Genotypic Characterization Of Virulence Factor Afa, Papc, Sfa And Molecular Detection Of Bla $_{TEM}$, Bla $_{SHV}$, Bla $_{CTX-M}$ Genes According To Phytogenetic Group In Extended Spectrum B- Lactamase (ESBL) *Escherichia Coli* Strains Isolated From Urinary Tract Infection In Kerala Patients

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Abstract

Aim: Drug resistance among gram positive *Escherichia coli* bacteria is a worldwide challenge. Due to the importance of antibiotic resistant *E.coli* strain in hospital acquired Urinary Tract Infection. The present study was determine the genotypic characterisation detection of virulence factor, molecular phylogenetic group and ESBL genes producing isolated obtained from hospitalized patients in Kerala

Material and method: In total (123/ 65) *Escherichia coli* isolated from years of April 2022 to May 2023 were collected from various infections genotypic identification of bacterial isolated was performed using standard method. Genotypic screening of virulence factor afa, papC, sfa and molecular detection ESBL genes bla$_{TEM}$, bla$_{SHV}$, bla$_{CTX-M}$ genes was also performed by the PCR & phylogenetic group method.

Results: The number of virulence factor genes afa, papC, sfa, eight isolated were multiplex PCR using amplification specific genes, five different UTIs genotypes patterns were observed and bla$_{TEM}$, bla$_{SHV}$, bla$_{CTX-M}$ was the most prevalent ESBL genotypes TEM (-890.46), SHV (1162.03), CTX-M (-2778.42) and nucleotides 41, 23, 50. This may be because of the reduction in initial mass taken for RNA upon antibiotic treatment. Expression of CTX-M and TEM was comparatively higher than SHV gene.

Conclusion: The emergence of ESBL – producing *E. coli* isolates with high antibiotic – resistant rates to commonly used antibiotics and increased predominance of major gene type's bla$_{CTX-M}$ is a serious concern to the clinicians and microbiologists. Periodic monitoring of antibiotic susceptibility and associated genes would help guide.

Keywords: Antimicrobial resistance, *Escherichia coli*, Extended-spectrum beta-lactamase, Molecular detection, Genotypic

INTRODUCTION

Urinary tract infections (UTIs) are one of the most common bacterial infections, and causative pathogen over 50% nosocomial UTI [1]. The production of ESBLs is a common resistant mechanism of UPEC [2]. *Escherichia coli (E. coli)* are one of the most significant members of the family Enterobacteriaceae which have been affected with the emergence of ESBLs. Similarly, some strains of E. coli disseminating high levels of β-lactamases by the mutations of genes have been considered as an important group globally. *E. coli* is an outstanding indicator species to investigate the transmission of antimicrobial resistance through faecal contamination of water [3].

Antibiotic resistance has formerly been considered as a clinical problem, but recently natural ecosystems have been viewed as a crucial reservoir of antibiotic-resistant genes. The occurrence of antibiotic-resistant bacteria in aquatic environments is increasing consistently [4]. The water bodies, after receiving huge amounts of urban wastewater, hospital waste, and animal waste, represent the repository of diverse *E. coli*. These water bodies play a pivotal role in transferring and spreading of the resistance genes in public health significant pathogens and emerging unique resistance mechanism in them [5]. A recent study indicated in vitro transmission of ESBL-encoding multidrug-resistant (MDR) plasmids from ESBL *E. coli* recovered from water to susceptible *E. coli* making them antimicrobial-resistant (AMR) organisms [6].
The most frequently detected and clinically important ESBLs belong to the Termoniera (TEM), Sulfhydryl variable (SHV) and Cefotaximase Munich (CTX-M) families. *Ecoli* strains are divided into three main phylogenetic groups TEM, SHV, CTX-M. Owing to these consequences, coupled with the increasing incidence it is important to carry out continuous surveillance on ESBL producing *Ecoli* in the hospital [7]. The virulence genes include adhesion (afa, sfa, papC). Cases of UTI caused by ESBL-producing *E.coli* are increasing. Among ESBL genes, CTX-M, TEM, SHV are the major clinical concern, and ESBL- producing *E.coli* is prevalent in several countries in Asia region [8]. Antibacterial choice is often complicated by multidrug resistance. There is an increasing association between ESBL production and multidrug resistance [9].

The burden of infection caused by these resistant strains is enormous with a higher mortality rate, increased length of hospital stay and increased health costs with a view to providing comprehensive and reliable epidemiological information which will be used in the improvement of patient care and advice on antibiotic stewardship in clinical practices. The purpose of this study was to asses' correlatives antibiotic resistant, virulence potential, molecular detection and phylogenetic groups an ESBL producing UTIs isolated from long team hospitality patients.

