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Prevalence of Genes Encoding Outer Membrane Virulence Factors Among Fecal *Escherichia coli* Isolates

Ahmad Rashki[1*], Hussein Ali Abdi[2], Milad Shookohi[3]

[1]Associate Professor in Molecular Genetics and Microbiology, Department of Pathophysiology, Faculty of Vet-Medicine, University of Zabol, Zabol, Iran
[2]PhD Student in Molecular Genetics, Faculty of Sciences, University of Sistan and Baluchestan, Zahedan, Iran
[3]MSc in Molecular Genetics, Department of Biology, Faculty of Basic Sciences, University of Zabol, Zabol, Iran

***Correspondence to**
Ahmad Rashki; Department of Pathophysiology, Faculty of Vet-Medicine, University of Zabol, Zabol, Iran.
Tel: +98-5424822250;
Fax: +98-5424822251;
Email: ah_rashki@usal.es

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Abstract

Objective: *Escherichia coli* is commensal bacterium of human intestine. The gut is a common pool of *E. coli* isolates causing urinary tract infections (UTIs). Some of fecal *E. coli* (FeEC) by the possession of certain virulence factors is able to cause diseases in human and other mammalian models. To evaluate the health threats coordinated with a given fecal source of *E. coli* strains, we determined the frequency of genes expressing virulence determinants in fecal *E. coli* isolates collected from human feces in Zabol, southeast of Iran.

Methods: *Escherichia coli* isolates (n=94) were separated from the feces of patients attending teaching hospitals, and screened for various virulence genes: *fimH*, *his*, *hlyA*, *ompT*, *irp2*, *iucD*, *iroN*, and *cnf1* by using the multiplex polymerase chain reaction (PCR) method.

Results: The prevalence of virulence genes was as follows: adhesins (*fimH,* 98% and *iha*, 26%), alpha-hemolysins (*hlyA,* 10%), outer membrane protease (*ompT,* 67%), aerobactin (*iucD,* 67%), iron-repressible protein (*irp2,* 91%) and salmochelin (*iroN,* 33%) and cytotoxic necrotizing factor 1 (*cnf1*). According to the diversity of different virulence genes, the examined isolates exhibited 29 different patterns.

Conclusion: Our results demonstrated that most of the assessed isolates harbored several virulence factors. Our findings propose possibility of human feces serving as a source for pathogenic organisms, supporting the notion that fecal materials of humans play a role in the epidemiological chain of extra-intestinal pathogenic *E. coli*. This is the first report of the frequency of virulence factors among *E. coli* isolates collected from human feces in Iran.

Keywords: Fecal *Escherichia coli,* Major virulence factors, Multiplex PCR

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Introduction

Escherichia coli represents as the most encountered etiology for urinary tract infections (UTIs) identified in the gastrointestinal tract of mammals. There is a well established belief that uropathogenic *E. coli* (UPEC) arises from the distal gut microbiota[1-3] In addition, *E. coli* is a diverse species regarding the genetic content and phenotypic and pathogenic traits.[4] The disease which generates strains of *E. coli* contains multiple virulence mediators participating in their pathogenesis (diarrhea, dysentery, septicemia, pneumonia, meningitis, and UTI). Also, commensal strains can lead to a disease in host with compromised immune system.[5] Some pathogenic strain of *E. coli* persist in gastrointestinal tract of human and are frequently associated with human diseases.

According to multiple virulence determinants, *E. coli* bacteria can be categorized into 3 major subclasses: commensal, intestinal pathogenic and extra intestinal pathogenic *E. coli* (ExPEC).[6] It has been shown that intestinal and ExPEC strains may be extended from commensal types through obtaining of virulence mediators.[7] ExPEC exhibit significant genetic heterogenicity and represents a wide spectrum of virulence-related factors such as adhesins, toxins, and iron up taking molecules, lipopolysaccharides, polysaccharide capsules, and invasions, which can be expressed on mobile genetic segments including plasmids, bacteriophages, and pathogenici-

ty-associated islands (PAIs).[8] The fecal flora from the hosts provides the most common source for infecting *E. coli* strain.[9] The UTI-causing strains that are commonly called UPEC are obtained by faces, the place of their entrance to the urinary tract via colonization of the vaginal introitus and the periurethral location.[10] *E. coli* strains residing within intestinal tract and promoting UTI typically contain virulence genes necessitated for colonization of the urinary tract. On the other hand, it seems that some of FeEC strains carrying many genes that encode virulence factors and can cause serious disease at diverse extraintestinal sites.

