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ORIGINAL RESEARCH

ESBL-Producing and Non-ESBL-Producing *Escherichia coli* Isolates from Urinary Tract Differ in Clonal Distribution, Virulence Gene Content and Phylogenetic Group

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Purpose: The objectives of this study are to determine the differences in clonality, virulence gene (VG) content and phylogenetic group between non extended-spectrum beta-lactamase-producing *E. coli* (non-ESBL-EC) and ESBL-EC isolates from urine.

Patients and Methods: This study characterized a total of 100 clinical *E. coli* isolates consecutively obtained from the inpatients hospitalized in The First Affiliated Hospital of Ningbo University in China by polymerase-chain reaction (PCR).

Results: Phylogenetic group B2 was found to be the most prevalent in both ESBL-EC and non-ESBL-EC group. Among 100 clinical isolates, the count of acquired virulence genes in group B2 was found to be significantly higher than that in group A, B1, and D (p <0.001). Additionally, the presence of content within virulence genes (the total number of virulence genes detected per isolate) in B2 of non-ESBL-EC and ESBL-EC showed a significant difference (p<0.001). ST131 was detected exclusively in ESBL-EC, while ST95 and ST73 were the main sequence types in non-ESBL-EC.

Conclusion: Our study demonstrated the different distribution of MLST, phylogenetic group in ESBL-EC and non-ESBL-EC group. The inverse association between beta-lactamase resistance and VG content performed in this study should get a lot more attention. At the same time, we should also be wary of the appearance of non-ESBL-EC isolates of group B2 harboring more virulence genes which will lead to high pathogenicity.

Keywords: *E. coli*, clonal structure, ST73, ST95, ST131, ST1193, virulence genes

Introduction

Escherichia coli is the primary cause of urinary tract infections (UTI).[1,2] Extended-spectrum beta-lactamases (ESBL)-producing strains, which are resistant to extended-spectrum cephalosporins and monobactams[3,4], play an important role in UTI.

The prevalence of ESBL-producing *E. coli* (ESBL-EC) is on the rise in the United States,[5] Africa,[6] and Vietnam regions,[7] despite variations in geography and population. In Jordan, the prevalence of ESBL-EC reached 62% in hospitalized patients with UTIs,[8,9] which is a concerning trend. Similarly, China has shown a high prevalence of ESBL-EC isolates in urinary tract infections.[10] Unfortunately, the common treatment for ESBL-EC involves parenteral aminoglycosides or carbapenems, which require hospitalization[11] and increase financial burden.[12] Therefore, it is of great significance to understand the clinical characteristics of *E. coli* causing UTI episodes, as they represent different target populations for prevention. In order to develop effective preventive strategies for UTIs caused by both non-ESBL-EC and ESBL-EC, it is important to gain thorough insight into their molecular epidemiology and clinical characteristics. These information can guide the development of targeted

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interventions, including vaccines currently in development.[13] Currently, there is limited evidence available on the differences in molecular epidemiology between ESBL-EC and non-ESBL-EC isolates from urine in China.

The present study provides data on the clonal distribution, virulence genes and phylogenetic groups distribution displayed by fifty ESBL-EC and fifty non-ESBL-EC strains.

Materials and Methods

Bacterial Isolates

A total of 100 non-duplicate isolates (one isolate per patient) randomly selected from the patients suffered UTI during 2022 in The First Affiliated Hospital of Ningbo University. These collections of isolates all came from urine and were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (BioMérieux, Marcy l'Etoile, FRANCE). And a random selection among them for that fifty isolates were positive for beta-lactamase production and fifty were negative.

Phenotypic Detection of ESBLs in *Escherichia coli*

The difference of phenotype between ESBL-EC and non-ESBL-EC was were detected by use of the double-disk synergy test in accordance with the phenotypic confirmation test recommended by Clinical and Laboratory Standards Institute (CLSI, 2019) (https://www.clsi.org). The confirmatory analysis for ESBL was determined as the zone of inhibition of cefotaxime and/or ceftazidime to ≥5mm from that of cefotaxime/clavulanicacid and/or ceftazidime/clavulanic acid. *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as positive and negative control strains for ESBLs detection, respectively.