MATERIALS AND METHODS

A total of 123/65 clinical sample sent to the microbiology laboratory were processed and cultured flowing standards microbiology techniques. UTIs patient urine sample isolated were obtained from various infection of hospitalized patient between April 2022 to May 2023 in Kerala.

DNA Isolation

All isolated were cultured on blood agar and incubated overnight at 37ºC. Genomic DNA was isolated from all strains with Wizard Genomic DNA purification kit (Promega, china) according to the manufactures instructions and used as template for PCR [10].

Virulence Factor Genes

Virulence genes were detected using a multiplex polymerase chain reaction assay developed by Johnson and Still [11]. This involved three primer pools with eight primers listed in order of decreasing amplicon size (bp) with in each pool as follows: pools, afa, papC, sfa. The genes encoding E.coli virulence genes were performed by single PCR as previously reported. The amplicons were electrophoresed in 2% agarose gels, stained with ethidium bromide, and detained with distilled water [12].

Molecular Detection

Molecular detection of bla $_{TEM}$, bla $_{SHV}$ and bla $_{CTX-M}$ were done by multiplex polymerase chain reaction (PCR). The extraction of the DNA was done with mini- prep kit (Jene Bioscience, Jena, Germany) according to manfacturers instructions. The primer used and their amplicon sizes. The PCR amplification was performed in 20 µl reaction mixture containing the Solis Biodyne hot start Master mix(ready –to-load) containing 200µM each deoxynucleoside triphosphates (Dntp), 2Mm $MgCl_2$,1×PCR buffer, 2 units of Taq DNA polymerase proof reading enzyme, 3µlof DNA (10-200ng), and sterile nuclease – free water wasused to make up the volume of the reaction mixture. The Thermal cycling was conducted in an eppendrorf thermal cycler (Nexus series) at an initial denaturation of 95ºC for 15 min, followed by 35 amplification cycles of 30 s at 95ºC; 30 s at 60ºC and I min at 72ºC. This was followed by a final extension step of 72ºC for 10 min. After amplification the product was separated on a 1.5% agarose gel electrophoresis and visualized by eithidium bromide staining. 100 base pair DNA ladders (Thermo Scientific) were used as DNA molecular weight standards. The DNA was analyzed using PCR for the presence of bla_{TEM} [13], bla_{SHV} [14], bla_{CTX-M} genes [15].

Phylogenetic Groups

Phylogenetic groups of the Ecoli isolates were determined using the polymerase chain reaction (PCR) - based method developed by Maximum likelihood method and Tamura Nei models [16]. Ecoli were categorized into one of the three main phylogenetic groups- TEM, SHV and CTX-M using three phylogenetic groups' markers A, B1, B2 and D. The groups were determined according to the different combinations of the three amplicons. Crude deoxyribonucleic acid (DNA) was prepared by lysis of colonies in 500µl of sterile distilled water at 100ºC for 15 min, followed by centrifugation. The lysis supernatant was used for the PCR. The PCR conditions were as follows: an initial activation at 94ºC for 4 min; then 30 cycles at 94ºC for 30 s, 65ºC for 30s, 72ºC for 30 s; and finally, extension at 72ºC for 5 min [17].

Role of ESBL Genes in Resistance to Antibiotics

The role of ESBL genes in resistance to antibiotics was studied using Real-Time PCR. The real-time PCR technique is used to study the variation in gene expression of 3 MDR genes CTX-M, HSV and TEM of the isolates grown in the (1) presence and (2) absence of the piperacillin antibiotic (5µg/ml). The gene expression of MDR genes of the selected isolate was compared with that of controls or housekeeping gene (16S rDNA) of the same isolate [18]. For gene expression studies, total cellular RNA was extracted from sample W51 using TRIzol reagent. The expression analysis was carried out by the Real Transcription Polymerase Chain Reaction (RT-PCR) method using cDNA synthesised from RNA isolated from cultures treated in antibiotics (test) and their respective untreated culture [19]. The Ct (cyclic threshold) values of each test were recorded. The relative expression of the genes is calculated and documented as follows.

Relative expression=$2-\Delta\Delta Ct$

Where, $\Delta\Delta$Ct = ΔCt (treated)- ΔCt (untreated)

ΔCt (treated) = Ct of target gene (treated)- Ct of housekeeping gene (treated)

ΔCt (untreated) = Ct of target gene (untreated)- Ct of housekeeping gene (untreated)

STATISTICAL ANALYSIS

Data were entered and percentage calculations were analysed using the statistical package for social science (SPSS) version 22. Continuous variables were compared with Chi- sequre and multivariate logistic regression analyses to identify associated risk factors to ESBL producing Ecoli infections<0.05 was considered statistically significant.

