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Original Research Article

MOLECULAR DETECTION OF ESBL-ENCODING GENES AMONG CLINICAL ISOLATES OF ESCHERICHIA COLI: A MULTIPLEX PCR–BASED STUDY

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Abstract: Extended-spectrum β-lactamase (ESBL)-producing Escherichia coli represent a major global threat due to increasing antimicrobial resistance and rapid dissemination of β-lactamase genes within hospital and community settings. This study aimed to determine the distribution of ESBL genes (CTX-M, SHV, TEM) among clinical isolates of E. coli using conventional multiplex PCR. A total of 72 isolates were processed using a standardized proteinase-K-based DNA extraction method followed by multiplex PCR targeting ESBL, OXA, shiga toxin, and intimin genes. Among ESBL genes, CTX-M was detected in 36.1% of isolates, TEM in 75%, while all isolates were negative for SHV. Co-existence of CTX-M and TEM was common, observed in 33.3% of isolates. OXA-type carbapenemase genes were also detected, with OXA-58 present in 40.2%, OXA-G23 in 12.5%, and OXA-G48 in 33.3% of isolates. Intimin was detected in 15.2% and Stx1 in 9.7% of isolates. The prevalence of ESBL genes in our setting aligns with findings from several Indian and international studies, though variations reflect regional antibiotic-selection pressure. The high proportion of TEM and CTX-M producers highlights the need for stringent antimicrobial stewardship and molecular surveillance. Continuous monitoring of β-lactamase genes is essential to guide empirical therapy and reduce resistance dissemination.

Keywords: Extended-spectrum β-lactamase, Escherichia coli, ESBL genes

INTRODUCTION

Escherichia coli is one of the most common human pathogens responsible for a wide range of infections, including urinary tract infections, bloodstream infections, diarrhoeal disease, and nosocomial sepsis[1]. In recent decades, the emergence of antimicrobial resistance—particularly extended-spectrum β-lactamase (ESBL)-producing strains—has severely limited available therapeutic options[2]. ESBLs are enzymes capable of hydrolyzing third-generation cephalosporins and monobactams while remaining inhibited by β-lactamase inhibitors[3]. Molecularly, ESBLs are predominantly encoded by the **TEM**, **SHV**, and **CTX-M** gene families, with the latter now recognised as the most widely distributed globally[4]. CTX-M–type ESBLs have largely replaced earlier TEM and SHV variants due to rapid plasmid-mediated dissemination[5]. These β-lactamase genes

are frequently associated with mobile genetic elements including plasmids, transposons, and integrons, allowing horizontal transfer between species[6]. The widespread use and misuse of broad-spectrum antibiotics in hospitals, agriculture, and community health settings has exacerbated the selection pressure leading to rapid expansion of ESBL-producing *E. coli* worldwide[7]. The epidemiology of ESBL-producing *E. coli* varies across regions, influenced by local antimicrobial consumption, infection control practices, and clonal spread[8]. Studies from India consistently report high prevalence rates, often exceeding 50% in tertiary-care settings[9]. International surveillance programs likewise highlight rising ESBL rates, particularly CTX-M-type enzymes, which now dominate in Asia, Europe, and South America[10]. Timely detection of ESBLs is crucial for appropriate clinical management. Phenotypic tests such as combination disk tests and automated systems may detect ESBL activity; however, they cannot differentiate between specific ESBL families nor identify co-existing resistance mechanisms[11]. Molecular methods such as multiplex PCR offer rapid, sensitive, and specific detection of ESBL genes, enabling enhanced epidemiological surveillance and guiding infection-control interventions[12]. In addition to ESBL determinants, *E. coli* may carry other resistance genes such as OXA-type carbapenemases, virulence factors including shiga toxins (Stx1, Stx2), and the intimin gene (eae) associated with enteropathogenic *E. coli*[13]. Understanding the coexistence of resistance and virulence genes provides insight into the pathogenic potential and therapeutic challenges posed by these strains. Given the clinical relevance and growing prevalence of ESBL-producing *E. coli*, this study aimed to determine the distribution of major ESBL genes—**CTX-M, SHV, and TEM**—among clinical isolates using conventional multiplex PCR. The study also evaluated the presence of OXA carbapenemase genes and select virulence markers. By comparing our results with national and international studies, we aim to contribute meaningful data to the regional antimicrobial resistance landscape and support informed antimicrobial stewardship.

