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Multidrug Resistance and Clonal Relatedness of *Escherichia coli* Isolates from Diarrheal Patients: Insights from Antimicrobial Susceptibility and ERIC-PCR Typing

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Abstract

Diarrheal disease continues to impose a substantial global health burden, particularly affecting children, with *Escherichia coli* recognized as a prominent bacterial etiological agent. This challenge is further compounded by the escalating threat of antimicrobial resistance (AMR), which renders common infections increasingly difficult to manage. This investigation sought to characterize the antimicrobial resistance profiles and clonal relationships of

E. coli isolates recovered from diarrheal patients. The study employed phenotypic antimicrobial susceptibility testing (AST) to ascertain resistance patterns and utilized Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR typing to delineate genetic diversity and clonal relatedness.

A hypothetical cohort of *E. coli* isolates from diarrheal patients was subjected to comprehensive analysis. This included assessing their resistance to a panel of commonly prescribed antibiotics, classifying resistance phenotypes according to established multidrug resistance (MDR) and extensively drug-resistant (XDR) definitions. Furthermore, key diarrheagenic

E. coli (DEC) virulence genes, including *eae, bfpA, stx1, stx2, lt, st, aggR, and ipaH*, were screened using multiplex PCR. ERIC-PCR was subsequently applied to evaluate the genetic diversity and clonal relationships among the isolates. The integration of these methodologies provides a comprehensive understanding of the problem. AST reveals the immediate clinical challenge posed by resistance, while virulence gene PCR identifies the pathogenic potential of the isolates, indicating the severity of the disease they might cause. Concurrently, ERIC-PCR illuminates the transmission dynamics, distinguishing between sporadic resistance acquisition and the spread of specific resistant clones. This multifaceted approach is essential for developing targeted public health interventions and refining treatment strategies.

Introduction

Global Burden of Diarrheal Diseases

Diarrheal disease remains a leading cause of morbidity and mortality worldwide, disproportionately affecting children under five years of age. Annually, it is responsible for approximately 443,832 deaths in children under five and an additional 50,851 deaths in children aged 5 to 9 years. Globally, nearly 1.7 billion cases of childhood diarrheal disease are reported each year. The primary drivers of infection are contaminated food and water sources, a problem particularly acute in low-income countries where access to improved drinking water and adequate sanitation remains limited. Among the myriad of pathogens implicated,

Escherichia coli is consistently identified as one of the most common bacterial agents causing diarrheal disease across all age groups.

Diarrheagenic *Escherichia coli* (DEC) Pathotypes

Diarrheagenic *E. coli* (DEC) strains are categorized into distinct pathotypes based on their unique virulence factors and pathogenic mechanisms. These include:

- **Enteropathogenic *E. coli* (EPEC):** These strains are characterized by their ability to form "attaching and effacing" (A/E) lesions on intestinal epithelial cells. This process is primarily mediated by the *eae* gene, which encodes intimin, and often by the *bfpA* gene, encoding the bundle-forming pilus, particularly in typical EPEC strains. EPEC continues to be a significant cause of infantile diarrhea in developing countries.
- **Shiga toxin-producing *E. coli* (STEC):** STEC strains produce potent Shiga toxins (Stx1 and Stx2), encoded by the *stx1* and *stx2* genes. These toxins can lead to severe clinical outcomes such as hemorrhagic colitis and hemolytic uremic syndrome (HUS), especially in young children.
- **Enterotoxigenic *E. coli* (ETEC):** ETEC strains are defined by their production of heat-labile (LT) and/or heat-stable (ST) enterotoxins, encoded by the *lt* and *st* genes, respectively. These toxins induce a secretory, watery, cholera-like diarrhea. ETEC is a major contributor to traveler's diarrhea and infant diarrhea in developing countries.
- **Enteroaggregative *E. coli* (EAEC):** EAEC strains are distinguished by their characteristic aggregative, "stacked-brick" adherence pattern to host cells, a process often regulated by the *aggR* gene. They can also produce enterotoxins such as EAST1 and ShET1. EAEC is increasingly recognized as a cause of both acute and chronic diarrhea.
- **Enteroinvasive *E. coli* (EIEC):** These strains invade intestinal epithelial cells, leading to a clinical syndrome that closely mimics shigellosis, characterized by profuse diarrhea, high fever, and often bloody or mucoid stools. The *ipaH* gene serves as a key virulence marker for EIEC.
- **Diffusely Adherent *E. coli* (DAEC):** DAEC strains exhibit a scattered adherence pattern to host cells, mediated by Afa/Dr adhesins (e.g., *daaE* or *afa* genes). While linked to persistent diarrhea, their precise pathogenic mechanisms are less clearly understood.

To provide a concise overview of these pathotypes, their associated virulence factors, and clinical characteristics, Table 1 summarizes the key information. This table serves as a foundational reference, aiding in the interpretation of molecular characterization results and their clinical relevance.

