

Molecular, phenotypic, and genomic characterization of UPEC/EPEC hybrid Escherichia coli strains isolated from urinary tract infections in Northeastern Brazil

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Abstract

Urinary tract infections (UTIs) are among the most prevalent bacterial infections worldwide, with uropathogenic *Escherichia coli* (UPEC) as the leading etiological agent. The high genetic plasticity of *E. coli* facilitates horizontal gene transfer and the emergence of hybrid pathogenic strains carrying virulence determinants from distinct pathotypes, including enteroaggregative *E. coli* (EAEC). In recent years, UPEC/EAEC hybrid strains have been increasingly reported in urinary infections, raising concerns regarding their pathogenic potential and clinical impact. However, data from Northeastern Brazil remain scarce. In this study, we investigated the occurrence and characterized the molecular, phenotypic, and genomic profiles of UPEC/EAEC hybrid strains among *E. coli* isolates obtained from urine samples of patients treated at two reference hospitals in Fortaleza, Ceará, Brazil. A total of 262 isolates were analyzed using quantitative real-time PCR targeting UPEC- and EAEC-associated virulence genes. Phenotypic assays included hemolytic activity, biofilm formation, and adherence to HeLa cells. Whole-genome sequencing was performed on hybrid strains, followed by in silico serotyping, multilocus sequence typing (MLST), core genome MLST (cgMLST), and phylogenetic analyses. Among the urinary *E. coli* isolates, 234 harbored the combination of virulence-associated genes (*chuA*, *fyuA*, *yfcV*, and *vat*), and eleven (4.2%) fulfilled the criteria for UPEC/EAEC hybrid strains by harboring virulence markers characteristic of both pathotypes. These hybrids exhibited heterogeneous virulence gene profiles, including the presence of *aggR*, *aap*, *aatA*, and *pic*, with variable distribution of aggregative adherence fimbriae genes. Phenotypic analyses revealed limited expression of classical EAEC traits, whereas hemolytic activity and biofilm formation were strain-dependent. Genomic analyses demonstrated substantial genetic diversity, with the identification of multiple sequence types, including ST69, ST38, ST95, ST10, and ST421, and distinct phylogenetic clustering patterns. Collectively, our findings demonstrate the circulation of genetically diverse UPEC/EAEC hybrid strains in Northeastern Brazil and highlight the complexity of their virulence and evolutionary trajectories. These results underscore the importance of continuous molecular and genomic surveillance of hybrid *E. coli* lineages associated with urinary tract infections.

INTRODUCTION

Escherichia coli is a Gram-negative bacterium widely distributed in nature and a constituent of the intestinal microbiota of humans and endothermic animals. Although many strains are commensal, this microorganism exhibits high genetic plasticity, which has favored the emergence of distinct pathotypes associated with different clinical conditions (Nataro & Kaper, 1998; Petersen & Hubbard, 2020; Corrao et al., 2024). Classically, *E. coli* strains are grouped into diarrheagenic pathotypes (DEC), responsible for intestinal infections, and extraintestinal pathotypes (ExPEC), associated with infections outside the gastrointestinal tract (Geurtsen et al., 2022).

Among ExPEC, uropathogenic *E. coli* (UPEC) stands out as the main etiological agent of urinary tract infections (UTIs), accounting for up to 90% of community-acquired cases (Nascimento et al., 2021; Pokharel, Dhakal & Dozois, 2023). UPEC pathogenesis is multifactorial and involves adhesins, iron acquisition systems, toxins, flagellar motility, biofilm formation, and capsule production. These factors enable colonization of the urinary tract, evasion of the host immune response, and progression to more severe clinical outcomes, such as pyelonephritis and sepsis (Bien, Sokolova & Bozko, 2012; Flores-Mireles et al., 2015; Whelan, Lucey & Finn, 2023).

Within the DEC group, enteroaggregative *E. coli* (EAEC) is recognized as an important agent of acute and persistent diarrhea, especially in children and travelers. This pathotype is characterized by an aggregative adherence pattern to epithelial cells and the presence of an aggregative adherence plasmid (pAA), which encodes essential virulence factors, such as aggregative adherence fimbriae (AAF), the dispersin protein (Aap), and the transcriptional regulator AggR (Nataro et al., 1987; Estrada-García & Navarro-García, 2012; Haugan et al., 2024).

The high genetic mobility of *E. coli*, combined with the localization of many virulence factors on mobile genetic elements, facilitates horizontal gene transfer and the emergence of hybrid pathogenic strains, also referred to as hybrid pathogenic *E. coli* (HyPEC) (Braz, Melchior & Moreira, 2020; Santos et al., 2020). These strains harbor combinations of virulence factors typical of different pathotypes and may exhibit enhanced pathogenic potential. High-impact clinical events, such as the outbreak caused by an EAEC/STEC hybrid strain in Germany in 2011, highlighted the public health relevance of these lineages, which was attributed to the combination of the aggregative adherence pattern with the production of Shiga toxin 2 (Santos et al., 2020).

In recent years, several studies have identified UPEC/EAEC hybrid strains associated with urinary tract infections, suggesting that the acquisition of EAEC virulence factors by uropathogenic strains may enhance adhesion capacity, biofilm formation, and persistence in the urinary tract (Abe et al., 2008; Boll et al., 2013; Lara et al., 2017; Nascimento et al., 2021; Nascimento et al., 2022; Tanabe et al.,

2022; Schüroff et al., 2022). In Brazil, these investigations have been conducted mainly in the Southeast and Midwest regions, with more recent reports from the North of the country (Bigli et al., 2025). However, to date, there are no studies focused on the characterization of UPEC/EAEC hybrid strains in the Northeast region.

In this context, the present study aimed to characterize UPEC/EAEC hybrid strains isolated from urine samples obtained from Hospital Universitário Walter Cantídio and Maternidade Escola Assis Chateaubriand, in Fortaleza, Ceará, Brazil, contributing to the understanding of the molecular, clinical, and epidemiological profiles of these strains in Northeastern Brazil.

