

Genetic Characterization of ESBL-Producing *Escherichia coli* Isolates from Nosocomial Infections, Healthcare Workers, and Sewage in a Tertiary Hospital: Insights into the Emergence of the High-Risk Clone ST8881

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Article

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Abstract

Objectives

Extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* (*E. coli*) poses a significant clinical challenge due to its resistance to commonly used beta-lactam antibiotics. Recent studies have highlighted the emergence of high-risk clones, particularly ST8881, in both hospital settings and among healthcare workers. This study aimed to investigate the genetic features and molecular epidemiology of ESBL-producing *E. coli* within a healthcare environment, focusing on the high-risk ST8881 clone.

Methods

A descriptive cross-sectional study was conducted from June 2021 to October 2021, involving 371 *E. coli* isolates from hospitalized patients (n = 246), healthcare workers' feces (n = 100), and hospital wastewater (n = 25). ESBL production was screened using the combination and double disk methods. Genotypic characterization included multilocus sequence typing (MLST) for sequence type identification and PCR detection of ESBL resistance genes (*CTX-M*, *shv*, and *tem*).

Results

ESBL-producing *E. coli* was isolated from 20% of samples, with the highest prevalence in hospital wastewater (60%), followed by healthcare workers (25%) and hospitalized patients (14.2%). MLST revealed that ST8881 was the predominant strain. Notably, the *ctx-M* gene was detected in 97% of ESBL-producing isolates.

Conclusions

Our study highlights the widespread distribution of ESBL-producing *E. coli*, particularly the high-risk ST8881 clone, in a healthcare environment, including hospital workers and wastewater. The high prevalence of *ctx-M* and other ESBL genes underscores the need for enhanced infection control measures and targeted antibiotic stewardship strategies.

Introduction

Extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) have emerged as a critical public health problem worldwide. These organisms produce enzymes that hydrolyze penicillins, aztreonam, and broad-spectrum cephalosporins (first- to third-generation) (1), rendering many common β -lactam antibiotics ineffective. ESBL-producing *E. coli* are now encountered both in healthcare and community settings(1, 2). Infections with ESBL-Ec are associated with worse clinical outcomes: patients tend to have higher morbidity, longer hospital stays, and increased healthcare costs(3). Indeed, the World

Health Organization (WHO) considers ESBL-producing Enterobacteriaceae among the most challenging pathogens of concern(4). Because so many standard antibiotics are inactivated by ESBLs, carbapenems are often the only remaining effective drugs(5). The global use of carbapenems against ESBL-Ec is rising, but this approach is unsustainable as carbapenem resistance (e.g., carbapenemase) is emerging (6).

Antimicrobial resistance (AMR) in general is recognized as one of the top global health threats: in 2019, bacterial AMR directly caused an estimated 1.27 million deaths and contributed to about 4.95 million more(7). All regions are affected, but low- and middle-income countries suffer the greatest burden(8). In these settings, factors like widespread antibiotic use and limited infection control favor the spread of resistant *E. coli*. Recent surveillance data underscore the high prevalence of ESBL-Ec carriage: a global meta-analysis found that roughly one-quarter of people carry ESBL-producing *E. coli* in their gut (pooled ~ 25.4%), with prevalence ~ 23.4% in healthy community populations and ~ 27.7% in hospitalized patients(9). This large reservoir of colonized individuals helps fuel both community and hospital infections. Hospitals, in particular, serve as important reservoirs and amplifiers for ESBL-Ec. With high antibiotic selection pressure, hospital patients frequently harbor ESBL strains and shed them into the environment(10). Antibiotics and their metabolites excreted by patients enter hospital sewage, creating a strong selection for a resistant clone(11). Multiple studies have isolated ESBL-producing *E. coli* from hospitalized patients (e.g., fecal or urine specimens) as well as from environmental sources like sinks, drains, and untreated wastewater. Notably, *E. coli* is the most common host of ESBL genes among Enterobacteriaceae(12), and the globally disseminated ST131 clone (often carrying CTX-M-type ESBLs) predominates in many settings(13). Genetic studies have found identical ESBL-Ec strains in patient samples and in the hospital sewage system, suggesting movement of bacteria between patients, staff, and the built environment. Resistant bacteria can then escape into municipal sewage and the wider community if not fully contained. Even healthcare workers may become colonized and serve as vectors; for example, one ICU study found 65% of staff carried ESBL-producing *E. coli*(14).