RESULTS

The plasmid DNA was prepared from eight selected isolates Fig.1. The DNA samples were further used for the amplification of ESBL genes. The presences of virulence factor genes in genomic DNA of eight isolates were checked by multiplex PCR using amplification of specific genes sfa, afa and papC. Fig.2 & Table. 1. Shows the results showed clear bands at around 594 bps for afa, 410 bps for sfa and 205 bps for papC in all the isolates. BLAST result of Afa, papC, sfa sequence of U51 Isolate.

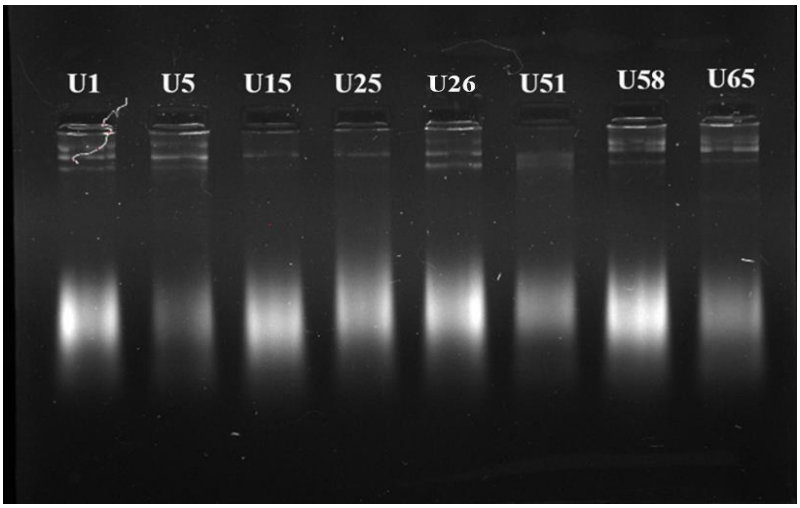

Fig.1. Plasmid DNA

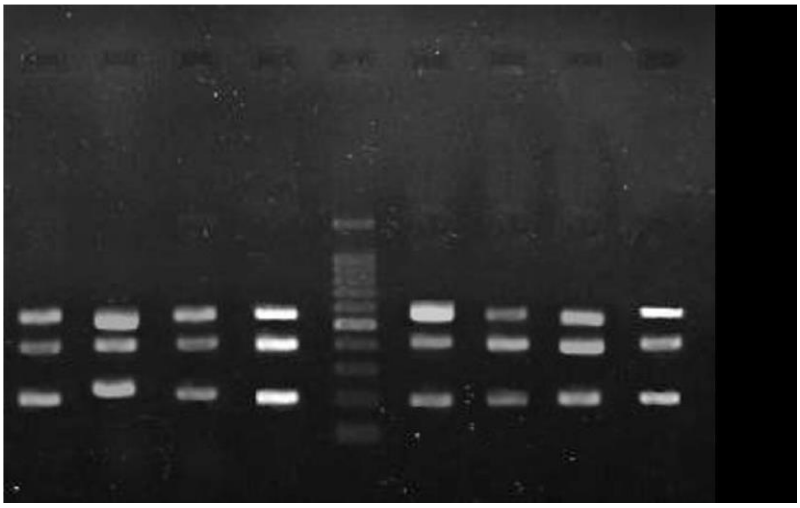

Fig.2. Amplification using Virulence Factor Genes Lane 1: U1 Lane 2: U5 Lane 3: U15 Lane 4: U25 Lane 5: 100 bp DNA Ladder Lane 6: U26 Lane 7: U51 Lane 8: U58 Lane 9: U65

Table. 1. BLAST result of Primers Virulent genes *Escherichia coli* U51 strain

SNo	Primers Virulent genes	Oligonucleotide sequence 5^{1}--3^{1}	Size (bp)	specificity
1	afa	F- GGAGCGTGAATATATTGCGCC R-CCGTGAATATCCGCTGCCTG	594	ON148408
2	papC	F- GCCGGCTACACTGACACGTTCAATG R- AGTGGCGGTGGACTGACA	410	ON148409
3.	sfa	F- CTGGCGAGGTTTAAGGCCACAC R-CAAATGGACAGGTA	205	ON148410

The presence of ESBL producing genes in eight isolates were checked using amplification of specific genes bla $_{TEM}$, bla $_{SHV}$ and bla $_{CTX-M}$. Fig.3, 4, 5, & Table.2. Shows the results clear bands at around 800bps for bla $_{TEM}$, 713 bps for bla $_{SHV}$ and 688 bps for bla $_{CTX-M}$ in all the isolates.