MATERIALS AND METHODS

Experimental design and samples

Escherichia coli isolates were obtained from urine samples of patients attended at Hospital Universitário Walter Cantídio (HUWC-UFC) and Maternidade Escola Assis Chateaubriand (MEAC-UFC), following medical requests for urine culture. Only isolates identified as *E. coli* were included in the study, while other uropathogenic microorganisms were excluded. The sample size was estimated considering $\alpha = 0.05$, 80% statistical power, and the previously reported prevalence of UPEC/EAEC hybrid strains in Brazil, resulting in a minimum of 262 isolates. The study was approved by the Research Ethics Committee of the Federal University of Ceará (CAAE No. 6.148.644).

Bacterial culture and identification

Urine samples were inoculated onto chromogenic agar and incubated at 37°C for up to 48 h. Isolates showing growth $\geq 10^5$ CFU/mL were subjected to bacterial identification and antimicrobial susceptibility testing using the Vitek 2 Compact system (bioMérieux), according to the manufacturer's recommendations and BrCAST criteria. The strains were cultured in tryptic soy broth (TSB) at 37°C for 18 h under agitation. UPEC and EAEC reference strains were cultivated under the same conditions.

Primer design and validation

Genes associated with UPEC (*fyuA*, *yfcV*, *chuA*, and *vat*) and EAEC (*aggR*, *aap*, *aatA*, *aggA*, *aafA*, *agg3A*, *agg4A*, *agg5A*, *CS22*, *pet*, and *pic*) were selected for molecular identification (Table 1). Gene sequences were retrieved from the BV-BRC and ENA databases, aligned to identify conserved regions, and primers were designed using Primer-BLAST (NCBI). Primer validation included efficiency, specificity, and limit of detection analyses by qPCR using serial dilutions of genomic DNA. Only primers with amplification efficiencies between 90–110% and a single peak in melting curve analysis were used in subsequent assays.

Table 1
– qPCR primer sequence for the evaluated genes.

Gene	Primers	qPCR product	TM (°C)	Reference
Virulence markers of UPEC				
<i>fyuA</i>	(F) TGGTCGGGCCGCGATTATTTTC (R) GCTGCCGTTCATGTAGCCAT	167 pb	65°C	This study
<i>yfcV</i>	(F) TTAAAGCCTGGCTGGTGGG (R) TGGAATGTGGTCAGGGTTTCG	71 pb	65°C	This study
<i>chuA</i>	(F) TAATGCGCAAAACACGGGGA (R) CCCGGATGTTGTTCTGACG	153 pb	60°C	Queiroga, 2023
<i>vat</i>	(F) TGGAACGACAGTGAATGGA (R) ACCCCACACCGTAAAGCATA	238 pb	61°C	Vigil et al., 2011
Virulence markers of EAEC				
<i>aggR</i>	(F) ATCGGCTGTAAGCTTCTTTTCCG (R) CTGCTTTGCTCATTCTTGATTGCAT	185 pb	60°C	This study
<i>aap</i>	(F) GGACCCGTCCCAATGTATAA (R) CCATTCCGTTAGAGCACGAT	250 pb	60°C	Havt et al., 2017
<i>aatA</i>	(F) CGGCTTATGAAGCAAAAATGCAGA (R) CGAGCATCAACATCAGGTGAGA	156 pb	60°C	This study
<i>aggA</i>	(F) TCTATCTRGGGGGGCTAACG (R) ACCTGTTCCCCATAACCAGAC	218 pb	65°C	Jønsson et al., 2017
<i>aafA</i>	(F) CTACTTTATTATCAAGTGGAGCCGCTA (R) TAGGAGAGGCCAGAGTGWATCC	292 pb	65°C	Jønsson et al., 2017
<i>agg3A</i>	(F) TTGCGAGTCTGGTATTCAGCTTG (R) TTAATGCTCCTCGTCCTGGCT	186 pb	65°C	This study
<i>agg4A</i>	(F) TGGCGGTCTTATTTCTGGCAT (R) GCTGTGGTTTTTGTGGCCCT	132 pb	65°C	This study
<i>agg5A</i>	(F) CTTTAATGACGACGTACGGTGA (R) ACGGTATATTGTCCGGGTTTGTA	145 pb	60°C	This study
<i>CS22</i>	(F) ACCATCAACGCAACCTGGAC (R) GACCTGCAGCATTTTAAATGGGA	181 pb	60°C	This study
<i>pet</i>	(F) ATTTGGCTCCGCTGACAAGG (R) TACTGCCGGTGGAGTTCAGT	188 pb	60°C	This study
<i>pic</i>	(F) TGGTATGCCGGTGCTGAAAC (R) AGCGGAATGTTTTCCCGGAC	109 pb	60°C	This study

Sequences of the primers used in qPCR reactions for the detection of virulence markers associated with the uropathogenic (UPEC) and enteroaggregative (EAEC) pathotypes of *Escherichia coli*. The table presents the target genes, the sequences of the forward (F) and reverse (R) primers, the expected size of the amplification product (in base pairs), the annealing temperature (Tm) used in the reactions, and the respective bibliographic references.

DNA extraction and qPCR

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega) and quantified by spectrophotometry (NanoDrop ND-1000). qPCR reactions were performed using GoTaq® qPCR Master Mix (Promega) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Amplification parameters followed standardized protocols, and product specificity was confirmed by melting curve analysis. The hybrid UPEC/EAEC *E. coli* strain HSP60 (Nascimento et al., 2021) was used as the positive control for qPCR, while *E. coli* strain CN066, obtained from the strain collection analyzed in this study, was used as the negative control.

