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# Molecular Identification of TEM and SHV Genes in Extended Spectrum Beta-lactamase Producing *Escherichia coli* and *Klebsiellae pneumoniae* Isolates in a Tertiary Care Hospital, Bangladesh

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**Extended spectrum β-lactamases (ESBLs) are enzymes encoded by mutation of a class of genes such as TEM, SHV, OXA and CTX etc. in gram negative *Enterobacteriaceae*. In this study, the first report was focusing on the multidrug resistance in *Escherichia coli and Klebsiella pneumoniae* producing extended spectrum β-lactamases (ESBLs) encoded from mutated TEM and SHV genes. Out of 108 bacterial isolates, 79% were found as ESBL positive and 21% were ESBL negative; 42% were *E. coli*, 37% were *K. pneumoniae* by chromogenic agar plate method. Further in disk diffusion method, 75% were ESBLs positive strains and 25% were ESBLs negative. The 50 strains (25 *E. coli* and 25 *K. pneumoniae*) were examined for Multiplex PCR to detect TEM and SHV genes. Out of 50 samples, 41 strains were PCR positive. In case of *E. coli,* 36% contained both TEM and SHV genes; 40% contained TEM gene; 8% contained SHV gene; 16% were PCR negative and contained others genes. In case of *K. pneumoniae,* 40% strains contained both TEM and SHV genes; 28% contained TEM gene; 12% contained SHV gene; 20% were PCR negative for and contained others genes. The length of TEM gene was around 1100-1200 bp and SHV around 450-500 bp determined comparing with 1 kb and 200 bp ladder respectively. ESBLs production is significant in phenotypic laboratory screening. Presence of both TEM and SHV genes in ESBLs producing bacteria was more common. Length of TEM gene was higher than SHV gene.**

**Keywords:** ESBLs, Cephalosporins , Monobactams, DNA, PCR, TEM and SHV.

Bacteria including the Enterobacteriaceae family have been proclaimed globally as etiologic factors of many nosocomial and community infections [1]. ESBLs are rapidly evolving group of β-lactamase and morally recognized in all the family of Enterobacteriaceae. These enzymes are most commonly produced by *Klebsiella* sp., *Escherichia coli* and other species such as *Proteus sp., Speudomonus sp., Morganella sp., Providencia sp.* etc[2]. ESBLs are the key regulator of resistance to beta-lactam antibiotics, cephalosporins and oxyimino monobactams and permit hydrolysis of it[3]. These enzymes are known to either plasmid or chromosomal encoded (as a result of transposons) and derived from the point mutation of classical genes resulting substitution of one or more amino acids around

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the active site of the parent beta-lactamase (TEM-1, TEM-2 and SHV-1) can induce a horizontal spreading of antibiotic resistance among bacterial strains[4,5,6]. Others genes such as OXA, CTX etc. are also responsible in ESBLs production. The comprehensive, excessive and uncontrolled use of antibiotics (3rd generation cephalosporins and oxyimino monobactams) is virtually believed to be the fundamental cause of mutation in the classical TEM and SHV genes[7]. The Enterobacteriaceae producing ESBLs have become a severe problem of public health in hospitals and community and reported worldwide but there have been limited data of the molecular evidence in Bangladesh. The first ESBLs were described in Germany in 1983. It was isolated from *E. coli* in patient named Temoniera and TEM was arrived from. The name of SHV beta-lactamase is due to sulfhydryl variable group in the active site[2,3,8]. Infections caused by Enterobacteriaceae rods are crucial to manage because of the reduction of therapeutic possibilities, resulting from constantly increasing resistance of these organisms to antibiotics[3]. More than 200 different types of ESBLs have been reported around the world so far. ESBLs are mostly found in *E. coli* and *K. pneumoniae*[2,3]. ESBLs producing bacteria cause many serious infections including urinary tract infections, peritonitis, cholangitis and intra-abdominal abscess[9,10].

Treatment of the infections caused by these organisms is a major challenge for health care facilities and preventive strategies[11]. Phenotypic methods cannot discriminate ESBL types. Molecular methods like isoelectric focusing, PCR and DNA sequencing need to be used for detection and typing of different ESBL enzymes. In this study, PCR based gene identification was implicated[12]. The study was undertaken to look for TEM and SHV genes in ESBLs producing *E. coli* and *K. pneumoniae* isolated from in patients and outpatients suffering from infections of blood, urinary tract and wound etc. in a tertiary care hospital, Bangladesh.

