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Full Length Research Paper

Molecular Screening of Sideropohore Genes in Extraintestinal Pathogenic Escherichia coli Isolated From Clinical and *Escherichia coli* Isolated Food Samples in Turkey

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Abstract

Iron is an essential element for Escherichia coli growth and survival in the host and the external environment, but its availability is generally low due to the poor solubility of its ferric form in aqueous environments and the presence of iron-withholding proteins in the host. Most E. coli can enhance access to iron by excreting siderophores such as enterobactin, which has a very strong affinity for Fe3+. A smaller proportion of isolates appeared to generate up to 3 additional siderophores associated with pathogenesis; aerobactin, salmochelin, and yersiniabactin. The aim of this study was to compare the presence of siderophore genes in E. coli strains isolated from clinical and food samples. Sixty five and thirty five of E. coli strains were isolated from the clinical and food samples, respectively. The prevalence of the siderophore-related genes, which was determined in the E. coli strains by using PCR method, was as follow: enterobactin receptor (Fep A), 18%; enterobactin biosynthesis (Ent A), 18 % ; yersiniabactin receptor (Fyu A), 17%; heme receptor (Chu A) 16%; aerobactin receptor (iut A),12%; aerobactin biosynthesis (iuc A), 15%; and salmochelin receptor (iro N), 3%, and iron-responsive element (ire A), 1%. The siderophore-related genes are considered as main factors in the growth and survival of the clinical strains of E. coli and adaptation to their low-iron environment. Our results strongly supported that and found the prevalence of siderophore genes is significantly higher among clinical isolates than among food isolates.

Keywords: Siderophore, PCR, Iron, Food, Clinical.

INTRODUCTION

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains appeared to be the cause of most community and hospital developed extraintestinal *E. coli* infections, encompassing the urinary tract infections, bloodstream, and other anatomical sites (Russo and Johnson 2003, ALBARRI, Var *et al.,* 2017). Virulence factors, which might aid pathogens in colonizing the host, comprise a wide variety of substances including bacterial toxins, adherence factors, protective capsules, and siderophores, enhance bacterial potential to cause disease. Iron is described as a significant micronutrient

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for essentially all living organisms, excluding lactic acid bacteria where manganese and cobalt are employed instead of iron. Under aerobic circumstances, the abundance of free iron is restricted by the poor solubility of ferric hydroxide (Weinberg 1997). It plays an essential role as a cofactor for the redox-dependent enzymes encompassed in the most cellular processes, such as electron transfer, RNA synthesis, and resistance to reactive oxygen intermediates (Braun 1997). Under physiological circumstances, iron might occur in the reduced ferrous form (Fe^{2+}) or the oxidized ferric form (Fe^{3+}). The redox potential of Fe^{2+}/Fe^{3+}renders iron tremendously resourceful when it is combined into proteins as a catalytic centre or as an electron carrier (Imbert and Blondeau 1998).

Early microorganisms were capable to employ soluble ferrous iron (Fe^{2+}) that was plentiful due to an oxygen-poor atmosphere; however, as oxygen-rich circumstances arose, ferrous iron was oxidized to in soluble ferric iron (Fe^{3+}), detaching an easily bioavailable source of iron. Responding to this problem, microorganisms developed siderophores – small ferric iron (Fe^{3+})-chelating compounds(Braun and Killmann 1999).

In a pathogenic context, microbes produce siderophores to obtain and solubilize ferric iron from the host. Most *E. coli* can enhance access to iron by secreting siderophores such as enterobactin, which showed a very strong affinity for Fe^{3+}. A smaller proportion of isolates can produce up to 3 extra siderophores associated with pathogenesis; aerobactin, salmochelin, and yersiniabactin. In this study, the prevalence of siderophore genes in both *E. coli* isolated from food samples and in Extraintestinal pathogenic *E. coli* (ExPEC) isolated from clinical samples was investigated.

MATERIAL AND METHOD

Material

A total of 48 samples, including 16 samples of vegetables, 16 samples of chicken, 16 samples of meat, were collected between September and October 2016 from retail vegetable stores and markets. All samples were randomly collected in sterile plastic bags, labelled and transported to the Microbiology laboratory/ department of food engineering of Cukurove University for analysis. On the other hand clinical samples were collected from urinary tract infections, surgical site infections, and pneumoniapatientsbetween 2014-2016 from Cukurova university Balcali Hospital.

