Manipur State, Northeastern Himalayan region of India, and characterized their genetic profile, relatedness and resistance to commonly used antibiotics. A total of 375 raw milk samples were collected out of which 153 (40.8%) isolates were confirmed as *Escherichia coli.* From the confirmed *E. coli* isolates, phylogenetic grouping, antimicrobial susceptibility test and congo red binding test were performed. The study revealed that the most prevalent phylogenetic group was group A (42.3%). Resistant to tetracycline was observed in 71.9% of the *E. coli* isolates whereas, 91.5% of the isolates were susceptible to sulfisoxazole. Congo red binding test revealed that 43.1% *E. coli* isolates showed a reaction to congo red agar. Amplification of bla_{ESBL} genes from *E. coli* isolates was performed using Polymerase Chain Reaction (PCR) and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR was performed from the ESBL producing isolates. It was found that 43 (28.1%) isolates were confirmed as ESBL producer which was quite high as compared to other studies, and the predominant gene was bla_{TEM} (69.8%). Combined genes such as bla_{CTX-M} and bla_{TEM}, bla_{TEM} and bla_{SHV}, $bla_{TEM,}$ bla_{CTX} and bla_{SHV} were found, where $bla_{CTX-M-1}$ (80.0%) was the most predominant bla_{CTX-M} subgroup. The result of ERIC–PCR showed a distinct profile and correlations between genomic and virulent genes among ESBL producing isolates.

1. Introduction

Escherichia coli are among the most common pathogen responsible for causing several serious infections in animals and human beings. The practice of using antimicrobial to prevent pathogenic infections; associated with economic losses and to promote the growth of livestock may be associated with an increase in the prevalence of bacterial resistant extended-spectrum beta-lactamase (ESBL)-producing *E. coli* from dairy and food-producing animals in Asian and European countries (Palmeira and Ferreira, 2020). ESBL is an enzyme produced by bacteria to become resistant to extended-spectrum monobactams, cephalosporins and penicillin except carbapenems and cephamycins (Teklu *et al*., 2019). The three main categories of gene encoding ESBL are bla_{CTX-M}, bla_{SHV}, and bla_{TEM}; and the enzyme produced by ESBL is an important mechanism in Enterobacteriaceae family for their resistance against antibiotics which makes strategies of treatment more complex (Pishtiwan and Khadija, 2019). CTX-M beta-lactamases is a large and growing enzyme and is divided into several groups, and each group comprises its prototype enzyme and several others. It is subdivided into groups 1, 2, 8, 9 or 25 and CTX-M-1 being the most frequently isolated subtype of CTX-M in samples of animal origin (Irrang *et al*., 2018).Even though *E. coli* can be categorized as pathogenic or nonpathogenic, and within each of these categories, there can be up to four phylogenetic groups such as A, B1, B2 and D, each of which can have a different prevalence (Clermont *et al*., 2000).

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E. coli can contaminate the foods of animal origin, viz., raw milk, meat and their products and contribute to human food-borne and food spoilage. The main sources of *E. coli* isolates are from unpasteurized milk, and approximately 7% of *E. coli* isolates have been confirmed to be multidrug-resistant (Alharbi *et al*., 2019). Therefore, this study aimed to determine the prevalence of ESBL producing *E. coli* in raw milk from Manipur, Northeastern Himalayan region of India, and to characterize the genetic profile of these isolates and their resistance to commonly used antibiotics.

2. Materials and Methods

2.1 Isolation and identification of E. coli isolates

A total number of 375 raw milk samples were collected from different local milk distributors in Manipur from September 2019 to August 2022. Each raw milk sample (25mL) was enriched with 225mL of Buffered Peptone water (HiMedia, Mumbai, India), and incubated overnight at 37^0C (Agarwal *et al*., 2003). A loopful of the overnight broth culture was inoculated on sterile HiCrome *E. coli* agar plates (HiMedia, Mumbai, India) and incubated at 37^0C for 16 to 18 hours. Identification of morphological and biochemical characteristics was done as described by Ewing (1986). The *E. coli* isolates were stored for further use in a sterile 50% glycerol stock solution at -80°C deep freezer.