Despite its importance, the epidemiology of ESBL-*E. coli* in low-resource hospitals remains poorly characterized. Few studies have systematically sampled all potential reservoirs (patients, staff, wastewater) and performed detailed genotyping. In particular, there is a need to link molecular data and epidemiologic information to understand transmission pathways. Modern molecular-typing methods (MLST, plasmid, and resistance-gene profiling) can fill this gap, revealing how clones spread within hospitals and between community and healthcare settings.

In this study, we address these gaps by sampling *E. coli* from three sources within a hospital: clinical specimens from inpatients, fecal swabs from healthcare workers, and untreated hospital wastewater. ESBL production was confirmed phenotypically, and isolates were characterized by multilocus sequence typing (MLST). In addition, we performed PCR assays for common ESBL genes CTX-M, *shv*, and *tem-1* in each isolate. By combining this molecular epidemiology with the source data, we aim to elucidate the prevalence, genetic diversity, and transmission routes of ESBL-producing *E. coli* in the hospital. The aim of the study was to determine the molecular profiles and distribution of ESBL-producing *E. coli* from

hospitalized patients, health care workers, and hospital wastewater, in order to understand how resistant strains disseminate in the hospital environment.

Methods

a. Sample Collection

This cross-sectional study was conducted over a four-month period, from June to October 2021, at Valiasr Hospital, a major referral and teaching hospital in Arak, Iran. A total of 371 non-duplicate bacterial isolates were obtained from three different sources, representing both clinical and environmental reservoirs within the hospital setting.

Clinical samples from hospitalized patients (n = 246):

The majority of isolates were recovered from urine specimens (n = 162; 65.9%), followed by wound swabs (n = 62; 25.2%) and respiratory tract specimens, including sputum and tracheal aspirates (n = 22; 8.9%). These samples were collected from patients with suspected infections, based on clinical diagnosis and physician request.

Samples from healthcare workers (n = 100):

To assess possible colonization and carriage among hospital staff, fecal samples were collected from consenting healthcare workers across different wards of the hospital. This group included physicians, nurses, and ancillary staff who were directly or indirectly involved in patient care.

Environmental samples (n = 25):

To evaluate the hospital environment as a potential reservoir for bacterial contamination and transmission, sewage samples were obtained from different sections of the hospital wastewater system. These samples were collected under aseptic conditions and immediately transported to the microbiology laboratory for culture and isolation.

All samples were processed using standard microbiological techniques. Isolates were identified through conventional biochemical tests and confirmed using appropriate diagnostic methods. To minimize bias, duplicate isolates from the same patient or sample source were excluded from the study.

This study was reviewed and approved by the Ethical Committee of Arak University of Medical Sciences under the reference number IR.ARAKMU.REC.1400.027. Written informed consent was obtained from healthcare workers prior to sample collection. For patient specimens collected as part of routine diagnostic procedures, the requirement for written informed consent was waived by the aforementioned Ethical Committee due to the use of anonymized residual samples that posed no additional risk to patients. Patient confidentiality and ethical considerations were strictly maintained throughout the study.

b. Bacterial Culture and Identification

All collected specimens, including clinical, fecal, and environmental (wastewater) samples, were immediately transported under aseptic conditions to the microbiology laboratory of Valiasr Hospital, Arak, for processing. Each sample was inoculated onto MacConkey agar plates (Condalab, Spain) to selectively isolate Gram-negative bacteria. Plates were incubated aerobically at 35–37 °C for 18–24 hours.