Detection of Phylogenetic Group

Whole-cell DNA of uropathogenic *Escherichia coli* (UPEC) colonies was extracted by boiling lysis method at 90°C for 10 minutes. Polymerase Chain Reactions were performed to detect the *gadA, chuA*, and *yjaA* genes for the classification of main phylogenetic groups (A, B1, B2, D). All primers[14] used in this study are shown in Table S1.

The PCR reaction volumes consisted of 0.5ul forward primer, 0.5ul reverse primer, 5ul of reaction mixture reagent (Yeasen Biotechnology Inc., Shanghai, China), 5ul of distilled water, and 1ul of DNA product extracted by boiling, for a total of 12ul. Thermal cycling was performed in GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA, USA) under the following conditions: denaturation for 5 minutes at 95°C; 30 amplification cycles of 1 minute at 95°C, 1 minute at 60°C, and 5 minutes at 72°C; and a final extension of 10 minutes at 72°C. Expected amplicon sizes for *gadA, chuA, yjaA and TSPE4.C2* was 373-, 281-, 216- and 158-base-pair (bp) respectively. Finally, PCR products were subjected to electrophoresis and photography under ultraviolet trans-illumination.

The results of PCRs that $chuA^{-}TSPE4.C2^{+}$, $chuA^{+}yjaA^{+}$, $chuA^{-}TSPE4.C2^{-}$, $chuA^{+}yjaA^{-}$ were identified to group B1, B2, A and D respectively. The function of the *gadA* gene was a control for internal amplification.

Multilocus Sequence Typing

Based on a previously standardized protocol about multilocus sequence typing (MLST) of *E. coli*,[15] seven housekeeping genes were analyzed from 100 strains of UPEC by PCR reactions. And the amplification products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) with forward primer. The sequencing results were submitted to a web database (http://mlst.ucc.ie/mlst/dbs/Ecoli) and compared on line to obtain the allelic profile so that the result sequence type were obtained. The PCR amplification settings and materials were identical to those described above for the phylogenetic group assay and the primer sequences utilized for MLST were listed in Table S1.

Detection of Virulence Genes

In this work, 19 virulence genes were analyzed by PCR to investigate the adhesion, protection, toxin, and iron uptake-related capabilities of UPEC. The virulence gene count was calculated by summing the number of virulence genes found in each isolate. The primers of detecting virulence genes were listed in Table S2.

Statistical Analysis

The data were analyzed by using the one-way ANOVA test and the non-parametric Mann Whitney test with SPSS for Windows version 21.0 (SPSS Inc., Chicago, IL, USA). *P* values <0.05 were considered statistically significant.

Results

Detection of Phylogenetic Groups

The main phylogenetic group of the two groups was B2 (52%-non-ESBL-EC vs 56%-ESBL-EC) came after other three groups: B1 (12% vs 14%), D (14% vs 28%), and A (22% vs 2%). Besides, group D was found to be more prevalent in the non-ESBL-EC group, and group A in the ESBL-EC was more common. The differences were statistically significant (Figure 1).

Sequence Types

Forty-nine STs were identified among the 100 studied isolates, 68% of the ESBL-EC group isolates corresponded to the following STs: ST1193 (24%), ST131 (14%), ST648 (8%), ST38 (8%), ST69 (6%), ST75 (4%) and ST1011 (4%). And 48% of the non ESBL-EC group isolates were ST73 (20%), ST95 (14%), ST216 (6%), ST409 (4%) and ST58 (4%). The main sequence types of the two groups were totally different (Figure 2).

Virulence Genes

Of the 19 virulence genes were detected, the virulence genes (VG) count ranged from 2 to 16. The median of VG among non-ESBL-EC (11, IQR 6–15) were higher compared to ESBL-EC (10, IQR 8–12) (Figure 3). From the graph (Figure 4), VG count varied between different phylogenetic groups for that the highest in group B2 and the lowest in group A. Specifically, the average of VG belonging to group B2, B1, A and D were 12.35, 7.31, 6.58 and 8.57, respectively. The distribution of virulence genes in group B2 differed significantly from that in groups B1, A, and D, respectively ($p< 0.001$). What 's more, ESBL-negative ST73 (median 15, IQR 14–15) and ST95 (median 13, IQR 10–16) isolates had higher average VG count and ESBL-positive ST 38 isolates had the lowest count (median 8, IQR 7.5–8) (Figure 5). Additionally, in group B2 which most strains belonged to, average VG count of non-ESBL-EC was higher than ESBL-EC and the difference between the B2 group made a great sense (Figure 6).