Method

Isolation of *E. coli* from clinical samples

A total of 65 ExPEC isolates, including 54 (83%) isolates from urine, 5 (8%) isolates from wound, 3 (4%) isolates from blood and 3(4%) isolates from aspiration, were used in this study. The majority of ExPEC *strains* were isolated from urine samples as Figure 1. There were 46 (71%) women and 19 (29%) men (Figure 2), their age ranged between 0 and 87 years as follow : twenty of patients were less than 10 years of age (30.7%), seventeen of patients were between 11-50 years of age (26.1%) and the remaining patients were between 50-90 years of age (N=28,43.04%), (Figure 3).

Isolation of *E. coli* from Food samples

Bacterial isolates

48 food samples inclulded meat (n=16), Chicken (n=16) and vegetable (n=16). Each 10-g sample of meat,chicken or vegetable was homogenized for 2 min in 90 ml of peptone water, we applied the fluorocult laurly sulfate broth (FLSB) using most probable number (MPN) to isolate *E. coli* (ALBARRI, Var *et al.,* 2017).

Genomic DNA extraction and molecular identification of *E.coli* strains

We have used uidA gene, which is encoded beta-D-glucuronidase, for molecular identification of *E. coli*. uidA gene has used as positive control in our study for amplification Table 3 (Henrissat 1991). Firstly we extracted genomic DNA according to thaw and freeze protocol (Squires and Hartsell 1955)**.**Polymerase chain reaction (PCR) was used to detect uid A gene.5 µl of DNA was used in a 25 µl reaction volume containing 12.5 µl master mix, 0.5µl uidA-F, 0.5µl uidA-R and 6.5 µl distilled water (AL-zuwainy and Abid, ALBARRI and Var 2018). Amplification for uid A PCR was as follow:1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and 1 cycle at 72°C for 10 min. The product of PCR loaded in 2% gel of agarose in electrophoresis device and the bands evaluated in imaging device (Lee, Costumbrado *et al.,* 2012).

2.2.4 Detection of of siderophore genes in *E. Coli* strains by using polymerase chain reaction (PCR)

The presence of 8 siderophore geneswas assessed by PCR amplification (**Table 1**). DNA extraction was performed according to thaw and freeze protocol (Squires and Hartsell 1955). Amplified products were run on 2% agarose gels and the bands evaluated in imaging device [10].5µl of DNA was used in a 25 µl reaction volume containing 12.5 µl master mix, 0.5µl of each primer and 6.5 µl distilled water. Amplification for each PCR was as follows: 1 cycle at 95°C 5 for min,35 cycles at 95°C for 30s, 55°C for 30s, 72°C for 1 min, and 1 cycle at 72°C for 5 min.