Hemolytic activity

Hemolytic activity was assessed on blood agar plates containing 5% sheep blood. Standardized bacterial suspensions (0.5 McFarland) were inoculated and incubated at 37°C for 24 h (Beutin, 1991). Hemolysis was classified into α -hemolysis (partial hydrolysis of red blood cells), β -hemolysis (complete hydrolysis of red blood cells), and γ -hemolysis (absence of hydrolysis) (Lad et al., 2022). The *Staphylococcus aureus* ATCC 38591 strain was used as a positive control (β -hemolysis), and the *Enterococcus faecalis* ATCC 29212 strain was used as a negative control (γ -hemolysis). The UPEC strain CFT073 was used as a positive control for UPEC.

Biofilm production

Biofilm formation was assessed using the crystal violet microtiter plate assay. Briefly, bacterial strains were cultured on MacConkey agar at 37°C for 16 h and then inoculated into Brain Heart Infusion (BHI) broth supplemented with 1% glucose. The bacterial suspension was adjusted to a 6.0 McFarland standard, and 200 μ L was transferred to flat-bottom 96-well polystyrene microplates. After incubation at 37°C for 48 h, the wells were washed with PBS, fixed with 99% methanol for 15 min, stained with 0.3% crystal violet for 10 min, washed again with PBS, and air-dried. The bound dye was solubilized with 33% (v/v) acetic acid, and the optical density (OD) was measured at 490 nm. Wells containing culture medium without bacterial inoculum served as the negative control. Biofilm production was classified according to the method described by Stepanović et al. (2007). The OD cut-off value (OD_c) was calculated as the mean OD of the negative controls plus three standard deviations. Based on the OD_c, strains were classified as non-biofilm producers (OD $\leq$ OD_c), weak (OD_c < OD $\leq$ 2×OD_c), moderate (2×OD_c < OD $\leq$ 4×OD_c), or strong biofilm producers (OD > 4×OD_c).

Cell adhesion assays

Adherence patterns were evaluated using HeLa cells cultured in RPMI supplemented with 10% fetal bovine serum. The cells were incubated at 37°C in an incubator with 5% CO₂ until they reached 70% confluence. The hybrid strains were cultured in TSB at 37°C for 18 h. After this period, 20 μ L of the bacterial suspension was added to each well containing HeLa cells. The plate was incubated at 37°C for three hours. Subsequently, the medium was removed, and the cells were washed with PBS (pH 7.2) (Rodrigues et al., 1996). The adherent cells and bacteria were fixed on the coverslips with 1 mL of analytical-grade methanol for 10 minutes and stained with May-Grünwald/Giemsa for 15 minutes. Strain EAEC 042 was used as a positive control for aggregative adherence, strain UPEC CFT073 served as the UPEC control, and *E. coli* K-12 MG1655 was used as a negative (non-adherent) control.

Whole-genome sequencing and bioinformatics analyses

Whole-genome sequencing was performed on the Illumina NextSeq 1000/2000 platform using the Illumina DNA Prep kit. Read quality was assessed with FastQC, and preprocessing was carried out using Fastp. De novo genome assembly was conducted with SPAdes and evaluated using QUAST. Molecular typing was performed using classical MLST and cgMLST/wgMLST approaches with chewBBACA. In silico serotyping was carried out using SerotypeFinder.

Phylogenetic analysis

Phylogenetic analysis was based on core genome alignment using Parsnp. The phylogenetic tree was inferred by maximum likelihood with IQ-TREE, applying the GTR + G model, and statistical support was assessed by ultrafast bootstrap (1,000 replicates) and SH-aLRT. Final visualization was performed using FigTree.

RESULTS

Primer validation by in silico analysis and qPCR

Primers designed for genes associated with the UPEC and EAEC pathotypes were initially evaluated by in silico analysis. Sequences retrieved from the BV-BRC and ENA databases showed wide variability in the number of available records, reflecting the differential distribution of these genes among *Escherichia coli* strains. BLAST analysis demonstrated that all primers exhibited 100% coverage and identity with their target sequences, as well as E-values close to zero, indicating high specificity

A CFU count (Table S1) was performed on the UPEC and EAEC positive control strains to determine the bacterial load detectable by the primers used in this study. DNA was extracted from the bacterial suspension used for serial dilutions and subsequently diluted to a concentration of approximately 50 ng/μL.

Experimental validation by qPCR demonstrated adequate performance for all primers evaluated, with amplification efficiencies ranging from 90% to 93% and coefficients of determination (R^2) ≥ 0.997 . Melting curve analysis revealed a single peak for each reaction, confirming the absence of nonspecific amplification. Primers associated with UPEC (*fyuA* (Figure S1), *yfcV* (Figure S2), *chuA* (Figure S3), and *vat* (Figure S4)) showed equivalent limits of detection, being able to detect DNA concentrations as low as 0.001718 ng/μL, corresponding to approximately 2.7×10^3 CFU/mL. Similarly, primers targeting EAEC detection (*aggR* (Figure S5) and *aap* (Figure S6)) exhibited comparable sensitivity, with a minimum estimated detection of 1.31×10^4 CFU/mL.

Molecular characterization and frequency of virulence genes

Although all isolates (n = 262) were recovered from urine samples and were therefore considered uropathogenic *E. coli*, 234 (89.3%) isolates carried the combination of virulence genes described by Spurbeck et al. (2012) as being strongly associated with UPEC strains. Which considers the presence of the *fyuA* gene in association with *yfcV*, *chuA*, or *vat*. Among the observed genetic profiles, the combinations *fyuA* + *yfcV* + *chuA* and *fyuA* + *yfcV* + *chuA* + *vat* were the most frequent (Table 2).

Table 2
– Distribution of samples according to combinations of virulence genes.

Combinations of virulence genes	Quantity of samples
<i>fyuA</i> + <i>yfcV</i>	7
<i>fyuA</i> + <i>chuA</i>	33
<i>fyuA</i> + <i>vat</i>	0
<i>fyuA</i> + <i>yfcV</i> + <i>chuA</i>	123
<i>fyuA</i> + <i>chuA</i> + <i>vat</i>	1
<i>fyuA</i> + <i>yfcV</i> + <i>chuA</i> + <i>vat</i>	70
Negatives	28

Frequency of combinations of the genes *fyuA*, *yfcV*, *chuA*, and *vat* among the analyzed samples. The predominance of the *fyuA*+*yfcV*+*chuA* profile (n = 123) and *fyuA*+*yfcV*+*chuA* + *vat* (n = 70) was observed, while 28 samples did not present any of the evaluated genes.