**Sample collection**

A total 108 samples (blood, swab and urine) were collected from the infected patients resistant to 2nd and 3rd generation antibiotics at the department of Microbiology in a tertiary care Hospital, Bangladesh from April 2014 to September 2014.

**Isolation of ESBLs producing strains**

Samples primarily were grown in nutrient agar media. Then ESBLs strains were isolated by two methods i.e. chromogenic agar plate method and disc diffusion test. In chromogenic agar plate method, colony grown in nutrient agar medium were streaked in HiCrome ESBL agar base media (HiMedia, India) containing supplemented antibiotics (three cephalosporins i.e ceftazidime (1.5mg/500ml), cefotaxime (1.5mg/500ml), ceftriazone (1 mg/500ml), and oxymino monobactam aztreonam (1 mg/500ml) and one antifungal fluconazole (5 mg/500ml) were used to avoid the contamination. ESBLs strains were grown and non-ESBLs strains were inhibited. Reference strain was used as control.

*E. coli* and *K. pneumoniae* react with chromogenic substances from the ESBL medium and developed respective color according to instruction of the medium. Chromogenic mixture acts as indicator to select the ESBLs producing bacteria. Control strains were *K. pneumoniae* ZJ-02 and *E. coli* ATCC 25922 and act as negative control were inhibited. *E. coli* appeared pink to purple, *K. pneumoniae* was bluish green colony in the ESBL medium.

Colony grown in HiCrome ESBL agar base media was further screened by disc diffusion method. In this test same antibiotics were used but different doses according to CSLI guideline[13]. In this method disc were prepared as ceftazidime 30 µg, cefotaxime 30 µg, ceftriazone 30 µg, and aztreonam 30 µg. The standard antibiotic disc were obtained from Mast Diagnostics (Mast Group, UK). According to the CLSIs guidelines, isolates show zone of inhibition of ≤ 22 mm with ceftazidime (30 µg), ≤25 mm with ceftriaxone (30 µg), ≤ 27 mm with cefotaxime (30 µg) , ≤ 27 mm with aztreonam (30 µg), was identified as potential ESBL producers and short listed for confirmation of ESBL production[13].

**Preparation of genomic DNA**

50 strains (25 *E. coli* and 25 *K. pneumoniae*) were randomly selected for genomic DNA isolation. Genomic DNA was isolated from ESBLs bacterial broth culture using Jena Bioscience kit. The DNA was run on 0.8% agarose gel and observed under UV light trans-illuminator.

**PCR for ESBLs encoding genes**

TEM and SHV genes were detected by PCR analysis. Primer used in this experiment were *bla*-TEM F (5'AAAATTCTTGAAGACG 3'); *bla*-TEM R (5'TTACCAATGCTTAATCA3') and *bla*-SHV F (5'TTAACTCCCTGTTAGCCA 3'); *bla*-SHV R (5'GATTTGCTGATTTCGCCC 3'). Primers obtained from Invent Technology, UK. A total 50 µl was prepared; DNA template 2 µl, master mix 25 µl, primer forward 1 µl, primer reverse 1 µl, nuclease free water 21 µl. Amplification were performed in sure cycler 8800 (Agilent Technology). The PCR mixture (Promega, USA) consisted of 10 µmol of each primer, 1µl DNA sample (3 µg/ml), 3 mM $MgCl_2$, 400 µM each dNTP and 50U Taq DNA polymerase in the total number of 50 µl of PCR reaction.

The parameters were Hot start at 95°C for 3 min, denaturation at 95°C for 30 seconds, annealing at 51°C for TEM for 30 seconds and 53°C for SHV for 30 seconds , Elongation 72°C for 30 seconds, Final elongation at 72°C for 7 minutes. The PCR products were run on 1.5% agarose gel by 1 kb ladder (Roth, Germany) and 200 bp ladder (Jena Bioscience) respectively. Photograph of bands was captured by using gel documentation system (Protein Simple). It was done to find out the gene either present or absent in the ESBL strains. Band of PCR product present on the gel indicates the gene responsible for ESBLs production was present in the strain and band absent indicates the absence of the gene.

