Virulence factors and pathogenicity islands in uropathogenic*E. coli* in children

Abstract :

Escherichia coli (*E. coli*) is a Gram-negative bacillus responsible for intestinal and extraintestinal infections, being the main cause of urinary tract infection (UTI). Pathogenic strains have several virulence factors (VF), encoded by genes that are located on the bacterial chromosome in specific regions called pathogenicity islands (PAI). In addition to VF, *E. coli* has other resistance mechanisms, such as the production of extended spectrum ß-lactamases (ESBL).
Aims: Investigate the frequency of VF from *E. coli* isolated in the urine of children with suspected UTI in the community, investigate the types of ESBL, compare the frequency of virulence factors and PAI between strains that produce and do not produce ESBL, and perform the phylogenetic classification of the strains.
Study design: This is an observational, descriptive and cross-sectional study carried out through the analysis of positive urine cultures from children aged 0 to 12 years old.
Place and Duration of Study: The samples were collected in the city of Londrina, state of Paraná (Brazil) and processed from May 1, 2021, to September 9, 2022.
Methodology: Of the samples collected, 89 *E. coli samples* from urine cultures were included, of which 44 were samples that showed ESBL and 45, as a control, did not. DNA extraction was carried out using the boiling method and the polymerase chain reaction (PCR) was subsequently carried out. The PCR method was used for the genotypic identification of the main virulence factors of ExPEC.
Results: Among VF, the *iutA*iron acquisition system was the most prevalent (77.5%). There was a statistical difference in the two groups ESBL or non-ESBL in relation to the *fimH*and *hlyA.* The ESBL most prevalent was CTX-M2. PAI IV536 was the most prevalent among all isolates (52.8%) and the only pathogenicity island with a significant result. Among the isolates, the most prevalent phylogenetic classification group was B2 (41.6%).
Conclusions: *E. coli* strains have an arsenal of VF that allow them to induce infection, with the statistical difference between the ESBL and non-ESBL groups in relation to VF. PAI IV536 was the most prevalent among all isolates, in the same way as phylogenetic group B2, indicating high pathogenicity of the isolates and corresponding to the virulence potential elucidated. The finding of CTX-M2 like the more prevalent type of ESBL agrees with other studies in the region.

Key-words: ExPEC; UTI pediatrics; Virulance Factors; Phylogenetic Classification.

1. INTRODUCTION

The bacterium Escherichia coli (*E. coli*) is a Gram-negative bacillus inhabiting the common microbiota of the human gastrointestinal tract, belonging to the order *Enterobacterales*, family *Enterobacteriaceae*(ROZWADOWSKI; GAWEL, 2022). E. *coli* presents a genetic diversity that includes non-pathogenic commensal intestinal isolates and isolates that can cause intestinal and extraintestinal infections. Pathogenic *E. coli* strains can cause extraintestinal diseases, such as urinary tract infection (UTI), bacteremia, neonatal meningitis and other non-intestinal infections (BIRAN; RON, 2018).

They are classified into seven phylogenetic groups: A, B1, B2, C, D and F, and clade I. Commensal strains are part of the human intestinal microbiota and play an important role in digestion and the synthesis of certain vitamins. Such strains do not have virulence factors and generally belong to groups A or B1. Pathogenic strains are classified into intestinal *E coli* (intestinal pathogenic*E. coli* - IPEC) and extraintestinal (extraintestinal*E.* coli - ExPEC). IPEC strains responsible for intestinal infections belong to phylogenetic groups A, B1 or D, while ExPEC strains responsible for extraintestinal infections are part of groups B2 or D (SAROWSKA, J. et al, 2019).