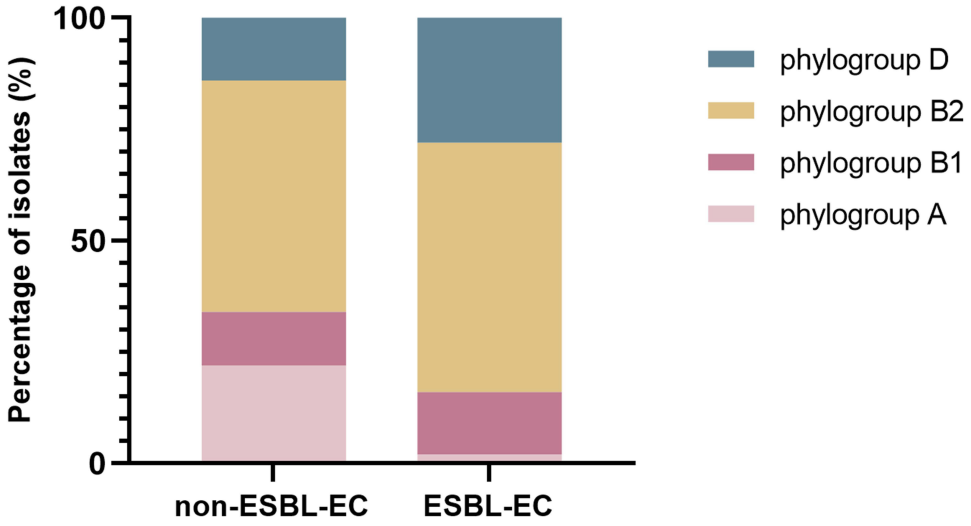

Figure 1 Comparison of the distribution of phylogenetic groups between non-ESBL-EC and ESBL-EC.
Abbreviations: ESBL, extended-spectrum beta-lactamase; ESBL-EC, ESBL-producing*E. coli*; non-ESBL-EC, non-ESBL-producing*E. coli*; phylogroup, phylogenetic group.

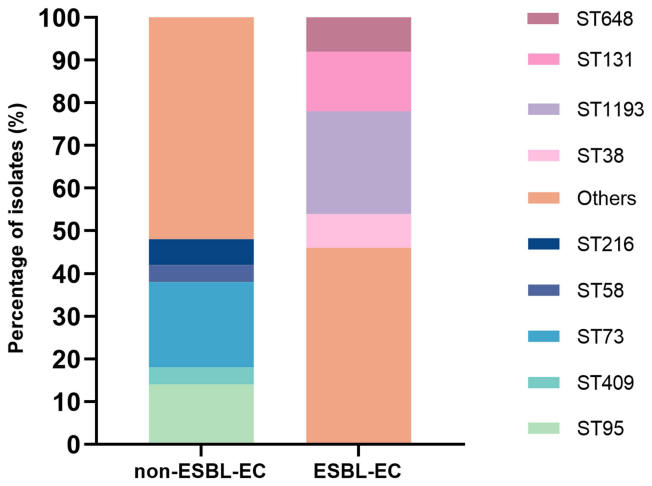

Figure 2 Comparison of the distribution of sequence types between non-ESBL-EC and ESBL-EC. Others, STs that occurred <5% with non-ESBL-EC or ESBL-EC were categorized as "others".
Abbreviations: ESBL, extended-spectrum beta-lactamase; ESBL-EC, ESBL-producing*E. coli*; non-ESBL-EC, non-ESBL-producing*E. coli*; ST, sequence type.