Multiple studies have been done in different parts of Iran in order to investigate the frequency of virulence genes among UPEC.[11-13] However, there is no information about the frequency of virulence mediators in FeEC isolated from human in Iran. Therefore, this study was conducted to examine the distribution of 8 virulence factors, including type 1 fimbriae (*fimH*), iron-regulated gene homologue adhesion (*iha*), alpha-hemolysin (*hlyA*), outer membrane protease (*ompT*), aerobactin (*iucD*), yersiniabactin (*irp2*), salmochelin receptor (*iroN*) and cytotoxic necrotizing factor 1 (*cnf1*) in *E. coli* strains isolated from human feces in Zabol, southeast of Iran, by using the multiplex polymerase chain reaction (PCR) method.

Methods

Bacterial Isolates

The sample size was calculated as described by Charan and Biswas.[14] Swabs were collected directly from stool samples of the patients with diarrhea admitted to teaching hospitals in Zabol, Iran during July 2014 through October 2014. Samples were suspended into Cary-Blair transport media (Laboratorios Conda, S.A., Spain) and transported to laboratory on ice where one loop from each sample was streaked directly on MacConkey agar (HiMedia Laboratories) within 4 hours after collection. Plates were incubated at 37°C for 18–24 hours, and up to 3 colonies with typical appearance of *E. coli* were selected and subjected to biochemical tests including oxidase, indole, methyl red, Voges-Proskauer, nitrate reduction, urease production, Simmons' citrate agar (HiMedia Laboratories), and various sugar fermentation.[15,16]

Extraction of DNA

The bacteria were isolated from 1 mL of the *E. coli* culture grown for 18 hours at 37°C. The bacterial DNA was extracted by boiling methods.[17] Briefly, all *E. coli* isolates were cultivated overnight (16 hours) in 5 mL Luria-Bertani (LB) broth (HiMedia Laboratories) in a shaking incubator (200 rpm) at 37°C. Two milliliters of bacterial isolates were then pelleted, suspended in 200 µL of sterile double-distilled water and boiled at 95°C for 10 minutes. The mixture was cooled on ice (5 minutes), and the supernatant was collected following centrifugation (13 000 rpm 5 minutes). After centrifugation, the supernatants were kept as DNA at -20°C until applied for PCR.

Detection of Putative Virulence Genes Using the Multiplex PCR Method

Prevalence of putative virulence genes in FeEC isolates was determined by multiplex PCR.[3] Details of primer sequences, target genes and products size are shown in Table 1. Amplification of selected genes was performed by setting a net volume of 25 µL. The reaction contained 2 µL of DNA, 12.5 µL of Taq DNA Polymerase Master Mix Red (amplicon, A/S, Denmark), 1 µL of primers (30 pmol concentration for each) (Pishgam, Iran) and 8.5 30 pmol ddH_2O. The multiplex PCR was performed considering an initial phase of denaturation (94°C, 5 minutes), followed by 35 cycles consisting of denaturation (94°C for 30 seconds), annealing (59°C for 50 seconds) and extension (72°C for 70 seconds), and followed by a final extension step at 72°C for 5 minutes. Amplification was performed

