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Original Article

Presence of virulence genes and pathogenicity islands in extraintestinal pathogenic *Escherichia coli* isolates from Brazil

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Abstract

Introduction: Extraintestinal pathogenic *Escherichia coli* (ExPEC) is associated with various diseases such as urinary tract infections, neonatal meningitis and septicemia. There are many virulence factors (VF) encoded by genes in ExPEC, including *pap*C, *pap*G, *ecp*A, *iro*N, *fyu*A, *iut*A, *omp*Tp, *tsh*, *hly*F, *hly*A and *iss*. These virulence genes may be present in pathogenicity islands (PAI) or plasmids.

Methodology: In this study, we analyzed the presence of VF encoding genes, PAI sequences and phylogenetic groups of 96 ExPEC strains isolated from the urine and blood of patients at the University Hospital of Londrina, and we compared them with 50 faecal commensal strains from healthy individuals.

Results: The VF *fyu*A (65.60%) was detected in pathogenic strains and commensal strains (46%). A comparison of the distribution of ExPEC and commensal strains in the phylogenetic groups showed that more ExPEC strains belonged to group B2 whereas more of the commensal isolates belonged to group A. The distribution of the seven PAI sequences between commensal strains and ExPEC strains showed that PAI IV_{536} was common in both ExPEC and commensal isolates.

Conclusions: These results showed that the ExPEC strains that belonged to group B2 had more PAI sequences compared to those of the other groups, especially group B1, which had virulence genes but the lowest percentage of PAI sequences, which leads us to conclude that the virulence of ExPEC strains characterized as B2 is likely attributed to PAI encoded genes, whereas the virulence of ExPEC strains belonging to phylogenetic group B1 is likely due to plasmid encoded virulence genes.

Key words: ExPEC; commensal *Escherichia coli*; virulence factors; PAI sequences; phylogenetic groups..

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Introduction

Among *Escherichia coli* pathotypes, extraintestinal pathogenic *E. coli* (ExPEC) are important etiological agents of bacteremia and other systemic infections. ExPEC are often classified in different groups according to the anatomical site of infection, with uropathogenic *E. coli* (UPEC) being associated with urinary tract infections (UTIs) [1]. UTIs are one of the most common infections in the general population globally [2], and *E. coli* are the most commonly isolated etiological agent of UTIs, accounting for approximately 70%-85% of urinary infections.

There are many virulence genes associated with ExPEC, including *pap*C (P fimbrial usher), *pap*G (P fimbrial adhesin), *ecp*A (extracellular adhesive fimbriae), *iro*N (salmochelin siderophore receptor), *fyu*A (yersiniabactin siderophore receptor), *iut*A (aerobactin siderophore receptor), *omp*Tp (episomal outer membrane protein OmpT), *tsh* (temperature sensitive hemagglutinin), *hly*F (hemolysin F), *hly*A (hemolysin) and *iss* (serum resistance associated protein) [3-5]. These factors are commonly found in ExPEC isolated from UTIs and are less common in most fecal commensal *E. coli* [6]. However, as UPEC originate from the intestinal tract, some fecal commensal strains may also contain these virulence genes.

In addition to the aforementioned virulence genes, genomic islands (GI), which include pathogenicity islands (PAIs), have been identified in ExPEC that

carry blocks of genes encoding several virulence factors. These, may have been acquired by plasmids and bacteriophages and can encode the molecular arsenal that enables these strains to colonize the urinary tract and survive in extraintestinal sites. In UPEC, the most studied PAIs are: PAI I and II identified in *E. coli* J96 and CFT073, and PAI I and IV in *E. coli*, with PAI IV_{536} being more common in UPEC [6, 7]. The presence of PAIs in UPEC is a feature associated with clinically severe forms of infection [8].

Furthermore, *E. coli* can be classified phylogenetically into four main groups: A, B1, B2, and D [9-11]. Commensal isolates belong mainly to phylogenetic groups A and B1 [12]; intestinal pathogens are spread among groups B1, D, and less frequently belong to group A [5]; whereas human ExPEC are mostly members of group B2 and, to a lesser extent, group D [13].

The goal of this study was to investigate the presence of genes encoding virulence factors and pathogenicity island associated sequences, and to compare the phylogenetic groups of ExPEC isolated from humans to the phylogenetic groups of faecal commensal isolates in southern Brazil.