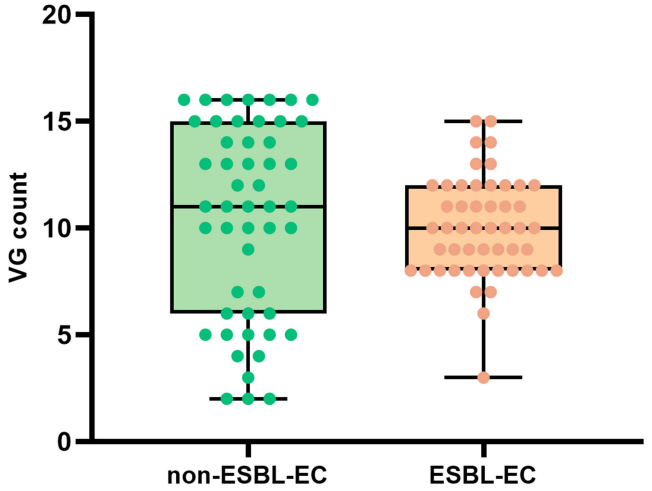

Figure 3 Comparison of the total number of VG detected per isolates between non-ESBL-EC and ESBL-EC. Every dot represents a single isolate.
Abbreviations: ESBL, extended-spectrum beta-lactamase; ESBL-EC, ESBL-producing*E. coli*; non-ESBL-EC, non-ESBL-producing*E. coli*; VG count, the number of virulence genes.

There was an inverse correlation between β-lactamase producing and the number of VG count. Wherein, eleven types of virulence genes were more prevalent in isolates from non-ESBL-EC group. Statistical significance was observed in the genes for *hlyA, papC, papGII, cnf1, iroN, vat and sfa* ($p<0.05$) (Table 1).

Discussion

In this study, we have noted discernible distinctions with regard to clonal distribution, virulence gene count, and phylogenetic group between ESBL-EC and non-ESBL-EC strains that were isolated from urine.

Compared to prior analogous investigations on *E. coli* isolates from bloodstream[16–18] the clonal distribution exhibited by ESBL-EC urine isolates stands in stark contrast to their non-ESBL-EC counterparts. As per a recent investigation, the two most prevalent sequence types of *E. coli* urine isolates tested for the presence of ESBL genes via PCR were ST131 and ST1193,[19] a result that aligns with our own research findings. The observed discrepancy in clonal distribution between ESBL-positive and non-ESBL *E. coli* urine isolates may be primarily attributed to the presence of multidrug resistance (MDR) in a subset of the former isolates. In recent times, the ST131 clone has emerged as the foremost and highly potent multi-drug resistant (MDR) clone on a global scale, serving as a major etiological factor for community-

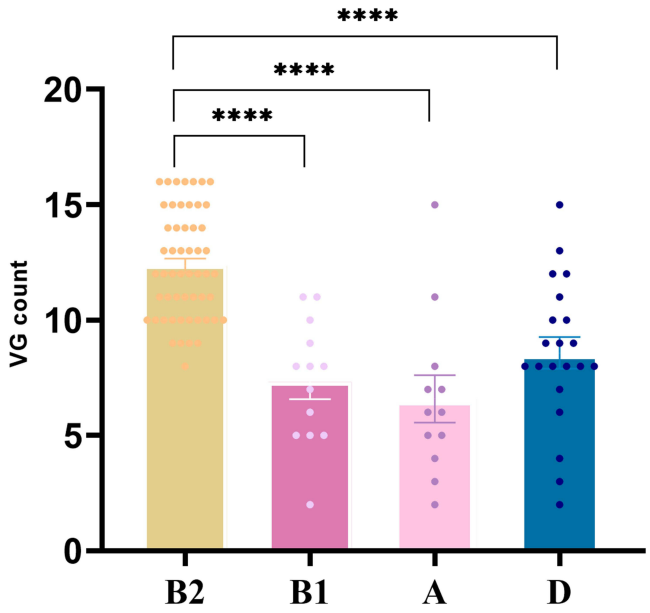

Figure 4 Comparison of the distribution of virulence factors in four main phylogenetic groups. B2, phylogenetic group B2; B1, phylogenetic group B1; A, phylogenetic group A; D, phylogenetic group D; VG count, the number of virulence genes; every dot represents a single isolate; *p* value of comparison about the distribution of virulence factors in four main phylogenetic groups, calculated with analysis of ANOVA, one-way analysis of variance, SPSS 21.0. *P* <0.0001 (marked as ****) was defined as extremely significant.