MATERIALS & METHODS:

Study Design and Setting: This laboratory-based observational study was conducted at the Central Research Laboratory (CRL), Maratha Mandal's NGH Institute of Dental Sciences and Research Centre, Belagavi, Karnataka. The study aimed to detect ESBL-encoding genes (CTX-M, TEM, SHV) among *Escherichia coli* clinical isolates using conventional multiplex polymerase chain reaction (PCR). Additional gene targets included OXA carbapenemase genes (OXA-58, OXA-G23, OXA-G48) and virulence-associated genes (Stx1, Stx2, eae). The procedures adhered strictly to standard operating protocols followed at the CRL.

A total of 72 *E. coli* isolates were included in the study. These isolates were received as slant cultures from the Department of Microbiology. Though phenotypic susceptibility data were not available, the isolates were processed specifically for molecular characterization of β-lactamase and virulence genes. All isolates were stored at 4°C until further processing and sub-cultured onto nutrient agar to ensure viability prior to DNA extraction. Genomic DNA was extracted using a modified Proteinase-K method as described in the CRL protocol. This method provides high-quality DNA suitable for PCR applications and is particularly useful when processing bacterial isolates from stored slants.

Sample Preparation

Each slant culture was transferred into a microcentrifuge tube containing Tris–EDTA (TE) buffer. Samples were vortexed and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded, and 500 µL of fresh TE buffer was added. This washing step was repeated 3–4 times to remove impurities and media components that could inhibit PCR.

Cell Lysis: After the final wash, the supernatant was discarded and the cell pellet was resuspended in 50 µL of Lysis Buffer I. The mixture was vortexed and incubated briefly for 5 minutes. Then, 50 µL of Lysis Buffer II and 10 µL of proteinase-K (10 mg/mL) were added. Tubes were vortexed vigorously to ensure thorough mixing and incubated in a water bath at 60°C for 2 hours to facilitate enzymatic digestion of proteins. Subsequently, tubes were transferred to a boiling water bath for 10 minutes to inactivate proteinase-K and lyse remaining cells.

DNA Recovery and Storage: The mixture was centrifuged briefly, and the supernatant containing purified genomic DNA was transferred to a fresh sterile tube. DNA samples were stored at −20°C until use.

PCR Primer Selection: Three separate multiplex PCR assays were performed for the detection of ESBL genes, OXA carbapenemase genes, and virulence genes. Primer sequences from the established CRL protocol were used to amplify specific gene targets with predetermined amplicon sizes: CTX-M (593 bp), SHV (747 bp), TEM (445 bp), OXA-58 (599 bp), OXA-G23 (482 bp), OXA-G48 (286 bp), Stx1 (180 bp), Stx2 (255 bp), and eae (384 bp)

PCR Reaction Preparation: PCR reactions were prepared using Amplicon Taq DNA Polymerase 2X Master Mix (RED), which contains Taq polymerase, dNTPs, $MgCl_2$, loading dye, and reaction buffer optimized for robust amplification.
For each 20 µL reaction:

- PCR Master Mix: 10 µL
- Forward primer (10 pmol): 1 µL
- Reverse primer (10 pmol): 1 µL
- Template DNA: 2 µL (10 pg–1 µg)
- Nuclease-free water: to make 20 µL

Reaction mixtures were vortexed gently, centrifuged, and placed on ice until amplification.

PCR Cycling Conditions

1. ESBL Gene Multiplex (CTX-M, SHV, TEM)

- Initial denaturation: 95°C × 5 min
- 30 cycles of:
  - Denaturation: 95°C × 30 sec
  - Annealing: 60°C × 30 sec
  - Extension: 72°C × 2 min
- Final extension: 72°C × 10 min
- Hold: 4°C

2. OXA Gene Multiplex (OXA-58, OXA-G23, OXA-G48)

- Initial denaturation: 95°C × 5 min
- 35 cycles of:
  - Denaturation: 95°C × 30 sec
  - Annealing: 60°C × 45 sec
  - Extension: 72°C × 1 min
- Final extension: 72°C × 5 min
- Hold: 4°C

3. Virulence Gene Multiplex (Stx1, Stx2, eae)

- Initial denaturation: 95°C × 5 min
- 35 cycles of:
  - Denaturation: 95°C × 1 min
  - Annealing: 66°C × 1 min
  - Extension: 72°C × 90 sec
- Final extension: 72°C × 5 min
- Hold: 4°C

PCR was performed using an Applied Biosystems Thermal Cycler.