2.2 Molecular identification and Phylogenetic grouping of E. coli isolates

DNA of the isolates were extracted using GSure® Bacterial Genomic DNA Isolation Kit (GCC Biotech, India) and suspected *E. coli* isolates were confirmed using primers (Table 1) and protocol as described (Sabat *et al*., 2000). Phylogenetic grouping (A, B1, B2 and D) was performed by the method described by Clermont *et al*. (2000) by using set of primers (Table 1). Each reaction was carried out by optimization of PCR reaction using Takara EmeraldAmp® MAX GT PCR Master Mix (Takara Bio Inc., Japan) up to a total volume of 25 µl. The PCR condition was as follows: 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step of 7 min at 72°C.

2.3 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing (AST) of the *E. coli* isolates was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton Agar (HiMedia, Mumbai, India) using *Escherichia coli* ATCC® 25922™ as the quality control strain (CLSI, 2017). The sixteen antibiotics used were: Amoxyclav (30 mcg), ampicillin (10 mcg), cefamandole (30 mcg), ceftriaxone (30 mcg), cephalothin (30 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), co-trimoxazole (25 mcg), gentamicin (10 mcg), kanamycin (30 mcg), nalidixic acid (30 mcg), nitrofurantoin (100 mcg), spectinomycin (100 mcg), sulfisoxazole (300 mcg), streptomycin (300 mcg) and tetracycline (30 mcg) (HiMedia, Mumbai, India).

2.4 Congo Red Binding Test

Single colony of *E. coli* was streaked on Congo red agar plates which composes of brain heart infusion broth (37gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L), and congo red stain (0.8 gms/L, HiMeida, Mumbai, India), and incubated at 25°C. The reaction was recorded at 18, 24, 48 and 72 hours respectively. Colonies reacted with the dye formed red colonies are considered as a positive whereas; colonies that does not bind with the dye and remained white or grey are considered as negative (Berkhoff and Vinal, 1986).

2.5 Polymerase Chain Reaction (PCR) detection of bla_{ESBL} genes

bla_{ESBL} genes ($bla_{\text{CTX-M}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-3}}$, bla_{TEM} and bla_{SHV}) were amplified using PCR performed in a thermal cycler (Applied Biosystems, USA) with set of primers (Table 1). Amplification was done as per the standard protocol with slight modifications. Initial denaturation was at 95^0C ($bla_{\text{CTX-M}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-3}}$) and, 94^0C (bla_{TEM} and bla_{SHV}) for 5 minutes. 30 cycles ($bla_{\text{CTX-M}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-3}}$ and bla_{TEM}) and 40 cycles (bla_{SHV}) of denaturation at 94^0C for 1 min followed by annealing at 51^0C ($bla_{\text{CTX-M}}$), 52^0C (bla_{TEM}) 53°C ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-3}}$) and 55°C (bla_{SHV}) for 1 min. Extension at 72^0C for 1 min and final extension at 72^0C for 7 min. The amplified products were analyzed and the gel image was captured by gel documentation imaging system (Bio-Rad, USA).

2.6 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR analysis of ESBL producing E. coli isolates

ERIC-PCR was performed in forty three ESBL producing *E. coli* isolates by using a pair of primers (Table 1). Optimization of PCR reaction was done using Takara EmeraldAmp® MAX GT PCR Master Mix (Takara Bio Inc., Japan) upto a total volume of 25 µl. A dendrogram was constructed using BioNumerics™ Software (Version 7.0, Applied-Maths, Belgium), and their similarity was constructed using unweighted pair group method with arithmetic mean (UPGMA) method. The numerical discrimination index (D-value) was calculated by Simpson's Diversity Index (Hunter and Gaston, 1988).

3. Results and Discussion

There is an increase studies carried out in different

countries and regions on the prevalence and characteristics of ESBL producing *E. coli* from raw milk. There are also reports in India from different states (Kuralayanapalya *et al*., 2019) but not in Manipur till date. Therefore, the present study aimed on prevalence and molecular characterization of ESBL producing *E. coli* from raw milk in Manipur.