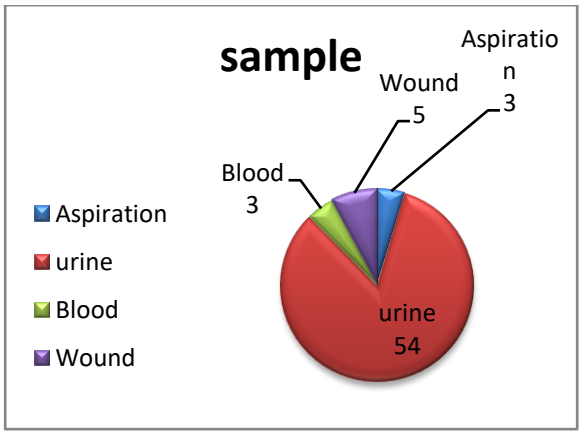

Figure 1. Clinical Material

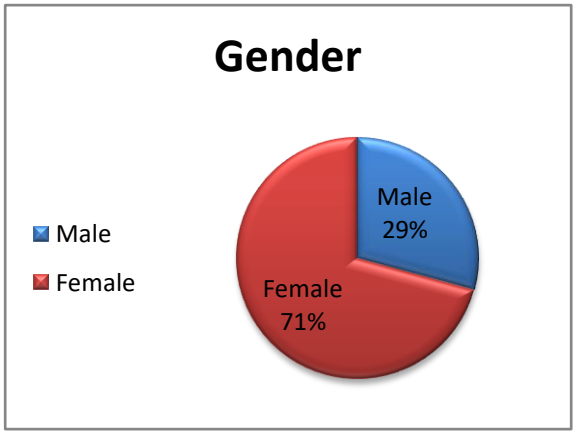

Figure 2. Clinical Material

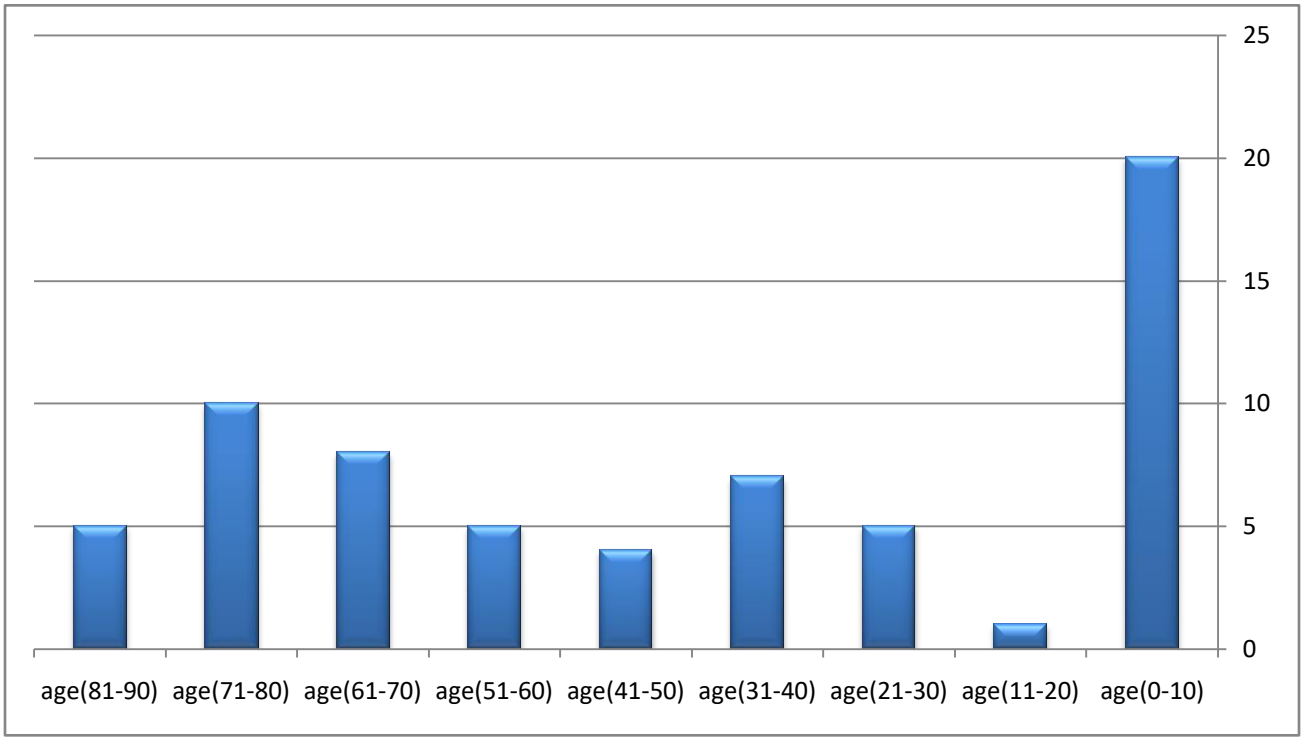

Figure 3. Age composition

Yersiniabactin and aerobactin PCRs had slight alterations, with the annealing temperature raised to 60°C for the yersiniabactin PCR and elongation step shortened to 40 s for the aerobactin PCR. Amplification for IRE A PCR was 1 cycle at 95°C for 5 min, 25 cycles at 95°C for 15s, 55°C for 1 min, 72°C for 30 s, and 1 cycle at 72°C for 5 min. Amplification for Chu A PCR was 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 1 cycle at 72°C for 10 min.

Statistical analysis

Differences between proportions were tested for significance by the Friedman Test and p values <0.05 were considered statistically significant.