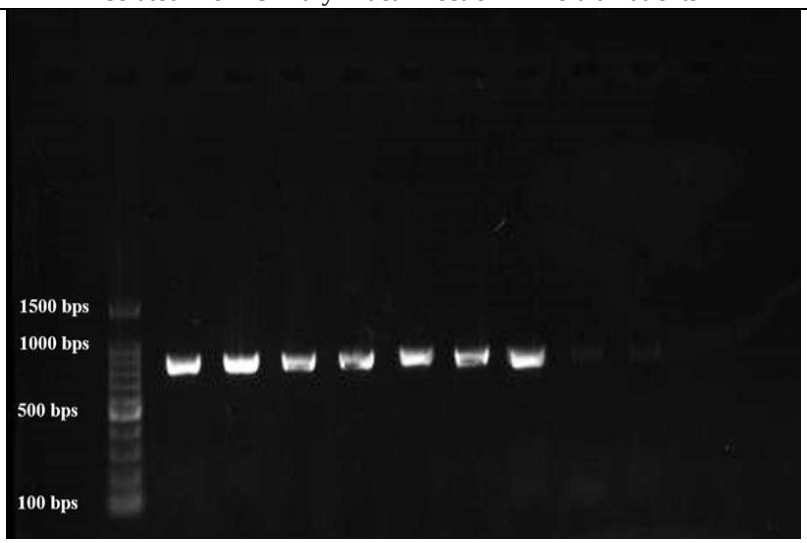

Fig.3. Molecular detection and Amplification using TEM Primer of ESBL genes Lane 1: 100 bp DNA Ladder Lane 2: U1 Lane 3: U5 Lane 4: U15 Lane 5: U25 Lane 6: U26 Lane 7: U51 Lane 8: U58 Lane 9: U65

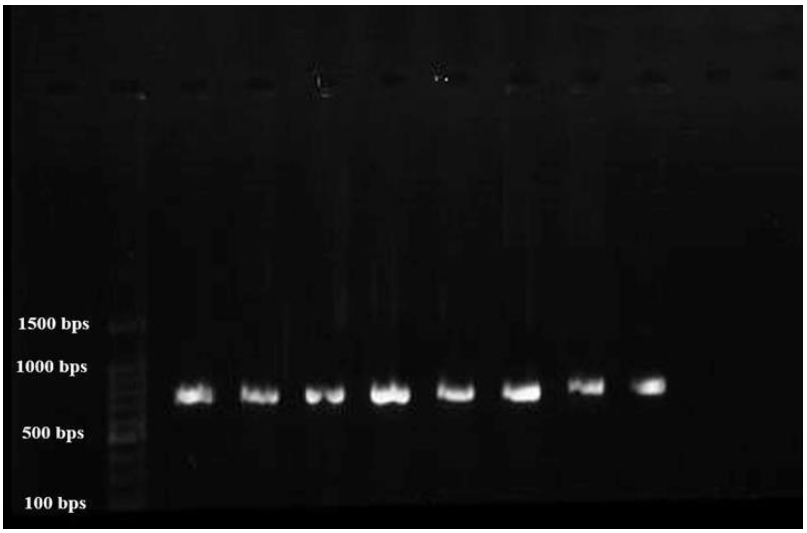

Fig.4. Molecular detection and Amplification using SHV Primer of ESBL genes Lane 1: 100 bp DNA Ladder Lane 2: U1 Lane 3: U5 Lane 4: U15 Lane 5: U25 Lane 6: U26 Lane 7: U51 Lane 8: U58 Lane 9: U65