After incubation, representative colonies were selected based on morphology and pigmentation. Pure cultures were obtained by subculturing on fresh agar plates. Identification of bacterial isolates to the species level was carried out using conventional biochemical tests, including oxidase, catalase, triple sugar iron (TSI), citrate utilization, indole, urease, motility, and methyl red/Voges-Proskauer (MR/VP) tests, in accordance with standard microbiological procedures. Where necessary, isolates with ambiguous biochemical profiles were further confirmed by additional diagnostic assays.

c. ESBL Phenotypic Detection

To determine extended-spectrum β -lactamase (ESBL) production, presumptive Enterobacteriaceae isolates were subcultured on blood agar plates (Merck, Germany) and incubated overnight at 35–37 °C to obtain fresh growth. Phenotypic detection of ESBLs was performed according to the Clinical and Laboratory Standards Institute (CLSI) M100, 30th edition (2020) guidelines using two complementary methods:

Combination Disk Test (CDT):

Each isolate was tested with cefotaxime (30 μ g) and ceftazidime (30 μ g) disks, both alone and in combination with clavulanic acid (10 μ g). An increase of ≥ 5 mm in the inhibition zone diameter around the disk containing the antibiotic–clavulanic acid combination compared to the antibiotic alone was interpreted as ESBL-positive.

Double-Disk Synergy Test (DDST):

Antibiotic disks containing third-generation cephalosporins (cefotaxime, ceftazidime, and aztreonam) were placed at a distance of 20–30 mm (center to center) from a disk containing amoxicillin–clavulanic acid (20/10 μ g) on Mueller–Hinton agar. After incubation, an enhanced inhibition zone ("keyhole effect") toward the clavulanic acid disk indicated synergy and confirmed ESBL production.

All tests were performed in duplicate to ensure reproducibility. Appropriate quality control strains, including *Escherichia coli* ATCC 25922 (negative control) and *Klebsiella pneumoniae* ATCC 700603 (positive control), were included in each batch of testing to validate the results.

d. DNA Extraction

Genomic DNA was extracted from each phenotypically confirmed isolate using the Favorgen Genomic DNA Extraction Kit (Favorgen Biotech Corp., Taiwan), following the manufacturer's recommended

protocol. Briefly, overnight bacterial cultures were harvested, lysed, and processed through silica membrane spin columns for purification. The extracted DNA was eluted in nuclease-free water and stored at -20°C until further use.

DNA concentration ($\text{ng}/\mu\text{L}$) and purity ($\text{A}_{260}/\text{A}_{280}$ ratio) were determined using a NanoDrop[™] spectrophotometer (Thermo Fisher Scientific, USA). Integrity of the genomic DNA was additionally assessed by 1% agarose gel electrophoresis, ensuring the presence of high-molecular-weight bands without significant shearing or degradation. Only DNA samples with an $\text{A}_{260}/\text{A}_{280}$ ratio between 1.8–2.0 were considered suitable for downstream molecular analyses.

e. PCR Amplification of Housekeeping Genes

To perform molecular typing, seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) were amplified according to the Achtman multilocus sequence typing (MLST) scheme for *Escherichia coli*.

Polymerase chain reaction (PCR) assays were carried out in $25\ \mu\text{L}$ reaction mixtures using a PEQLAB thermocycler (Germany). Each reaction contained:

$12.5\ \mu\text{L}$ of $2\times$ Super Master Mix (Pishgam Biotechnology, Iran),

$0.2\ \mu\text{M}$ of each forward and reverse primer (synthesized by a commercial supplier),

$50\text{--}100\ \text{ng}$ of template DNA, and nuclease-free water to adjust the final volume.

The PCR cycling conditions were as follows:

Initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, Final extension at 72°C for 7 minutes.

PCR products were analyzed by electrophoresis on 1.5% agarose gels (Sina Clone Company, Iran) prepared in $1\times$ TBE buffer, containing Safe Stain nucleic acid dye (Sina Clone Company, Iran). Samples were mixed with $6\times$ loading buffer prior to loading. A 100 bp DNA ladder (Thermo Fisher Scientific, USA) was used as a molecular size marker. Successful amplification was confirmed by visualization of bands under UV illumination using a gel documentation system.

