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Original Article

Distribution of Genes Encoding Iron Uptake Systems among the *Escherichia coli* Isolates from Diarrheal Patients of Iran

Aida Hajihosein-Tabrizi[1], Mehri Habibi[1], Mohsen Tabasi[1,2], Mohammad Reza Asadi Karam[1*]

[1]Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran; [2]Iranian Legal Medicine Research Center, Legal Medicine Organization, Tehran, Iran

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Introduction: Diarrheagenic *Escherichia coli* (DEC) including enteropathogenic (EPEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), and shiga toxin producing *E. coli* are among the most common agents of diarrhea. There are various classes of iron uptake receptors, but there is not much data on the presence of these iron receptors in DEC isolates. The present study aimed to evaluate the presence of iron receptor genes and also hemolysis activity in these isolates. **Methods**: Totally, 88 DEC isolates (EAEC, ETEC, STEC, and EPEC) from a previous microbial collection were included in this study. The isolates were tested for the production of hemolysin on blood agar plates. Then, Polymerase Chain Reaction (PCR) was used for detection of iron acquisition genes, including *chuA*, *hma*, *iroN*, *fyuA*, *iutA* and *ireA*. **Results**: Our results showed that 8 (66.7%), 25 (89.3%), 17 (44.4%) and 10 (83.4%) of EPEC, STEC, ETEC and EAEC isolates, respectively had hemolytic activity. All the EPEC isolates were negative for *hma* gene, and *iroN* and *ireA* genes were absent in the EAEC isolates. The frequency of *chuA*, *hma* and *fyuA* genes in the STEC and EAEC isolates was higher, whereas EPEC and ETEC isolates revealed a higher frequency of *iroN* gene than the STEC and EAEC isolates. **Conclusion**: This study reports the presence of various iron receptor genes with a significant hemolysin activity in DEC isolates from Iran. The presence of these genes may contribute to the increased pathogenesis of these isolates in the intestinal tract. *J Med Microbiol Infec Dis, 2018, 6 (1): 25-30.*

Keywords: Diarrheagenic, *Escherichia coli*, Iron receptors, Hemolysin, PCR.

INTRODUCTION

Escherichia coli bacteria are the most common microorganisms of the human and animals' intestine. These bacteria, through horizontal gene transfer from other pathogenic agents, have acquired various virulence genes becoming the causative agents of a wide variety of intestinal or extraintestinal infections in humans [1, 2]. Based on the acquired virulence determinants, the *E. coli* are divided into pathotypes of diarrheagenic *E. coli* (DEC) and non-diarrheagenic *E. coli* [3]. The DEC strains is further categorized into enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Vero toxin-producing/Shiga toxin-producing *E. coli* (VTEC/STEC) including the subgroup enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) [4].

Diarrheal illness is a severe public health problem with high morbidity and mortality especially in infants and young children [5]. Annually, it is estimated that diarrhea cause ≈2.5 million deaths of children worldwide [4, 6] and enteropathogenic *E. coli* strains are considered as one of the significant cause of deaths in children under five years of age in Iran [7, 8].

Numerous virulence factors such as adhesins, invasins, toxins, and secretion systems are involved in pathogenicity of *E. coli* [9]. Iron is an essential factor for pathogenicity of *E. coli* strains in the human body, and these strains must have advanced strategies for acquiring iron from the host to produce disease. One of them is an iron-chelating small organic molecule called siderophore [10]. The enterobactin as siderophore receptor is among the most common iron acquiring systems of pathogenic and nonpathogenic *E. coli*. The involvement of this receptor in the pathogenesis of *E. coli* strains is not apparent yet [10]. Furthermore, *E. coli* strains synthesize at least three additional types of siderophores including salmochelin (iroN), yersiniabactin (fyuA) and aerobactin (iutA) [11]. Two heme uptake systems in *E. coli* facilitate the uptake of heme via the receptors ChuA and Hma and the use of heme from hemoglobin. Mutation in these genes reduces the iron uptake which results in reduced bacterial growth. Also, the heme acquisition via the receptors ChuA and Hma was demonstrated to contribute to the pathogenesis of extra-intestinal *E. coli* such as uropathogenic *E. coli* [12].