## RESULTS

In phenotypic ESBLs screening by chromogenic agar plate method, 79% (85 of 108) were found as ESBL positive; 42% (36 of 85) was *E. coli*, 37% (31 of 85) was *K. pneumoniae* and 21% (18 of 85) were others ESBLs positive strains such as *Proteus sp., Morganella sp., Providencia sp.* etc, 21% (23 of 108) non-ESBL producing strains can't grow on ESBL agar base medium. In this study, *E. coli* appeared purple to pink colored colony and *K. pneumoniae* was bluish green after using chromogenic mixture.

In ESBLs conformation test by disc diffusion test, zone of inhibition by 30 µg dose of cephalosporins was less than 22 mm and these strains were found as ESBL producing. Some strains exhibit more than 40 mm clear zone of inhibition by the recommended dose as ceftazidime 30 µg, cefotaxime 30 µg, ceftriazone 30 µg and aztreonam 30 µg which were shown as ESBLs positive by chromogenic agar plate method (i.e. were grown on Hicrome ESBL agar base). So, these were the non ESBLs producing strain, zone of inhibition was more than 40 mm by recommended dose.

In disc diffusion test, the number of ESBLs producing strains isolated somewhat different from chromogenic agar plate method. In this method, 75% were ESBLs positive (81 strains of 108) and 25% were ESBLs negative (27 strains of 108).

The 50 strains (25 of *E. coli* and 25 of *K. pneumoniae*) were randomly selected for genomic DNA isolation among all ESBLs positive strains and PCR for TEM and SHV genes were carried out. Annealing temperature in our analysis for TEM gene was 51°C and that for SHV was 53°C. In PCR analysis, out of 50 samples, 41 strains were PCR positive. In case of *E. coli*, 36% (9 of 25) strains contained both TEM and SHV genes, 40% strains (10 of 25) contained only TEM gene, 8% strains (2 of 25) contained only SHV gene, 16% strains (4 of 25 were PCR negative for TEM and SHV genes) contained others genes may be OXA, CTX, AmpC etc. In case of *K. pneumoniae,* 40% (10 of

**Table 1.** Phenotypic screening of ESBLs producing strains in Chromogenic Method

| Bacteria | ESBLs positive strains | Non-ESBL strains |
|---|---|---|
| Total | 79% (85 of 108) | 21% (23 of 108) |
| *E. coli* | 42% (36 of 85) | n/a |
| *Klebsiella pneumoniae* | 37% (31 of 85) | n/a |
| Other ssp. | 21% (18 of 85) | n/a |

**Table 2.** Incidence of TEM and SHV genes in ESBLs producing *E. coli* and *K. pneumonia*

| Sample | Name of Strains | Only TEM gene | Only SHV gene | Both TEM & SHV genes | PCR Negative; (OXA, CTX, etc.) |
|---|---|---|---|---|---|
| 1. S-1 | *K. pneumoniae* | - | - | Yes | - |
| 2. S-2 | *K. pneumoniae* | - | - | Yes | - |
| 3. S-3 | *E. coli* | Yes | - | - | - |
| 4. S-4 | *K. pneumoniae* | - | - | Yes | - |
| 5. S-5 | *K. pneumoniae* | - | - | - | Yes |
| 6. S-6 | *E. coli* | Yes | - | - | - |
| 7. S-7 | *K. pneumoniae* | - | | Yes | - |
| 8. S-8 | *E. coli* | Yes | - | | - |
| 9. S-9 | *E. coli* | - | | - | Yes |
| 10. S-10 | *K. pneumoniae* | - | - | Yes | - |
| 11. S -15 | *K. pneumoniae* | Yes | - | - | - |
| 12. S-17 | *E. coli* | - | - | Yes | - |
| 13. S-19 | *K. pneumoniae* | - | - | Yes | n/a |
| 14. S -20 | *E. coli* | - | - | - | - |
| 15. S-21 | *E. coli* | - | - | Yes | - |
| 16. S-23 | *K. pneumoniae* | Yes | - | - | - |
| 17. S -24 | *E. coli* | - | - | - | Yes |
| 18. S -26 | *E. coli* | Yes | - | - | - |
| 19. S -27 | *K. pneumoniae* | | | | Yes |
| 20. S -29 | *E. coli* | - | - | Yes | - |
| 21. S-30 | *K. pneumoniae* | - | - | Yes | - |
| 22. S -32 | *K. pneumoniae* | - | Yes | - | - |
| 23. S-33 | *E. coli* | - | - | Yes | - |
| 24. S-41 | *E. coli* | Yes | - | - | - |
| 25. S-46 | *E. coli* | - | - | - | Yes |
| 26. S-47 | *K. pneumoniae* | - | Yes | - | - |
| 27. S-50 | *E. coli* | - | - | Yes | - |
| 28. S-51 | *K. pneumoniae* | Yes | - | - | - |
| 29. S -55 | *K. pneumoniae* | | - | - | Yes |
| 30. S -56 | *E. coli* | Yes | | - | - |
| 31. S -59 | *E. coli* | | Yes | - | - |
| 32. S-61 | *K. pneumoniae* | - | - | Yes | - |
| 33. S -62 | *E. coli* | Yes | - | - | - |
| 34. S-64 | *K. pneumoniae* | - | - | Yes | - |
| 35. S-65 | *E. coli* | Yes | - | - | - |
| 36. S-68 | *K. pneumoniae* | Yes | - | - | - |
| 37. S -70 | *E. coli* | Yes | - | - | - |
| 38. S-71 | *E. coli* | - | - | Yes | - |
| 39. S-72 | *K. pneumoniae* | - | Yes | - | - |
| 40. S -74 | *K. pneumoniae* | Yes | - | - | - |
| 41. S-77 | *E. coli* | - | - | Yes | - |
| 42. S -79 | *E. coli* | - | - | Yes | - |
| 43. S-82 | *K. pneumoniae* | - | - | - | Yes |
| 44. S-84 | *E. coli* | - | Yes | - | - |
| 45. S-87 | *E. coli* | Yes | - | - | - |
| 46. S-91 | *K. pneumoniae* | | - | Yes | - |
| 47. S-88 | *K. pneumoniae* | Yes | - | - | - |
| 48. S-96 | *K. pneumoniae* | - | - | - | Yes |
| 49.S-100 | *E. coli* | - | - | Yes | - |
| 50. S-107 | *K. pneumoniae* | Yes | - | - | - |