Amplified products were stored at -20°C until sequencing.

f. Multilocus Sequence Typing (MLST)

Thirty isolates were selected for MLST, including six each from neurosurgery, ICU-B, orthopedics, male surgery, and hospital sewage. PCR products for each housekeeping gene were purified and confirmed by gel electrophoresis. Amplicons were sent on ice (cold chain) to Pishgam Biotechnology Co. (Tehran,

Iran) for Sanger sequencing using the primers listed in Table 1. The resulting sequences were assembled and analyzed using the PubMLST database (<https://pubmlst.org>) to assign sequence types.

Table1 Gene names and primers used in the MLST method

Gene name	Sequence of primers 5' 3' 	Product length
<i>adk</i>	F: ATTCTGCTTGGCGCTCCGGG R: CCGTCAACTTTCGCGTATTT	583 bp
<i>fumC</i>	F: TCACAGGTGCGCCAGCGCTTC R: GTACGCAGCGAAAAAGATTC	806 bp
<i>gyrB</i>	F: TCGGCGACACGGATGACGGC R: ATCAGGCCTTCACGCGCATC	911 bp
<i>icd</i>	F: ATGGAAAGTAAAGTAGTTGTTCCGGCACA R: GGACGCAGCAGGATCTGTT	878 bp
<i>mdh</i>	F: ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG R: TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	932 bp
<i>purA</i>	F: CGCGCTGATGAAAGAGATGA R: CATACGGTAAGCCACGCAGA	816 bp
<i>recA</i>	F: CGCATTCGCTTTACCCTGACC R: TCGTCGAAATCTACGGACCGGA	780 bp

g. Data Availability Statement

The datasets generated and/or analyzed during the current study are not publicly available due to patient privacy and ethical restrictions but are available from the corresponding author on reasonable request.

Results

A total of 371 *Escherichia coli* isolates were successfully recovered from the three sources included in this study: hospitalized patients (n = 246), healthcare workers’ fecal samples (n = 100), and hospital sewage (n = 25).

Among these isolates, 75 (20.2%) were identified as extended-spectrum β-lactamase (ESBL) producers based on phenotypic confirmatory tests. The distribution of ESBL-producing *E. coli* varied considerably across the different sources:

- Clinical isolates (patients): Of the 246 isolates obtained from hospitalized patients, 35 (14.2%) were confirmed as ESBL producers.
- Healthcare workers: From the 100 fecal samples analyzed, 25 isolates (25%) showed ESBL production, suggesting a relatively higher carriage rate among hospital staff compared to patient-derived isolates.
- Hospital wastewater: Among the 25 isolates recovered from sewage, 15 (60%) demonstrated ESBL production, representing the highest proportion among the studied groups.

These findings indicate that the prevalence of ESBL-producing *E. coli* is not limited to clinical cases but is also notable in healthcare workers and the hospital environment, particularly in wastewater, which may act as an important reservoir for resistant strains.

Table 2: Prevalence of ESBL-producing *E. coli* isolated from different sources

Source of Isolation	Total Number of Isolates	ESBL-producing Isolates n (%)
Hospitalized patients	246	35 (14.2%)
Healthcare workers' fecal samples	100	25 (25.0%)
Hospital wastewater	25	15 (60.0%)
Total	371	75 (20.2%)

a. Multilocus Sequence Typing (MLST) Results

Out of the 75 ESBL-producing *E. coli* isolates, 20 representative strains were selected for multilocus sequence typing (MLST) analysis. These isolates were chosen from the three distinct sources (hospitalized patients, healthcare workers, and hospital wastewater) to capture the diversity and potential epidemiological relationships among circulating ESBL-producing strains.

MLST analysis revealed that the isolates clustered into two sequence types (STs): ST8881 and ST320. Of the 20 isolates analyzed, 22 (73.3%) were identified as ST8881, while 6 (20%) belonged to ST320. ST8881 emerged as the predominant lineage, detected across all three sources of isolation, whereas ST320 was restricted to specific hospital wards and wastewater.