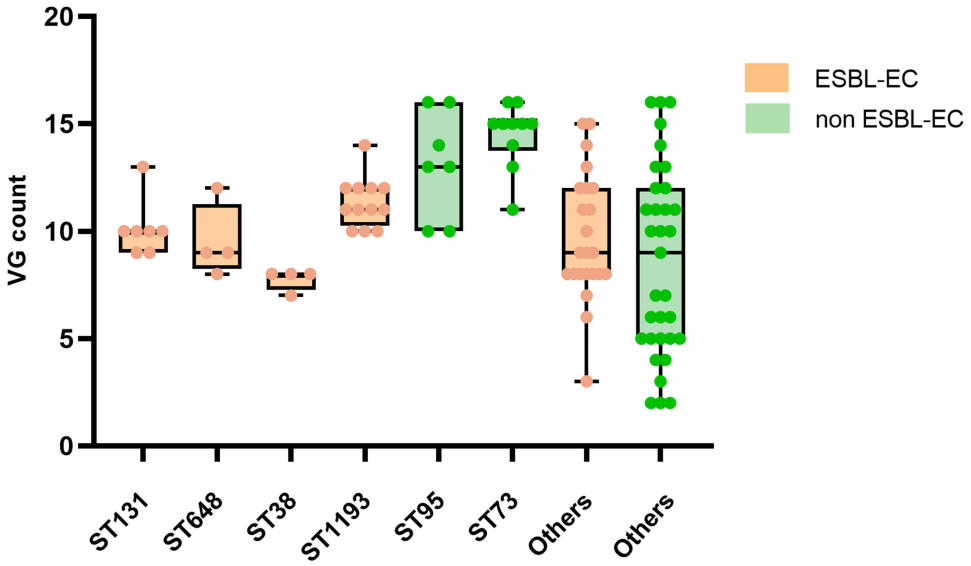

Figure 5 UPEC-associated VG count in different STs, stratified for ESBL-positivity[a]. [a]ESBL-positivity based on the phenotypic ESBL production; Boxplots display median VG count and inter quartile range (IQR); every dot represents a single isolate; only STs that occurred >5% with non-ESBL-EC or ESBL-EC were grouped into main groups, the rest were categorized as "Others".

Abbreviations: ESBL, extended-spectrum beta-lactamase; ESBL-EC, ESBL-producing*E. coli*; non-ESBL-EC, non-ESBL-producing*E. coli*; ST, sequence type; VG, virulence gene.

acquired urinary and bacteremic infections.[20,21] ST1193 clone follows closely due to its prevalent overuse of Fluoroquinolone (FQ) and third-generation cephalosporin.[22] Birgy et al[23] revealed that ST1193 constituted one of the most prevalent clones during the final study phase, among 218 ESBL-producing *E. coli* infections that resulted in febrile urinary tract infections in children between 2014 and 2017.

In our study, ST73 and ST95 were absent in ESBL-EC group, and it is established that ST73 is vulnerable to antibiotics.[24] The ST distribution phenomena shows that in UPEC, the typically antimicrobial-susceptible STs: ST73 and ST95 have been replaced by strong antibiotic pressure. Most striking was that ST73 and ST95 which were both negative in producing extended beta-