ExPEC comprises isolates from infections outside the intestinal tract and are grouped into uropathogenic*E. coli* (UPEC), neonatal meningitis-associated *E. coli (NMEC)* and *E. coli* that causes sepsis (SEPEC) (RAEISPOUR; RANJBAR, 2018). UPEC is capable of invading the bladder and generating substantial changes in its morphology, with a robust immune response. In addition, intracellular bacterial communities (IBC) form within the surface cells of the bladder. IBC are made up of thousands of bacteria, similar to a biofilm, and are important in leading to later infection (YANGY et al., 2019).

UTI is among the most frequent bacterial infections in the community and in the hospital environment and, as it presents significant morbidity and mortality, it generates high economic costs for the health system. It is estimated that there are more than 150 million UTI occurrences worldwide annually, impacting countries' health systems (SAHU et al., 2019). In the USA, the total cost of healthcare used to treat UTI is more than 3.5 billion dollars, of which approximately 630 million dollars are for pediatric UTIs (MILLNER; BECKNELL, 2019).

ExPEC strains have several virulence factors (VF) that make them capable of colonizing the host and spreading. VR are encoded by genes located on the bacterial chromosome, where they are often located in specific regions called pathogenicity islands (PAI), or in mobile elements, such as plasmids. Virulence factors include adhesion molecules (adhesins), iron acquisition systems, mechanisms to escape the immune system and toxins (DESVAUX, M. et al., 2020).

In addition to the acquisition of FV and its effects on pathogenicity, the acquisition of resistance genes plays an important role in therapeutic failure and increased mortality. One of the most prevalent resistance mechanisms is extended-spectrum ß-lactamases (ESBL) produced by enterobacteria (REHAB, MA et al, 2020).

The ability of *E. coli* to cause mutation, to acquire and transmit plasmids and other mobile genetic elements that encode resistance genes (including ESBL) has represented a rapidly expanding problem (SAROWSKA, J. et al, 2019).

The main objective of this study was to investigate the frequency of ExPEC virulence factors from the urine of communitypediatric patients with suspected UTI. Other objectives were to investigate the types of ESBL in the bacteria studied, compare the frequency of virulence factors of ESBL-producing strains with non-ESBL-producing strains, compare the frequency of PAI of ESBL-producing strains with non-ESBL-producing strains, and carry out phylogenetic classification of the strains studied.

2. MATERIAL AND METHODS

The samples were collected from positive urine cultures of children with suspected UTI aged 0 to 12 years old, carried out in the laboratory of the Municipal Health Authority of the Municipality of Londrina (CentroLab), from May 1, 2021, to September 9, 2022. These samples were collected from children who were treated in the primary care services of the Municipal Health Authority in the city of Londrina, a city in the state of Paraná (Brazil). Of the samples collected, 89 *E. coli samples* from urine cultures were included, of which 44 were samples that showed ESBL and 45, as a control, did not. To identify the isolates, the VITEK ® 2 GN ID card and the VITEK ® 2 AST 239 card were used to evaluate the antimicrobial susceptibility test.

DNA extraction was carried out using the boiling method and the polymerase chain reaction (PCR) was subsequently carried out.

A total of 7 genes of the main virulence factors were analyzed, namely: hemolysin (*hlyA*), aerobactin (*iutA*), yersiniobactin (*fyuA*), type 1 fimbria (*fimH*), P fimbria (*papC*), serum resistance (*traT*), polysaccharide capsule (*KpsMT II*), described in Table 1. The PCR method was used for the genotypic identification of the main virulence factors of ExPEC.

The characterization of ESBL was carried out using the multiplex PCR technique using specific primers for the detection of the *bla*CTX -M genes as described by Woodford, Fagan and Ellington (WOODFORD, FAGAN and ELLNGTON). The PCR reaction was performed using the TopTag ® Master Mix Kit (QUIAGEN).

A total of 3 described UPEC pathogenicity islands were researched (Table 2) and the PCR method was also used for this (SABATÉ et al., 2006).