Notably, ST8881 isolates were found simultaneously in hospitalized patients, healthcare workers, and hospital wastewater, suggesting a possible transmission link between the hospital environment and human reservoirs. For instance, in both the ICU B and Neurosurgery Department, ST8881 strains were isolated from patients and healthcare workers, indicating potential cross-transmission within clinical wards. Similarly, ST8881 was also recovered from hospital sewage, further supporting its persistence and circulation within the hospital ecosystem.

In contrast, ST320 isolates were mainly confined to the Orthopedic and Male Surgery Departments and were also detected in wastewater samples, indicating that this clone may be restricted to certain clinical units but still contributes to environmental contamination.

Table 3 Distribution of MLST sequence types among ESBL-producing *E. coli* isolates from different hospital departments.

Hospital Department	Isolates from Healthcare Workers (n)	Isolates from Patients (n)	Detected Sequence Types
Neurosurgery	ST8881 × 3	ST8881 × 3	ST8881
ICU B	ST8881 × 3	ST8881 × 3	ST8881
Orthopedic	ST8881 × 3	ST320 × 3	ST8881, ST320
Male Surgery	ST8881 × 3	ST320 × 3	ST8881, ST320

Note: Four isolates were identified as ST8881 from hospital wastewater, and two isolates were identified as ST320.

This analysis highlights the dominance of ST8881 as a successful ESBL-producing clone in both clinical and environmental settings, whereas ST320 appears to circulate in more limited hospital niches. The overlap between isolates from patients, healthcare workers, and wastewater strongly suggests clonal dissemination within the hospital environment.

b. Prevalence of ESBL Resistance Genes

Molecular screening of the 75 ESBL-producing *E. coli* isolates revealed a high frequency of β-lactamase resistance genes (Table 4). The *ctx-M* gene was the most prevalent, detected in 73 isolates (97%), followed by *tem* in 53 isolates (70%) and *shv* in 42 isolates (56%).

A considerable proportion of isolates carried multiple resistance determinants simultaneously, indicating co-existence of ESBL genes within the same strain. The predominance of *ctx-M* suggests that *ctx-M*-type enzymes represent the principal driver of ESBL-mediated resistance in the studied population.

Table 4 Prevalence of ESBL resistance genes among ESBL-producing *E. coli* isolates (n = 75)

Gene detected	No. of positive isolates	Prevalence (%)
<i>ctx-M</i>	73	97%
<i>tem</i>	53	70%
<i>shv</i>	42	56%

Discussion

Our study found that 20.2% of all *E. coli* isolates (75/371) were ESBL producers, a figure closely matching recent pooled estimates for sub-Saharan Africa (20.76%)(2). However, stratification by source revealed notable disparities. Among clinical patient isolates, only 14.2% were ESBL-positive. This is lower than many reports from African hospitals. For example, a systematic review found an average ESBL carriage of 21.1% among hospitalized patients worldwide(15). Even more strikingly, a study in rural Tanzania detected ESBL-PE in 70.9% of UTI patients' stools(16), suggesting extremely high local rates. Our lower rate may reflect differences in patient selection, specimen types, or local antibiotic use. Regardless, the 14.2% prevalence in hospitalized patients, though substantial, is below some published figures from high-burden settings.

Among healthcare workers (HCWs), we observed a 25.0% ESBL carriage rate (25/100 fecal samples). This is considerably higher than reports from high-income countries. For example, a large Swiss study found only a 5.4% rectal ESBL-E carriage rate among > 1,200 hospital employees(17). The elevated rate in our HCW cohort suggests substantial occupational or community exposure in our setting. It indicates that HCWs could serve as a hidden reservoir, potentially facilitating nosocomial transmission. To our knowledge, few studies have quantified HCW carriage in similar settings; the disparity with European data(17) Highlights the need for more surveillance in low-income hospitals.