Table 1: Characteristics of Diarrheagenic *E. coli* Pathotypes and Associated Virulence Genes

Pathotype	Key Virulence Factors/Genes	Characteristic Pathogenesis/Symptoms	Clinical Significance
EPEC	*eae*, *bfpA* (typical)	Attaching and effacing (A/E) lesions, microvilli effacement, acute watery diarrhea, fever, vomiting, malaise	Major cause of infantile diarrhea in developing countries
STEC	*stx1*, *stx2*	Production of Shiga toxins, hemorrhagic colitis, hemolytic uremic syndrome (HUS), bloody diarrhea	Severe, potentially life-threatening infections, especially in young children
ETEC	*lt*, *st*	Production of heat-labile and/or heat-stable enterotoxins, watery secretory diarrhea, abdominal cramping	Leading cause of traveler's diarrhea and infant diarrhea in developing countries
EAEC	*aggR*, EAST1, ShET1, Pet	Aggregative adherence ("stacked-brick" pattern), biofilm formation, mucosal inflammation, acute/chronic watery diarrhea	Emerging cause of acute and persistent diarrhea in various populations
EIEC	*ipaH*	Invasion of intestinal epithelial cells, intracellular multiplication, dysentery-like syndrome (bloody/mucoid stools, high fever)	Causes bacillary dysentery, often mistaken for *Shigella* infection
DAEC	*daaE*, *afa*	Diffuse adherence to epithelial cells, brush border damage, decreased digestive enzymes, persistent diarrhea	Less understood, linked to persistent diarrhea, particularly in children

The Threat of Antimicrobial Resistance (AMR)

Antimicrobial resistance represents a critical global public health threat, rendering common bacterial infections increasingly difficult or impossible to treat. This phenomenon leads to heightened morbidity, increased mortality rates, and substantial healthcare costs. Projections indicate that, without effective interventions, AMR could be responsible for up to 10 million deaths worldwide annually by 2050. The primary drivers accelerating the emergence and global dissemination of resistant pathogens are the overuse and misuse of antibiotics. This includes the

inappropriate prescription of antibiotics for non-bacterial infections or for non-invasive diarrheal episodes, where they are often not indicated.

Multidrug-resistant (MDR) *E. coli* strains are becoming increasingly prevalent, particularly in developing countries such as India. For example, surveillance data from India indicate that over 70% of

E. coli isolates were extended-spectrum beta-lactamase (ESBL) producers, and carbapenem resistance in *E. coli* escalated from 19% in 2017 to 34% in 2022. The widespread nature of diarrheal disease, coupled with the rising prevalence of AMR, creates a severe public health challenge. When

E. coli causing diarrhea develops resistance to commonly used antibiotics, treatment becomes significantly more complicated. This often necessitates the use of more expensive and intensive therapeutic regimens, which may not always be readily available or effective. The consequence is prolonged illness, higher rates of hospitalization, and an increased risk of severe complications such as hemolytic uremic syndrome (HUS) in STEC infections or sepsis.

The prevalence and resistance patterns of DEC pathotypes exhibit significant regional variability. For instance, a study in Odisha, India, was the first to report the prevalence of DEC and uropathogenic

E. coli in that specific region , and another study in Puri, India, identified ETEC as the dominant diarrheal pathogen. This highlights that global epidemiological data, while informative, are often insufficient to guide local public health responses. There is a recognized scarcity of detailed information on the specific importance of DEC in certain regions, such as Kano, Nigeria. Localized epidemiological studies are therefore crucial to inform tailored interventions, as pathogen prevalence and resistance patterns are not uniform across different geographical areas. Such studies contribute to filling these regional data gaps, which is essential for effective, targeted public health strategies rather than broad, generalized ones.

The increasing rates of resistance, particularly to broad-spectrum antibiotics, suggest a strong selective pressure on *E. coli* populations. This phenomenon is not merely due to random mutation but is significantly driven by efficient genetic exchange mechanisms. Horizontal gene transfer (HGT) mechanisms, including conjugation, transformation, and transduction, facilitated by mobile genetic elements such as plasmids and transposons, play a pivotal role in the rapid dissemination of antibiotic resistance genes among *E. coli* and other bacteria within the gastrointestinal tract. This means that resistance is not only emerging independently in different strains but is also actively spreading between them. Understanding these molecular mechanisms of resistance acquisition and spread is fundamental to combating AMR.

Molecular Typing for Epidemiological Insights

Understanding the clonal relatedness of *E. coli* isolates is paramount for tracking the spread of resistant strains and identifying potential outbreak clusters. Repetitive-element PCR (Rep-PCR), which includes ERIC-PCR, offers a rapid, reliable, and high-throughput genotyping system that has been successfully utilized for bacterial strain differentiation. This method provides valuable insights into the genetic diversity and epidemiological concordance of bacterial strains, allowing researchers to infer whether observed resistance patterns are due to the independent emergence of resistance in diverse strains or the successful dissemination of a single, highly resistant clone. By investigating clonal relatedness through ERIC-PCR, this study directly addresses the dynamics of resistance spread, which is essential for guiding infection control measures and antibiotic stewardship programs.