Target class	Gene	Sequence (forword)	Sequence (Reverse)	Size (bp)	R
Yersiniabactin	FyuA	GGGAATGTGAAACTGCGTCT	CGGGTGCCAAGTTCATAGTT	791	(Searle, Méric et al. 2015)
Salmochelin	IroN	CTTCCTCTACCAGCCTGACG	GCTCCGAAGTGATCATCCAT	648	(Searle, Méric et al. 2015)
Aerobactin	IucA	ATAAGGGAAATAGCGCAGCA	TTACGGCTGAAGCGGATTAC	212	(Searle, Méric et al. 2015)
	IutA	GGCTGGACATCATGGGAACTGG	CGTCGGGAACGGGTAGAATCG	300	(Yun, Kim et al. 2014)
Heme receptor	ChuA	GACGAACCAACGGTCAGGAT	TGCCGCCAGTACCAAAGACA	279	(Russo, Carlino et al. 2001)
Enterobactin	FepA	TTTGTCGAGGTTGCCATACA	CACGCTGATTTTGATTGACG	349	(Searle, Méric et al. 2015)
	EntA	GTGCGCTGTAATGTGGTTTC	CAGAGGCGAGGAACAAAATC	184	(Searle, Méric et al. 2015)
Iron-responsive element	IreA	TGGTCTTCAGCTATATGG	ATCTATGATTGTGTTGGT	415	(Yun, Kim et al. 2014)
Beta-D-glucuronidase	UidA	ATGCCAGTCCAGCGTTTTTGT	AAAGTGTGGGTCAATAATCAGGAAGTG	120	(Koga, Tomazetto et al. 2014)

RESULT AND DISCUSSION

Prevalence of siderophore genes

Eight of siderophore-related genes, which are enterobactin receptor (Fep A), 18%; enterobactin biosynthesis (Ent A), 18 %; yersiniabactin receptor (Fyu A), 17%; heme receptor (Chu A) 16%; aerobactin receptor (IutA),12%; aerobactin biosynthesis (Iuc A),15%; and salmochelin receptor (iro N), 3%, and iron-responsive element (Iire A) was 1 % , were determined in all food, and human clinical *E. coli* isolates by the PCR method (Figure 4). Among the (66) human clinical isolates, ExPEC, the prevalence of individual siderophores genes ranged from 15% Iro N to 95% FepA Table 3.Seven out of eight genes encoding siderophores were detected in at least one isolate of ExPEC**.** While IreA is not, detected in ExPEC isolates**,** the prevalence of Fep A, Ent A, Fyu A, Chu A, Iut A, Iuc A; and Iro N were 97%, 91%, 86%, 72%, 71%, and 15%, respectively. On the other hand, the occurrence of the siderophore-related genes among 35 *E. coli* strains isolated from food ranged from 9 % Ire A to 63% Fep A. Eight of siderophore encoding genes were detected in at least one isolate of *E. coli*, as follows: Fep A (63%), Ent A (60%), Iuc A (60%), Chu A (54%), Fyu A (49%), Iut A (23%), Iro N (14%), and Ire A (9%). All the siderophore-related genes occurred, significantly, more frequently among ExPEC strains than food strains except Ire A which was detected only in food samples (Table 4, Figure 5)**.**

Extraintestinal E. coli (ExPEC) are a diverse group of pathogenic E. coli strains which have high ability to colonize several extra-intestinal sites such as blood stream, meninges, and urinary tract. The great differences have been shown through comparison of whole-genome of E. coli strains which cause different diseases (i.e. intestinal and extraintestinal infections) (16). These differences are also present within ExPEC strains which are a cause of the same disease and infect the same host tissues (Dobrindt, Blum-Oehler *et al.,* 2002). ExPEC have a high ability to effectively infect the gastrointestinal tract (Johnson and Stell 2000), and this ability depends up on their virulence factors (VFs) which enable the strains to avoid or subvert local and systemic host defense mechanisms, colonize host mucosal surfaces, injure or invade the host, scavenge essential nutrients such as iron, and stimulate a noxious inflammatory response (Bower, Eto *et al,*. 2005).