Table 1. Primers for the Multiplex PCR Assays

Virulence Factor	Target gene	Primer	Primer Sequences (5'-3')	Size of Product(bp)
Cytotoxic necrotizing factor 1	*cnf1*	cnf1-F	AGGCAGGAATAAACCAGGAGGT	1286
		cnf1-R	ACGAGCAGAATTTGACACACGA	
Outer membrane protease	*ompT*	ompT-F	TGCGATCAGCTCTTTTGCTTCT	144
		ompT-R	AGTTGACTGACTTTTCGGCCTC	
Yersiniabactin	*irp2*	irp2-F	AGCATCGCCTGCTAAAACTGAA	623
		irp2-R	CAGACGATGCAGGGCGTTATTA	
Iron-regulated gene homologue adhesion	*iha*	iha-F	CTGGAAGTCAGCATTCGTGGAA	934
		iha-R	GATGCCACTCATCCTCAGCAAA	
Alpha-hemolysin	*hlyA*	hlyA-F	GTTAGCGGGTGTCACCAGAAAT	1361
		hlyA-R	GTGTGATTACCCTGCCGTCTTT	
Salmochelin receptor	*iroN*	iroN-F	CGGTTCCTGGCACGAATATCAT	1048
		iroN-R	TTTTGGGATTTCCCCAACCTGG	
Aerobactin	*iucD*	iucD-F	ATGGCATCACTGCCGATTCTTT	534
		iucD-R	AGTGAGTTAAAGCAGCAGCCTC	
Type 1 fimbriae	*fimH*	fimH-F	ATTCCTCACAATCAGCGCACTT	170
		fimH-R	ATCAGCAGTACAGCAAACAGGG	

using a gradient Eppendorf's Mastercycler® Pro (Eppendorf, Germany). The multiplex PCR products were separated by agarose gel 2% electrophoresis and visualized under UV-induced fluorescence. A 100 bp DNA ladder (Fermentase) was used as size standard (Figure 1). Amplification identities were confirmed by restriction analysis.

Restriction Analysis

Amplified fragments of selected genes were confirmed by restriction analysis. Restriction patterns of 8 sequences were obtained with the Webcutter 2.0, online software (http://rna.lundberg.gu.se/cutter2/). The *hlyA, iha, irp2* and *iroN* gene sequences were restricted with *Tfi*I endonuclease followed *ompT, iucD* and *fimH* sequences restricted with *Alu*I endonuclease. *MspI* endonuclease was chosen for restriction of *cnf1* sequence. Restriction conditions were identical in all cases. Each 30 µL reaction mixture contained 1 µL of restriction endonuclease, 8 µL of PCR product, 3 µL of specific endonuclease enzyme buffer and 18 µL of sterilized dH_2O. After overnight incubation, the restriction products were determined by electrophoresis of the digested DNA in 2% agarose gel.

Results

The frequency of virulence genes collected from *E. coli* isolates of fecal samples is shown in Figure 2. In total, 94/94 (100%) of FeEC isolates represented with at least one of the studied virulence genes, of which 1 (1%), 18 (19%), 10 (11%), and 29 (31%) were detected harboring 1, 2, 3, and 4 virulence factors, respectively. Twenty-nine different virulence combinations were found among fecal *E. coli* isolates (Table 2). FeEC11 pattern was determined by the presence of the *irp2, ompT, iucD* and *fimH* genes which presented the most identified pattern, detected in 19 (20%) isolates. *fimH* was the frequent virulence gene identified in all 92(98%) FeEC isolates. Among the iron-acquisition genes, *irp2* was the most prevalent gene and was identified in 86 (91%) isolates, while *iucD* and *iroN* genes were detected in 63 (67%) and 31 (33%) isolates respectively. The secretory virulence genes including *hly* and *cnf* were present in 9 (10%) and 3 (3%) FeEC isolates, respectively. The *ompT* gene was identified in 63 (67%) and the *iha* was detected in 24 (26%) isolates. The association of 7 genes was recognized in FeEC1 and FeEC2 patterns (3 isolates). One isolate harbored *fimH* only (FeEC29). The 2 FeEC3 and FeEC4 patterns included isolates possessing a combination of 6 amplified genes (Table 2).