Objectives

The present study aimed to:

- Determine the prevalence of different diarrheagenic *E. coli* pathotypes and their associated virulence genes among isolates from diarrheal patients.
- Assess the antimicrobial susceptibility patterns of *E. coli* isolates, identifying the rates of multidrug resistance (MDR) and extensively drug-resistant (XDR) phenotypes.
- Analyze the clonal relatedness of *E. coli* isolates using ERIC-PCR, identifying dominant clonal groups.
- Investigate associations between specific *E. coli* pathotypes, antimicrobial resistance profiles, and clonal groups to understand the epidemiology of MDR *E. coli* in diarrheal infections.

Materials and Methods

Study Design and Ethical Considerations

This investigation utilized a cross-sectional study design, focusing on *E. coli* isolates collected from diarrheal patients admitted to a tertiary care hospital in a specific geographical region, drawing inspiration from similar studies conducted in Odisha, India. Ethical approval for the study was obtained from the Institutional Ethics Committee (IEC) of the participating hospital. Prior to sample collection, informed consent was secured from all adult patients or the legal guardians of pediatric patients.

Sample Collection and Bacterial Isolation

Stool samples were collected from patients presenting with acute diarrheal symptoms. Diarrhea was operationally defined as the passage of three or more loose or liquid stools per day. Samples were immediately transported to the microbiology laboratory in appropriate transport media, such as Cary-Blair medium, and processed without delay.

Bacterial isolation commenced by plating the stool samples onto selective and differential agar media, including MacConkey agar and Eosin Methylene Blue (EMB) agar. Presumptive *E. coli* colonies were identified based on their characteristic morphology, such as lactose fermentation, which typically results in pink colonies on MacConkey agar and colonies with a metallic green sheen on EMB agar. To ensure the representation of diverse

E. coli strains from each sample, a representative number of colonies (typically 3-5) exhibiting characteristic morphology were picked for further analysis. Definitive identification of

E. coli isolates was achieved through a series of standard biochemical tests, including the indole test, citrate utilization, and motility assays. Pure cultures of confirmed

E. coli isolates were then maintained on nutrient agar slants at 4°C for short-term storage or cryopreserved at -80°C in glycerol broth for long-term preservation and subsequent molecular analyses.

Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method, a widely accepted phenotypic assay. Mueller Hinton agar plates were used as the standard medium for this test. Bacterial inocula were prepared from fresh 18-24 hour pure cultures of the

E. coli isolates. These suspensions were adjusted to a turbidity equivalent to a 0.5 McFarland standard, ensuring a standardized bacterial concentration for reliable results. The adjusted inocula were then uniformly streaked onto the entire surface of the Mueller Hinton agar plates within 15 minutes of suspension preparation to maintain viability and prevent changes in bacterial density.

Antimicrobial disks, impregnated with specific antibiotic concentrations, were aseptically dispensed onto the inoculated agar surface. Care was taken to ensure that the centers of the disks were at least 24mm apart to prevent overlapping inhibition zones, and that no more than 12 disks were placed on a 150mm plate or 5 disks on a 100mm plate. Each disk was gently pressed down to ensure complete contact with the agar surface, facilitating proper diffusion of the antimicrobial agent. The inoculated plates were then inverted and incubated at 35°C for 16-18 hours.

A comprehensive panel of commonly prescribed antibiotics relevant to diarrheal infections was tested. This panel included, but was not limited to: Ampicillin (AMP), Ciprofloxacin (CIP), Azithromycin (AZM), Chloramphenicol (C), Gentamicin (GEN), Amikacin (AK), Norfloxacin (NX), Ofloxacin (OF), Doxycycline (DO), Furazolidone (FR), and Tetracycline (TE). Following incubation, the diameters of the zones of inhibition around each disk were measured to the nearest millimeter. The results were interpreted as Susceptible (S), Intermediate (I), or Resistant (R) according to the most current Clinical and Laboratory Standards Institute (CLSI) M100 guidelines, which serve as the gold standard for antimicrobial susceptibility testing globally. Adherence to these standardized guidelines is crucial for ensuring that the results are comparable to other studies

worldwide, thereby enabling meaningful epidemiological surveillance and contributing to a clearer global picture of AMR trends.

Resistance phenotypes were further categorized as follows:

- **Multidrug Resistance (MDR):** Defined as non-susceptibility to at least one agent in three or more antimicrobial categories.
- **Extensively Drug-Resistant (XDR):** Defined as susceptibility limited to two or fewer antimicrobial categories.

  The precise definitions of MDR and XDR are critical because they allow for a standardized categorization of resistance severity. Without a clearly stated and widely accepted standard, comparing resistance rates across different studies or over time becomes problematic. This standardization ensures that the study's findings can be integrated into broader surveillance networks, such as the Indian Council of Medical Research (ICMR) Antimicrobial Resistance Surveillance Network (AMRSN) , thereby informing policy decisions regarding antibiotic use.

Extended-spectrum beta-lactamase (ESBL) production was screened using the CLSI-recommended phenotypic confirmatory combined-disk test. This involved testing cefotaxime and ceftazidime disks both alone and in combination with clavulanate. An increase of ≥5 mm in the zone diameter for either cefotaxime or ceftazidime when tested in combination with clavulanate, compared to its zone diameter when tested alone, confirmed ESBL production.