The DEC strains may produce more than one type of siderophore and other iron utilization systems, however, few studies have examined the distribution of iron acquisition systems among these pathogens [13, 14].

***Correspondence:** Mohammad Reza Asadi Karam
Department of Molecular Biology, Pasteur Institute of Iran, No. 69, Pasteur Ave, Tehran, Iran, 1316943551.
Email: m_asadi12@yahoo.com
Tel: +98 (21) 66953311 **Fax:** +98 (21) 66492619

Thus, in the present study, distribution of the genes encoding six different iron acquisition systems among EPEC, ETEC, STEC, and EAEC isolates from diarrheagenic patients was examined. Furthermore, the phenotypic virulence property of the isolates including the hemolysis was evaluated. This study could provide a better insight into understanding the role of different iron acquisition systems and other virulence factors in the pathogenesis of DEC strains in humans.

MATERIAL AND METHODS

Collection of DEC isolates. Eighty-eight diarrheagenic *E. coli* strains were randomly selected from a previous collection available in the Molecular Biology Department of Pasteur Institute of Iran. These isolates had previously been obtained from acute diarrhea patients and identified as DEC by biochemical and molecular methods as described by others [15, 16]. Further molecular studies defined 12 isolates as EPEC, 28 STEC, 36 as ETEC and12 as EAEC.

Detection of hemolytic activity. DEC strains were tested for the production of hemolysin on blood agar plates. In this assay, prepared defibrinated sheep blood was washed three times with PBS and then added to Trypticase Soy Agar (TSA; Merck, Germany) at a final concentration of 5%. Production of hemolysis was characterized by the formation of a clear zone around the bacterial colonies after overnight incubation at 37°C. Total hemolysis or partial lysis of erythrocytes corresponded to positive hemolysis. The absence of hemolytic activity in the plates was defined as non-hemolytic *E. coli* strains.

DNA extraction. All the *E. coli* isolates were grown on Luria-Bertani broth (Merck, Germany) overnight at 37°C and DNA was extracted using the phenol-chloroform protocol as described previously [17]. The bacterial cell pellets obtained by centrifugation (5000rpm, 5 min, 4°C) were suspended in 200 µl of the same buffer containing lysozyme (final concentration 5 mg/ml), and the samples were incubated at 37°C for 60 min. Proteinase K and SDS (Sodium Dodecyl Sulphate) were added to final concentrations of 2 mg/ml and 1% (v/w), respectively, and the mixtures were incubated at 56°C for 2 h. Then, 200 µl of phenol (saturated with 10 mMTris/HCl, pH 8.0) was added to each mixture, and the samples were vortexed and centrifuged at 14,000 rpm for 1 min. The aqueous phase was collected and mixed with an equal volume of chloroform/isoamyl alcohol (24:1), vortexed and centrifuged at 14000 rpm for 30 min. Nucleic acids from the recovered supernatant were precipitated with isopropanol. The pellet was washed with 70% ethanol and resuspended in TE (20 µl) containing RNase. The samples were stored at -20°C until further use. Finally, electrophoresis on 1% agarose gel and a spectrophotometer were used to check the quality and quantity of the extracted DNAs.

Detection of iron acquisition genes by PCR assay. We designed primers for amplification of iron acquisition genes including *chuA*, *hma*, *iroN*, *fyuA*, *iutA* and *ireA* genes (Table 1).

Table 1. The primers used for amplification of iron receptor genes

No	Gene	Primer sequences (5′-3′)	Annealing Temp (°C)	Expected band (bp)
1	*fyuA*	$FyuA_F$: ATGAAAATGACACGGCT $FyuA_R$: GAAGAAATCAATTCGCG	56	2200
2	*ireA*	$IreA_F$: ATGAAGAACAAATATATC $IreA_R$: GAAGGATACTCTTACATT	53	2500
3	*hmaA*	Hma_F: ATGGTTAAAGATACAATC Hma_R: CCACTGATAACGGGTAT	56	1850
4	*iroN*	$IroN_F$: AAGTCAAAGCAGGGGTTGCCCG $IronN_R$: GACGCCGACATTAAGACGCAG	55	700
5	*chuA*	$ChuA_F$: ATGTCACGTCCGCAAT $ChuA_R$: CCATTGATAACTCACGAA	57	2000
6	*iutA*	$IutA_F$: ATGGCGCAGCGGCAG $IutA_R$: ACGTAACAGAGACAGAACA	58	1750