25) strains contained both TEM and SHV genes, 28% strains (7 of 25) contained only TEM gene, 12% strains (3 of 25) contained only SHV gene, 20% were strains (4 of 25 were PCR negative for TEM and SHV genes) contained others genes may be OXA, CTX, AmpC etc.

Gene length of TEM was around 1100-1200 bp compared with 1 kb ladder which will be confirmed accurately after sequencing PCR product of the gene. In this way, gene length of SHV around 450-500 bp compared with 200 bp ladder which will be confirmed accurately after sequencing PCR product of the gene (Figure 4-11).

## DISCUSSION

Resistance to beta-lactam antibiotics, remarkably 3rd generation cephalosporins, of gram negative bacteria isolated from clinical samples has been increased worldwide[14,15]. ESBL is now a momentous problem in hospitalized patients throughout the world. The prevalence of ESBLs among clinical isolates varies worldwide and patterns are rapidly changing over time[16].

In phenotypic chromogenic agar plate method of ESBLs screening, out of the 108 isolates included in our study 79% (85 of 108) were found as ESBL positive. Bazzaz *et al.* revealed the incidence of ESBLs producing organism were 59.2%[17] where Chandra *et al.* exhibit 64.10% (100/156) of the isolates were ESBL producers with highest incidence of *K. pneumoniae*. In our study, the highest incidence of *E.coli* was noticed 42% (36 of 85) followed by *K. pneumoniae* 37% (31 of 85). Bogaerts *et al.,* acknowledged the similar result. In disc diffusion test, the number of ESBLs producing strains isolated somewhat different from chromogenic agar plate method. In comparison, 79% was ESBL positive and 29% ESBL negative in agar plate method and 75% ESBL positive and 25% ESBL negative in disc diffusion method.

A number of previous studies have showed the high prevalence of ESBL producing *E. coli* and *K. pneumoniae*[10]. In India, high prevalence of ESBLs producing *K. pneumoniae* strains has been reported by various groups[18,19,20]. Our result was collaborated with the work done by Rahman *et al.,* 2016 showed 43.21% *E. coli* ESBL producers[21]. So the rate of ESBL producing *E. coli* in these studies in Bangladesh is most similar. In our study,