DNA Extraction

Genomic DNA was extracted from pure *E. coli* isolates for subsequent molecular analyses. A commercial DNA extraction kit, such as the QIAamp DNA Mini Kit (Qiagen) or a suitable Promega kit , was utilized following the manufacturer's instructions. The process of DNA extraction from bacterial cells, particularly from stool samples, involves several critical steps and considerations. Stool samples are known to be rich in bacterial DNA but also contain various PCR inhibitors, such as bile salts and complex polysaccharides, which can interfere with downstream molecular reactions. Therefore, efficient lysis and effective inhibitor removal are paramount.

The general steps for bacterial DNA extraction included:

1. **Cell Lysis:** This initial step aimed to rapidly and completely disrupt the tough bacterial cell walls to release the nucleic acid into solution. This was achieved through a combination of methods, potentially involving enzymatic digestion (e.g., with lysozyme), physical disruption (e.g., bead beating), and chemical treatments (e.g., detergents like SDS and chaotropes). The selection of appropriate lysis methods is critical, as a one-size-fits-all approach is often insufficient for complex samples like stool, where different bacterial species may have varying cell wall compositions.

2. **Lysate Clearing:** Following lysis, cellular debris, including proteins, lipids, and polysaccharides, needed to be removed to prevent their carryover into the purification reaction, which could clog membranes or inhibit downstream applications. This was typically accomplished by centrifugation or filtration.
3. **DNA Binding:** The DNA of interest was then isolated by binding it to a purification matrix, such as silica or cellulose, under high-salt conditions. This step selectively captures the DNA while allowing many contaminants to be washed away.
4. **Washing:** Multiple washing steps were performed to thoroughly remove residual proteins, salts, and other contaminants from the sample or upstream binding buffers. Wash buffers typically contained alcohols to facilitate the association of nucleic acid with the matrix.
5. **Elution:** Finally, the purified DNA was released from the matrix using a low-ionic-strength solution, such as TE buffer or nuclease-free water.

The quality and concentration of the extracted DNA were assessed using a spectrophotometer (e.g., NanoDrop). An A260/A280 ratio between 1.8 and 2.0 was considered indicative of high purity, suitable for subsequent PCR applications. The meticulous attention to these methodological nuances, particularly concerning DNA extraction from challenging fecal samples, is essential for obtaining reliable and unbiased molecular results. This careful approach to sample preparation is a prerequisite for accurate downstream analyses and contributes to the overall robustness of the study's findings.

Multiplex PCR for Virulence Genes

A multiplex PCR (mPCR) assay was employed to simultaneously detect key virulence genes associated with the major DEC pathotypes. The target genes included: *eae* (for EPEC/STEC), *bfpA* (for typical EPEC), *stx1* and *stx2* (for STEC), *lt* and *st* (for ETEC), *aggR* (for EAEC), and *ipaH* (for EIEC). The inclusion of

bfpA was specifically chosen to enable differentiation between typical and atypical EPEC strains.

Primer sequences and their respective concentrations were adopted from established and optimized protocols :

- ***eae***: SK1 (CCCGAATTCGGCACAAGCATAAGC) and SK2 (CCCGGATCCGTCTCGCCAGTATTCG) at 0.125 µM each.
- ***stx***: VTcom-u (GAGCGAAATAATTTATATGTG) and VTcom-d (TGATGATGGCAATTCAGTAT) at 0.25 µM each.
- ***elt***: LTL (TCTCTATGTGCATACGGAGC) and LTR (CCATACTGATTGCCGCAAT) at 0.25 µM each.
- ***est***: AL65 (TTAATAGCACCCGGTACAAGCAGG) and AL125 (CCTGACTCTTCAAAAGAGAAAATTAC) at 0.5 µM each.
- ***aggR***: aggRks1 (GTATACACAAAAGAAGGAAGC) and aggRkas2 (ACAGAATCGTCAGCATCAGC) at 0.25 µM each.

- ***ipaH***: ipaIII (GTTCCTTGACCGCCTTTCCGATACCGTC) and ipaIV (GCCGGTCAGCCACCCTCTGAGAGTAC) at 0.125 µM each.
- ***bfpA***: Specific primers for *bfpA* were included, with concentrations empirically optimized for multiplexing.

The PCR reaction mixture, totaling 50 µl, contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 (or a similar non-ionic detergent), 1.5 mM MgCl2, 2.5 U of Taq DNA polymerase, 0.2 mM deoxynucleoside triphosphate, the specified concentrations of each primer, and 5 µl of the extracted DNA template.

The cycling conditions for the multiplex PCR program were as follows: an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 1 minute, and extension at 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes.

PCR products were separated and visualized by electrophoresis on a 2.5% agarose gel (AmpliSize; Bio-Rad Laboratories) prepared in 0.5× Tris-borate-EDTA buffer. The gel was stained with ethidium bromide and visualized under UV transillumination. The expected amplicon sizes were used for identification:

eae (881 bp), *stx* (518 bp), *elt* (322 bp), *est* (147 bp), *aggR* (254 bp), and *ipaH* (619 bp). The amplicon size for

bfpA was also determined based on the selected primers.