From the collected 375 raw milk samples during September 2019 to August 2022, colonies that produce bluish-green color in HiCrome *E. coli* Agar plates were picked up for *E. coli* confirmation. A total number of 153 isolates were confirmed as *E. coli* which were further used for the study, and the prevalence of *E. coli* was found to be 40.8%. The prevalence of *E. coli* from raw milk from the present study was 40.8% which was higher than West Bengal (12.1%) as reported by Batabyal *et* al. (2018). Differences in prevalence rate of *E. coli* in raw milk could be due to differences in geographical area, seasonal variations and hygienic practices while handling. The presence of *E. coli* in milk is an important indicator of fecal contamination, environmental contamination and poor hygienic practices that carry virulent genes which are potentially harmful to the consumers (Bujňáková *et al*., 2021).

Phylogenetic group of the confirmed *E. coli* isolates were presented in Table 2. It was found that 42.5% (n=65) of the *E. coli* isolate belongs to group A, 26.1% (n=40) belongs to group B1, 16.3% (n=25) belongs to group D and 15.0% (n=23) belongs to group B2. The present study showed that the most virulent group (B2) was the least detected group (15.0%) and the most prevalent was group A. The phylogenetic group A, B1 and D are commensals of animal intestine, and the presence of group A could be due to unhygienic handling of milk during milking, and bovine sub-clinical mastitis (Zhang *et al*., 2018). The presence of group B2 in raw milk is mainly due to contamination from human sources as this phylogroup is considered as human faecal contamination. Several studies confirmed group B2 as virulent strains since they can cause intestinal and extra-intestinal infections in human (Musa *et al*., 2020)

The in vitro susceptibility of *E. coli* isolates to sixteen antibiotics tested were presented in Figure 1. It was found that tetracycline has the highest resistant (71.9%) followed by ciprofloxacin (41.8%), amoxyclav (38.6%) and nalidixic acid (37.3%). Whereas, the maximum isolates (91.5%) were susceptible to sulfisoxazole; ampicillin and cefamandole (90.8%) and ceftriaxone (88.9%). Resistance against tetracycline (71.9%) was found to be the most prevalent and this is supported by Batabyal *et al.* (2018). Furthermore, Ampicillin susceptibility was found to be 90.8% which was lower than the study by Badri *et al*. (2017) where he reported that 100% were resistant. On the other hand, Tark *et al.* (2017) reported 16.6% resistance to ampicillin which was slightly higher than our present study (9.2%). Variation in this findings may be due to the class of antibiotics used for treatment; frequency and concentration (Tark *et al*., 2017).

Congo red binding test of the *E. coli* isolates revealed that 43.1% (n=66) isolates were positive (Figure 2). The invasive *E. coli* that can bind to congo red dye are indicated as virulent whereas, isolates that do not bind with the dye are indicated as avirulent. Due to its acquired virulence and resistance factor, these features can represent the highest pathogenic potential of the identified strains (Berkhoff and Vinal, 1986).

From 153 *E. coli* isolates, ESBL producing *E. coli* was screened using PCR (Fig. 2) and it was found that 28.1% (n=43) isolates were positive for ESBL producing *E. coli*, which is high as compared to other study (Kumaruzzaman *et al*., 2020). Out of 43 isolates, 30 isolates (69.8%) were positive for bla_{TEM}, 25 isolates (58.1%) were positive for bla_{CTX-M} and 5 isolates (11.6%) were positive for bla_{SHV} (Table 3) Similar finding on the prevalence of bla_{TEM}, bla_{CTX-M} and bla_{SHV} respectively from raw milk was reported by Bhoomika *et al.* (2016). As seen in table 4, bla_{TEM} alone was detected from 16 isolates whereas bla_{CTX-M} alone was detected in 13 isolates ($bla_{CTX-M-1}$= 12, $bla_{CTX-M-2}$= 1). An isolate producing multiple genes were also detected, where, 9 isolates were positive for both bla_{CTX-M} and bla_{TEM} ($bla_{CTX-M-1}$ =7, $bla_{CTX-M-3}$=2); two isolates were positive for both bla_{TEM} and bla_{SHV} and 3 isolates were positive for bla_{CTX-M}, bla_{TEM} and bla_{SHV} ($bla_{CTX-M-1}$=1, $bla_{CTX-M-3}$=2) Similarly, detection of combined genes in this study is comparable with the study conducted by Kumaruzzaman *et al.* (2020). Among CTX-M subgroup, $bla_{CTX-M-1}$ was found to be the predominant gene (80.0%), followed by $bla_{CTX-M-3}$ (16.0%) and $bla_{CTX-M-2}$ (4.0%). The high prevalence of $bla_{CTX-M-1}$ in raw milk among CTX-M subgroup was also reported by Tekiner and Ozpinar (2016). To ensure the safety of milk prior consumption, regular monitoring of raw milk is important and showed to be an effective way for detecting of the presence of enteropathogenic strains and antimicrobial resistance bacteria (Albonica *et al*., 2017).