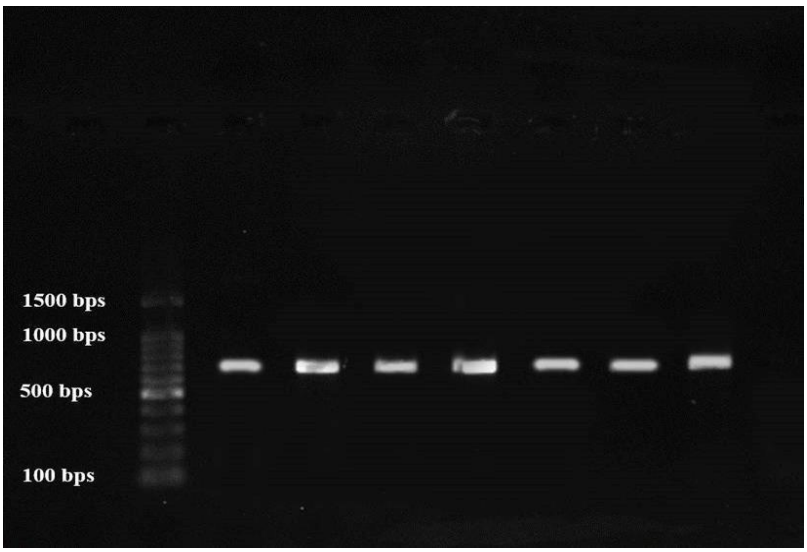

Fig. 5. Molecular detection and Amplification using CTX-M Primer of ESBL genes Lane 1: 100 bp DNA Ladder Lane 2: U1 Lane 3: U5 Lane 4: U15 Lane 5: U25 Lane 6: U26 Lane 7: U51 Lane 8: U58 Lane 9: U65

Table. 2. BLAST result of Primers molecular detection *Escherichia coli* U5 strain

SNo	Primers molecular detection	Oligonucleotide sequence $5^{1-}3^{1}$	Size (bp)	spescificity
1	TEM	F- AAAGTAAAAGATGCTGAAGATCAG R- CGCCTTGATCGTTGGGAACCGGAGCTGA	800	OM965357
2	SHV	F- GAGCAAATTAAACTAAGCGAAAGC R- TGGCCAGCGGCCGCACGCTGAC	713	OM965358
3.	CTX-M	F- AGGCAGACTGGGTGTGGCAT R- TCGTGCTGATGAGCGCTTTGCGA	688	OM965359

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood for bla $_{TEM}$ (-890.46), bla $_{SHV}$ (-1162.03), bla $_{CTX-M}$ (-2778.42) is shown Fig.6, 7, 8. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 41, 23, 50 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 342,656,582 positions in the final dataset. Evolutionary analyses were conducted in MEGA –X.