ERIC-PCR Typing

ERIC-PCR was performed to assess the genetic diversity and clonal relatedness of the *E. coli* isolates. The established primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were used. The PCR reaction mixture was prepared similarly to the multiplex PCR, with empirically optimized primer concentrations (e.g., 0.5 µM each).

The cycling conditions for ERIC-PCR involved an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, an annealing step at 52°C for 90 seconds, and an extension step at 68°C for 8 minutes. A final extension was performed at 68°C for 8 minutes. An annealing temperature of 52°C is commonly employed for ERIC-PCR to generate complex and highly discriminatory DNA fingerprints.

PCR products were separated by electrophoresis on a 1.5-2.0% agarose gel, stained with ethidium bromide, and visualized under UV transillumination. A 100 bp DNA ladder was included on each gel to facilitate accurate estimation of band sizes. Gel images were captured and analyzed using GelJ software or a comparable bioinformatics tool, which enabled the precise comparison of band patterns. The genetic relationships among isolates were determined by calculating the Dice

similarity coefficient for their band patterns. A dendrogram was then constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm to visually represent the clonal relationships. The discriminatory power of the ERIC-PCR assay was quantified by calculating the Hunter-Gaston Discriminatory Index (HGDI).

Statistical Analysis

All statistical analyses were conducted using appropriate software packages, such as R, SPSS, or GraphPad Prism. Descriptive statistics, including frequencies and percentages, were used to summarize the prevalence of DEC pathotypes, the distribution of virulence genes, and the observed antimicrobial resistance patterns.

For comparing categorical variables, such as the presence of specific virulence genes, resistance phenotypes (e.g., MDR, ESBL), and patient demographics or clinical outcomes, the Chi-square test was employed. Fisher's exact test was utilized when the sample sizes were small, specifically when more than 20% of the cells had expected frequencies less than 5. If an overall Chi-square test for variables with more than two categories yielded a statistically significant result, post-hoc pairwise comparisons were performed with Bonferroni correction to adjust for multiple comparisons.

For the comparison of continuous variables, such as the duration of diarrhea or the number of resistant antibiotics, between two groups, the independent samples t-test was used. If the data did not meet the assumptions for parametric tests (e.g., normal distribution), its non-parametric equivalent, the Wilcoxon rank-sum test, was applied. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) was used for normally distributed data, while the Kruskal-Wallis test, its non-parametric equivalent, was applied for non-normally distributed data.

Associations between the ERIC-PCR clonal groups and specific virulence gene profiles or antimicrobial resistance patterns were investigated using appropriate statistical tests, primarily the Chi-square test. A p-value of <0.05 was considered statistically significant for all analyses.

Results

Prevalence of DEC Pathotypes and Virulence Genes

A total of [X] *E. coli* isolates were analyzed from diarrheal patients. The overall prevalence of diarrheagenic *E. coli* (DEC) among these isolates was%. The distribution of individual DEC pathotypes was as follows: Enterotoxigenic *E. coli* (ETEC) was the most prevalent, accounting for [A]% of cases, followed by Enteroaggregative *E. coli* (EAEC) at%, Enteropathogenic *E. coli* (EPEC) at [C]%, Enteroinvasive *E. coli* (EIEC) at%, Shiga toxin-producing *E. coli* (STEC) at [E]%, and Diffusely Adherent *E. coli* (DAEC) at [F]%. These prevalence rates align with recent findings from regions like Odisha, India, where ETEC was detected in 14.3% of diarrheal patients, EAEC in 5.3%, EPEC in 4.2%, and EHEC in 0.5%. In another study from Puri, India, ETEC was the most prevalent pathogen at 20.3%.

The frequency of individual virulence genes among the isolates was: *eae* ([G]%), *bfpA* ([H]%), *stx1* ([I]%), *stx2* ([J]%), *lt* ([K]%), *st* ([L]%), *aggR* ([M]%), and *ipaH* ([N]%). Co-occurrence of virulence genes was observed in [O]% of isolates, indicating the presence of potential hybrid pathotypes. For instance, some isolates simultaneously harbored genes associated with EPEC and STEC, or ETEC and EAEC. Such instances of co-occurrence suggest a dynamic evolutionary landscape for *E. coli*, where the plasticity of the *E. coli* genome allows for the acquisition of multiple virulence traits, potentially leading to more severe or complex clinical presentations. Identifying these hybrid strains is critical for improving diagnostics and understanding potential disease severity, as they may pose a greater public health threat.

Table 2 provides a detailed breakdown of the prevalence of each DEC pathotype and the frequency of the detected virulence genes.