The primers were designed based on the gene sequences available in Genbank database (NCBI, www.ncbi.nlm.nih.gov) and were checked using the Primer-Blast Tool (blast.ncbi.nlm.nih.gov/Blast.cgi). A Polymerase Chain Reaction (PCR) was used for amplification of six virulence genes in the DEC isolates. The optimized 25 µL mixtures contained 1 µl of DNA template, 2.5 µl of 10x reaction buffer, 200 µM dNTPs, 2 mM of $Mgcl_2$, 10 pmol of each primer (Gen FanAvaran, Tehran, Iran), and 1U of *Taq* DNA polymerase (CinnaGen, Tehran Iran). The amplification was performed in a thermal cycler (Eppendorf, USA) programmed for an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 50°C to 63°C for 1 min (Table 1), and 72°C for 1 min and a final DNA extension at 72°C for 10 min. PCR products were electrophoresed (120 V/208 mA) on a 1% agarose gel, stained with ethidium bromide 0.5 µg/mL and visualized under a UV Gel Doc (Bio-Rad, USA).

RESULTS

Hemolytic activity. The phenotypic hemolysis activity of the DEC isolates was evaluated by the culture on the blood agar medium as described elsewhere. Our results showed that 8 (66.7%), 25 (89.3%), 17 (44.4%) and 10 (83.4%) of EPEC, STEC, ETEC and EAEC isolates, respectively had the hemolytic activity on the blood agar.

Quality and quantity of the extracted DNA from DEC isolates. Our results showed the extractions were of high quality and quantity by the spectrophotometry (OD 260/280 nm~1.8) and gel electrophoresis (Fig. 1).

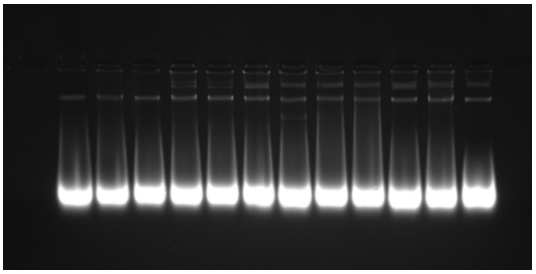

Fig. 1. The quality and quantity of DNAs extracted from the EAEC isolates by Phenol and Chloroform method