The detected virulence genes were confirmed by restriction analysis: *iha, iroN, irp2* and *hlyA* amplicons (934, 1084, 623 and 1361 bp respectively) were restricted with *TfiI* endonuclease enzyme and yielded fragments of 465, 267, 129 and 73 bp; 1004 and 80 bp; 426 and 197 bp; and 1030 and 331bp respectively. The *cnf1* amplicon (1286 bp) was restricted with *MspI* and yielded fragments of 467, 404, 261 and 154 bp. The detected virulence genes were confirmed by restriction analysis: *iucD, ompT* and *fimH* amplicons (534, 144 and 170 bp respectively) and were restricted with *AluI* endonuclease enzyme and yielded fragments of 386 and 148 bp; 135 and 9 bp; and 109 and 61bp respectively (Figure 3).

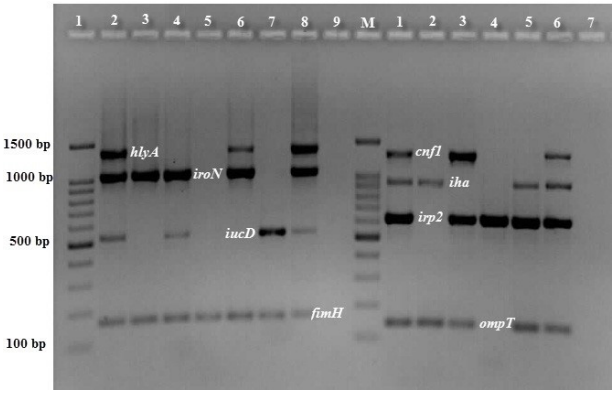

Figure 1. Electrophoresis of Virulence Selected Genes Among FeEC Isolate Obtained by Multiplex PCR.
Abbreviations: FeEC, fecal *Escherichia coli;* PCR, polymerase chain reaction.
Each band is indicated by the names of the virulence genes. Lines 1 and "M" denote 100 bp DNA marker.

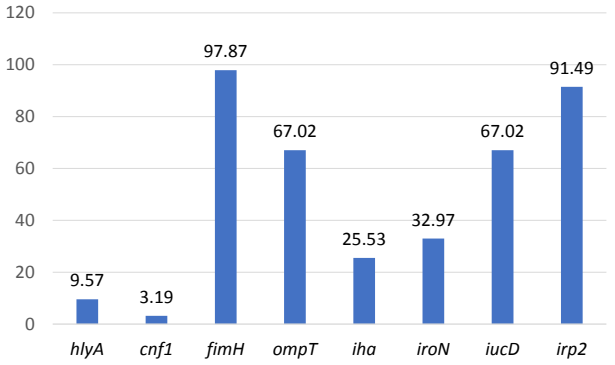

Figure 2. Prevalence of Virulence Genes Among 94 FeEC Isolates Collected From Human Feces.
Abbreviation: FeEC, fecal *Escherichia coli*.

Discussion

The lower gastrointestinal tract is considered as the richest pool of UTI generating organisms. Some strains can colonize the vagina and urinary tract.[18] The variety and heterogeneity of virulence factors, including adhesins, toxins and siderophores emerge to be momentous for *E. coli* strains and make the development of multiplex PCR method especially significant. In this work, to our knowledge for the first time in Iran, we assessed the prevalence of virulence gene profile in 94 FeEC isolates Understanding the distribution of virulence determinants in FeEC isolates is important to evaluate their relative contribution to the extraintestinal infection. We also assessed multiplex PCR assays to detect virulence factors utilizing a combination of primers previously reported. [3]

Our findings showed that *FimH* adhesion was the most prevalent virulence factor detected. This factor occurred in 92 (98%) FeEC isolates as seen in Table 2. Similar results were obtained by previous studies,[18, 19] whereas the