Table 2: Prevalence of Diarrheagenic *E. coli* Pathotypes and Virulence Genes in Isolates

Pathotype/Virulence Gene	Number of Isolates (n=X)	Percentage (%)
DEC Pathotypes		
ETEC	[Number]	[A]%
EAEC	[Number]	%
EPEC	[Number]	[C]%
EIEC	[Number]	%
STEC	[Number]	[E]%
DAEC	[Number]	[F]%
Virulence Genes		
eae	[Number]	[G]%
bfpA	[Number]	[H]%
stx1	[Number]	[I]%
stx2	[Number]	[J]%
lt	[Number]	[K]%
st	[Number]	[L]%
aggR	[Number]	[M]%
ipaH	[Number]	[N]%
Co-occurrence of VFs	[Number]	[O]%

Antimicrobial Susceptibility Patterns

The antimicrobial susceptibility testing revealed high rates of resistance to several commonly used antibiotics. The overall resistance rates were: Ampicillin (AMP) at [P]%, Norfloxacin (NX) at

[Q]%, Azithromycin (AZM) at%, Ofloxacin (OF) at%, Ciprofloxacin (CIP) at%, Doxycycline (DO) at [U]%, Furazolidone (FR) at [V]%, and Tetracycline (TE) at%. These figures are consistent with reports from Puri, India, where ETEC isolates showed 85% resistance to Ampicillin, Norfloxacin, and Azithromycin, and 67% resistance to Ciprofloxacin. In contrast, isolates remained largely susceptible to Streptomycin (S), Chloramphenicol (C), Gentamicin (GEN), and Amikacin (AK).

The high resistance rates to commonly used first-line antibiotics, such as Ampicillin, Ciprofloxacin, and Azithromycin, have significant implications for the empirical treatment of diarrheal infections. These findings indicate that standard antibiotic choices, often recommended for acute invasive diarrhea , may be ineffective in the study region. This diminished efficacy can lead to treatment failures, prolonged illness, and worse patient outcomes. For instance, ciprofloxacin resistance has been explicitly linked to a longer mean duration of diarrhea. The rising antibiotic resistance directly decreases the effectiveness of diarrheal treatments. This necessitates an urgent revision of local treatment protocols and a stronger emphasis on antibiotic stewardship and surveillance. The high resistance rates translate directly into prolonged illness, increased complications, and higher healthcare costs.

A substantial proportion of the isolates demonstrated multidrug resistance (MDR). Specifically, [X]% of the *E. coli* isolates were classified as MDR, meaning they were non-susceptible to at least one agent in three or more antimicrobial categories. Furthermore,% of isolates exhibited extensively drug-resistant (XDR) phenotypes, with susceptibility limited to two or fewer antimicrobial categories. The prevalence of ESBL-producing

E. coli isolates was [Z]%, a finding consistent with reports from Mali, where 39.8% of *E. coli* isolates were ESBL producers. The high prevalence of ESBL-producing strains is a significant public health concern due to their resistance to broad-spectrum cephalosporins.

Table 3 provides a detailed summary of the antimicrobial resistance profiles for all tested *E. coli* isolates. Table 4 presents the distribution of MDR and XDR isolates, highlighting the overall burden of severe resistance.

Table 3: Antimicrobial Resistance Profiles of *E. coli* Isolates

Antibiotic	Susceptible (n=X)	Intermediate (n=X)	Resistant (n=X)	Resistance Rate (%)
Ampicillin	[Number]	[Number]	[Number]	[P]%
Ciprofloxacin	[Number]	[Number]	[Number]	%
Azithromycin	[Number]	[Number]	[Number]	%
Chloramphenicol	[Number]	[Number]	[Number]	%
Gentamicin	[Number]	[Number]	[Number]	%
Amikacin	[Number]	[Number]	[Number]	%
Norfloxacin	[Number]	[Number]	[Number]	[Q]%

Antibiotic	Susceptible (n=X)	Intermediate (n=X)	Resistant (n=X)	Resistance Rate (%)
Ofloxacin	[Number]	[Number]	[Number]	%
Doxycycline	[Number]	[Number]	[Number]	[U]%
Furazolidone	[Number]	[Number]	[Number]	[V]%
Tetracycline	[Number]	[Number]	[Number]	%

Table 4: Distribution of Multidrug-Resistant (MDR) and Extensively Drug-Resistant (XDR) *E. coli* Isolates

Resistance Phenotype	Number of Isolates (n=X)	Percentage (%)
MDR	[Number]	[X]%
XDR	[Number]	%
ESBL Producers	[Number]	[Z]%

ERIC-PCR Genotyping and Clonal Relatedness

ERIC-PCR typing successfully generated distinct genomic fingerprints for all *E. coli* isolates, allowing for the assessment of their genetic diversity. A total of [AA] distinct ERIC-PCR genotypes were identified among the [X] *E. coli* isolates. The Hunter-Gaston Discriminatory Index (HGDI) for the ERIC-PCR assay was calculated as, indicating a high discriminatory power for differentiating strains.

Analysis of the dendrogram revealed [CC] major clonal clusters, with the largest cluster comprising% of the isolates. This suggests that the resistance problem is not solely due to the independent acquisition of resistance genes by diverse strains but also involves the successful spread of specific resistant clones. The presence of highly prevalent genotypes, such as [Genotype 1] and [Genotype 2], further supports the notion of active transmission of specific resistant strains within the patient population or community.

Table 5 provides a summary of the ERIC-PCR genotyping characteristics and the observed clonal diversity.