Methodology

Isolation and identification of E. coli strains

In this study, we studied 96 ExPEC isolates (90 from the urine of patients with a UTI; 6 from blood of patients with bacteremia) collected between 2008-2012. As a comparative control, 50 *E. coli* isolates were also collected from faecal samples of healthy volunteers. The participants in this study, were all 18 years of age or older, and were seen by the outpatient service and emergency department of the University Hospital (UH) and Clinic Hospital (CH), Universidade Estadual de Londrina (UEL/PR). This study was approved by the Human Research Ethics Committee - UEL (Process 23335/08). *E. coli* strains were isolated and identified by the Phoenix automated method, and samples were stored in Brain Heart Infusion (BHI) (Difco, Livonia, USA) plus 20% glycerol (Sigma Aldrich, St. Louis, USA) media at -80 °C. ExPEC strains RS218, V27 [14], J96 [6] and EPEC 2348/69 [15] were used as reference strain controls.

Antimicrobial susceptibility testing

ExPEC and commensal strains were tested for susceptibility against a large panel of antimicrobials by referencing the guidelines set forth by the Clinical and Laboratory Standards Institute [CLSI] (2012) [16]. Susceptibility to the following antimicrobials was determined by microdilution on the automated Phoenix system: amikacin, aztreonam, cefazolin, cefepime, cefotaxime, ceftazidime, ciprofloxacin, ertapenem, gentamicin, imipenem, levofloxacin, meropenem, nitrofunrantoin, piperacillin + tazobactam, and trimethoprim-sulfamethoxazole.

Table 1. Primers for detection of virulence genes.

Genes	Sequences (5´ to 3´)	Size of product (bp)
*iro*N	AATCCGGCAAAGAGACGAACCGCCT GTTCGGGCAACCCCTGCTTTGACTTT	553
*omp*T	TCATCCCGGAAGCCTCCCTCACTACTAT TAGCGTTTGCTGCACTGGCTTCTGATAC	496
*hly*F	GGCCACAGTCGTTTAGGGTGCTTACC GGCGGTTTAGGCATTCCGATACTCAG	450
iss	CAGCAACCCGAACCACTTGATG AGCATTGCCAGAGCGGCAGAA	323
*iut*A	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	302
tsh	GGTGGTGCACTGGAGTGG AGTCCAGCGTGATAGTGG	620
*ecp*A	TGAAAAAAAAGGTTCTGGCAATAGC CGCTGATGAGGAGAAAGTGAA	482
*hly*A	AACAAGGATAAGCACTGTTCTGGC ACCATATAAGCGGTCATTCCCGTC	1177
*fyu*A	TGATTAACCCCGCGACGGAA CGCAGTAGGCACGATGTTGTA	880
*pap*C	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328
*pap*G	CTGTAATTACGGAAGTGATTTCTG ACTATCCGGCTCCGGATAAACCAT	1070

Detecting virulence genes using PCR

Primers used for amplification of DNA fragments corresponding to virulence genes *iro*N, *omp*Tp, *hly*F, *iss*, *iut*A [4], *tsh* [17], *hly*A, *fyu*A, *pap*G [14], *pap*C [18] and *ecp*A [19] are shown in Table 1. Bacterial DNA was amplified using 2.5 µl of supernatant in 12.5 µl of reaction mixture, which contained 1.5 U *Taq* polymerase (Invitrogen, Carlsbad, USA), 0.2 mM of each deoxynucleotide triphosphate (dNTP, Invitrogen), 2.5 mM $MgCl_2$, PCR Buffer 10X (Invitrogen) and the appropriate primers (20 pmol/ µl).

Ten microliters of each reaction mixture were subjected to electrophoresis on a 2% agarose gel followed by staining with 1 µl Gel Red 20X (Biotium, Hayward, USA) (1:500) and visualization on a UV transilluminator. A 1 Kb DNA ladder (Invitrogen) was run on each gel.

Detecting PAI sequences

Specific sequences corresponding to seven different pathogenicity islands were analyzed by PCR: PAI I_{536}, PAI II_{536}, PAI III_{536}, PAI IV_{536}, PAI I_{J96}, PAI I_{CFT073} and PAI II_{CFT073}. The isolates were subjected to PCR in a thermal cycler (Applied Biosystems, Carlsbad, USA) with an annealing temperature of 55°C as described by Sabaté *et al*. [6].