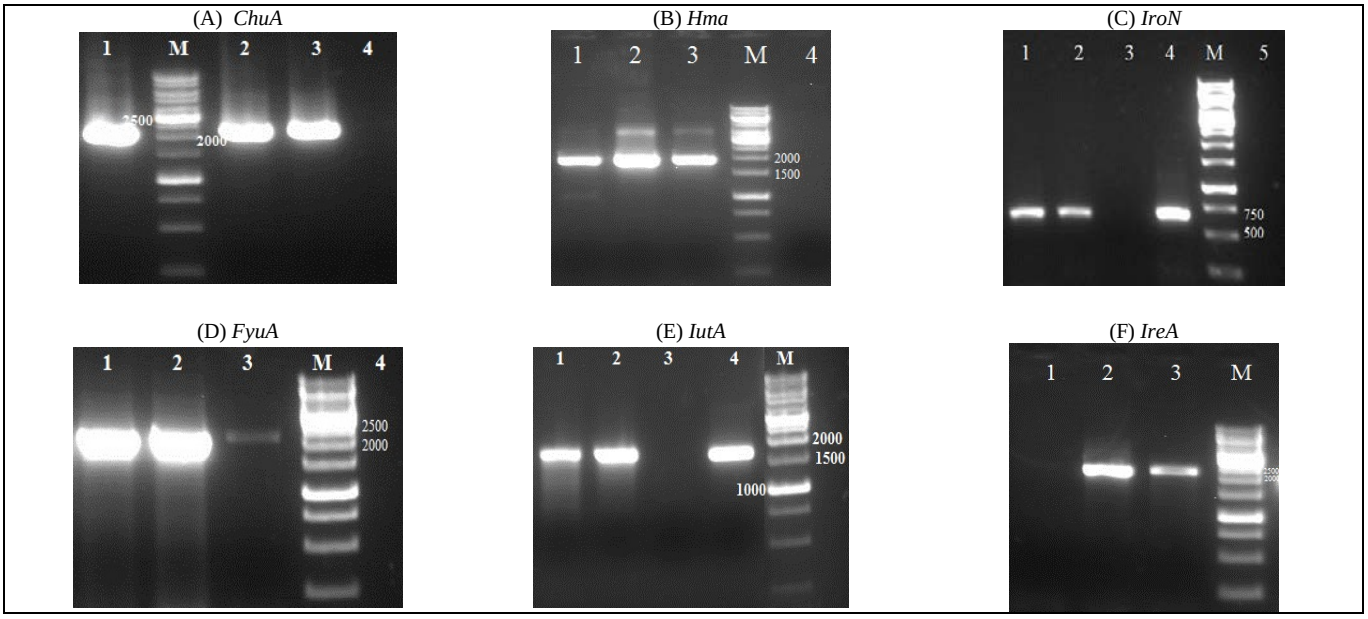

Fig. 2. Amplification of iron genes in DEC isolates. (A) *chuA* gene, line 1, positive control EPEC E234869; lines 2 and 3, positive isolates; line 4, negative control; (B) *Hma* gene, line 1, positive control EHEC O157:H7; lines 2 and 3, positive isolates; line 4, negative control; (C) *IroN* genes, line 1, positive control EPEC E234869; lines 2 and 4, positive isolates; line 3, negative control; line 5, negative isolate; (D) *FyuA* gene, line 1, positive control EPEC E234869; lines 2 and 3, positive isolates; line 4, negative control; (E) *IutA* gene, line 1, positive control EHEC O157:H7; lines 2 and 4, positive isolates; line 3, negative control; (F) *IreA* gene, line 2, positive control EHEC O157: H7; line 3, positive isolate; line1, negative control. The *E. coli* K12 was used as negative control and 1 kb ladder mix as DNA marker (M) in all PCR reactions.

Table 2. Frequency of virulence genes in DEC isolates

Virulence genes	No. (%) of positive isolates			
	EPEC (n=12)	STEC (n=28)	ETEC (n=36)	EAEC (n=12)
chuA	6 (50%)	20 (71.5%)	16 (44.5%)	8 (66.7%)
Hma	0 (0%)	12 (42.8%)	8 (22.3%)	4 (33.3%)
iroN	4 (33.3%)	4 (14.3%)	8 (22.3%)	0 (0%)
fyuA	8 (66.7%)	24 (85.7%)	22 (61.2%)	12 (100%)
iutA	3 (25%)	8 (28.5%)	16 (44.5%)	8 (66.7%)
ireA	2 (16.7%)	4 (14.3%)	4 (11.2%)	0 (0%)

Detection of iron receptor genes. The frequency of the iron receptor genes in four classes of DEC isolates was evaluated by PCR (Fig. 2). As reflected in Table 2, all the EPEC isolates were negative for *hma* gene, and *iroN* and *ireA* genes were absent in the EAEC isolates. The iron scavenger receptor *fyuA* showed the highest frequency and was observed in all EAEC isolates (100%). Our results showed a higher frequency of *chuA*, *hma* and *fyuA* genes among STEC and EAEC isolates, while the frequency *iroN* gene in EPEC and ETEC isolates was higher than STEC and EAEC isolates.

DISCUSSION

The iron acquisition systems of DEC strains can maximize the chance of these strains for successful persistence and pathogenesis in the host [18]. For example, production of different siderophores helps DEC strains to compete with the commensal *E. coli* strains in the intestinal tract [19]. Also, the iron receptors could have other roles in these strains. For instance, a new adhesion factor as IrgA homolog adhesin (Iha) in *E. coli* strains showed significant homology with siderophore receptors IreA and IroN [20].