Agarose Gel Electrophoresis: PCR products were analysed by 2.5% agarose gel electrophoresis using 1X TAE buffer. Ethidium bromide (0.5 µg/mL) was added to the gel for nucleic acid staining. A 100 bp DNA ladder was loaded to determine amplicon sizes. Twenty microliters of each amplified product was mixed with 2 µL loading dye and loaded into individual wells.

Electrophoresis was carried out at 80V for 90 minutes. Gels were visualized under a UV transilluminator using a gel documentation system (Major Science, USA). Bands corresponding to expected gene sizes were interpreted as positive.

Data Tabulation and Analysis: Results were tabulated gene-wise for all 72 isolates as per the PCR report. ESBL genes were the primary focus. Data were analysed for frequency distribution, co-existence of gene combinations, and comparison with external studies. No statistical software was used, as the study involved descriptive analysis only.
Ethical Considerations: The study involved archived bacterial isolates without patient identifiers. Ethical approval was deemed not required; however, institutional permission was obtained.

RESULTS

1. Overview of Gene Detection

A total of **72 clinical isolates of** *Escherichia coli* were analysed by multiplex PCR for ESBL genes (CTX-M, TEM, SHV), OXA carbapenemase genes, and select virulence factors. All isolates yielded interpretable PCR results.

2. Detection of ESBL Genes

2.1 CTX-M gene

CTX-M was detected in **26 of 72 isolates (36.1%)**.
These isolates showed clear amplification at 593 bp.
CTX-M positivity was often associated with concurrent TEM gene detection (details below).

2.2 TEM gene

TEM gene showed the highest prevalence among all ESBL markers.
It was detected in **54 of 72 isolates (75.0%)**.
This indicates that TEM is the predominant ESBL determinant in the present study population.
/

2.3 SHV gene

SHV was **not detected in any isolate (0%)**.
Absence of SHV suggests that this gene family does not significantly contribute to ESBL production in the current local epidemiology.

3. Co-existence of ESBL Genes

3.1 CTX-M + TEM dual positivity

A total of **24 isolates (33.3%)** were positive for both CTX-M and TEM.

This suggests frequent plasmid-mediated co-carriage, consistent with global trends of multi-ESBL gene dissemination.

3.2 TEM only

TEM alone (without CTX-M or SHV) was found in **30 isolates (41.6%)**, emphasizing its dominant role in β-lactam resistance.

3.3 CTX-M only

CTX-M alone (TEM-negative) was found in **2 isolates (2.7%)** — less common than dual ESBL gene carriage.

4. Detection of OXA Carbapenemase Genes

Although the primary focus of this study is ESBLs, the multiplex PCR also screened for OXA gene variants, yielding the following results:

OXA gene	n (%) positive
OXA-58	**29/72 (40.2%)**
OXA-G23	**9/72 (12.5%)**
OXA-G48	**24/72 (33.3%)**

Many isolates carried **multiple OXA genes**, suggesting possible early evolution toward carbapenem resistance, even though phenotypic resistance was not assessed.

5. Detection of Virulence Genes

Three virulence-associated markers were screened.

Virulence gene	n (%) positive
Stx1	7/72 (9.7%)
Stx2	2/72 (2.7%)
eae (intimin)	11/72 (15.2%)

The presence of these genes indicates that a minor subset of isolates may possess diarrheagenic *E. coli* virulence profiles.

6. Gene Co-existence Patterns

6.1 **ESBL + OXA**

A significant proportion of ESBL-positive isolates co-carried OXA variants. Co-occurrence of any ESBL gene with any OXA gene was observed in **41 isolates (56.9%)**

This highlights extensive accumulation of β-lactamase determinants.

6.2 ESBL + Virulence Genes

A smaller subset carried both resistance and diarrheagenic-associated virulence markers:

- **Stx1 + TEM** frequently occurred
- **eae + CTX-M or TEM** was detected in multiple isolates

This suggests simultaneous virulence–resistance evolution.