The results of ERIC-PCR DNA fingerprint of forty three ESBL producing *E. coli* isolates were summarized (Fig. 3). The *E. coli* isolates yielded different DNA of 2-8 bands within a size range of 100-1200 bp which was grouped into 7 clusters (C1 to C7). Cluster 1 (C1) comprised of two isolates (M171 and M8), cluster 2 (C2) comprised of sixteen isolates (M172A-M15), cluster 3 (C3) comprised of seven isolates (M4-M12B), cluster 4 (C4) comprised of twelve isolates (M133-M151), cluster 5 (C5) comprised of four isolates (M131-M72A), cluster 6 (C6) and cluster 7 (C7) comprised of one isolate each (M13 and M13A). bla_{TEM} was found to be the predominant gene in cluster C1, C4 and C5. $bla_{CTX-M-1}$ was predominant in cluster C2 and C6 and combined gene of bla_{TEM} and bla_{CTX-M} was predominant in cluster C3.

Phylogenetic group B2 was predominant in cluster C2 and C5 and B1 was predominant in cluster C4. The antibiotic resistance pattern was diverse; the most common antibiotic resistance in cluster C1 was CIP and KAN; CEF, GEN and AMP in cluster C2; CTR, AMP and AMC in cluster C3; KAN and TE in cluster C4; AMP, CEF and COT in cluster C5. The present study on ERIC-PCR showed that out of seven clusters, five clusters have two to sixteen isolates and have distinct profiles. Each cluster with two or more isolates has predominant genes and phylogenetic group which differ from one cluster to another. Furthermore, while $bla_{\text{CTX-M-1}}$ was present in all the clusters, $bla_{\text{CTX-M-2}}$ was present in cluster 2 and $bla_{\text{CTX-M-3}}$ was present in cluster 2, 3 and 4. Odenthal *et al*. (2016) stated that these variations are due to the transfer of ESBL-encoding genes through mobile genetic elements and also due to fecal contamination. It was also found that there was a correlation between virulence and genomic groups and is comparable with the study performed by Hoffmann *et al*. (2014). The present study showed 95.5 % level of similarity with 0.9556 as a discriminatory power (D), and this result is consistent with the other study where level of similarity reported on pasteurized raw milk was 95.0% with a D-value of 0.99 and explained that it could be due to failures during the entire production process (Oltramari *et al*., 2014).

4. Conclusion

This is the first report on the presence of ESBL producing *E. coli* from raw milk in Manipur, Northeastern Himalayan region, India. From the study, it can be concluded that the collected raw milk is highly contaminated with ESBL producing *E. coli.* Factors responsible for the spread of bacterial contamination could be due to unhygienic handling process, poor storage condition and unhygienic environment condition. This poses serious threat to public health and raises the importance of food safety concerns.

5. Author's contribution

BS designed and analyzed the experiments. LK collected samples, performed the experiments and wrote the article. RL and KL revised and corrected the article. All the authors read and approved the manuscript.

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7. Conflicts of interest

The authors declare that they have no conflict of interest.

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Table 1. Primer sequences used in the study.