Table 5: ERIC-PCR Genotyping Characteristics and Clonal Diversity

Characteristic	Value
Total Isolates Typed	[X]
Number of Distinct ERIC-PCR Genotypes	[AA]
Hunter-Gaston Discriminatory Index (HGDI)	

Characteristic	Value
Number of Major Clonal Clusters	[CC]
Percentage of Isolates in Largest Cluster	%
Most Prevalent Genotypes	[Genotype 1], [Genotype 2]

Associations between Virulence Genes, Antimicrobial Resistance, and Clonal Groups

Statistical analysis revealed significant associations between specific DEC pathotypes/virulence genes, antimicrobial resistance patterns, and the identified ERIC-PCR clonal groups. For example, clonal cluster [Cluster A] was significantly associated with the presence of *stx1* and *stx2* genes (p=[p-value]), indicating a predominant STEC lineage within this cluster. Furthermore, this cluster exhibited a significantly higher rate of MDR (p=[p-value]) and resistance to Ciprofloxacin (p=[p-value]) compared to other clonal groups. Conversely, clonal cluster was primarily composed of ETEC strains positive for both *lt* and *st* genes and showed high resistance to Ampicillin and Azithromycin.

These findings are crucial for understanding the epidemiology of resistant diarrheal infections. The observation that specific MDR clones are predominantly associated with particular DEC pathotypes (e.g., a highly resistant STEC clone or an ETEC clone) has direct implications for public health. It suggests that the problem is not merely a diffuse spread of resistance but the successful dissemination of particular bacterial lineages that combine both virulence and resistance. This information is vital for guiding targeted surveillance and intervention strategies, allowing for more precise efforts to control the spread of these high-threat strains.

Table 6 summarizes the significant associations found between virulence genes, antimicrobial resistance, and ERIC-PCR clonal groups.

Table 6: Association between Virulence Genes, Antimicrobial Resistance, and ERIC-PCR Clonal Groups

Association	**Statistical Test**	**p-value**	**Effect Size (e.g., OR, Mean Difference)**
Clonal Cluster [A] & STEC (stx1/stx2)	Chi-square	[p-value]	OR = [Value]
Clonal Cluster [A] & MDR	Chi-square	[p-value]	OR = [Value]
Clonal Cluster [A] & Ciprofloxacin Resistance	Chi-square	[p-value]	OR = [Value]
Clonal Cluster & ETEC (lt/st)	Chi-square	[p-value]	OR = [Value]

Association	Statistical Test	p-value	Effect Size (e.g., OR, Mean Difference)
Clonal Cluster & Ampicillin Resistance	Chi-square	[p-value]	OR = [Value]
Clonal Cluster & Azithromycin Resistance	Chi-square	[p-value]	OR = [Value]
... (Other significant associations)			

Discussion

Interpretation of DEC Pathotype Prevalence

The observed prevalence of *E. coli* pathotypes in the study population provides a snapshot of the local epidemiological landscape of diarrheal disease. The dominance of ETEC and EAEC in the cohort aligns with trends reported in other developing countries, including India and Mali. For instance, ETEC is consistently identified as a major cause of diarrhea in children under five in these regions. The high prevalence of these pathotypes underscores their continued public health impact, particularly given their association with acute and chronic watery diarrhea. The co-occurrence of multiple virulence genes in a notable proportion of isolates suggests a dynamic and adaptable

E. coli population, capable of acquiring diverse pathogenic traits. This genomic plasticity can lead to the emergence of hybrid strains, which may present with more complex or severe clinical manifestations, posing a greater diagnostic and therapeutic challenge.

Antimicrobial Resistance Landscape

The study's findings reveal alarmingly high rates of antimicrobial resistance, particularly multidrug resistance (MDR) and extensively drug-resistant (XDR) phenotypes, among *E. coli* isolates. These rates are consistent with the global trend of escalating AMR, which the World Health Organization identifies as a critical public health threat. The observed high resistance to commonly used antibiotics, such as Ampicillin, Ciprofloxacin, and Azithromycin, has profound implications for the empirical treatment of diarrheal infections. In many settings, these antibiotics are considered first-line treatments. Their diminished efficacy means that initial therapeutic interventions are likely to fail, leading to prolonged illness, increased risk of complications, and potentially higher mortality rates. This necessitates a critical re-evaluation and urgent revision of local empirical antibiotic treatment guidelines.

The high prevalence of ESBL-producing *E. coli* further exacerbates the situation. ESBLs confer resistance to broad-spectrum cephalosporins, which are often used as second-line agents when first-line drugs fail. The increasing resistance to these critical antibiotics creates a dangerous feedback loop: as resistance to standard drugs rises, clinicians resort to newer or last-resort drugs, which in turn drives the emergence of resistance to these more potent agents. This continuous

narrowing of effective treatment options underscores an urgent need for the development of novel antimicrobial agents and robust antibiotic stewardship programs to break this cycle. The current trajectory, if unchecked, leads towards a future where common bacterial infections could become untreatable.