7. Frequency Summary Table

Gene category	Gene	Positive n (%)
ESBL genes	CTX-M	26 (36.1%)
	TEM	54 (75.0%)
	SHV	0 (0%)
Carbapenemase genes	OXA-58	29 (40.2%)
	OXA-G23	9 (12.5%)
	OXA-G48	24 (33.3%)

Gene category	Gene	Positive n (%)
Virulence genes	Stx1	7 (9.7%)
	Stx2	2 (2.7%)
	eae	11 15.2%)

Overall Interpretation: High prevalence of TEM (75%) indicates it is the dominant ESBL gene locally. CTX-M (36.1%) remains significant but is not the dominant ESBL type here, unlike many other regions where CTX-M predominates. SHV absent (0%) suggests regional variation in ESBL epidemiology. OXA-type genes widely present, which may indicate emerging carbapenem resistance reservoirs. Virulence gene carriage, although low, is epidemiologically relevant, indicating potential circulation of hybrid pathogenic/resistant strains. The over all summary of the genes were depicted in Table 1.

Table 1 : Distribution of ESBL genes among clinical isolates of E.coli

Gene Category	Gene	Amplicon Size (bp)	Number Positive (n = 72)	Percentage (%)
ESBL Genes	CTX-M	593 bp	26	36.1%
	TEM	445 bp	54	75.0%
	SHV	747 bp	0	0%
Co-existence (ESBL)	CTX-M + TEM	—	24	33.3%
	TEM only	—	30	41.6%
	CTX-M only	—	2	2.7%
Carbapenemase Genes	OXA-58	599 bp	29	40.2%
	OXA-G23	482 bp	9	12.5%
	OXA-G48	286 bp	24	33.3%
Virulence Genes	Stx1	180 bp	7	9.7%
	Stx2	255 bp	2	2.7%
	eae (Intimin)	384 bp	11	15.2%
Overall Gene Burden	≥1 ESBL gene detected	—	54	75%
	ESBL + OXA co-carriage	—	41	56.9%
	Any virulence gene detected	—	13	18.0%

DISCUSSION

Extended-spectrum β-lactamase (ESBL)–producing Escherichia coli represent a major therapeutic challenge due to their ability to hydrolyse β-lactam antibiotics and disseminate rapidly via plasmids. In this study, molecular detection using multiplex PCR revealed a high prevalence of TEM (75%), followed by CTX-M (36.1%), with no SHV genes detected. These findings provide insight into the local molecular epidemiology of ESBL determinants and reflect the ongoing evolution of β-lactamase gene distribution.

The predominance of TEM-type ESBLs in this study contrasts with the now globally dominant CTX-M lineage but aligns with several Indian studies. For example, Paterson et al. reported TEM as the most frequent ESBL determinant in northern India[1], while

Grover et al. observed TEM in 72% of E. coli isolates in a tertiary care center[2]. Similarly, a study from Karnataka by Ranjini et al. reported TEM prevalence of 68% among ESBL producers[3]. Two additional Indian reports—by Singh et al. (Delhi)[4] and Sharma et al. (Hyderabad)[5]—also documented high TEM frequencies (65–70%). Collectively, these findings suggest that while CTX-M has expanded nationwide, some regions continue to harbour dominant TEM-carrying plasmids. The 75% frequency in the current study is consistent with this regional pattern.

In contrast, several international studies reveal an opposite trend, with CTX-M overwhelmingly dominating. A large European surveillance report by Canton et al. noted CTX-M prevalence exceeding 80% among ESBL-producing E. coli[6]. A Chinese multicentric study identified CTX-M in 85% of isolates, with CTX-M-15 and CTX-M-14 being the most prevalent[7]. Similarly, research from Turkey reported CTX-M prevalence of 78%[8], while studies from Brazil[9] and Canada[10] also documented CTX-M as the predominant ESBL gene. Compared with the present study's CTX-M prevalence of 36.1%, these global figures highlight substantial geographical variation, likely influenced by antimicrobial practices, infection control programmes, and clonal spread of high-risk lineages such as ST131.

The absence of SHV genes in all isolates is also noteworthy. Although SHV-type ESBLs were historically associated with Klebsiella species, they have been reported sporadically among E. coli. Indian studies have typically reported SHV in only 5–20% of isolates[2,5]. Internationally, SHV prevalence remains similarly low, rarely exceeding 10–15%[10]. Thus, the complete absence of SHV genes in the present study aligns with the global trend of SHV declining in E. coli populations as CTX-M continues to dominate worldwide.

The co-existence of ESBL genes, particularly the CTX-M + TEM combination seen in 33.3% of isolates, has significant clinical and epidemiological implications. Co-carriage enhances resistance levels, broadens the substrate spectrum, and may facilitate horizontal transfer of multi-resistance plasmids. Indian studies have reported similar co-existence patterns: up to 40% dual ESBL gene carriage in reports from Vellore and Chennai[5]. International studies mirror this trend, with dual ESBL genes reported in 30–50% of isolates, especially in high-resistance settings[7,9].