Analysis of virulence gene frequency according to sex revealed no statistically significant differences for most markers, except for the *yfcV* gene, which was more frequent in samples from female patients.

Eu acho que faltou apresentar primeiro os resultados dos genes de EAEC detectados nos 262 isolados. Talvez uma tabela igual a anterior (Table 2). Depois caberia a frase abaixo.

EAEC-associated genes (*aggR* and *aap*) were detected exclusively in samples from female patients, although without statistical significance.

The distribution of genes according to age group did not show significant differences among the analyzed groups. However, a higher concentration of virulence genes was observed in the 20–39-year age group, which also reflected the greater sample representativeness of this group. Analysis by sector of origin demonstrated a high frequency of the *chuA* gene across all evaluated sectors, whereas *aggR* and *aap* showed sporadic distribution among sectors (Table 3).

Table 3
– Frequency of virulence genes according to the sector of origin of the samples.

<i>fyuA</i>	Emergency MEAC (%) (n = 71)	Urodynamics MEAC (%) (n = 31)	Maternal-fetal outpatient clinic (%) (n = 29)	Post-Kidney Transplant (%) (n = 17)	MEAC Station I (%) (n = 15)	Other Sectors (%) (n = 99)
	94,36	90,32	79,31	100	80	88,8
<i>yfcV</i>	77,4	74,19	75,86	88,23	86,66	90,9
<i>chuA</i>	95,77	100	96,55	100	93,33	92,92
<i>vat</i>	26,76	29	31	29,41	6,67	25,25
<i>aggR</i>	8,45	0	3,44	5,88	0	3,03
<i>aap</i>	5,63	0	3,44	0	0	1,01

Frequency of positivity for each gene according to the sector of origin of the samples.

Identification and genetic profile of UPEC/EAEC hybrid strains

Based on the simultaneous detection of UPEC markers (*fyuA* associated with *yfcV*, *chuA*, or *vat*) and EAEC markers (*aggR* and *aap*), eleven UPEC/EAEC hybrid strains were identified, corresponding to a frequency of 4,2% among the analyzed isolates. These strains additionally harbored genes associated with aggregative adherence fimbriae and other EAEC-associated virulence factors, such as *aatA* and *pic*, with variable profiles among isolates (Table 4).

Table 4
– Virulence gene profile of UPEC/EAEC hybrid strains found in this study.

CN066	<i>fyuA</i>	<i>yfcV</i>	<i>chuA</i>	<i>vat</i>	<i>aggR</i>	<i>aap</i>	<i>aatA</i>	<i>aggA</i>	<i>aafA</i>	<i>agg3A</i>	<i>agg4A</i>	<i>agg5A</i>	CS22	<i>pet</i>	<i>pic</i>
	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
HSP60	+	-	+	+	+	+	+	-	-	-	-	-	-	-	+
HIB167	+	+	+	-	+	+	+	-	-	+	-	-	-	-	+
HIB168	+	+	+	-	+	+	+	-	-	+	-	-	-	-	+
HIB169	+	+	+	-	+	+	+	-	-	+	-	-	-	-	+
HIB170	+	+	+	+	+	+	+	-	-	+	-	-	-	-	+
HIB172	+	+	+	-	+	+	+	-	-	+	-	-	-	-	+
HIB202	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+
HIB204	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+
HIB205	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+
HIB206	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+
HIB226	+	+	+	+	+	+	+	-	-	+	-	-	-	-	+
HIB229	+	-	+	-	+	+	+	-	-	+	-	-	-	-	+

Molecular characterization of the 11 hybrid strains regarding the presence of enteroaggregative *E. coli* (EAEC) virulence genes. The table presents the individual genetic profile of each isolate, demonstrating the distribution of EAEC-specific virulence markers among the hybrid strains identified in the study.

Phenotypic characterization of hybrid strains

Evaluation of hemolytic activity revealed that six hybrid strains exhibited β -hemolysis, whereas the remaining strains displayed an α -hemolytic pattern (Fig. 1). The UPEC control strain CFT073 showed β -hemolysis.

Regarding biofilm formation, most hybrid strains were classified as non-producers. Four isolates showed strong (HIB172, HIB202 and HIB226) to moderate (HIB206) biofilm-forming capacity, with no statistically significant differences compared to control strains. The CN066 strain, used as a negative control, exhibited strong biofilm-forming capacity.

In HeLa cell adherence assays, three hybrid isolates (HIB168, HIB169, and HIB205) (Fig. 2B, 2C and 2H) exhibited the classical aggregative adherence (AA) pattern characteristic of enteroaggregative *E. coli*. The remaining isolates showed only sparse, non-organized bacterial adherence, without a defined adherence pattern.

Hemolytic activity of representative hybrid UPEC/EAEC isolates cultured on 5% sheep blood agar. The upper plate shows isolate HIB172 together with the positive control (*S. aureus*), negative control (*E. faecalis*), and the reference UPEC CFT073 strain. The lower plates show representative hybrid isolates (CN066, HIB167, HIB168, HIB169, HIB170, HIB202, HIB204, HIB205, HIB206, HIB226, and HIB229) evaluated for hemolysin production. *S. aureus* and *E. faecalis* were included as positive and negative controls, respectively.

Representative adhesion patterns of hybrid UPEC/EAEC isolates on HeLa cells following May–Grünwald–Giemsa staining. Panels correspond to isolates HIB167 (A), HIB168 (B), HIB169 (C), HIB170 (D), HIB172 (E), HIB202 (F), HIB204 (G), HIB205 (H), HIB206 (I), HIB226 (J), and HIB229 (K). Panels L, M and N shows the prototype enteroaggregative *Escherichia coli* strain EAEC 042, used as the positive control; UPEC control strain CFT073 and *E. coli* strain K12 MG1655 (non-adherent control), respectively. Images were acquired by light microscopy under the same magnification. Scale bar = 100 μ m.