Fig.6. Phylogenetic Grouping of TEM Gene

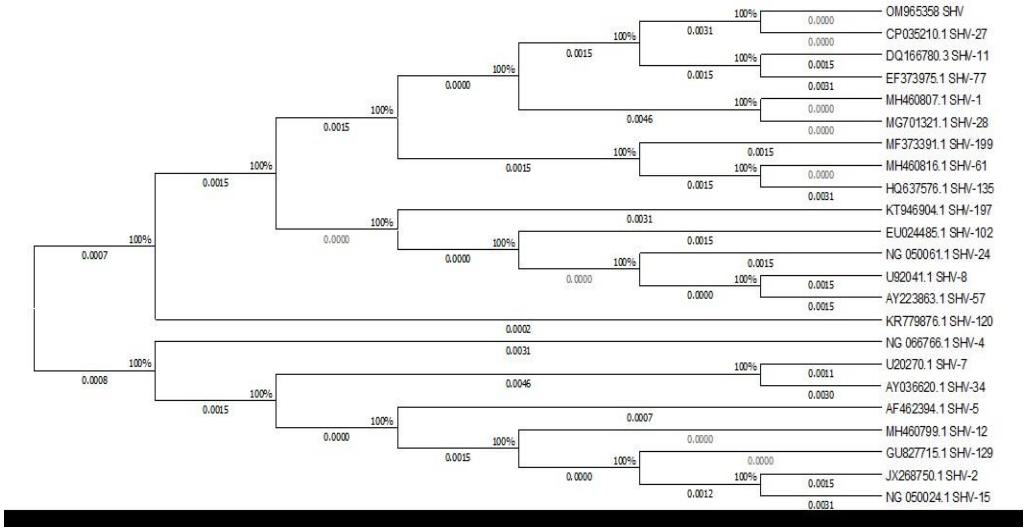

Fig.7.Phylogenetic Grouping of SHV Gene

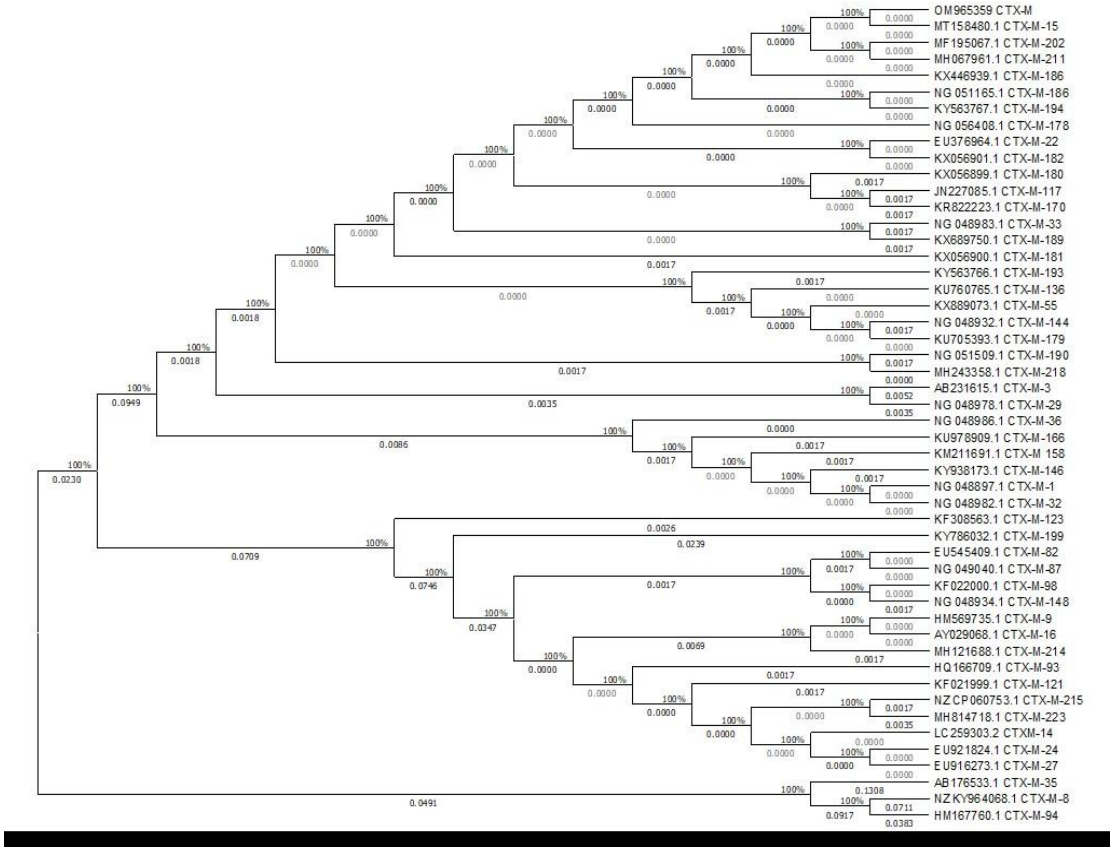

Fig.8. Phylogenetic grouping of CTX-M Gene

The relative expression level was measured using Ct values obtained from amplification plots and expression chart is documented. The Ct values indicate that the gene expression was less and delayed in treated test compared to that of treated control. Gene expression of treated was less compared to the untreated in all the three genes indicated. This may be because of the reduction in initial mass taken for RNA upon antibiotic treatment. Expression of bla $_{CTX-M}$ and bla $_{TEM}$ was comparatively higher than bla $_{SHV}$ gene Fig. 9, 10, 11, 12.