The samples were classified into 7 phylogenetic groups (A, B1, B2, C, D, E, F and clade I), based on the presence of the genes *chuA , yjaA , arpA , trpA*and DNA fragments (*TSPE4.C2*) (Table 3) using the PCR method. *E. coli* strains that do not fall between these groups are described as classification unknown.

To perform the statistics, qualitative and independent variables were used on the IBM® Statistical *software platform.*Package for the Social Sciences (SPSS)® version 22 for Windows. The *Chi -Square Test* was used to measure the degree of association between the variables. The *P-value* used as a significant difference between groups was 0.05. To evaluate the intensity of association between the variables, *oddsratio* was usedwith a 95% confidence interval.

Table 1. Genes encoding virulence factors

Genes	Sequences (5'-3')	coded FV	Base pairs
fimH	TGC AGA ACG GAT AAG CCG TGGGCA GTC ACC TGC CC TCC GGT A	Fimbriaetype 1	508
papC	GAC GGC TGT ACT GCA GGG TGT GGC G ATA TCC TTT CTG CAG GCA GGG TGT GGC	P Fimbriae	328
hlyA	AAC AAG GAT AAG CAC TGT TCT GGC ACC ATA TAA GCG GTC ATT CCG GTC	Hemolysin	1,177
iutA	GGC TGG ACA TCA TGG GAA CTG G CGT CGG GAA CGG GTA GAA TCG	Aerobactin siderophore receptor	300
fyuA	TGA TTA ACC CCG CGA CGG AA CGC AGT AGG CAC GAT CTT GTA	Yersiniobactin siderophore receptor	880
traT	GGT GTG GTG CGA TGA GCA CAG GGT GTG GTG CGA TGA GAC CAG	Serumresistanc e	290
kpsMTII	GCG CAT TTG CTG ATA CTG TTG CAT CCA GAC GAT AAG CAT GAC CA	Group 2 of capsular antigens	272

Source: Johnson and Stell, 2000

Table 2. Genes encoding Pathogenicity Islands

Pathogenicity Islands	Sequences (5'-3')	Base pairs
PAI I_{CFT073}	GGA CAT CCT GTT ACA GCG CGC A TCG CCA ATC ACA GC GAA C	930
PAI II_{CFT073}	ATG GAT GTT GTA TCG CGC ACG AGC ATG TGG ATC TGC	400
PAI IV_{536}	AAG GAT TCG CTG TTA CCG GAC TCG GGC AGC GTT TCT TCT	300

Source: Sabaté et al., 2006

Table 3. Genes used for phylogenetic classification

Genes	Sequences (5'-3')	Base pairs
chuA	ATG GTA CCG GAC GAA CCA AC TGC CGC CAG TAC CAA AGA CA	288
yjaA	CAA ACG TGA AGT GTC AGG AG AAT GCG TTC CTC AAC CTG TG	211
TspE4.C2	CAC TAT TCG TAA GGT CAT CC AGT TTA TCG CTG CGG GTC GC	152
arpA	AAC GCT ATT CGC CAG CTT GC TCT CCC CAT ACC GTA CGC TA	400
trpA	CGG CGA TAA AGA CAT CTT TCA AC GCA ACG CGG CCT GGC GGA AG	301
arpA (group E)	GAT TCC ATC TTG TCA AAA ATA TGC CC GAA AAG AAA AAG AAT TCC CAA GAG	290

Source: Johnson and Stell, 2000

3. RESULTS

3.1 Virulence factors

Among the virulence factors analyzed, the *iutA*iron acquisition systemwas the most prevalent among the isolates, with a total percentage of 77.5%. The *fyuA* iron acquisition systemit was the second most prevalent gene with a total of 75.3%. Next was the *traT gene*(70.8% of the total), a virulence factor responsible for serum resistance.Only in the *fimH*, *kpsMTII genes*and *papC*the percentages of *E. coli* producers were lower than non-ESBL producers.