Phylogenetic classification

The phylogenetic classification into groups was carried out according to the method developed by Clermont *et al.* [9] and modified by Kotlowski *et al.* [10]. The samples were subjected to PCR in a thermal cycler (Applied Biosystems) with an annealing temperature of 54°C. Ten microliters of each reaction mixture were subjected to electrophoresis on a 2% agarose gel followed by staining with 1 µl Gel Red (Biotium) and visualization in a UV transilluminator. A 1 Kb DNA ladder (Invitrogen) was run on each gel.

Southern blot and DNA probe tests

Native plasmids from 10 isolates were extracted and analyzed as described by Kado and Liu [20]. We selected only the strains that had the genes *omp*Tp and *hly*F to check if they had the native plasmid and then tested for other genes present in what has been described as the conserved virulence plasmidic (CVP) region (having 8 genes or operons: *iro*, *iuc*, *sit, omp*Tp, *cva, hly*F, *ets* and *iss*) [21] to confirm the presence of the native plasmid. Plasmidic DNA was separated by agarose gel electrophoresis and transferred to nylon membranes, Hybond-N+membrane (Amersham, GE Healthcare Life Sciences, Cleveland, USA) by capillary blotting. The probes for Southern hybridization were synthesized by PCR using specific primers for detection of virulence genes *ompT*p, *hlyF*, *sitA,* and APEC strain χ7122 [22] was used as positive a control. The amplified product was labeled with DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the manufacturer's instructions. The blot was hybridized, blocked, incubated with anti-digoxigenin antibodies and developed by chemiluminescence according to the manufacturer's recommendations.

Statistical analysis

Comparison of frequencies among different groups was analyzed by the Fisher exact test. The threshold for statistical significance was a P value < 0.05. The test was performed with the statistical program BioEstat version 5.3.

Results

Analysis of the distribution of extraintestinal infections

Among the 96 patients infected with ExPEC, 90 (93.8%) were from urinary tract infections that were categorized as cystitis (91.1%), urosepsis (6.7%) and pyelonephritis (2.2%) (Table 2). Using the records of patients, we were able to identify that 20% of the cases were recurrent infections. Six patients had bacteremia with positive blood cultures. Furthermore, UTIs were more frequent in female patients (71.9%). We noticed that the age of the patients with UTIs was most commonly between 18 and 30 years of age (30.4% of cases), while male patients were more often found in the >54 years age group, representing 57.1% of cases.

Antimicrobial susceptibility testing

In the analysis of antimicrobial susceptibility testing performed on ExPEC isolates, we observed that among the 96 tested, 42 (43.75%) were resistant to at

Table 2. Gender distributed among *E. coli* uropathogenic infections.

	Cystitis	Pyelonephritis	Urosepsis
Female	63*†	2	4
Male	19*.	0	2
Total	**82 (91.1%)**	**2 (2.2%)**	**6 (6.7%)**

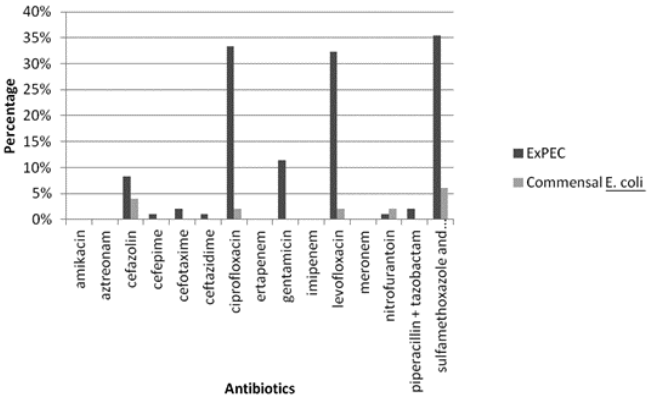
Data are reported as a number; * $p < 0.05$ Comparison within the same gender: † $p < 0.05$ Comparison between genders.

least one antimicrobial. In addition, 27 (28%) of the ExPEC isolates showed resistance to three or more of the 15 antimicrobials tested. Antimicrobials for which ExPEC were found to be commonly resistant included sulfamethoxazole-trimethoprim (35.41%), ciprofloxacin (33.33%), levofloxacin (32.29%) and cefazolin (8.33 %) (Figure 1). Of the 50 faecal *E. coli* isolates used as a case control, only 6 (12%) were resistant to one or more antibiotics. As such, the ExPEC strains were significantly more resistant to antimicrobials ($p < 0.01$) than the faecal control strains.