Hospital sewage harbored the highest proportion of ESBL-producing *E. coli*. In our 25 wastewater isolates, 60.0% were ESBL-positive (15/25). This is comparable to other studies showing very high ESBL frequencies in hospital effluent. For instance, one report from Benin found 100% of *E. coli* from hospital effluent to be ESBL producers(18), and a study in Nepal found ~ 57.1% of sewage *E. coli* to be ESBL-positive(19). These concordant findings imply that hospital wastewater is a concentrated source of resistant bacteria. The presence of ESBL-*E. coli* in effluents is concerning: it likely reflects inadequate waste treatment and poses a risk of environmental dissemination(18). Resistance genes from such wastewater can enter community waterways and farms, creating "hotspots" for AMR spread.

The very high ESBL rate in hospital sewage (60%) highlights an environmental pathway for resistance. Sewage often contains antibiotic residues and fecal bacteria, driving selection of resistant strains. Without effective wastewater treatment, these bacteria can contaminate surface waters or agricultural fields. Indeed, others have noted that untreated hospital effluents act as "hotspots" for resistance gene exchange (18). Our findings reinforce calls for improved waste management and environmental surveillance to prevent spillover of ESBL-*E. coli* into the community and ecosystem. In fact, experts argue that coordinated action (in clinics, agriculture, and the environment) is needed to curb ESBL spread(2). For example, policymakers should consider routine screening of wastewater and carriers, as well as investments in sanitation infrastructure, as part of AMR control.

Notably, identical ST8881 strains were recovered simultaneously from patients and healthcare workers in the same wards (e.g., both ICU B and Neurosurgery), and the same ST8881 types were also found in hospital sewage. This co-occurrence of ST8881 in humans and the hospital environment strongly suggests a single clonal lineage circulating through different reservoirs. Similarly, the ST320 lineage,

though less widespread, was present in both clinical isolates (Orthopedic and Male Surgery) and in wastewater. Such patterns of finding the same clone across patients, staff, and environment mirror previous reports of high-risk clones disseminating broadly (for example, *E. coli* ST131 has been found in patient samples, hospital sewage, and even downstream rivers(2).

MLST classifies isolates into sequence types (STs) based on allelic profiles of housekeeping genes(20). Here, each ST (such as ST8881 or ST320) corresponds to a unique combination of gene alleles. The allele profiles are highly reproducible and allow comparison of strains across studies(20). Thus, the finding that 73% of isolates share ST8881 indicates a highly clonal population.

The dominance of ST8881 in our setting is notable. Other genomic studies have recently identified ST8881 as a *putative emerging high-risk clone*. For example, whole-genome analyses of ESBL-*E. coli* from hospitals in Qatar have described ST8881 as a “putative emerging high-risk clone” characterized by multiple resistance and virulence factors(21). Its widespread presence in patients, staff, and wastewater here is consistent with those reports and raises concern that ST8881 is successfully spreading within the hospital ecosystem.

The simultaneous detection of ST8881 in clinical isolates and in sewage suggests direct or indirect transmission links between patients, staff, and the hospital environment. In other words, the hospital wastewater may serve as a reservoir or conduit for these strains. This echoes findings from environmental surveillance: for instance, a recent study showed *E. coli* ST131 (another epidemic clone) in every sampling location from patients to rivers, confirming “transmission of resistant strains through wastewater treatment plants to surface waters” (22). Thus, our data on ST8881 and ST320 similarly imply that hospital plumbing and sewage are part of the clonal network that sustains these ESBL lineages.

In contrast, ST320’s presence only in Orthopedic and Male Surgery wards (and in sewage) suggests a more localized clonal niche. Its restriction to certain departments implies limited spread – yet its recovery from wastewater shows it is still leaving the hospital. Even clones confined to particular units can contribute to wider environmental contamination if not contained.

The finding of the same clone in patients and healthcare workers indicates possible silent transmission. Indeed, other investigations have shown that routine screening can uncover such hidden spread. For example, an *E. coli* ST131 outbreak in a neonatal ICU was detected only after active surveillance, and researchers noted that “active surveillance was ... critical for detecting the silent spread of ESBL-producing Gram negatives in a critical healthcare setting” (23). In our context, regular screening of patients and staff (and even environmental sampling) could help track the entry and movement of ST8881 or ST320 in the hospital.