Antimicrobial susceptibility profile

Analysis of antimicrobial susceptibility profiles demonstrated heterogeneity among the hybrid strains. Most isolates showed broad susceptibility, whereas three strains exhibited multidrug-resistant profiles, with resistance to multiple antimicrobial classes, including

Genomic and phylogenetic characterization of hybrid strains

MLST analysis revealed genetic diversity among the hybrid strains, with predominance of ST69, identified in two isolates, while other STs were observed sporadically (Table 6). In four strains, it was not possible to assign a valid ST using classical MLST. cgMLST analysis enabled the typing of all strains, providing higher resolution for genetic differentiation among isolates (Fig. 2A).

Table 6
– Sequence types and serotypes
of hybrid strains analyzed in this
study.

Strains	ST	Serotype
CN066	-	O?: H19
HIB167	-	O86:H2
HIB168	69	O86:H2
HIB169	38	O153:H9
HIB170	95	(O2/O50):H7
HIB202	10	O3:H2
HIB204	421	O1:H7
HIB205	-	NA
HIB206	-	O9a:H10
HIB226	-	O9a:H10
HIB229	69	O86:H2
HSP60	69	O15:H2

Molecular characterization of *Escherichia coli* strains, showing sequence type (ST) determined by multilocus sequence typing (MLST) and serotype identified by in silico analysis. A dash (–) indicates that the sequence type could not be assigned, and NA indicates that the serotype was not determined.

In silico serotyping revealed predominance of serotypes O86:H2 and O9a:H10, as well as unique or partially typeable serotypes (Table 6). Core genome–based phylogenetic analysis revealed distinct clustering among the isolates, confirming the genomic diversity of the analyzed hybrid strains and their relationship with previously described UPEC, EAEC, and hybrid reference strains (Fig. 2B).

Phylogenetic relationships among *Escherichia coli* strains analyzed in this study. (A) Minimum spanning tree (MST) based on core genome multilocus sequence typing (cgMLST), where nodes represent individual strains and numbers on the branches indicate the absolute allelic distances between them. (B) Maximum-likelihood phylogenetic tree inferred from core genome single nucleotide polymorphisms (SNPs), illustrating the evolutionary relationships among the strains and reference genomes. Strains analyzed in this study are highlighted in blue.

DISCUSSION

In this study, we systematically investigated the occurrence of UPEC/EAEC hybrid strains among urinary isolates obtained from reference hospital in Northeastern Brazil, integrating molecular, phenotypic, epidemiological, and genomic analyses. The identification of strains simultaneously harboring classical UPEC and EAEC markers confirms the circulation of hybrid profiles in this regional setting and reinforces the high genetic plasticity of *Escherichia coli*, which is capable of acquiring and maintaining virulence determinants associated with distinct pathogenic niches.

The standardization and validation of primers specific to genes associated with the UPEC and EAEC pathotypes represented an essential step to ensure the methodological robustness of the study. Considering the limitations described in the literature for previously used primers, particularly regarding qPCR efficiency (Spurbeck et al., 2012; Oh et al., 2014), experimental validation allowed the establishment of reliable limits of detection and ensured accurate interpretation of molecular data obtained from clinical samples. This approach enabled consistent correlation of the identified genetic profiles with epidemiological and phenotypic data.

From an epidemiological perspective, the predominance of isolates from female patients and young adults is consistent with the classical profile of urinary tract infections, as widely described in the literature (Kaur & Kaur, 2021; He et al., 2025). The concentration of samples from the Maternidade Escola Assis Chateaubriand, particularly from sectors such as the Emergency Department and the

Maternal–Fetal Outpatient Clinic, reflects a clinical context in which physiological and healthcare-related factors may favor both the occurrence and recurrence of urinary tract infections, underscoring the relevance of microbiological surveillance in this setting.

The high frequency of the *chuA*, *fyuA*, and *yfcV* genes among isolates classified as UPEC is consistent with national and international studies (Lara, 2014; Rigvava et al., 2023). The predominance of *chuA*, detected in more than 95% of the samples, highlights the importance of the heme-mediated iron acquisition system in urinary pathogenesis, as well as its role in the formation of intracellular bacterial communities (Spurbeck et al., 2012). In contrast, the *vat* gene showed lower prevalence, corroborating evidence that this factor is more closely associated with dissemination and cytotoxicity than with the initial establishment of urinary tract infection (Nichols et al., 2016; Krawczyk & Wityk, 2025).

The identification of 11 UPEC/EAEC hybrid strains (4,2%) falls within the range reported in Brazilian studies, which tend to show less variability compared with international data (Lara et al., 2017; Nascimento et al., 2021; Tanabe et al., 2022). This consistency suggests greater national methodological standardization in the definition of hybrid strains, in contrast to international studies that adopt heterogeneous criteria and often rely on small sample sizes, resulting in highly variable frequencies (Santos et al., 2020; Bighi et al., 2025).

Detailed analysis of the genetic profiles of the hybrid strains revealed substantial heterogeneity among isolates. All hybrid strains carried the *aggR*, *aap*, *aatA*, and *pic* genes, reinforcing the acquisition of an EAEC-associated genetic backbone. However, the distribution of aggregative adherence fimbriae (AAF) variants was variable. Strains HIB167, HIB168, HIB169, HIB170 and HIB172 carried exclusively the *agg3A* variant (AAF/III), whereas HIB202, HIB204, HIB205, and HIB206 exhibited combinations involving *aggA* (AAF/I) and *agg3A*, and in the case of HIB206, also *agg4A* (AAF/IV). The concomitant presence of multiple AAF variants, as observed in HIB205 and HIB206, although uncommon, has been previously described and may reflect independent gene acquisition events rather than functional redundancy (Boisen et al., 2020; Freire et al., 2022).