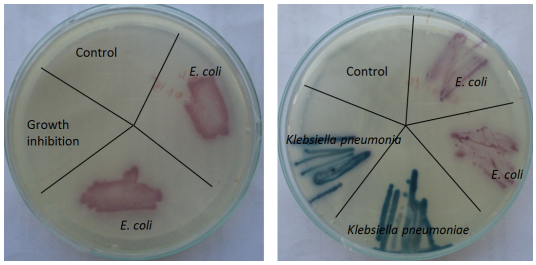


**Fig. 1.** Streaking of bacterial samples in Hicrome ESBL agar base medium

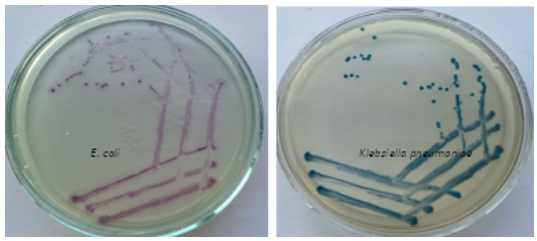


**Fig. 2.** Isolation of single colony of *E. coli* and *Klebsiella pneumoniae* in Hicrome ESBL agar base medium

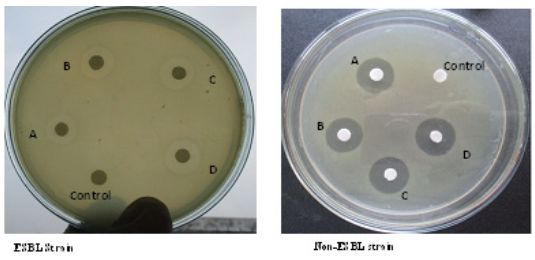


**Fig. 3.** ESBL conformation test by disk diffusion method as dose 30 µg and less than 22 mm zone of inhibition were found for ESBL producing strains. A= Ceftazidime 30 µg, B= Cefotaxime 30 µg, C= Ceftriazone 30 µg, D= Aztreonam 30 µg

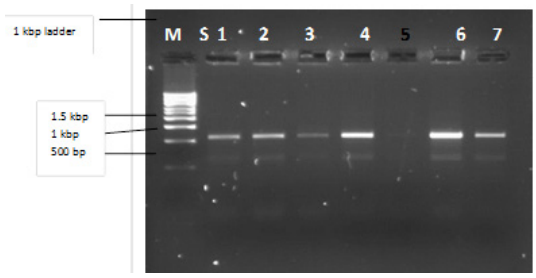


**Fig. 4.** PCR products (on 1.5% agarose gel) result of TEM gene of strain 1-7 by 1 kb ladder ( S= strain)

71% ESBLs positive was reported in disc diffusion method and 79% in agar plate method. Zaniani *et al.* noticed in his experiment that 43.9% of *E. coli* and 56.1% of *K. pneumoniae* produced ESBLs.

According to the CLSIs guidelines, isolates display zone of inhibition was less than 22 mm by the dose of 30 µg and these strains were counted as potential ESBLs producers in disk diffusion test. Some strains non ESBLs exhibit more than 40 mm clear zone of inhibition by the recommended dose as ceftazidime 30 µg, cefotaxime 30 µg, ceftriazone 30 µg and aztreonam 30 µg which were shown as ESBLs positive by chromogenic agar plate method (i.e. were grown on Hicrome ESBL agar base). So, these were the non ESBLs producing strain. There were some probable causes of the variation of two methods that are false positive result arising from high concentrated colony was picked and streaked, antibiotic can't be longer its activity in aqueous medium, during ESBL media preparation antibiotic must added when the media temperature exactly be 45-50°C[11].

ESBL positive *E.coli* rates were also relatively high in China (55.0%) and Thailand (50.8%) and Australia with higher rates of 20% to 50% in other parts of the continent[4,22]. The good news is that 100% ESBL producers were still susceptible to meropenem 5 µg and 10 µg.

In our study, genomic DNA was isolated instead of plasmid DNA, because some strains show negative result for TEM and SHV gene which showed positive result when genomic DNA used as PCR sample. Sharma *et al.* exposed that both plasmid DNA and chromosomal DNA were responsible for ESBLs production[23]. Amplification of whole genomic DNA increased the positivity of detection compared to amplification of plasmid DNA alone, suggesting β-lactemase expression controlled by both chromosomal and plasmid DNA[24,25,26].