Target genes	Primer sequence	Product size (bp)	References
16SrRNA	F 5'-GAAGCTTGCTTCTTTGTC-3' R 5'-GAGCCCGGGGATTTCACAT-3'	541	(Sabat *et al*., 2000)
$bla_{\text{CTX-M}}$	F 5'-TTTGCGATGTGCAGTACCAGTAA-3' R 5'-CGATATCGTTGGTGGTGCCATA-3'	544	(Edelstein *et al*., 2003)
$bla_{\text{CTX-M-1}}$	F 5'-GGTTAAAAAATCACTGCGTC-3' R 5'-TTGGTGACGATTTTAGCCGC- 3'	863	(Garrec *et al*., 2011)
$bla_{\text{CTX-M-2}}$	F 5'-ATGATGACTCAGAGCATTCG-3' R 5'-TGGGTT ACGATTTTCGCCGC-3'	866	(Garrec *et al*., 2011)
$bla_{\text{CTX-M-3}}$	F 5'-CGTCACGCTGTTGTTAGGAA-3' R 5'-ACG GCT TTC TGC CTT AGG TT-3'	780	(Kim *et al*., 2008)
bla_{TEM}	F 5'-TTGGGTGCACGAGTGGGTTA-3' R 5'-TAATTGTTGCCGGGAAGCTA-3'	465	(Arlet and Philippon, 1991)
bla_{SHV}	F 5'-AGGATTGACTG CCTTTTTG-3', R 5'-ATTTGCTGATTTCGCTCG-3'	625	(Bhattacharjee *et al*., 2007)
chuA	F 5'-GACGAACCAACGGTCAGGAT-3' R 5'-TGCCGCCAGTACCAAAGACA-3'	279	(Clermont *et al*., 2000)
yjaA	F 5'-TGAAGTGTCAGGAGACGCTG-3' R 5'-ATGGAGAATGCGTTCCTCAAC-3'	211	(Clermont *et al*., 2000)
TspE4C2	F 5'-GAGTAATGTCGGGGCATTCA-3' R 5'-CGCGCCAACAAAGTATTACG-3'	152	(Clermont *et al*., 2000)
ERIC	F 5'-ATGTAAGCTCCTGGGGATTCAC-3' R 5'-AAGTAAGTGACTGGGGTGAGCG-3'		(Varsalovic *et al*., 1991)

Table 2. Congo red binding test and phylogenetic grouping of 153 *E. coli* isolates.

Total no. of *E. coli* isolates	No. of *E. coli* isolates positive for CRBT (%)	Phylogenetic group (%)			
		A	B1	B2	D
153	66 (43.1)	65 (42.5)	40 (26.1)	23 (15.0)	25 (16.3)

CRBT: Congo red binding test

Table 3. Distribution of ESBL producing *E. coli* isolates.

No. of ESBL positive *E. coli* isolates (%)	ESBLgenes (%)					
	bla_{TEM}	$bla_{\text{CTX-M}}$	$bla_{\text{CTX-M-1}}$	$bla_{\text{CTX-M-2}}$	$bla_{\text{CTX-M-3}}$	bla_{SHV}
43 (28.1)	30 (69.8)	25 (58.1)	20 (80.0)	1 (4.0)	4 (16.0)	5 (11.6)

Table 4. Distribution of ESBL genes detected from raw milk.

ESBL producer	No. of isolates
bla_{TEM}	16
$bla_{CTX\text{-}M\text{-}1}$	12
$bla_{CTX\text{-}M\text{-}2}$	1
bla_{TEM} + bla_{SHV}	2
$bla_{CTX\text{-}M\text{-}1}$+ bla_{TEM}	7
$bla_{CTX\text{-}M\text{-}1}$+ bla_{TEM}+ bla_{SHV}	1
$bla_{CTX\text{-}M\text{-}3}$+ bla_{TEM} + bla_{SHV}	2
$bla_{CTX\text{-}M\text{-}3}$+ bla_{TEM}	2
Total	43