Clonal outbreaks like the one mentioned above were successfully interrupted by standard measures: strict contact precautions, patient/staff cohorting, thorough disinfection of equipment and surfaces, and training of personnel. In our setting, the presence of a dominant clone argues for similarly rigorous

steps. For instance, seeing ST8881 in an ICU should prompt immediate reinforcement of hand hygiene, barrier precautions (gowns, gloves), and perhaps restriction of admissions or movements until spread is halted. Past experience confirms that such interventions “were successful in interrupting the outbreak” of an ESBL *E. coli* clone(23).

Healthcare personnel can become colonized with ESBL-producing strains and unknowingly carry them between patients. In one recent survey in Switzerland, about 5.4% of hospital employees carried ESBL-producing Enterobacterales, and *E. coli* ST131 was the most common type detected(17). This underscores that staff can be part of the transmission chain. Ongoing education on antimicrobial stewardship and hygiene (e.g., safe handling of antibiotics, proper glove/gown use, hand sanitation) is therefore crucial. Periodic screening of HCWs may be considered during an outbreak to identify carriers.

The overwhelming predominance of *ctx-M*β-lactamase genes in our isolates (97%) is consistent with global trends. *ctx-M*-type ESBLs have rapidly emerged since the late 1990s and are now the most widespread ESBL enzymes in both human and animal *E. coli*(24). In our data, virtually all ESBL-producing strains carried a *ctx-M* gene, mirroring other recent reports. For example, Parzygnat *et al.* found that 100% of ESBL-positive *E. coli* from U.S. backyard poultry harbored a *ctx-M* gene (predominantly *ctx-M*-1) (25). Likewise, a large clinical study in Thailand reported *ctx-M* in 83.6% of ESBL-*E. coli* isolates, noting that *ctx-M*-15 was the single most common allele(26). The frequent recovery of *ctx-M* here suggests that *ctx-M*-type enzymes are the principal drivers of cephalosporin resistance in the population studied(24). These enzymes (“ctx” for cefotaximase) are particularly effective at hydrolyzing third-generation cephalosporins, limiting the utility of key antibiotics.

In contrast, *tem* and *shv* genes were detected at lower but still substantial rates (70% and 56%, respectively). These proportions align with other reports of co-circulation of multiple ESBL families. For instance, Gundran *et al.* found *tem* in ~ 58% and *shv* in ~ 28% of *E. coli* from broiler farms(24). Notably, clinical isolates often show high *tem* prevalence as well – one study of Thai patients found *tem* in 81% of ESBL-*E. coli*(24). In general, *shv*-type genes are less common than *tem* or *ctx-M*, but are still detected widely. Our findings indicate that multiple β-lactamase families co-exist in this setting; even though *ctx-M* dominates, the high frequencies of *tem* and *shv* suggest that these genes continue to circulate and contribute to the ESBL phenotype. Importantly, the concurrent presence of several genes in one strain broadens its resistance spectrum.

Indeed, co-occurrence of ESBL genes was a prominent feature of our isolates. A large fraction of strains carried more than one ESBL gene determinant. This multi-gene carriage has been reported elsewhere and is typically due to plasmids bearing arrays of resistance genes. For example, Gundran *et al.* observed that 73.5% of broiler *E. coli* carried multiple *ctx-M* subgroups, and several isolates simultaneously harbored *ctx-M* together with *tem* and *shv*(27). In our data, we similarly saw multiple resistance determinants in many isolates. Such co-existence is particularly concerning because it facilitates horizontal transfer: conjugative plasmids can move whole resistance cassettes between bacteria. Highly successful *E. coli* clones (e.g., ST131) are known to carry multiple bla genes (often including *ctx-M*

15plus others)(27), which can accelerate the dissemination of combined resistance traits. Thus, the presence of multiple ESBL genes per isolate suggests a genetic context primed for rapid spread of multidrug resistance.