In PCR analysis, for amplification of beta-lactemase genes, two set of primers of bla-TEM and bla-SHV were used. The importance of molecular diagnostics will increase as they are a more reliable method than phenotypic testing[27]. The present report is the first report of isolation of bla TEM and bla SHV genes from 50 ESBL strains of *E.coli* and *K. pneumoniae* isolated from the tertiary care hospital. Sometimes multiple genes are responsible for production of ESBLs

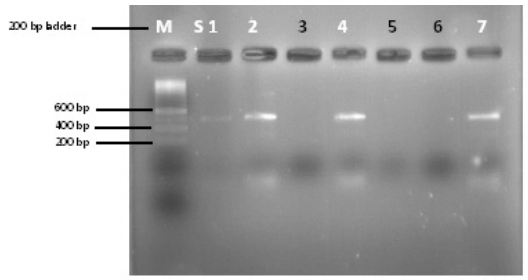


**Fig. 5.** PCR products (on 1.5% agarose gel) result of SHV gene of strain 1-7 by 200 bb ladder

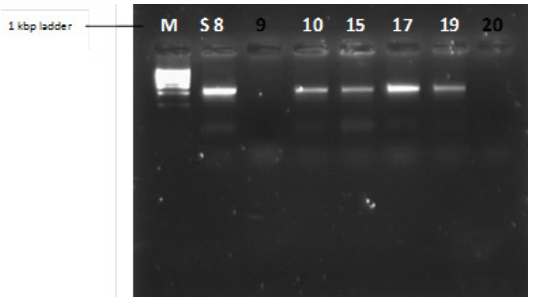


**Fig. 6.** PCR product (on 1.5% agarose gel) result of TEM gene of strain 8~20by 1 kb ladder

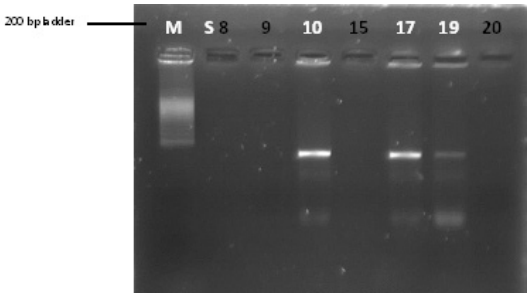


**Fig. 7.** PCR product (on 1.5% agarose gel) result of SHV gene of strain 8~20by by 200 bb ladder

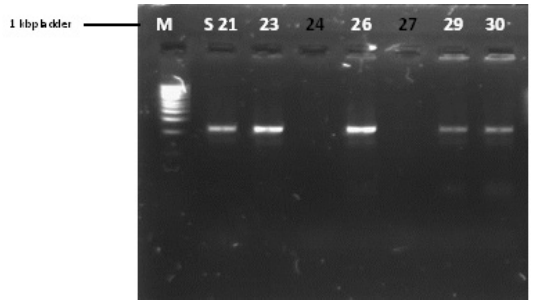


**Fig. 8.** PCR product (on 1.5% agarose gel) result of TEM gene of strain 21~30by 1 kb ladder

in a single isolates[28]. Although several studies addressed the issue of the emergence of ESBL producing Enterobacteriaceae worldwide, very few data of genotypic prevalence have been published from this area still now. The genotypic methods help us to confirm the genes responsible for ESBL production.

In PCR analysis, 41 strains were PCR positive out of 50 samples. In case of *E. coli,* 36% strains contained both TEM and SHV genes, 40% strains contained only TEM gene, 8% strains contained only SHV gene. In case of *K. pneumoniae,* 40% strains contained both TEM and SHV genes, 28% strains contained only TEM gene, 12% strains contained only SHV gene. Our finding bears the majority of the ESBLs producing bacteria were *E. coli and K. pneumoniae* bearing both TEM and SHV genes were more common than other ESBLs producing genes. This first molecular characterization study to repeat ESBL types TEM and SHV genes in Enterobacteriaceae isolates from the Medical College Hospitals revealed that TEM gene is highly prevalent among the isolates. Mutation of both TEM and SHV genes was more common. Our study was corroborated with other studies done in India[2]. The TEM gene has high frequency compared to SHV gene a fact which is similar to previous study[29,30].

Some ESBLs producing bacteria showed negative results in PCR method for TEM and SHV genes based on our study, therefore other beta-lactamases genes may be involved in ESBL resistance. However, further studies are required for finding the other genes in ESBL producing *E. coli* and *K. pneumoniae*.

Annealing temperature in our analysis for TEM gene was 51°C and that for SHV was 53°C. Amita Jain *et al.* exhibit 52°C annealing temperature for both TEM and SHV genes[31]. Jyoti Sharma *et al.* showed 50°C for both TEM and SHV genes[23].