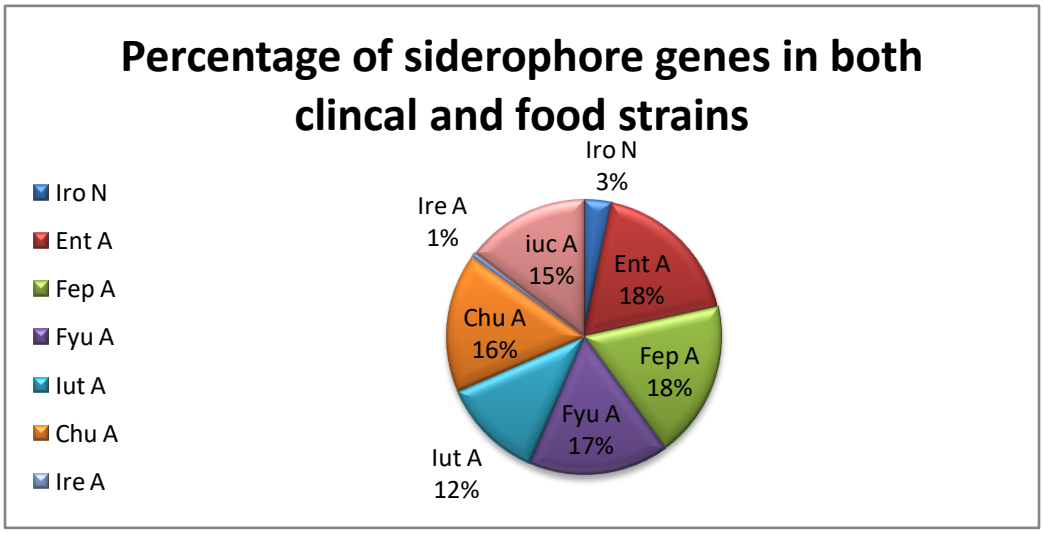

Figure 4. The Frequency of siderophore-related genes among E. coli strains

The components of the siderophore genes are more common in females than males except Iut A which was found more frequently in males than in females and Ire A that was similar in both males and females (Table 4, Figure 6)

Table 3: Distribution of siderophore-associated genes in urine, blood wound, and pneumonia samples among 66 *Escherchia coli* isolates.

	Urine	**Blood**	**Wound**	**Aspiration**	**Total**	**Woman**	**Man**
	N=54	**N=3**	**N=5**	**N=3**	**N=65**	**46(100%)**	**19**
Iro N	9	0	1	0	10	8 (18%)	2(11%)
Ent A	52	3	5	3	63	45 (98%)	18(95%)
Fep A	52	3	5	3	63	45(98%)	18(95%)
Fyu A	50	3	5	1	59	42(92%)	17(90%)
Iut A	38	2	5	2	47	31(68%)	16(85%)
Iuc A	35	3	5	3	46	33(72%)	13(69%
Chu A	46	2	5	3	56	36 (87%)	16(85%)
Ire A	0	0	0	0	0	0	0

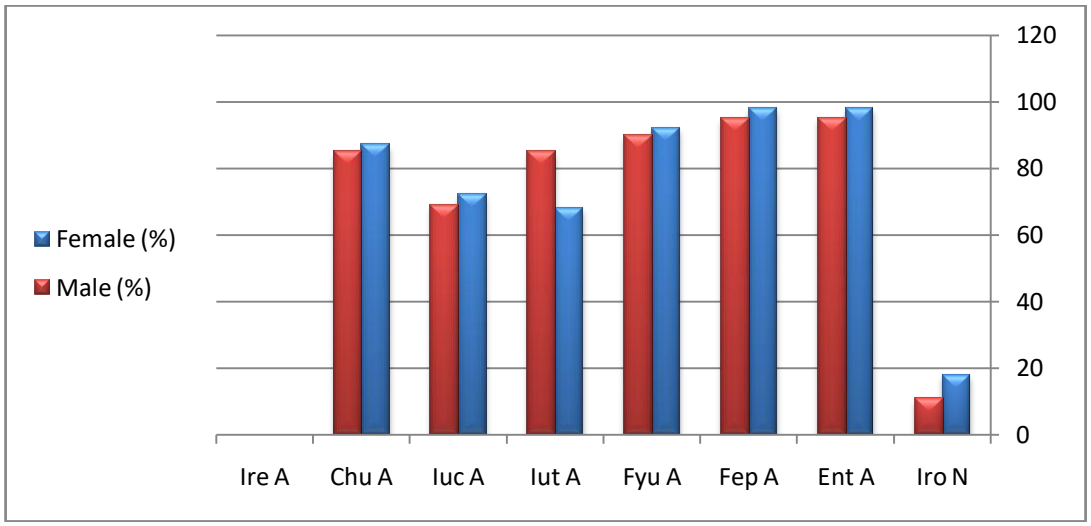

Figure 6. The comparison between the distributions of siderophore related genes in women and men