Table 2. Virulence Gene Patterns Identified Among the Studied Isolates

Patterns	Urovirulence Genes								No. of Strains
	cnf1	*iha*	*irp2*	*ompT*	*hlyA*	*iroN*	*iucD*	*fimH*	
FeEC1	-	+	+	+	+	+	+	+	2
FeEC2	+	-	+	+	+	+	+	+	1
FeEC3	-	-	+	+	+	+	+	+	2
FeEC4	-	+	+	+	-	+	+	+	3
FeEC5	-	+	+	-	+	+	-	+	1
FeEC6	-	-	+	+	-	+	+	+	12
FeEC7	-	-	+	-	+	+	-	+	1
FeEC8	-	+	+	-	-	+	+	+	1
FeEC9	-	-	+	+	-	-	-	+	4
FeEC10	-	+	+	-	-	-	+	+	6
FeEC11	-	-	+	+	-	-	+	+	19
FeEC12	-	+	+	+	-	-	-	+	1
FeEC13	-	-	+	+	-	+	-	-	1
FeEC14	-	+	+	-	-	-	-	+	1
FeEC15	+	-	+	+	-	+	-	+	1
FeEC16	+	-	+	+	-	-	+	+	1
FeEC17	-	+	-	+	-	+	+	+	1
FeEC18	-	-	+	+	+	+	-	+	1
FeEC19	-	-	+	+	-	+	-	+	2
FeEC20	-	-	+	-	+	-	+	+	1
FeEC21	-	+	+	+	-	-	+	+	8
FeEC22	-	-	+	-	-	+	-	+	1
FeEC23	-	-	+	-	-	-	+	+	4
FeEC24	-	-	+	-	-	-	-	+	11
FeEC25	-	-	+	-	-	-	+	-	1
FeEC26	-	-	-	-	-	+	-	+	1
FeEC27	-	-	-	-	-	-	+	+	1
FeEC28	-	-	-	+	-	-	-	+	4
FeEC29	-	-	-	-	-	-	-	+	1
Total	3	24	86	63	9	31	63	92	94

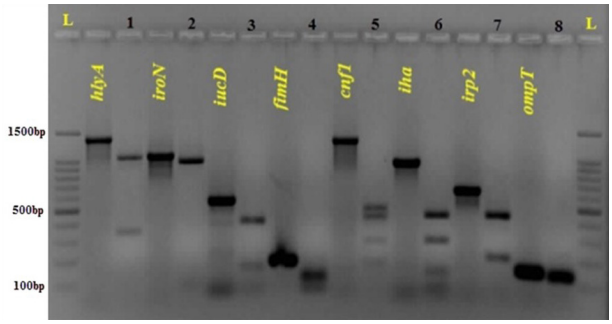

Figure 3. Gel Electrophoresis for Digestion Results of Virulence Genes in FeEC Isolates.
L: Lader; **Lane 1.** Digest of *hlyA* (1361bp) with *Tfi*I enzyme and production of fragments 331 bp and 1030 bp; **Lane 2.** Digest of *iroN* (1084 bp) with *TfiI* enzyme and production of fragments 80 bp and 1004 bp; **Lane 3.** Digest of *iucD* (534 bp) with *AluI* enzyme and production of fragments 148 bp and 386 bp; **Lane 4.** Digest of *fimH* (170 bp) with *AluI* enzyme and production of fragments 61 bp and 109 bp; **Lane 5.** Digest of *cnf1* (1286 bp) with *MspI* enzyme and production of fragments 154 bp, 261 bp, 404 bp and 467 bp; **Lane 6.** Digest of *iha* (934 bp) with *TfiI* enzyme and production of fragments 73 bp, 129 bp, 267 bp and 465 bp; **Lane 7.** Digest of *irp2* (623 bp) with *TfiI* enzyme and production of fragments 197 bp and 426 bp; **Lane 8.** Digest of *ompT* (144 bp) with *AluI* enzyme and production of fragments 9 bp and 135 bp

frequency of the *iha* gene, as other adhesion, was 26% (24/94). Our laboratory previously showed that the prevalence of *iha* gene was 29% among UPEC isolates.[20] However, no significant differences were observed in relation to *iha* gene. According to our knowledge, to date, no reports have been published about the prevalence of *iha* gene among FeEC isolates. The findings of our study indicated that the presence of the *hlyA* and *cnf1* genes was 10% and 3% respectively.