Analysis of presence of virulence genes among E. coli strains

Among the genes tested for by PCR, *ecp*A (94.8 %) and *fyu*A (65.6 %) were the most commonly identified, and both were more prevalent in strains belonging to phylogenetic groups B2 and D. As shown in Table 3, however, the *ecp*A gene was also more common in faecal isolates (96%). For the *iro*N and *iut*A genes, 35%-50% of strains were positive, and these genes were present among all phylogenetic groups. The *iro*N gene is more frequent in group B2, and *iut*A was prevalent in all groups except group A. The genes *omp*Tp, *tsh*, *hly*F and *hly*A were less prevalent (10%-20%) and were distributed among all phylogenetic groups, though *hlyA* was not prevalent in group B2. The *iss* gene, was present in 20% of ExPEC strains and was more distributed in group B1, which is statistically significant when compared to group A. Finally, *pap*C, and *pap*G were detected in 20-22% of ExPEC strains, which was not significant when compared to faecal strains ($p > 0.05$), and they were more common in strains from groups B2 and D. These results are shown in Table 3. In faecal commensal *E.coli* strains, the majority of these genes were less

Figure 1. Profile of resistance to 15 antimicrobial for 96 EXPEC strains and 50 faecal comensal isolates collected from patients at UH and UEL-HC in the period of 2008-2012.



common, with the exception of *ecp*A, or were not found, as is the case for the *iut*A gene ($p < 0.01$). Therefore, in commensal strains, group A was prevalent especially for the *fyu*A, *hly*A and *ecp*A genes. When compared to ExPEC strains, most genes were present in 0-15% of the commensal faecal isolates.

Out of the 96 ExPEC strains, 18 (18.75%) belonged to group A, 19 (19.79%) belonged to group B1, 33 (34.38%) were from group B2 and 26 (27.08%) were classified as group D.

Analysis of the distribution of PAI sequences in ExPEC and commensal strains

Of the 96 ExPEC strains analyzed in this report, 75 (78%) were positive for PAI sequences and 26 (52%) of 50 faecal *E. coli* isolates contained PAI sequences. A comparison of the distribution of PAI sequences in faecal commensal strains and ExPEC strains are shown in Figure 2. PAI III_{536} sequences were present in only one strain (1.04%), whereas PAI I_{J96} was not

Table 3. Distribution of virulence associated genes between phylogroups within ExPEC strains and faecal commensal strains.

Genes	Phylogenetic groups in ExPEC					Phylogenetic groups in Commensal				
	B2	B1	D	A	Total	B2	B1	D	A	Total
***iro*N**	48.5*(16)	24.3 (8)	15.2 (5)	12.1 (4)	34.4* (33)	040.0 (2)	0.0 (0)	0.0 (0)	060.0 (3)	10.0 (5)
***hly*F**	26.3 (5)	42.1 (8)	16.0 (3)	16.0 (3)	19.8* (19)	066.7 (2)	0.0 (0)	0.0 (0)	033.3 (1)	6.0 (3)
***iut*A**	30.0 (12)	17.5 (7)	37.5 (15)	15.0 (6)	41.7* (40)	00.0 (0)	0.0 (0)	0.0 (0)	00.0 (0)	0.0 (0)
***omp*Tp**	27.3 (6)	36.5 (8)	22.7 (5)	13.6 (3)	22.9*(22)	100.0 (2)	0.0 (0)	0.0 (0)	00.0 (0)	4.0 (2)
iss	30.0 (6)	40.0 (8)	20.0 (4)	10.0 (2)	20.8 (20)	025.0 (1)	0.0 (0)	0.0 (0)	075.0 (3)	8.0 (4)
***fyu*A**	47.6*(30)	6.3 (4)	34.9* (22)	11.1 (7)	65.6*(63)	08.7 (2)	0.0 (0)	0.0 (0)	091.3* (21)	46.0 (23)
tsh	33.3 (3)	22.2 (2)	33.3 (3)	11.1 (1)	9.4 (9)	00.0 (0)	0.0 (0)	0.0 (0)	100.0 (1)	2.0 (1)
***hly*A**	66.7*(12)	22.2 (4)	5.6 (1)	5.6 (1)	18.8* (18)	00.0 (0)	0.0 (0)	0.0 (0)	100.0* (4)	8.0 (4)
***pap*C**	36.4*(8)	9.1 (2)	40.9* (9)	13.6 (3)	22.9 (22)	033.3 (2)	0.0 (0)	0.0 (0)	066.7 (4)	12.0 (6)
***pap*G**	50.0*(10)	5.0 (1)	35.0* (7)	10.0 (2)	20.8 (20)	033.0 (2)	0.0 (0)	0.0 (0)	067.0 (4)	12.0 (6)
***ecp*A**	36.3 (33)	17.6 (16)	28.6 (26)	17.6 (16)	94.8 (91)	010.4 (5)	10.4 (5)	6.3 (3)	072.9* (35)	96.0 (48)