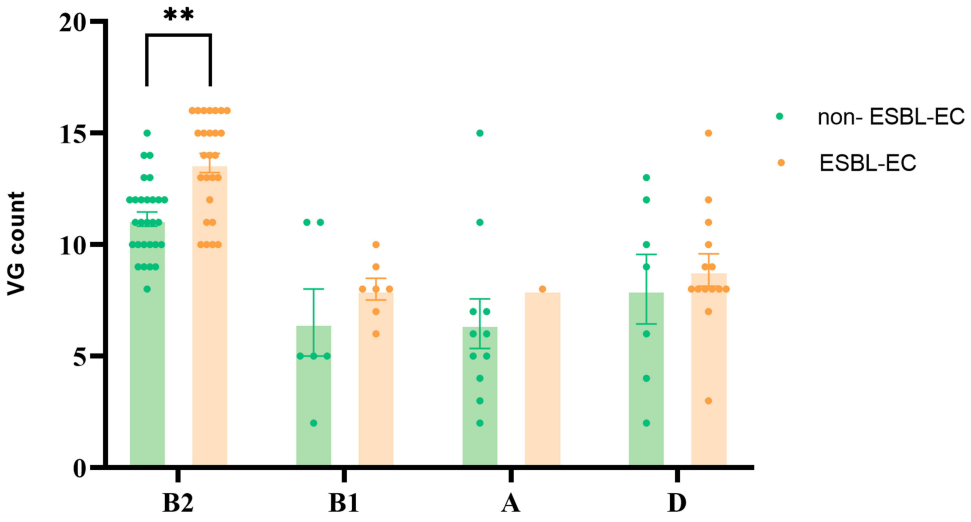

Figure 6 Comparison of acquired virulence genes count per phylogenetic group. Every dot represents a single isolate; B2, phylogenetic group B2; B1, phylogenetic group B1; A, phylogenetic group A; D, phylogenetic group D. *P* <0.001 (marked as***) was defined as extremely significant.
Abbreviations: VG count, the number of virulence genes; ESBL, extended-spectrum beta-lactamase; ESBL-EC, ESBL-producing*E. coli*; non-ESBL-EC, non-ESBL-producing*E. coli*.

lactamase had the highest VG content among other STs. Besides, the average of VG content related to group B2 of ESBL-EC was also lower than non-ESBL-EC. The result ties well with a former study performed by Horcajada et al.[25] These findings were unexpected and reflect the inverse association previously described[26] similarly between antimicrobial resistance and VG content. As for the other A, B1 and D groups poor in inverse association, we speculate that the limited strains is responsible for this result. Notably, the four primary sequence classifications identified in this investigation, namely ST131, ST1193, ST73, and ST95, happen to coincide with the sequence types previously demonstrated to disseminate from UTI to *E. coli* Bacteremia.[19] Such a coincidence may be due to the phenomenon that the aforementioned prevailing clones were renowned UPEC clones.[27]

Studies undertaken in South Korea,[28] Ethiopia,[29] and Mexico[30] revealed similar distributions, which are consistent with the majority of findings on the phylogenetic grouping of UPEC. These studies have shown that the phylogenetic group B2 primarily encompasses the bulk of *E. coli* isolates. In this study, we have also discovered that B2 was prevalent in both ESBL-EC and non-ESBL-EC patients. This emphasizes the need of addressing the appearance of isolates linked to this group. The information shown in Figure 4 supports this and indicates that group B2 demonstrates a high number of virulence determinants. Genetically stable isolates with much lower recombination rates make up the commensal assemblages of *E. coli*. This trait gives the population a clonal structure and allows the major phylogenetic groups to be classified.[31] Commensal *E. coli* isolates make up the majority of phylogenetic group A, in light of numerous past investigations. This observation remains true for both human commensal *E. coli* and UPEC strains, based on the reports of Duriez et al[32] and Khairy et al,[33] where phylogenetic group A accounts for the most significant fraction of phylogenetic groups. Depending on several reports, phylogenetic group A is the most prevalent phylogenetic group among UPEC isolates.[33–36] According to our research, almost 22% of non-ESBL-EC isolates fell into phylogenetic group A, which is much higher than the similar number reported in earlier studies carried out in South Korea (3.44%).[28] The aforementioned finding suggests that the gastrointestinal system is the primary source of ESBL negative strains that colonize the urinary tracts since phylogenetic group A, which is normally associated with commensal infections, is prevalent among UPEC isolates.[37]