When comparing the two types of ESBL-producing and non-ESBL-producing UPEC, there was a statistical difference in the two groups in relation to the *fimH* virulence factors, with a predominance of non-ESBL strains (*P* <0,001) and *hlyA* (*P*=0,007), with a predominance of strains ESBL, which were 4 times more likely to express the gene than non-ESBL-producing E. coli (Table 4).

Table 4. Percentage of FV of *E. coli* isolated from children with suspected UTI

Genes	ESBL producers n (%)	Non-ESBL producers n (%)	Total	*P*	OR (CI 95%)
iutA	36 (81.8)	33 (73.3)	69 (77.5)	0.447	1.64 (0.60-4.50)
fyuA	38 (86.4)	29 (64.4)	67 (75.3)	0.130	0.30 (0.05-1.54)
traT	34 (77.3)	29 (64.4)	63 (70.8)	0.245	1.88 (0.73-4.77)
fimH	21 (47.7)	40 (88.9)	61 (68.5)	<0.001	0.11 (0.04-0.03)
kpsMTII	25 (56.8)	29 (64.4)	54 (60.7)	0.52	0.73 (0.31-1.70)

papC	12 (27.3)	13 (28.9)	25 (28.1)	1.00	0.92 (0.37-2.32)
hlyA	14 (31.8)	4 (8.9)	18 (20.2)	0.007	4.78 (1.43-15.99)

Source: author himself, 2022

3.2 ESBL classification

Of the 44 samples of ESBL-producing strains, 21 were identified as ESBL CTX-M2, 13 as ESBL CTX-M1 and 10 as ESBL CTX-M8.

3.3 Pathogenicity Islands

PAI IV_{536} was the most prevalent among all isolates (52.8%) and the only pathogenicity island with a significant result (*P*<0.001), with 13 times greater chances of being present in ESBL-producing *E. coli* when compared to the control group of non-producers.

The second most prevalent pathogenicity island in all isolates was PAI I_{CFT073} with 27.0%. Finally, PAI II_{CFT073} was less prevalent, with 10.1% of the total samples (Table 5).

Table 5. Percentage of *E.coli* PAIsisolated from children with UTI

Pathogenicity Islands	ESBL producers n (%)	Non-ESBL producers n (%)	Total	*P*	OD (95% CI)
PAIIV_{536}	36 (81.8)	11 (24.4)	47 (52.8)	<0.001	13.91 (4.99-38.73)
PAI I_{CFT073}	16 (36.4)	8 (17.8)	24 (27.0)	0.06	2.64 (0.99-7.05)
PAI II_{CFT073}	7 (15.9)	2 (4.4)	9 (10.1)	0.09	4.07 (0.80-20.80)

Source: author himself, 2022

3.4 Phylogenetic classification

Among the isolates, the most prevalent phylogenetic classification group was B2 (41.6%), followed by D (22.5%), E (19.1%), F (6.7%) and clade I (1,1%). However, none of the *E. coli* isolatesfrom children with UTI analyzed belonged to groups A, B1 and C. The clade I group presented only one isolate, which was non-ESBL producer. Group E had a higher percentage of ESBL-producing *E. coli*, but the other groups (B2, D, F and clade I) had a higher percentage of non-ESBL-producing samples. Furthermore, 9% of the isolates had an unknown classification (Table 6).

Table 6. Phylogenetic classification of *E.coli*isolated from children with UTI

Phylogenetic classification	ESBL producers, n (%)	Non-ESBL producers, n (%)	Total
A	-	-	-
B1	-	-	-
B2	18 (40.9)	19 (42.2)	37 (41.6)
C	-	-	-
D	4 (9.1)	16 (35.6)	20 (22.5)
E	14 (31.8)	3 (6.7)	17 (19.1)
F	1 (2,3)	5 (11.1)	6 (6.7)
Clade I	-	1 (2,2)	1 (1,1)

CD*	7 (15.9)	1 (2,2)	8 (9.0)