Data are reported as percent with number in parentheses; *$p < 0.05$, frequency of genes in each phylogenetic group compared to the frequency in all others (Fisher exact test).

detected in any of the isolates. The distribution of PAIs in ExPEC and faecal commensal isolates is presented in Figure 2.

The distribution of PAI sequences in ExPEC and commensal strains by phylogenetic group shows that most ExPEC strains contain PAI sequences (78%) and belong to group B2 (42.7%). In commensal strains that have PAI sequences (52%) most of them belong to group A (84.6%). Of the 96 ExPEC isolates, three or more PAI sequences were found in 30 ExPEC strains (31.25%). Strains that are considered to be most virulent, generally belonging to group B2, had three or more PAI sequences (86.7%), whereas strains that did not have PAI sequences, were more commonly distributed in group B1 (47.6%) (Table 4).

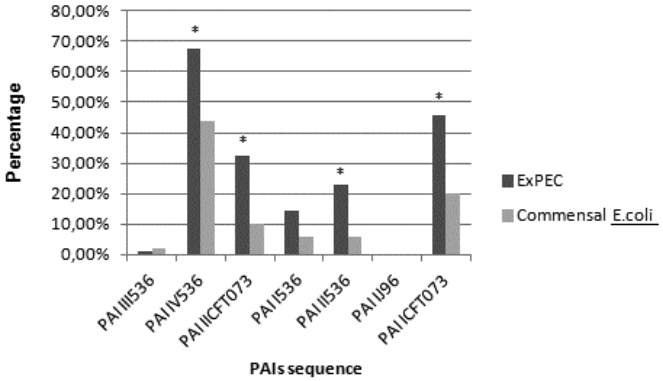
Southern blot and DNA probe tests

All 10 samples tested presented (1 to 5) plasmids of high molecular weight, and all the samples that had *hly*F and *omp*Tp genes were present in the higher molecular weight plasmids, as demonstrated by Southern blot using specific DNA probes labeled with digoxigenin. Of the 10 samples tested, fifty percent belonged to the B1 group and fifty percent harbored seven of eight genes presented in the CVP region (Table 5).

Discussion

There is a major concern regarding acquisition of resistance to several drugs used in the treatment of infections caused by *E. coli* causing UTIs [23].

Figure 2. Analysis of Pathogenicity Island associated sequences in 96 ExPEC and 50 commensal *E. coli* strains collected at UH and UEL-HC in the period of 2008-2012. *P < 0.01.



Antimicrobials that are widely prescribed for treatment of UTI episodes include beta-lactams, trimethoprim-sulfamethoxazole, quinolones, fluoroquinolones and aminoglycosides [24]. In Southern Brazil we have little recent data concerning the antimicrobial resistance of ExPEC. We had higher resistance profiles in ExPEC compared to commensal isolates, particularly with respect to resistance to fluoroquinolones (ciprofloxacin and levofloxacin) and trimethoprim-sulfamethoxazole (both at approximately 1/3 of strains being resistant) compared to other studies [24] (Figure 1). Among the ExPEC strains we tested, 28% were resistant to three or more antimicrobials. It is necessary to perform antimicrobial susceptibility testing because the *E. coli* strains show

Table 4. Proportion of isolates with zero, one, two or three or more PAI sequences in ExPEC strains.

PAIs	Phylogenetic groups in ExPEC			
	B2	B1	D	A
3 or more	86.7%	00.0%	06.7%	06.7%
1 or 2	13.3%	20.0%	46.7%	20.0%
Zero	04.8%	47.6%	14.3%	33.3%

Table 5. Bacterial and molecular characteristics of 10 studied isolates of ExPEC.