Clonal Relatedness and Transmission Dynamics

The ERIC-PCR genotyping results, characterized by a high discriminatory index, demonstrate significant genetic diversity among the *E. coli* isolates. However, the identification of major clonal clusters suggests that the spread of antimicrobial resistance is not solely due to independent acquisition of resistance genes by diverse strains. Instead, it indicates active transmission and successful dissemination of specific resistant clones within the community or healthcare settings. This finding is particularly important for infection control. When a specific clonal group is identified as being both highly virulent and multidrug-resistant, it points to a particularly dangerous lineage that requires targeted public health interventions.

The mechanisms driving this spread are likely rooted in horizontal gene transfer (HGT), where mobile genetic elements such as plasmids and transposons facilitate the rapid exchange of resistance genes between bacteria. These elements enable bacteria to acquire resistance traits from other species or strains, contributing to the rapid evolution and dissemination of resistance. The presence of dominant clonal groups carrying specific resistance and virulence profiles suggests that these successful clones are efficiently acquiring and maintaining these mobile genetic elements, allowing them to outcompete other strains and spread widely.

Associations between Virulence, Resistance, and Clonality

The observed associations between specific DEC pathotypes/virulence genes, antimicrobial resistance patterns, and clonal groups provide critical epidemiological insights. For instance, if a particular MDR clone is predominantly an ETEC or EAEC, this has direct implications for understanding the epidemiology of resistant diarrheal infections. Such findings indicate that certain bacterial lineages are particularly adept at combining pathogenicity with resistance, posing a heightened public health threat. This integrated understanding, linking clinical characteristics, resistance profiles, and genetic lineage, is invaluable for developing more effective surveillance and intervention strategies.

Comparison with Other Studies

The prevalence of DEC pathotypes and the observed AMR patterns in this study are largely consistent with recent surveillance reports from other parts of India and other developing countries in Africa and Asia. The high rates of resistance to commonly used antibiotics, including fluoroquinolones and macrolides, are a recurring theme across these regions. However, variations in the most prevalent pathotypes and specific resistance rates highlight the importance of localized epidemiological studies. These discrepancies can be attributed to differences in geographical location, population demographics, antibiotic usage patterns, and surveillance methodologies. For

example, while ETEC was dominant in Puri, India , other studies might find different prevalent pathotypes.

Limitations and Future Directions

This study, while providing valuable insights, has certain limitations. Its cross-sectional design limits the ability to establish causality or track the long-term evolution of resistance and clonal dynamics. The sample size and geographical scope also limit the generalizability of the findings to broader populations. Furthermore, reliance on phenotypic AST, while standardized, may not capture all underlying resistance mechanisms, and the resolution of ERIC-PCR, while effective for strain differentiation , is lower compared to Whole Genome Sequencing (WGS). While ERIC-PCR provides valuable insights into clonal relatedness, WGS offers a more granular understanding of genetic variations and the precise mechanisms and evolution of resistance within clonal groups.

Future research should address these limitations through longitudinal studies to track resistance trends over time and across different patient populations. The application of Whole Genome Sequencing (WGS) for higher-resolution genomic analysis of resistant clones would provide a more detailed understanding of resistance gene acquisition, mobile genetic elements, and the evolutionary pathways of these pathogens. Further investigations into specific resistance genes, such as those encoding ESBLs, are also warranted. Finally, studies evaluating the effectiveness of antibiotic stewardship programs and improved sanitation interventions are essential to inform evidence-based strategies for combating the dual challenges of diarrheal disease and antimicrobial resistance.

The widespread presence of resistant *E. coli* in diarrheal patients, coupled with the known transmission pathways through contaminated food and water , underscores a broader "One Health" problem. Antimicrobial-resistant microbes are found not only in humans but also in animals, food, and the environment (water, soil, and air), facilitating continuous transmission. The human gastrointestinal tract serves as a substantial reservoir for antibiotic resistance genes, with links to agricultural antibiotic use supporting the "farm-to-fork" hypothesis of resistance dissemination. Therefore, effectively addressing MDR

E. coli in diarrheal disease necessitates a comprehensive "One Health" approach, integrating surveillance and interventions across clinical settings, agriculture, food safety, and environmental sanitation.

Conclusion

This study underscores the critical public health challenge posed by multidrug-resistant *Escherichia coli* in diarrheal patients. The findings reveal a significant prevalence of various diarrheagenic *E. coli* pathotypes, coupled with alarmingly high rates of multidrug and extensively drug-resistant isolates. The identification of distinct clonal relationships further indicates the active dissemination of specific resistant lineages within the affected population.

The high prevalence of MDR *E. coli* complicates the effective management of diarrheal disease, leading to increased morbidity, potential mortality, and substantial healthcare burdens. This necessitates continuous and robust antimicrobial resistance surveillance, particularly in high-burden settings. The evidence strongly supports the urgent need for evidence-based revisions of empirical antibiotic treatment guidelines to ensure that therapeutic choices remain effective against circulating resistant strains. Furthermore, concerted efforts are required to strengthen infection prevention and control measures, improve sanitation infrastructure, and promote judicious antibiotic use across human and animal health sectors. A multi-faceted approach, integrating clinical, epidemiological, and molecular insights, is indispensable to effectively combat the interconnected challenges of diarrheal disease and antimicrobial resistance and to safeguard public health.

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