Phenotypic evaluation showed that the expression of virulence factors varied substantially among hybrid strains. Hemolytic activity was observed in five isolates (HIB167, HIB168, HIB202, HIB204, and HIB229), suggesting that the presence of this virulence-associated is not a universal phenotype among hybrid strains. Biofilm formation was even more restricted, being moderately detected only in strains HIB202 and HIB226, while HIB206 exhibited weak biofilm formation. Notably, only strain HIB202 combined hemolytic activity with biofilm-forming capacity, indicating an increased potential for tissue damage and persistence in the urinary tract.

In cell adhesion assays, three hybrid isolates (HIB168, HIB169, and HIB205) exhibited the classical aggregative adherence (AA) pattern characteristic of EAEC, whereas the remaining isolates showed only sparse, non-organized bacterial adhesion. These findings indicate that the acquisition of EAEC-associated virulence determinants may result in the expression of the AA phenotype in only a subset of hybrid strains, suggesting that additional regulatory mechanisms influence the functional expression of adhesins. The phenotypic heterogeneity observed among the hybrids reinforces the notion that the combination of UPEC and EAEC virulence genes does not necessarily translate into the simultaneous expression of both parental phenotypes, but rather into distinct functional configurations.

Integration of phenotypic data with antimicrobial resistance profiles revealed relevant patterns. Strain HIB169, belonging to the ST38 complex, stood out for exhibiting the highest level of multidrug resistance, including ESBL production, despite not expressing hemolytic activity, biofilm formation, or distinctive adherence. This finding is consistent with the literature, which associates ST38 with high antimicrobial resistance and dissemination in hospital environments (Carvalho et al., 2020; Soncini et al., 2024). In contrast, strains such as HIB202 and HIB206 displayed multiple virulence phenotypes associated with specific clinical settings, such as the Maternal–Fetal Outpatient Clinic and the Pre-Transplant Outpatient Clinic, respectively, suggesting an influence of the clinical environment and selective pressure exerted by antimicrobial therapy.

Genomic analyses provided additional support for these observations. The recurrence of the ST69 complex in strains HIB168, HIB229, and the control strain HSP60, together with phylogenetic proximity and the sharing of related serotypes, suggests clonal dissemination of hybrid lineages belonging to this complex in Brazil, as previously described by Lara et al. (2017). Antigenic variation observed within this clonal context further reinforces the evolutionary dynamics of these strains, likely driven by recombination and horizontal gene transfer.

Other clonal complexes identified, such as ST95/ST421 (HIB170 and HIB204) and ST10 (HIB202), indicate that the emergence of UPEC/EAEC hybrid strains is not restricted to a single genetic background. While complexes such as ST69 and ST38 appear to represent already consolidated hybrid lineages, strains such as HIB206 and HIB226, which share serotypes and exhibit partial acquisition of EAEC-associated genes, may represent intermediate stages in this evolutionary process.

CONCLUSION

Taken together, the results of this study indicate that UPEC/EAEC hybrid strains circulating in Northeastern Brazil display high genotypic and phenotypic heterogeneity, reflecting multiple evolutionary trajectories. The integrated strain-by-strain analysis highlights that the emergence of these lineages involves distinct combinations of virulence, antimicrobial resistance, and clonal background, reinforcing the importance of continuous genomic surveillance and in-depth characterization of these isolates in the context of urinary tract infections.

Declarations

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Author Contribution

D.O.S. and A.H. conceived and designed the study. D.O.S. performed the laboratory experiments and F.C.O., L.F.B.L., A.E.S.A., R.N.D.G. and S.A.R contributed to the laboratory experiments. L.G.R., G.S.C., G.M.D., and I.F.N.L. were responsible for the collection of clinical isolates and clinical data. A.M.M.F., C.R.D., C.B.M., F.M., and L.G.Z.C. performed the whole-genome sequencing. D.O.S. analyzed the molecular, phenotypic, and genomic data and wrote the first draft of the manuscript. M.A.F.C. contributed to the bioinformatic analyses and critically revised the manuscript. A.H. supervised the study and revised the manuscript. All authors reviewed the manuscript, approved the final version, and agree to be accountable for the work.

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Table 5

Table 5 is available in the Supplementary Files section.