Although ESBL genes were the main focus, the study also revealed a high prevalence of OXA-type β-lactamase genes, particularly OXA-58 (40.2%) and OXA-G48 (33.3%). While OXA carbapenemases are classically associated with Acinetobacter species, plasmid-associated OXA variants in E. coli have been increasingly documented. The presence of these genes in over half the isolates raises concern about possible future carbapenem resistance. Indian reports on OXA genes in E. coli remain limited, but sporadic detection has been documented in Mumbai and Bangalore studies[11]. Internationally, OXA-48–like genes are well-established contributors to carbapenem resistance in Europe and the Middle East[12]. Although phenotypic carbapenem susceptibility was not assessed here, the genetic markers indicate potential risk for emergent carbapenemase activity.

The detection of virulence genes (Stx1 in 9.7%, Stx2 in 2.7%, eae in 15.2%) indicates that a minority of isolates may resemble diarrheagenic E. coli pathotypes. Co-carriage of ESBL and virulence genes is an emerging global threat, with several studies documenting hybrid strains that combine resistance with high pathogenicity. Such strains complicate therapy and may contribute to outbreaks. Indian studies from Vellore and Kolkata have reported 5–10% prevalence of shiga-toxin genes among extraintestinal isolates[13]. Internationally, shiga-toxin–producing ESBL E. coli strains have been reported in Europe and Japan[14], underscoring the need for ongoing surveillance.

The absence of phenotypic antimicrobial sensitivity data is a limitation. Nonetheless, molecular detection provides highly specific insight into the genetic reservoir of resistance. The high prevalence of TEM and substantial presence of CTX-M indicate a heavy β-lactamase burden, which likely translates into resistance to third-generation cephalosporins. The co-existence of OXA-type genes further suggests potential future carbapenem resistance even if not yet phenotypically evident.

The findings emphasize the need for robust antimicrobial stewardship policies. In many Indian settings, indiscriminate broad-spectrum antibiotic use—including third-generation cephalosporins, fluoroquinolones, and carbapenems—exerts strong selection pressure favoring ESBL and OXA-producing organisms. Regional molecular surveillance is essential to monitor trends, detect high-risk clones, and implement targeted infection control interventions.

Comparative analysis with both Indian and international literature reveals that the molecular epidemiology of ESBL genes is highly region-specific. While TEM predominance here contrasts with the global CTX-M surge, it aligns with patterns seen in some Indian states, suggesting that plasmid diversity and regional antibiotic practices may influence gene distribution. The lack of SHV genes

and moderate CTX-M rates highlight a unique molecular profile that warrants continued monitoring.

In conclusion, the study demonstrates a high prevalence of ESBL genes, particularly TEM, with significant co-existence of multiple β-lactamase determinants and substantial presence of OXA enzymes. The detection of virulence genes in a subset of isolates adds further clinical relevance. Together, these findings underscore the importance of integrating molecular diagnostics into routine surveillance to guide appropriate antibiotic policies and curb the spread of multidrug-resistant E. coli.

CONCLUSION:

The molecular profile emerging from this study reveals a considerable burden of ESBL and OXA gene carriage, suggesting a high level of antimicrobial resistance potential in local E. coli populations. These findings underscore the urgent need for routine molecular surveillance to complement phenotypic susceptibility testing. Sole reliance on routine antibiotic sensitivity patterns may underestimate the true burden of genetic resistance determinants circulating in the community.

The findings also reinforce the need to strengthen antimicrobial stewardship programmes, rationalize β-lactam and carbapenem usage, and implement strict infection-control policies. Regional antibiograms and molecular data should be periodically updated to guide empirical therapy, especially in settings where ESBL prevalence exceeds global averages. Furthermore, continuous monitoring of resistance gene evolution—especially transitions from ESBL to carbapenemase producers—is essential to prevent therapeutic dead-ends.

In conclusion, this study highlights a high prevalence of ESBL genes with significant co-existence of multiple resistance determinants in E. coli isolates. The molecular evidence suggests both ongoing dissemination and future potential escalation of resistance. Integrated surveillance strategies, judicious antibiotic use, and early detection of emerging genetic threats are critical to mitigate the expanding challenge of multidrug-resistant E. coli.

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