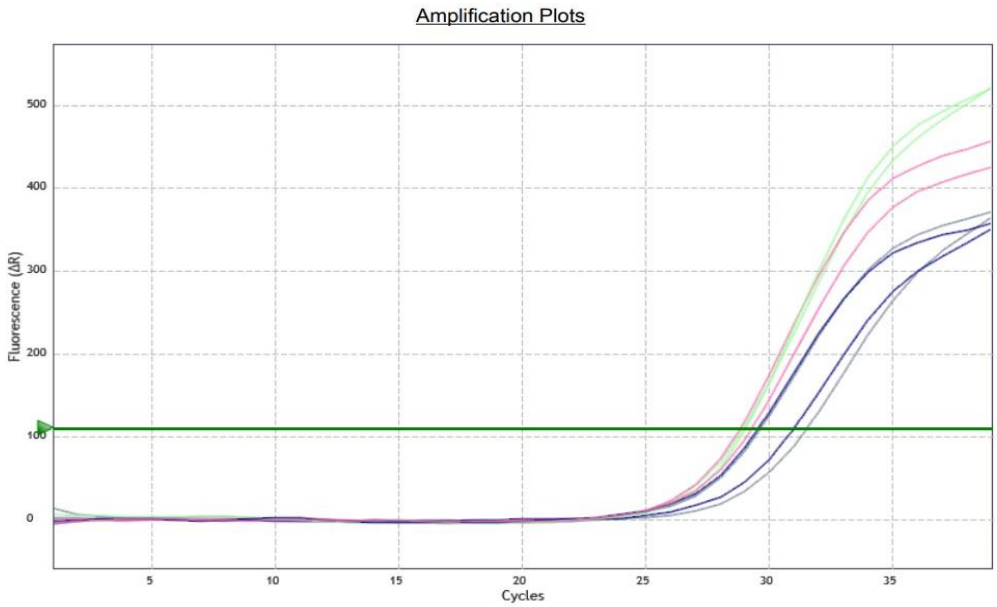

Fig.9. Gene Expression Using Quantitative Real Time PCR for TEM

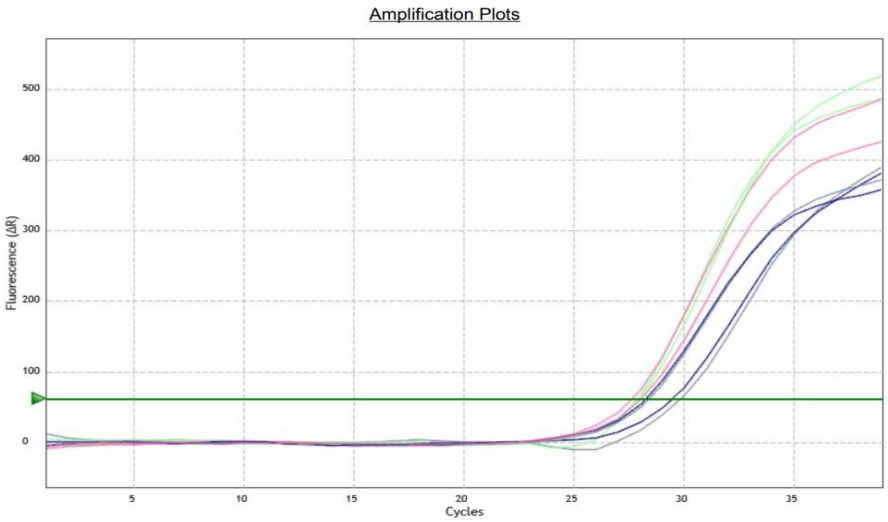

Fig.10. Gene Expression Using Quantitative Real Time PCR for SHV

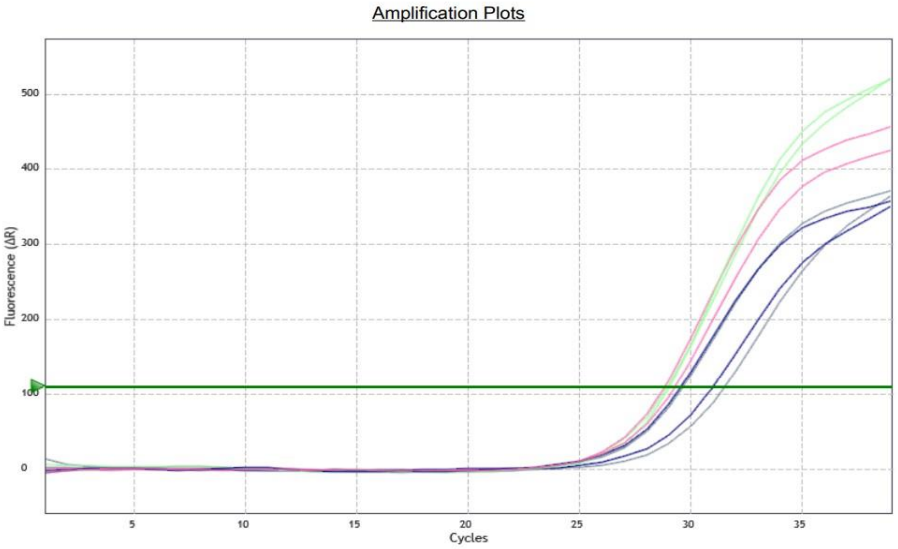

Fig.11. Gene Expression Using Quantitative Real Time PCR for CTX-M

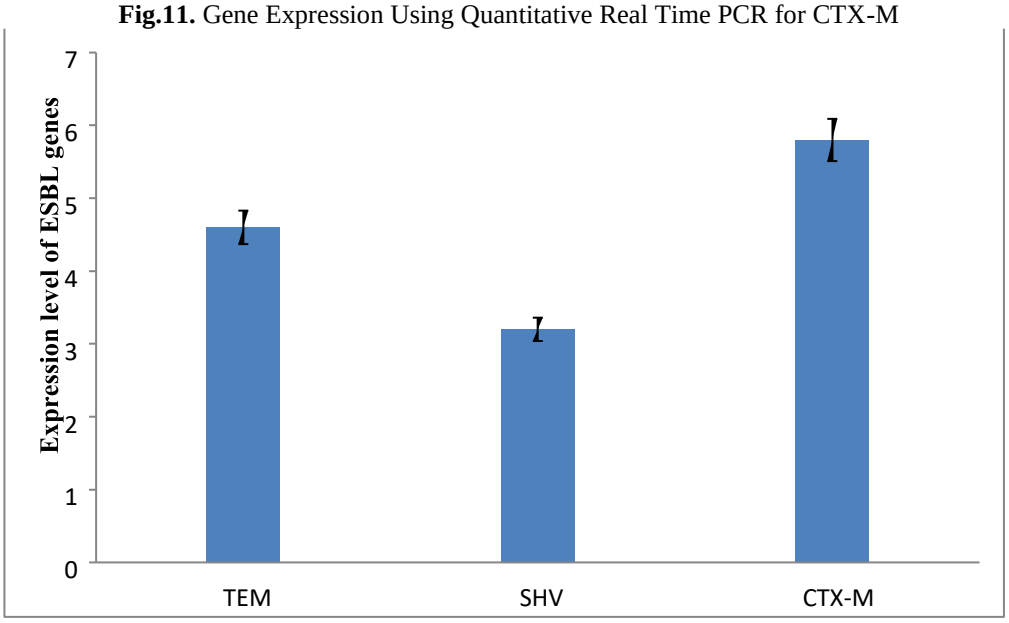

Fig. 12. Relative expression chart for ESBL genes

DISCUSSION

ESBL genotyping results showed that UTIs isolates carried different type ESBL genes, and 90.8% were afa, papC, sfa positive. Moreover, eight different ESBL genotype patterns were observed amongst them. Similar to other studies [22-24], we found that afa, papC, sfa type was the most prevalent ESBL genotype (47.5%, 53/123), and majority of UTIs isolates possess more than one ESBL genes. Therefore, the possible role of these genes either alone or in combination for ESBL cannot be ruled out. *E.coli* strains are divided into three main phylogenetic groups designed A, B & C and the most *E.coli* strains responsible for UTI belong to group C and A [11].

The most prevalent gene found in 15 ESBL E. coli isolates from water samples during the current study was bla $_{TEM}$ (40%), followed by bla $_{CTX-M}$ (33.33%) which concurs previous report from Pakistan (Irfan et al. 2021). However, bla $_{SHV}$ gene could not be detected in the tested ESBL E. coli isolates in this study. Similar occurrence of ESBL-encoding genes was reported in Germany except for bla $_{SHV}$ gene (Savin et al. 2020). However, one study in contrary to the current study reported bla $_{CTX-M}$ as the most prevalent gene in Pakistan (Abrar et al. 2018). ESBL E. coli isolates negative for all the genes indicated that ESBL-encoding genes other than the three tested in the current study are also present in them. However, we tested for only three genes due to their widespread occurrence and clinical relevance in human population (Tissera & Lee 2013). These findings indicated that the ESBL E. coli isolates should be analysed for other ESBL-encoding genes to understand the true distribution of ESBL-encoding genes in *E. coli* isolates.