**Unknown classification*
Source: author himself, 2022

4. DISCUSSION

In this study, virulence factors, pathogenicity islands and the phylogenetic classification of *E. coli isolates* from the urine of children with community UTIs were investigated. Virulence factors increase the ability of strains to cause infections, as they are associated with the pathogenicity of ExPEC (TERLIZZ et al, 2017). They can be chromosomally encoded and are usually located within pathogenicity islands or on plasmids. PAI are specific regions of the bacterial chromosome where virulence genes accumulate. PAI are common among *E. coli* strains that cause extraintestinal infections, mainly in ExPEC phylogroups B2 and D. There is a range of FV, and the combination between them occurs in different strains of ExPEC and can lead to many pathways for the extraintestinal virulence of *E. coli* (SAROWSKA, J. et al, 2019).

The most prevalent gene was *iutA*followed by the *fyuA gene*, both express virulence factors associated with the iron acquisition system. Iron, as an electron transporter, plays a key role in the process of cellular respiration, DNA replication and oxygen transport. Such virulence factors are fundamental in the pathogenesis of urinary tract infection, as they allow bacterial proliferation in the urinary tract, which is extremely limited in iron (SAROWSKA, J. et al, 2019).

In addition to *uitA and fyuA,* other frequent genes were *traT*, *fimH*and *kpsMTII.* According to a study carried out at the University Hospital of Londrina by Daga and others (2019), the virulence factors and pathogenicity islands of ExPEC isolated from different clinical materials were analyzed. In this study, urine isolates from inpatient UTI patients obtained the *fimH genes*(77.3%), *fyuA*(54.5%), *iutA*(45.5%), *traT*(40.9%), *kpsMTII*(34.1%), respectively with higher prevalence. For urine isolates from outpatients with UTI, there was a predominance of the genes *fimH* (98.0%), *fyuA* (67.3%), *traT* (51.1%), *kpsMTII* (51.1%) and iutA (46. 9%), corroborating the findings of the present study.

The expression of the *fimH*gene, which was significant in non-ESBL-producing strains in relation to ESBL-producing strains with a value of $p<0.001$ for the samples in this study, is relevant in UPEC due to its participation in the bacterial adhesion process. Adhesion is carried out by adhesins, virulence factors that recognize and bind to receptors present in the bladder epithelium, for the bacteria to establish themselves in the host and cause infection (DESVAUX et al, 2020). These adhesins are found at the ends of fimbriae, with type 1 fimbriae containing the adhesin*fimH*mediates the binding of UPEC with uroplakin present in the bladder epithelial cell, allowing the bacteria to resist the mechanical movements of peristalsis and urination (FLORES-MIRELES et al., 2015; MOBLEY, 2016). A possible explanation for this finding is that non-ESBL strains would need to express this virulence factor more frequently as they do not have the competitive advantage of this bacterial resistance mechanism (production of beta- lactamases). Some studies have shown that more resistant strains may express fewer virulence factors.

Another finding in this study was that ESBL-producing UPEC strains had a higher frequency of the *hlyA gene* than non-producers, a finding with statistical difference in this study. The toxins produced by *E. coli*, such as hemolysin A encoded by the *hlyA gene,* are virulence factors that contribute to cell lysis through the formation of pores in the membranes of urinary epithelial cells (SORA et al, 2021). It is reported in the literature that ESBL-producing *E. coli* is linked to the expression of the *hlyA gene*, according to a study by Hasan and Ibrahim (2022), 80% of UPEC samples from children with UTI had the *hlyA gene* and 64% of these were positive for ESBL.

Regarding the finding of three specific types of ESBL, CTX-M2, CTX-M1 and CTX-M8 with a predominance of the first, it agrees with other studies, which show that this is the main type of beta-lactamase in South American countries(BEVAN, JONES and HAWKEY, 2017).

This study showed that PAI IV_{536} was most prevalent among all isolates and the only pathogenicity island with a significant result ($p<0.001$), with ESBL strains having a 13 higher frequency than non-ESBL strains.