Despite the vital role of the iron utilization systems in the pathogenesis of DEC strains, there are not much data on these iron uptake genes in the strains [21].

To our knowledge, the present study is the first one to evaluate the prevalence of iron scavenger genes *chuA*, *hma*, *iroN*, *fyuA*, *iutA* and *ireA* in DEC strains simultaneously. The high prevalence of yersiniabactin (*fyuA*) and low rate of the locus encoding salmochelins (*iroN*) observed in the present study among the intestinal pathogenic *E. coli* especially STEC and EAEC isolates were in agreement with other reports from Nigeria and Germany [13, 22]. The presence of *fyuA* gene within a high pathogenicity island (HPI) seemed to be associated with the pathogenesis of DEC strains as its presence increased the growth of the strains in the intestinal tract [23], whereas IroN showed to be associated with extra-intestinal *E. coli*, and its role in the pathogenesis of intestinal pathogens has been not defined [24].

In this study, EPEC group harbored one or more iron-related genes. In this group, the *fyuA* gene had the highest prevalence, while *hma* gene was negative among the isolates. In comparison to the present study, the hem receptor *chuA* in EPEC group from Germany was higher [25], whereas a lower frequency of aerobactin *iutA* (19%) was found in the same group from Brazil [26]. Another finding from the UK indicated that ≈39% of EPEC isolates from diarrheal patients were able to produce aerobactin [27], which is higher than the rate in our study. Also, a report from the UK indicated utilization of haem by 80% of EPEC isolates and hemoglobin by 61% as iron sources during growth in a limited iron environment.

Among the STEC isolates, we detected 2 or more iron-related genes with the highest ratios for *fyuA* (85.7%) and *chuA* (71.5%) genes, whereas in similar studies in French [28] and United States [29] *fyuA* was detected only in 5% and 8.6% of STEC strains, respectively.

The prevalence of aerobactin (*iutA*) was found in more than 65% of EAEC isolates that was near to the range of *iutA* in EAEC isolated from Nigeria [13]. In this study, we could not detect *iroN* gene among the EAEC isolates, whereas in a similar study in Poland, a low prevalence of *iroN* gene (15.5%) was reported among EAEC strains [14]. Although, *chuA* as a haem utilization gene showed to be prevalent in highly pathogenic bacteria such as EHEC O157:H7 [30], we could detect this gene in the majority of EAEC Iranian isolates suggesting the role of *chuA* as an essential virulence factor in these isolates.

In accordance with a study from Colombia [31], the *fyuA* gene with the frequency of 75% was the most common detected gene in our ETEC isolates. This gene is known to be linked with the ETEC HPI pathogenicity island [22]. The differences in the prevalence of iron receptor genes among different pathotypes of DEC isolates may be related to the different geographical regions, sampling methods, public health status, and food diets.

Hemolysin is a well-known virulence factor among extra-intestinal *E. coli* isolates; it causes hemolysis by forming pores in the erythrocytes of the host [32], but the knowledge on the role of hemolysis in the pathogenesis of DEC strains is scarce. In the previous studies from Brazil and United States, production of α-hemolysin among the EAEC isolates showed to be associated with pathogenesis and the development of persistent diarrhea [33, 34]. The high prevalence of hemolysin was also demonstrated in Iranian EAEC isolates from Kerman [35]. We detected a high ratio of hemolytic activity in STEC isolates which was similar to findings in the United States and India [36, 37]. However, among EPEC isolated from children in Brazil, only 25% indicated the ability of hemolysin production [38] that was lower than the rate we obtained here in this study. Additionally, the rate of hemolysin activity in our ETEC isolates was close to the similar isolates from diarrhea patients in Iran and Brazil [35, 39]. The high prevalence of hemolysin activity in DEC isolates suggests its role in the increased pathogenicity of these strains. Furthermore, the differences in the prevalence of hemolysin in different studies could be attributed to the quality of life in these countries and other factors.

In conclusion, the DECs isolated from Iranian patients showed one or more iron scavenger receptors that could increase their pathogenesis in the intestinal tract. Furthermore, a significant hemolysin production among various groups of DEC isolates was observed that could improve their efficacy in the host.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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