In another study, Usein et al[21] demonstrated that the *hlyA* and *cnf1* genes were found in 35% of *E. coli* bacteria recovered from the fecal flora of healthy adult humans. The results showed the variation geographical distribution of these genes. The prevalence of the *cnf1* gene was different in fecal isolates studied by other investigators.[22,23] In accordance with our study, Obiet et al[24] also described that the *cnf1* gene was expressed in 3.5% of *E. coli* isolates collected from diarrheic stool samples in South Africa. In another study, our group demonstrated that *cnf1* gene was more frequently detected in UPEC (28%) in comparison with FeEC isolates (3%).[20] Particularly, ExPEC expresses a richness of apparently excessive iron obtaining systems, including the salmochelin, yersiniabactin, and aerobactin

siderophores. In accordance, we observed in our study a very high prevalence of the iron acquisition genes; *irp2* (91%), *iucD* (67%) and *iroN* (33%). The *irp* gene cluster is mapped within the high pathogenicity island (HPI) described primarily in *Yersinia* spp. and horizontal gene transfer has caused it to be present in intestinal and extra intestinal clinical *E. coli* strains.[25] The *iroN* gene, that is situated on the *iroA* gene cluster encodes a receptor which is responsible for iron uptake mediated by the siderophores salmochelins, contributes to the virulence of UPEC. This interaction facilitates transport of the complex into the bacterial cytosol.[26] The virulence genes of UPEC bacteria such as *iroN*, *iucD* and *irp2* have been previously recorded from Iran.[11,12] However, according to published data, there is no information on the occurrence of these genes in FeEC isolates.

The distribution of the *ompT* gene among the studied isolates was also similar to that in the previously reported data.[27] *OmpT* rather seems as a conserved protease executing in metabolism of *E. coli* derived from secretory proteins. This outer membrane protease contributes to the destruction of several proteins interacting with the outer membrane.[28] However, there is no report about the prevalence of *ompT* gene among FeEC isolates in Iran. The analysis of the association between the presences of different combinations of virulence genes among FeEC isolates, allowed us to divide the tested isolates into 29 virulence patterns noted FeEC1 to 29. Our results revealed the complexity of the properties of virulence markers in fecal *E. coli* isolates. The pattern FeEC6 included strains simultaneously positive for *irp2*$^+$, *ompT*$^+$, *iucD*$^+$, *iroN*$^+$ and *fimH*$^+$ (12 isolates). The prevalence of genes coding for the 2 adhesion pathways (type 1 fimbriae and *iha*) which confers the ability to colonization among 24 isolates were fit with those published by other investigators.[3,29] Among FeEC isolates, 89 isolates contain genes encoding an iron acquisition protein (yersiniabactin, aerobactin or salmochelin receptor). The maximum number of detected amplicons in 1 isolate was 6. The main novel finding is the high occurrence of genes of outer membrane virulence proteins among FeEC isolates in Zabol, southeast of Iran. Apart from the high occurrence of virulence genes, various virulence determinants were also revealed in fecal *E. coli* strains. In our analysis, 74% (70/94) of the bacteria were represented with various virulence genes (4 or more genes). It is comprehensible that the recurrence and prevalence of virulence features of fecal *E. coli* strains are different in other regions of Iran. Probably, geographical differences, cultural habitants, dietary features, public and hospital health policies, weather climate of each region and even sampling methods may exert high impacts on detection rate of virulence factors of FeEC isolates. Nonetheless, further research on the expression of virulence genes and molecular typing methods covering wider geographical areas in Iran is needed to determine the distribution pattern of virulence determinants and develop effective strategies to treat FeEC -induced diseases. This may constitute a limitation of our study.

Conclusion

The FeEC isolates expressing virulence genes may be regarded as an important organism for extra-intestine infections in Iran. The current work is the first report which identified virulence genes among FeEC isolates in Iran. The multiplex PCR designed in the present study successfully screened FeEC isolates for various *E. coli*-related virulence genes. Further studies in other parts of Iran are needed to identify virulent factors and to ascertain the pathophysiology of such infectious agents to consider possible prevention interventions.

Ethical Approval

We obtained informed consent form our participants.

Competing Interests

Authors declare that they have no conflict of interest.

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