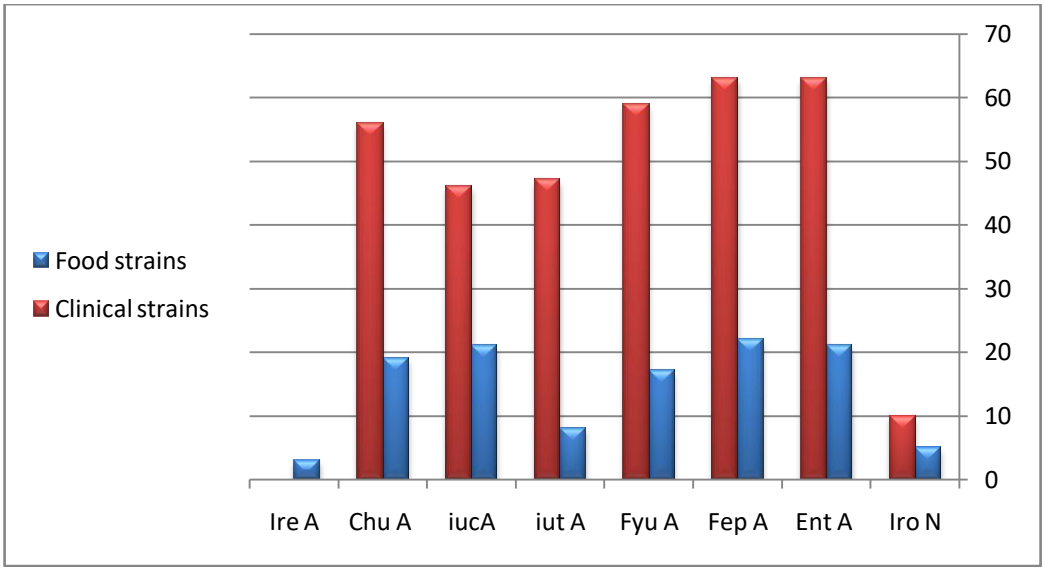

Figure 5. The comparison between the distributions of siderophore related genes in clinical strains and food strains

Table 4. Distribution of siderophore genes in clinical and food strains among 100 *Escherichia coli* isolates.

Function category	Gene Name	Number of strains ExPEC strains N=65(%)	Number of strains Food strains N=35(%)	P value
Siderophores	**Iro N**	**10(15%)**	**5(14%)**	.000[x]
	Ent A	**63(97%)**	**21(60%)**	.000[x]
	Fep A	**63(97%)**	**22(63%)**	.000[x]
	Fuy A	**59(91%)**	**17(49%)**	.000[x]
	Iut A	**47(72%)**	**8(23%)**	.000[x]
	Iuc A	**46(71%)**	**21(60%)**	.000[x]
	Chu A	**56(86%)**	**19(54%)**	.000[x]
	Ire A	**0(0%)**	**3(9%)**	.000[x]

In mammalian hosts, free iron concentrations are very low for the growth of pathogenic bacteria. Therefore, many bacteria, which include ExPEC, possess multiple ways of acquiring iron from the host by the expression of iron-acquisition systems. The iron acquisition systems genes are basic factors in the growth and survival of the ExPEC strains and their adaptation to the iron limiting environment. Our study have shown that, the prevalence of siderophore genes are as follow: FyuA (n=59, 91%), IutA (n=47, 72%), and ChuA (n=56, 86%) in ExPEC strains which are higher than those found by Safi et al., (2106) in Tunis who stated that the occurrence of FyuA, Iut A, and ChuAin urine E. coli isolates were 74%, 56%, and 65%, respectively. However, the prevalence of IroN was 59% which is higher than the average rate in our results.