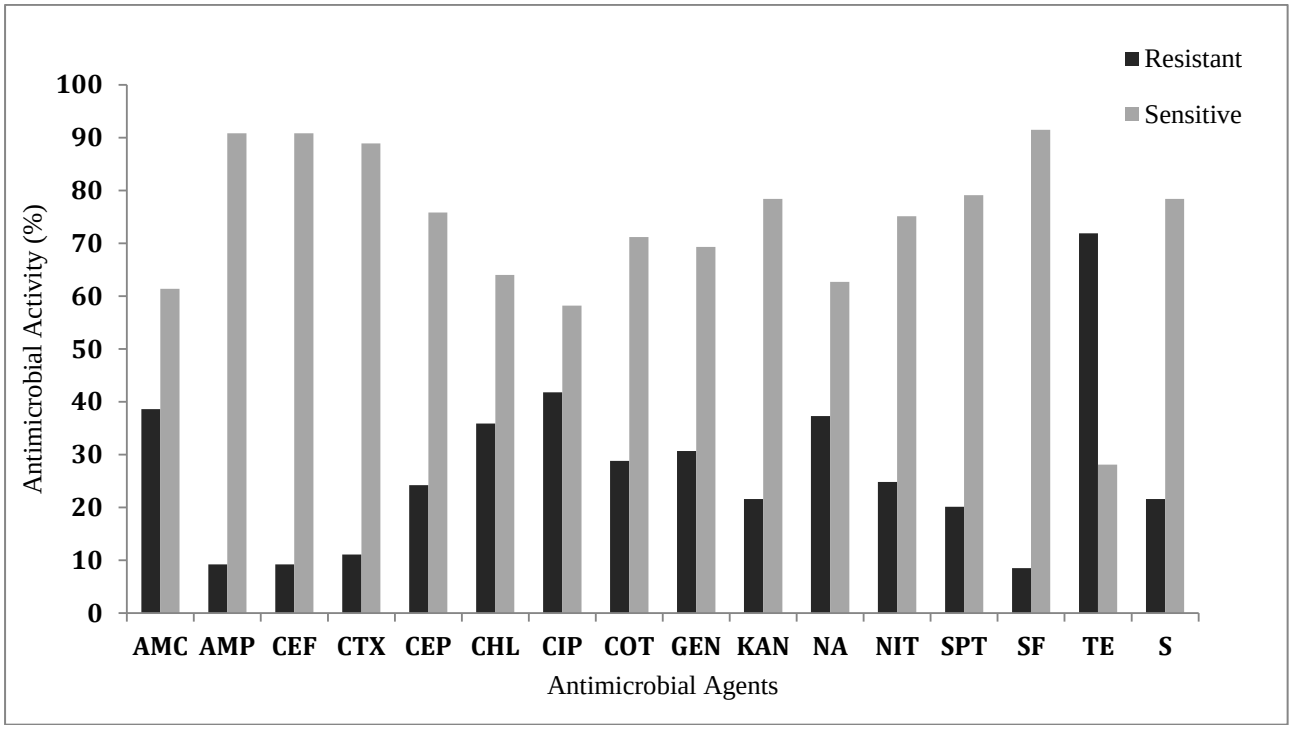

Figure 1. Antimicrobial susceptibility pattern of 153 *E. coli* isolates from raw milk. AMC: Amoxyclav, AMP: Ampicillin, CEF: Cefamandole, CTX: Ceftriaxone, CEP: Cephalothin, CHL: Chloramphenicol, CIP: Ciprofloxacin, COT: Co-Trimaxozole, GEN: Gentamicin, KAN: Kanamycin, NA: Nalidixic Acid, NIT: Nitrofuratoin, SPT: Spectinomycin, SF: Sulfisoxazole, TE: Tetracycline, S: Streptomycin.