Academically, our study highlights gaps in AMR surveillance. Data on ESBL carriage in hospital staff and sewage are sparse in many regions. Our findings suggest these areas deserve more research attention. Future studies could use molecular typing to trace transmission pathways between the environment, staff, and patients. Longitudinal monitoring is also needed to see if interventions (e.g., antibiotic stewardship, hygiene campaigns) reduce these carriage rates. In sum, by confirming high ESBL prevalence in understudied reservoirs, this work provides a basis for targeted interventions and underlines the importance of continued AMR research (in line with One Health recommendations). The epidemiological and clinical implications of these findings are significant. *ctx-M*-type ESBLs confer resistance to critical cephalosporins, and infections caused by *ctx-M*-producing *E. coli* are associated with worse outcomes, including higher mortality and delayed effective therapy. The need to use carbapenems or novel β -lactam/ β -lactamase inhibitor combinations to treat such infections has increased in settings where *ctx-M* is prevalent. Moreover, our observation of ESBL genes in what may include animal or environmental sources (as suggested by comparisons to poultry studies(25)) raises One Health concerns. Resistant *E. coli* from animals can enter the human food chain or environment, and plasmids carrying *ctx-M*, *tem*, and *shv* can move freely between species. Thus, the high *ctx-M* prevalence and frequent gene co-carriage underscore the importance of continued surveillance of ESBL genes, as well as prudent antibiotic use across human and veterinary medicine. In summary, our results – like many recent studies – highlight *ctx-M* enzymes as the dominant ESBL mechanism in *E. coli* and illustrate the complex genetic background of resistance in these bacteria. The MLST data reveal clonal spread of ESBL *E. coli* within the hospital. ST8881's ubiquity across patients, staff, and wastewater indicates a linked reservoir demanding integrated control measures. Effective infection control will require enhanced surveillance (including molecular typing), aggressive outbreak interventions (contact precautions, cleaning), and attention to environmental pathways. Citing analogous cases in the literature confirms that without such actions, high-risk clones can disseminate silently through healthcare settings.

Conclusion

The present study contributes significantly to the existing literature on the prevalence and distribution of ESBL-producing, particularly the emerging high-risk clone ST8881, in the hospital environment. This study investigated simultaneous factors among hospitalized patients, healthcare workers, and hospital wastewater; hence, it supplied the entire landscape of antibiotic resistance.

Key findings:

1. The prevalence of ESBL-producing *E. coli* was highest in the case of hospital wastewater (60%), followed by healthcare workers with a percentage of 25% and lastly hospitalized patients with a

percentage of 14.2%.

2. *ctx-M* gene predominance at 97% in ESBL-producing *E. coli* isolates, similar to global patterns.
3. One of First report of an emerging high-risk clone ST8881 in Iran, previously only identified in Qatar, showing likely international circulation.
4. Identification of asymptomatic carriers among the healthcare workers is often an overlooked route of transmission.

This study, though single-centered with no long-term follow-up, has contributed significantly to the existing knowledge of antibiotic resistance patterns and transmission dynamics in healthcare settings. It serves as a sort of clarion call to developing nations to take up this growing threat proactively. Any future studies of this nature must comprise the longitudinal surveillance of such high-risk clones as ST8881 and the molecular mechanisms of resistance transmission in hospitals. These efforts are needed to find out successful strategies to deal with the spread of antibiotic-resistant bacteria and the preservation of existing antibiotics.

Declarations

Ethics Declaration

This study was approved by the Ethics Committee of Arak University of Medical Sciences, Arak, Iran (Ethics code: **IR.ARAKMU.REC.1400.027**). The research was conducted in accordance with all relevant guidelines and regulations for the protection of human subjects in medical research, as approved by the Ethics Committee.

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Author Contribution

M.M.S. wrote the main manuscript text. M.A. and E.G.-R. reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to patient privacy and ethical restrictions but are available from the corresponding author on reasonable request.

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