Strains	UTI	Phylogenetic group	Virulence Genes							
			*hly*F	*sit*A	*omp*T	*cva*A	*ets*C	*iuc*C	*iro*N	*iss*
UPEC 16	Cystitis	B2	+	+	+	-	+	+	+	+
UPEC 31	Cystitis	B1	+	+	+	-	+	+	+	+
UPEC 33	Cystitis	B1	+	+	+	-	-	-	+	+
UPEC 40	Pyelonephritis	B2	+	+	+	-	+	+	+	+
UPEC 42	Cystitis	A	+	+	+	-	-	-	-	-
UPEC 59	Cystitis	B1	+	+	+	-	+	-	+	+
UPEC 95	Cystitis	B1	+	+	+	-	-	-	+	+
UPEC 97	Cystitis	B1	+	+	+	+	+	+	+	+
HEMO 81	Hemoculture	D	+	+	+	-	+	+	+	+
HEMO 114	Hemoculture	A	+	+	+	-	-	-	+	+

variability in their susceptibility to antimicrobials and because upwards of 43.75% of *E. coli* are resistant to first-line antimicrobials.

ExPEC are known to produce several virulence factors, contributing to their survival in the host urinary tract, blood, and other tissues. Among the eleven tested virulence genes, all of them were detected in some of the ExPEC strains and faecal commensal isolates, as shown by others [4,25]. The gene most commonly found in both ExPEC and commensal samples was *ecp*A (94.8% and 96%, respectively). Despite potentially playing a role in UTI virulence [26], as *ecpA* is highly conserved between both ExPEC and faecal commensal strains, it cannot serve as a marker to potentially differentiate ExPEC from commensal *E. coli*. However, in commensal strains, group A was prevalent especially for genes *fyu*A, *hly*A and *ecp*A. In strains belonging to group B2 a greater presence of all tested genes was expected because this group is characterized as being the most virulent [13]. In our study, the percentage of ExPEC belonging to group B2 was 34.4%. By contrast, although ExPEC group B2 predominated, ExPEC group B1 was also common (19.8%) in our study.

Figure 3. Study of pathogenicity islands, phylogenetic classification, and virulence genes in 96 ExPEC strains from human. In blue are the samples belonging to phylogenetic group B2; in orange, the samples belonging to group D; in green, the group B1; and finally in pink, samples related to group A.

The comparison of the presence of these virulence genes in commensal faecal *E. coli* isolates and ExPEC showed that some genes are significantly more present in ExPEC (Table 3). Genes such as *iro*N, *iut*A, *omp*Tp and *hly*F present in the CVP region [21] were significantly more frequent in clinical isolates compared to commensal faecal isolates. In this study, fifty percent of the ExPEC strains containing plasmid-encoded virulence-associated genes such as *omp*Tp and *hly*F belonged to phylogenetic group B1 (Table 5).

The PAI sequences were also present in some commensal faecal isolates although less often (52%) than in ExPEC strains (78%). The distribution of PAI sequences in ExPEC samples in this study also corroborates the results published by Sabaté *et al.* [6]. PAI $IV_{536,}$ reported as the most common in UPEC, was found most frequently in our strains (Figure 2). This PAI is quite stable in *E. coli* 536 (originally isolated from a patient with acute pyelonephritis) which could explain its high frequency in our strains as well. The PAI $III_{536,}$ which is classified as unstable, easily lost and not required for virulence [6], was present in a low percentage of ExPEC (1.04%). The majority of strains that had PAI sequences belonged mainly to phylogenetic group B2, and the strains that belonged to group B1 had few PAI sequences, which leads us to conclude that the virulence of ExPEC strains characterized as B2 is likely attributed to PAI encoded genes, whereas the virulence of ExPEC strains belonging to phylogenetic group B1 is likely due to plasmid encoded virulence genes (Figure 3).

Conclusion

In summary, we have found virulence genes in our ExPEC and commensal samples, but some genes (*iro*N, *iut*A, *omp*Tp and *hly*F) are significantly more enriched in ExPEC strains. We also observed that PAI sequences and the B2 phylogroup were highly prevalent among ExPEC strains. Our results suggest that continued research on virulence factors of ExPEC is necessary because there is no consensus regarding which genes would be ideal virulence markers, as all of these genes provide the bacteria more resistance against host defense mechanisms when compared to the commensal strains.

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