Figures

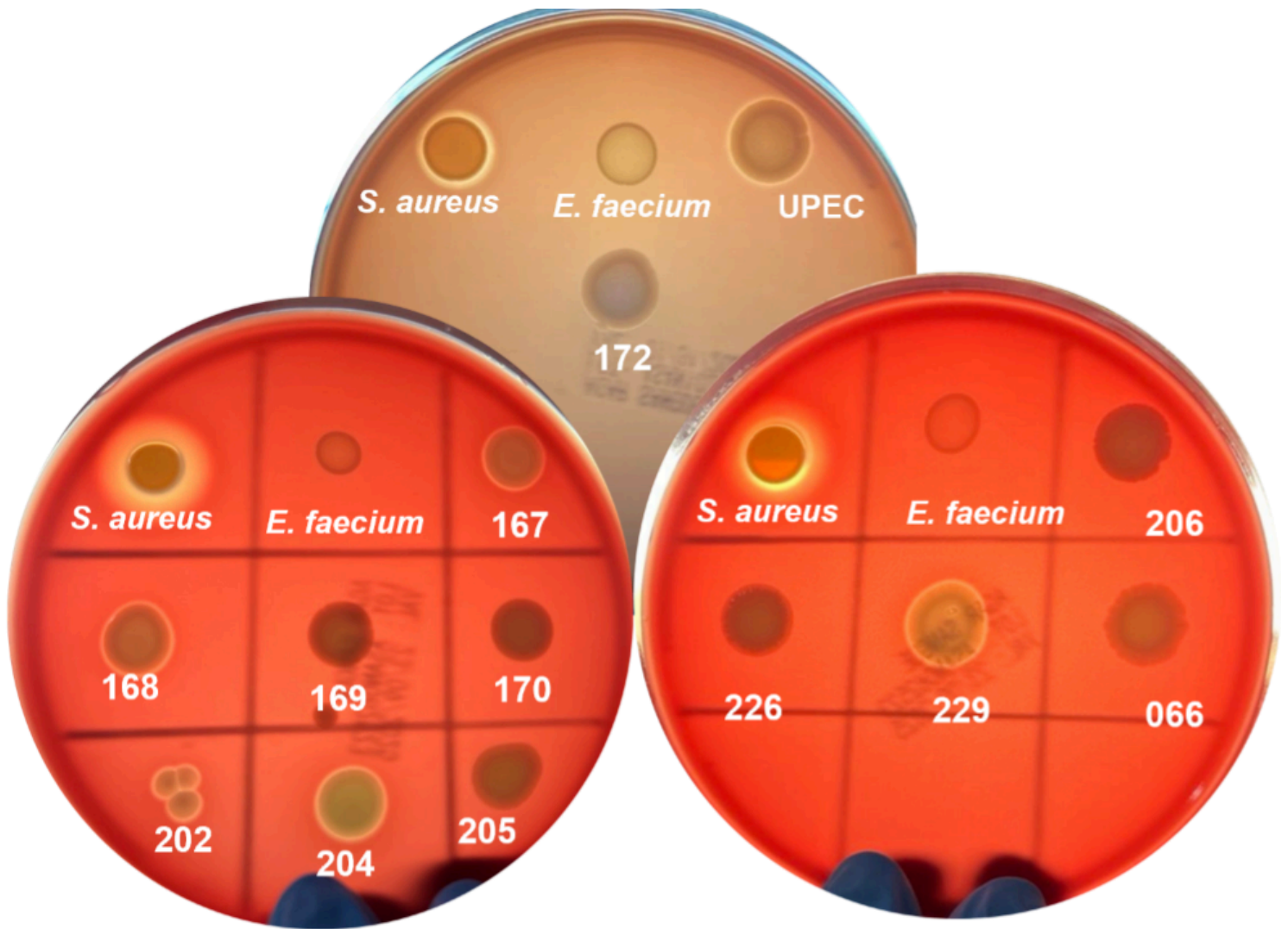


Figure 1

Hemolytic activity of hybrid UPEC/EAEC isolates on blood agar.

Hemolytic activity of representative hybrid UPEC/EAEC isolates cultured on 5% sheep blood agar. The upper plate shows isolate HIB172 together with the positive control (*S. aureus*), negative control (*E. faecalis*), and the reference UPEC CFT073 strain. The lower plates show representative hybrid isolates (CN066, HIB167, HIB168, HIB169, HIB170, HIB202, HIB204, HIB205, HIB206, HIB226, and HIB229) evaluated for hemolysin production. *S. aureus* and *E. faecalis* were included as positive and negative controls, respectively.

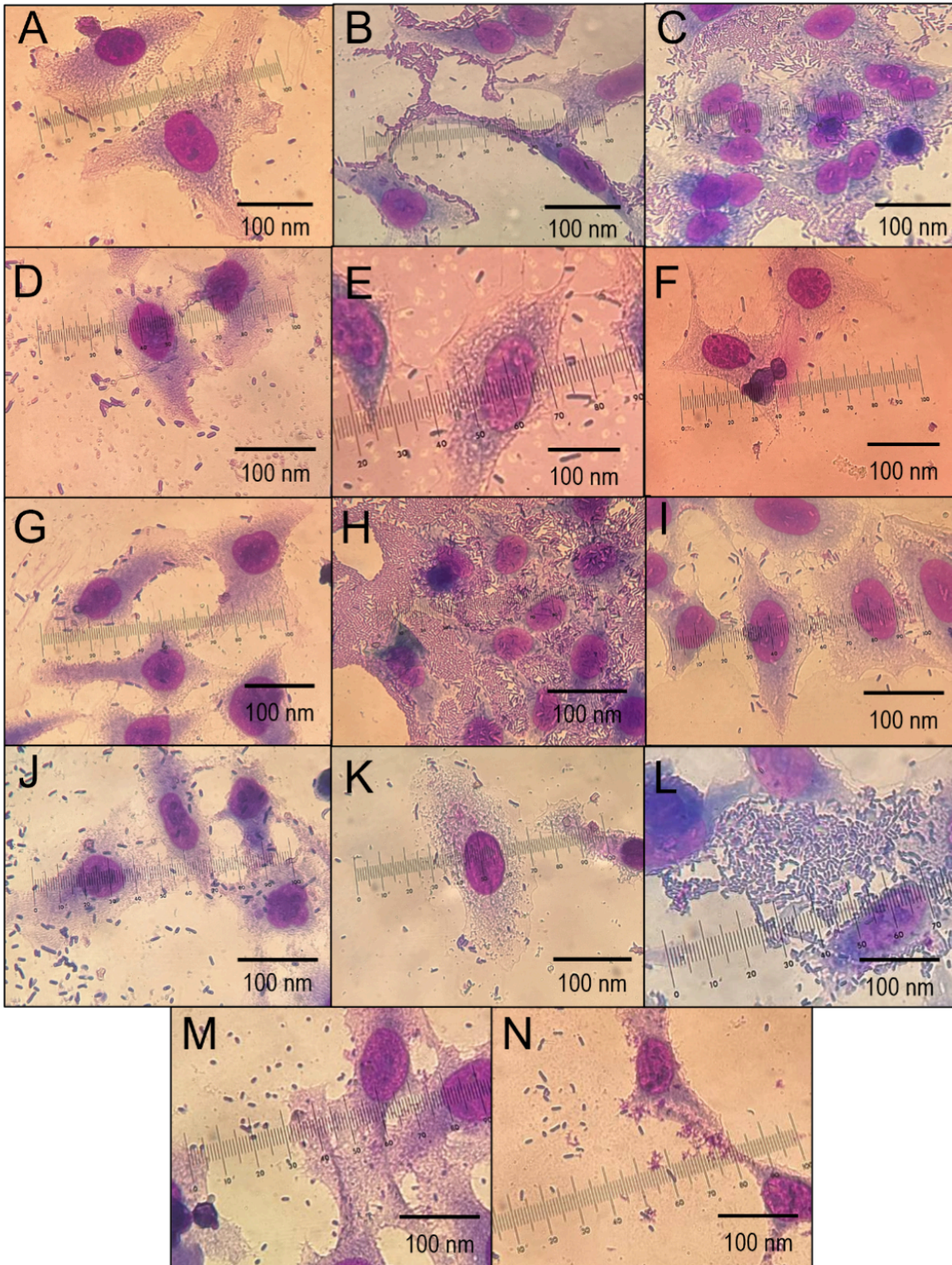


Figure 2

Adhesion patterns of hybrid UPEC/EAEC isolates on HeLa cells.