Pathogenicity islands are specific regions of the bacterial chromosome where virulence genes accumulate (SAROWSKA, J. et al, 2019). They are a group of integrative elements that encode one or more virulence genes that are absent in the genome of non-pathogenic bacteria of the same species or in related species. Unlike other integrative elements such as bacteriophages, plasmids or integrative and conjugative elements, PAI are not replicative and do not have the ability to self-mobilize. Many FV are encoded by PAI and involve fundamental steps in the infectious process (DESVAUX et al, 2020).

PAI IV_{536} encodes the siderophore systemyersiniabactin, responsible for iron uptake. As previously described, iron is essential for bacteria because of its involvement in many metabolic functions, such as oxygen and electron transport. The acquisition of iron is a fundamental need for the survival of UPEC in the urinary tract environment, which is extremely iron-limited. In addition to yersiniabactin, UPEC also expresses the siderophoressalmochelin and aerobactin (TERLLIZZI et al, 2017).

The fact that ESBL strains present more PAI IV_{536} than non-ESBL-producing strains is probably related to gene regulation through still unknown mechanisms of virulence stimulation and inhibition. It is proven that PAI not only encode virulence factors but are also involved in virulence regulation networks (DESVAUX et al, 2020).

According to the phylogenetic classification, phylogroup B2 was the most prevalent, followed by group D. According to a study carried out by Navidina et al. (2013), in which the presence of PAI markers and phylogenetic groups were compared in *E. coli* isolated from the urine of children with UTI and *E. coli* isolates from stool samples from healthy children, phylogenetic group B2 containing PAI IV_{536} was the predominant group found among UPEC samples. Phylogenetic groups B2 and D are the groups found most frequently among the ExPEC strains responsible for causing human infections, as they present several virulence genes that allow the induction of extraintestinal infections in immunocompromised and healthy hosts. (RUSSO; JOHNSON, 2000). Phylogroups A, C and B1 were not identified in the urine isolates analyzed from children with UTI, according to reports in the literature, isolates belonging to groups A and B1 are commonly belonging to commensal strains of the intestinal microbiota and do not have virulence determinants of causing intestinal or extraintestinal diseases. (SKJØT-RASMUSSEN et al., 2012). Group C is closely related to group B1 and therefore may not have been identified among the isolates (CLERMONT et al., 2011).

5. CONCLUSION

E. coli is the bacterial microorganism that causes the most prevalent UTI and is responsible for the high mortality and morbidity of UTIs in children, making its prevention and control essential in pediatrics. Therefore, it is important to carry out studies that investigate the possible molecular mechanisms that increase the ability of *E. coli to cause* extraintestinal diseases and their resistance mechanisms.

According to the results of this study, it was possible to conclude that *E. coli* from children with suspected UTI have an arsenal of virulence factors that allow them to induce infection, with the most expressed FV being related to the iron acquisition system, serum resistance, toxin formation and adhesin production. Such actions are fundamental for the survival, proliferation and pathogenesis of *E. coli* in the urinary tract.

There was a statistical difference between the ESBL and non-ESBL groups in relation to FV *fimH*and *hlyA*probably related to the existence of positive and negative relationships between antibiotic resistance and virulence. It was shown that non-ESBL strains had more of the adhesin gene*fimH*and ESBL strains had more of the *hlyA*gene, responsible for the formation of pores in host cell membranes). A possible explanation for this would be that ESBL strains, as they already have a system that protects against beta-lactam antibiotics, would not need this virulence factor as much. Hasan and Ibrahim also found the same result, with E. coli ESBL strains with a greater presence of *hlyA*.

Furthermore, PAI IV_{536} was the most prevalent among all isolates, in the same way as phylogenetic group B2, indicating high pathogenicity of the isolates and corresponding to the virulence potential elucidated.

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