ESBL producing isolates also appeared to be an important cause of infection among patients attended to in the medical and surgical facilities of the hospitals[32]. In the area of study, there is practice of sale of some antibiotics as over the counter drugs in some places as well as the indiscriminate practice of antibiotic prescription for patients with viral infections in the community and country. This selection pressure of use and overuse of antibiotics in the treatment of patients leads to the selection for new variants of the β-lactamases[32]. The selective pressure created by the use of third generation cephalosporins has also been described as one of the most important factors elsewhere[33]. Although there is still the need for sequencing of these ESBL producers, yet we report the first attempt to study the molecular characterization of ESBL subtypes and the epidemiology of ESBL producing *E. coli* and *K. pneumoniae* isolates in the tertiary care hospitals.

In our study, the length of TEM gene was found around 1100-1200 bp compared to 1

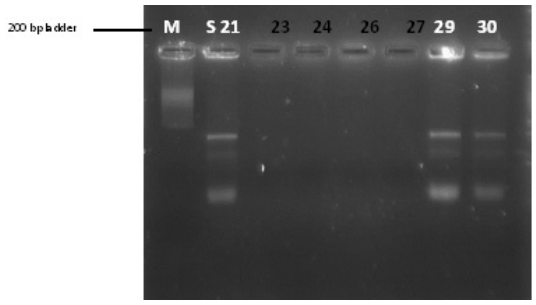


**Fig. 9.** PCR product (on 1.5% agarose gel) result of SHV gene of strain 21~30 by 200 bp ladder

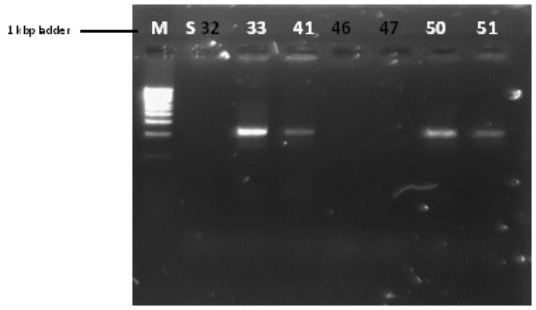


**Fig. 10.** PCR product (on 1.5% agarose gel) result of TEM gene of strain 32~51by 1 kb ladder

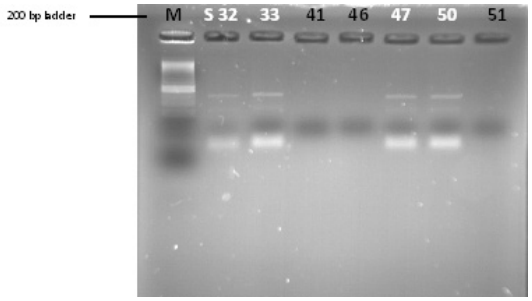


**Fig. 11.** PCR product (on 1.5% agarose gel) result of SHV gene of strain 32~51by 200 bb ladder

kb ladder and that of SHV 450-475 bps compared to 200 bps ladder. The length of TEM gene was 1079 bp and of SHV gene 930 bp[28]. Similar with PCR products with a length of 686 bp, 733 bp and 585 bp were purified from the agarose gel using Gel-Out Kit (A&A Biotechnology) and then sequenced using 3130xls Genetic Analyser (Applied Biosystems, USA)[33]. Amita Jain declared that genotypic characterization by PCR of all ESBL positive isolates of *klebsiella sp.* revealed that 867 bps and 930 bps amplified product were seen in isolates with bla-TEM and bla-SHV genes respectively[31]

ESBLs production is significantly high in phenotypic laboratory screening because of containing ESBLs producing gene such as TEM and SHV. The extent of resistance to 3rd generation cephalosporins was also high in these isolates. Presence of TEM and SHV genes in ESBLs producing bacteria was more common. It indicates that highly resistance resulting from misuse and excessive use of antibiotics and they are also affected by antibiotic residues used in animal or birds and antimicrobial preservatives in foods.

## CONCLUSION

In conclusion, we reported the presence of TEM and SHV genes in ESBL producing *E. coli* and *K. pneumoniae* isolates from infected patients both hospitalized and out patients in the tertiary care Hospital, Bangladesh. Drug resistance was high in isolates having both TEM and SHV genes. PCR showed TEM and SHV gene present in the strains. Length of TEM gene was higher than SHV gene. But point mutation which turns beta-lactamase into ESBLs will be known by sequencing of PCR product.

## ACKNOWLEDGEMENT

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