Representative adhesion patterns of hybrid UPEC/EAEC isolates on HeLa cells following May–Grünwald–Giemsa staining. Panels correspond to isolates HIB167 (A), HIB168 (B), HIB169 (C), HIB170 (D), HIB172 (E), HIB202 (F), HIB204 (G), HIB205 (H), HIB206 (I), HIB226 (J), and HIB229 (K). Panels L, M and N shows the prototype enteroaggregative *Escherichia coli* strain EAEC 042, used as the positive control; UPEC control strain CFT073 and *E. coli* strain K12 MG1655 (non-adherent control), respectively. Images were acquired by light microscopy under the same magnification. Scale bar = 100 μm.

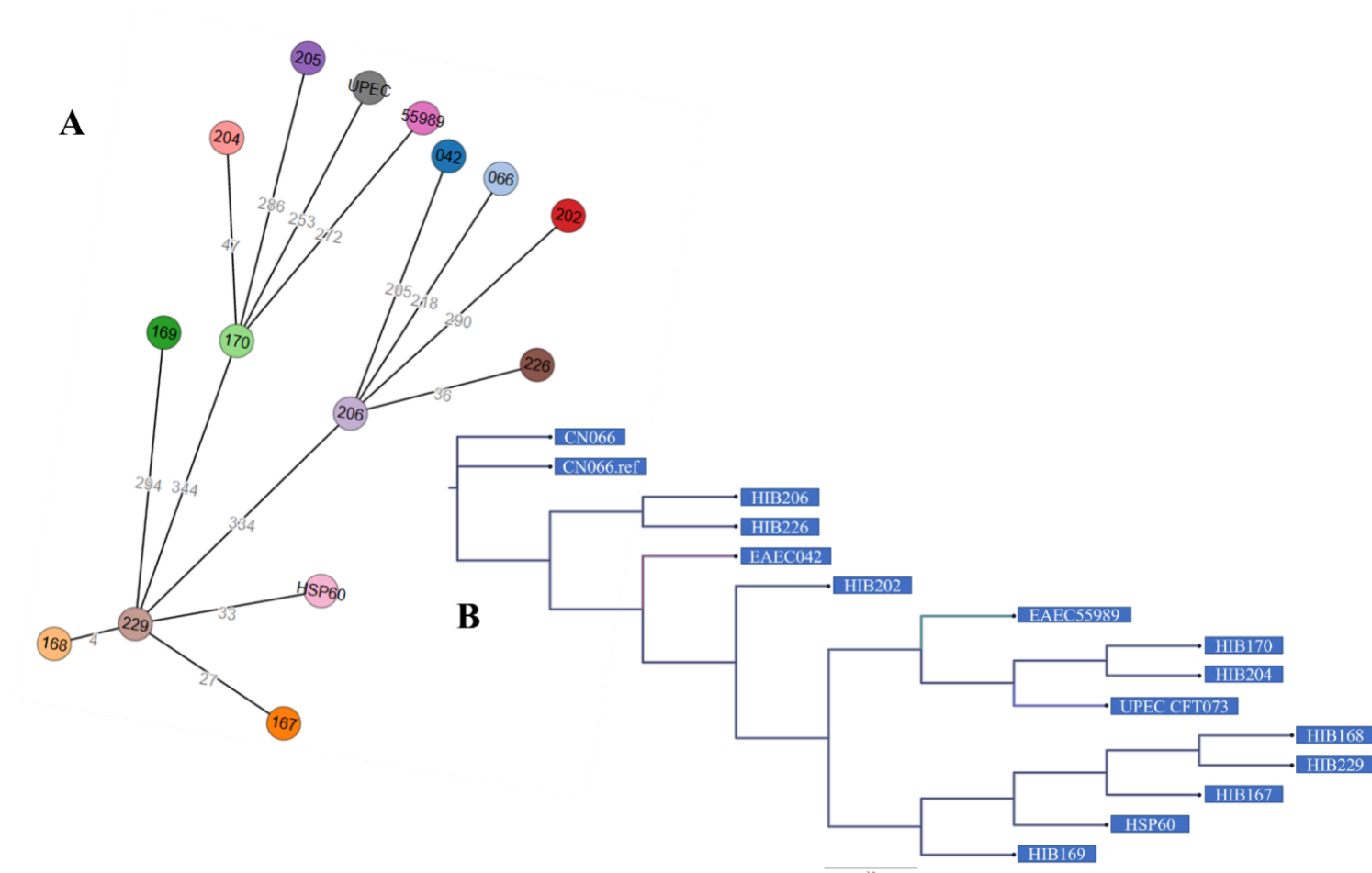


Figure 3

Genetic relatedness of hybrid strains based on cgMLST and core genome SNP analyses.

Phylogenetic relationships among *Escherichia coli* strains analyzed in this study. **(A)** Minimum spanning tree (MST) based on core genome multilocus sequence typing (cgMLST), where nodes represent individual strains and numbers on the branches indicate the absolute allelic distances between them. **(B)** Maximum-likelihood phylogenetic tree inferred from core genome single nucleotide polymorphisms (SNPs), illustrating the evolutionary relationships among the strains and reference genomes. Strains analyzed in this study are highlighted in blue.

Supplementary Files

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