The results of ESBL-encoding genes in the current study were quite interesting as the most prevalent gene was bla$_{TEM}$ in the current study. Previously, bla$_{CTX-M}$ is considered as the most predominant gene present in the region (Abrar et al. 2019). This change in genomic epidemiology of ESBL *E. coli* suggested that the bacterial population in the environment may be facing selection pressure. The situation of AMR may become worse if it is true and this world can face a new pandemic in the form of AMR in near future.

CONCLUSION(S)

This study indicates that UTIs *Ecoli* show multidrug resistance, and co-resistance to other ESBL antibiotics. Virulence factor genes are the most prevalent ESBL genotype and majority of UTIs *Ecoli* more than one ESBL genes. *E coli* belong mainly to phylogenetic group A and B, and most of the virulence genes are more prevalent in group B2.These results suggest that resistance, virulence and phylogenetic groups are three different mechanisms for the outcome of UTIs. Phylogenetic distribution of virulence genes among ESBL-producing UTIs
E.coli isolated from long-term hospitalized patients in the study enhanced our current knowledge of the resistance, the pathogenicity and genetic characteristics of UTIs. Moreover, determining the correlation of resistance, virulence and phylogenetic groups is crucial for the prevention and control of nosocomial UTI caused by ESBL-producing *E.coli*.

Limitation

This study was limited by short duration and moderate sample. Ultimately, it is recommended to performing of the other studies on other genes between control and case groups.

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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