These siderophore genes thought to have a main role in enabling iron uptake and contributing to virulence in the iron limiting environment. This was explained previously by the redundant function of some genes like IroN which contributed less in the virulence than other iron uptake genes (Safi, Achour *et al.,* 2016). Our finding have shown that Ire A gene was not detected in ExPEC strains, however, it was detected in 9% of food strains which is less than those documented by park et al.(2007) who found that IreA gene was found in only 5% (1/22) of UTI cases (Park, Jung et al., 2009). Fyu A and Iut A genes are of the bacterial iron acquisition systems. In our study, the prevalence of Fyu A (91 %) and Iut A (72%) genes in the ExPEC isolates were higher than food samples (FyuA 49%, and Iut A 23%).

This finding supports the importance of FyuA in virulence, particularly in the establishment of symptomatic urinary tract infection (UTI). The relationship between FyuA and invasive UTI had been suspected in previous studies (Cheng, Tsau et al., 2010), and our result was higher than those recorded by Yun *et al.,* (2014) who found that the prevalence of IutA and Fyu A were as follow: 53.1% (n = 34), and 45.3% (n = 29) (Yun, Kim *et al.,* 2014).Some bacteria have ability to produce siderophore in the bloodstream which is very important for bacterial survival.

The aerobactin is considered as an important virulence factor which contributes to bacterial growth in fluids and host tissues where the availability of iron is very limited (Garénaux, Caza et al. 2011).The aerobactin receptor (IutA) is commonly associated with ExPEC (Moreno, Planells *et al.,* 2005).Koga *et al.,* (2014)found that the most common virulence gene was Iut A which was detected in 65% of the tested isolates, while Iut A was not detected in commensal isolates and this finding is in agreement with our result.

Moreover, it has been reported that the presence of IroN and FyuA genes were found in 55% and 45% of isolates, respectively. However, our result has shown that Iro N and Fyu A were detected in 15% and 91% of isolates.

Our study has also shown that the prevalence of Iuc A gene is present in 71% of ExPEC isolates which is similar to what has been documented by Palma *et al.,* (2016), who also found that Iuc A was present in 67.7% of isolates, whereas Iut A and fyu A were detected in 83.1% and 49.2% of isolates, respectively (Palma, Gomes *et al.,* 2016). On the other study, Khan el al., (2015) reported that the presence of IutA, IreA, and Fyu A were 70.4%, 92.5%, and 77.5% , respectively (Khan, Zou *et al.,* 2017). Moreover, our study was in accordance with what has been found by Sobieszczan´ska *et al.,* 2008 who reported that Iro N was detected in 15.5% of isolates while enterobactin was present in 67.7% of isolates which was lower than our study (Sobieszczańska 2008).

The high level of siderophore-related genes among our isolates is in accordance with other descriptions (Burdet, Clermont et al., 2014), highlighting the relevance of this kind of virulence factor in the development of bacteraemia. This is related to the iron needs of bacteria, which contains approximately 105-106 Fe atoms, and to the difficulty to access iron within human fluids due to binding to transport molecules such as ferritin or lactoferrin (Bidet, Bonarcorsi *et al.,* 2012).The siderophores are produced by some bacteria and are able to capture the iron from these molecules then they are recaptured by specific bacteria receptors such as FyuA, IutA or ChuA (Braun, Pramanik et al., 2009). The siderophore-related genes are main factors in the growth and survival of the ExPEC strains and their adaptation to the iron limiting environment. This is supported by our findings which showed that the prevalence of siderophore genes was higher in ExPEC isolates than food isolates. ExPEC strains expressed different combinations of these genes, with some strains expressing all siderophores.

CONCLUSION

Iron is a vital element required by every living organism for numerous cellular processes. Under iron-deficient conditions, the growth of microorganisms becomes impaired. The microorganisms survive under such iron-limited conditions by secreting siderophores. In mammalian hosts, free iron concentrations are very low for the growth of pathogenic bacteria.

Therefore, many bacteria, which include ExPEC, possess multiple ways of acquiring iron from the host by the expression of iron-acquisition systems. Thus, we concluded that the high prevalence of siderophore genes were found in ExPEC isolates due to the difficulty to get enough iron from host because of its defense systems (Lipocalin 2). However the siderophore-related genes were also found in food isolates but it was lower than ExPEC these isolates can easily get iron from environment due to no defense system provided in its environment.

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