Additionally, these phylogenetic groupings have a noteworthy characteristic as a result of their comparatively increased quantity of virulence variables, making them extremely virulent clinical isolates and complicating efforts to cure them. Compared to other phylogenetic groups, the group B2 showed a significantly higher prevalence of virulence genes. Specifically, the isolates belonging to group B2 in non-ESBL-EC group harbored more virulence genes. One plausible justification for this observation could be the co-localization of the extended-spectrum beta-lactamase-producing gene and certain virulence genes on the same plasmids or chromosomes. This is consistent with the findings of earlier research from Denmark,[38] Pakistan, Ethiopia,[39] Mexico,[30] and Poland.[40]

Table 1 Virulence Genes Distribution Between ESBL-EC and Non-ESBL-EC Group of UPEC Isolates

Virulence Gene	ESBL-EC n=50 (%)	Non-ESBL-EC n=50 (%)	*p* value
sat	30 (60)	6 (12)	<0.001
fimH	47 (94)	46 (92)	1
hlyA	6 (12)	18 (36)	0.005
papC	8 (16)	22 (44)	0.002
papG-II	8 (16)	21 (42)	0.004
feoB	50 (100)	49 (98)	1
fliCD	28 (56)	37 (74)	0.059
chuA	43 (86)	32 (64)	0.011
traT	35 (70)	16 (32)	<0.001
kpsM	39 (78)	30 (60)	0.052
iutA	43 (86)	17 (34)	<0.001
yfcV	29 (58)	30 (60)	0.839
fyuA	43 (86)	36 (72)	0.086
OmpT	41 (82)	43 (86)	0.585
tsh	5 (10)	6 (12)	0.749
cnf1	5 (10)	20 (40)	0.001
iroN	14 (28)	33 (66)	<0.001
vat	20 (40)	35 (70)	0.003
sfa	6 (12)	31 (62)	<0.001

Notes: *P* values were calculated with chi-square test comparing the prevalence of the virulence gene in ESBL-EC and non-ESBL-EC group. Values significantly higher than the ESBL-EC group are showed in red boxes.
Abbreviations: ESBL, extended-spectrum beta-lactamase-producing positive *E. coli*; non-ESBL-EC, extended-spectrum beta-lactamase-producing negative *E. coli*.

This study focused on the clonal distribution, phylogenetic group, and virulence profiling of UPEC isolates based on their beta-lactamase production status. According to the findings above, more attention should be paid not only to phylogenetic group B2 that most isolates belonged to, but also non-β-lactamase producing strains harboring high virulence genes, which will lead to high pathogenicity.

It is noteworthy to mention that a limited number of *E. coli* isolates were selectively obtained from a singular tertiary-care hospital. Consequently, the findings are not generalizable to all UPEC isolates. Accordingly, it is our hope to conduct a wide-ranging molecular epidemiological study utilizing a multicenter approach in future endeavors.

In conclusion, our research indicates that there is relevant evidence between the molecular characteristics of *E. coli* isolates and the phenotype of beta-lactamase synthesis. Here, we found significant differences in the clonal distribution, virulence gene content, and phylogenetic groups of non-ESBL-EC and ESBL-EC urine isolates that outweigh their phenotypic ESBL-positivity. To avoid attributing resistant *E. coli* features to the overall *E. coli* population, future multicenter studies attempting to explain the molecular epidemiology of UTI should instead focus on *E. coli* without ESBL-positivity.

Conclusion

Non-ESBL-EC and ESBL-EC isolates from The First Affiliated Hospital of Ningbo University differed in clonal distribution, phylogenetic group and VG content. The inverse association between beta-lactamase resistance and VG content should get a lot more attention. At the same time, we should also be wary of the appearance of non-ESBL-EC isolates belonging to group B2.

Ethics Approval and Consent to Participate

Our research was reviewed and approved by Medical Ethics Committee of The First Affiliated Hospital of Ningbo University (Approval NO:2023053RS). The patient who is going to be hospitalized will sign an informed consent which implies that biological samples taken may be used for medical teaching and research. In this study, the sample were obtained from inpatients who freely and voluntarily signed the informed consent under the full explanation of the clinician. And for the patients who were actively screened, oral consent was also obtained.

Acknowledgments

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Disclosure

The authors declare that there is no conflict of interest in the publication of this paper.

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