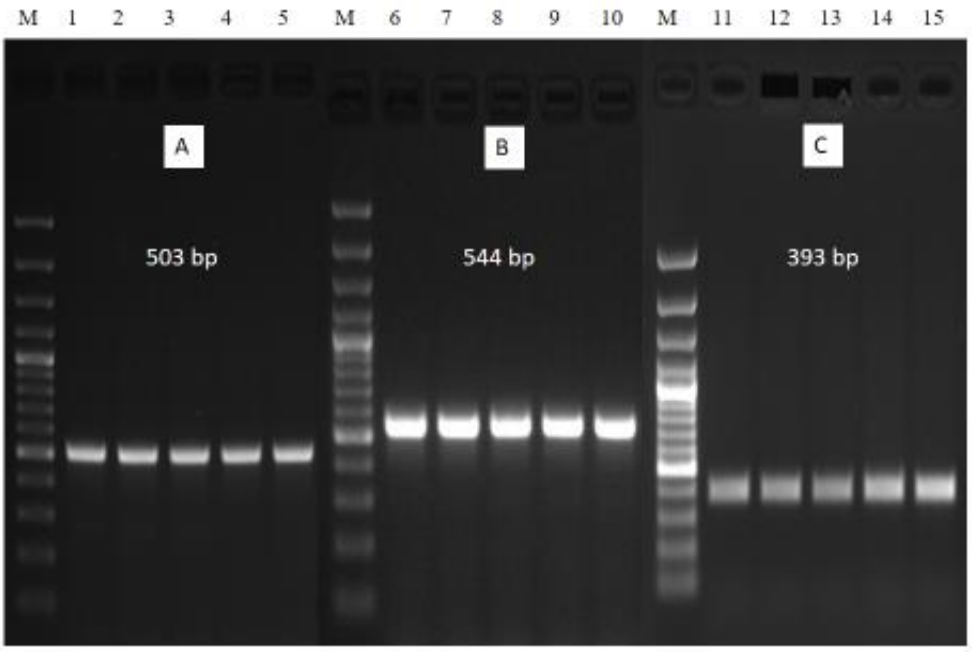

Figure 2. Detection of *bla*$_{TEM}$ (A), *bla*$_{CTX-M}$ (B) and *bla*$_{SHV}$ (C) genes in extended spectrum β-lactamase producing *Escherichia coli* isolated from raw milk samples by Polymerase Chain Reaction. Lane M: 100 bp ladder, lane 1 to 5, 6 to 10 and 11 to 15: Test samples.

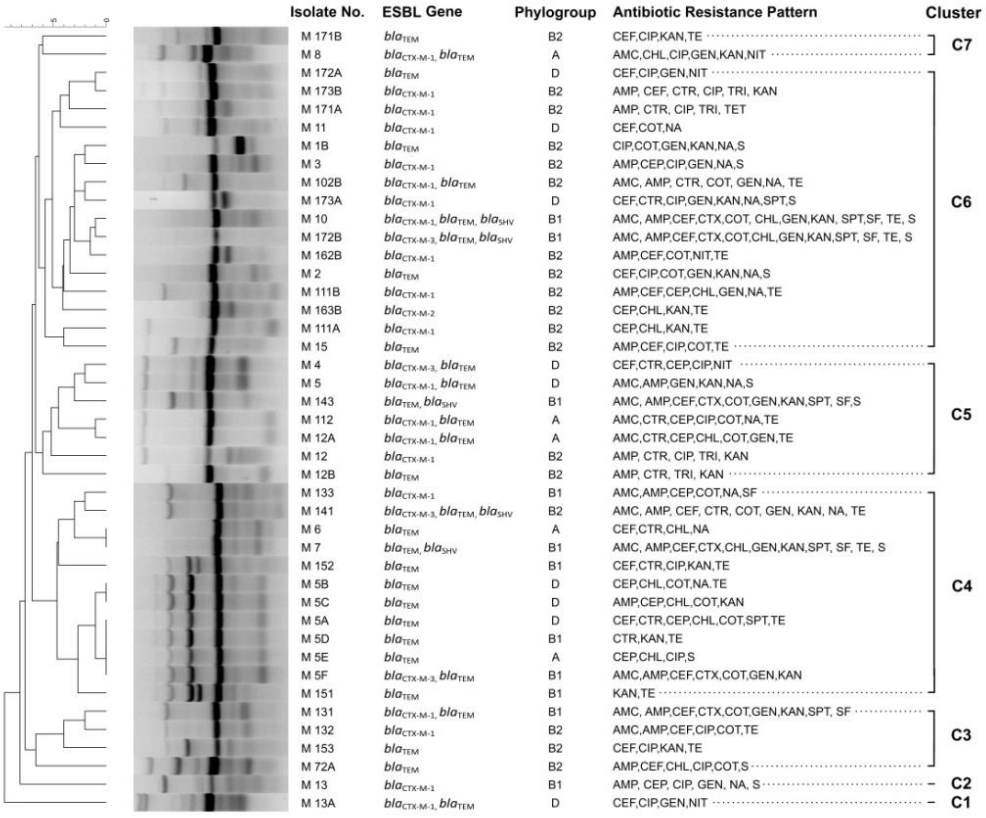

Figure 3. Dendrogram generated from ERIC-PCR of 43 ESBL producing *E. coli* isolates from raw milk showing relatedness and diversity of ESBL genes with phylogenetic group and antibiotic resistance pattern. AMC: Amoxyclav, AMP: Ampicillin, CEF: Cefamandole, CTX: Ceftriaxone, CEP: Cephalothin, CHL: Chloramphenicol, CIP: Ciprofloxacin, COT: Co-Trimaxozole, GEN: Gentamicin, KAN: Kanamycin, NA: Nalidixic Acid, NIT: Nitrofuratoin, SPT: Spectinomycin, SF: Sulfisoxazole, TE: Tetracycline, S: Streptomycin.