ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc THESIS

Osman ALBARRI

INVESTIGATION of THE OCCURRENCE OF SIDEROPHORE GENES IN *E.coli* ISOLATED FROM FOODBORNE AND CLINICAL SAMPLES AND IDENTIFICATION OF GENOTYPIC RELATIONSHIP OF *Escherichia coli* BY USING PFGE METHOD.

DEPARTMENT OF BIOTECHNOLOGY

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We certify that the thesis titled above was reviewed and approved for the award of the degree of the Master of Science by the board of jury on30/03/2018.

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ABSTRACT

MSc THESIS

INVESTIGATION of THE OCCURRENCE OF SIDEROPHORE GENES IN *E.coli* ISOLATED FROM FOODBORNE AND CLINICAL SAMPLES AND IDENTIFICATION OF GENOTYPIC RELATIONSHIP OF *Escherichia coli* BY USING PFGE METHOD.

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Iron is essential 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 increase access to iron by excreting siderophores such as enterobactin, which have a very strong affinity for Fe^{3+}. A smaller proportion of isolates can generate up to 3 additional siderophores linked with pathogenesis; aerobactin, salmochelin, and yersiniabactin. This study compared the presence of siderophore genes in *E.coli* strains isolated from clinical and food samples, and studied genotypic relationship of *Escherichia coli* strains. Sixty five of *E. coli* strains were isolated from the clinical samples and 35 of *E.coli* isolated from the food samples were our material in this study. The prevalence of the siderophore-related genes were determined in the *E.coli* strains by the PCR method which 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 genotypic relationship of *E.coli* strains have also studied by using PFGE method and the evaluation of PFGE analysis showed that 6 (6.8%) out of 89 isolates of human and foodborne isolates had a clonal relationship while there was no significant relationship between the other isolates. Conclusion: The siderophore-related genes are main factors in the growth and survival of the *E.coli* clinical strains and their adaptation to the iron limiting environment, our results supported that and found that siderophore genes are higher prevalence in *E.coli* isolated from clinical samples than food samples.

Key words: *E.coli*, siderophore, iron, PCR, PFGE

ÖZ

YÜKSEK LİSANS TEZİ

GIDA KAYNAKLI VE KLİNİK ÖRNEKLERDEN İZOLE EDİLEN *E.coli* DE SİDEROFOR GENLERİNİN OLUŞUMUNUN ARAŞTIRILMASI VE PFGE YÖNTEMI İLE *Escherichia coli* GENOTİPİK İLİŞKİSİNİN BELİRLENMESI.

Osman ALBARRI

ÇUKUROVA ÜNİVERSİTESİ
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Demir, konak hücrelerinde ve dış ortamda *Escherichia coli* büyümesi ve hayatta kalması için gereklidir,ancak ferrik formunun sulu ortamlarda zayıf çözünürlüğü ve konakta demir tutan proteinlerin varlığına bağlı olarak kullanılabilirliği genellikle düşüktür.Çoğu *E. coli*, Fe^{3+} 'ya karşı güçlü bir afiniteye sahip olan enterobactin gibi sideroforları oluşturarak demir erişimini artırabilir.İzolatların daha küçük bir kısmı, patogenezle bağlantılı olarak 3 ek siderofor oluşturabilir; Bunlar aerobaktin, salmochelin ve yersiniabaktindir.Bu çalışmada, klinik ve gıda örneklerinden izole edilen *E.coli* suşlarında siderofor genlerinin varlığı araştırılmış ve *Escherichia coli* suşlarının genotipik ilişkisi incelenmiştir.Çalışmamızda, klinik örneklerden izole edilmiş altmış beş *E. coli* suşu ile gıda örneklerinden izole edilen 35 *E.coli* suşu kullanılmıştır.*E.coli* suşlarında Sideroforla ilişkili genlerin prevalansı, enterobaktin reseptörü (Fep A),% 18; Enterobactin biyosentezi (Ent A),% 18; yersiniabactin reseptörü (Fyu A),% 17; heme reseptörü (Chu A)% 16; aerobaktin reseptörü (iut A),% 12; aerobaktin biyosentez (iuc A),% 15; ve salmochelin reseptörü (iro N),% 3 ve demir cevabı veren element (ire A),% 1 olarakPCR yöntemi ile belirlenmiştir.*E.coli* suşları arasındaki genotipik ilişki PFGE metodu kullanılarak incelenmiş ve PFGE analizinin değerlendirilmesinde, insan ve gıda kaynaklı izolatların 89'undan 6'sının (% 6.8) klonal bir ilişki sergilediği, diğerleri arasında anlamlı bir ilişki bulunmadığı görülmüştür.Sonuç olarak; klinik örneklerden izole edilen *E.coli* suşlarının gıda örneklerinden elde edilen suşlara göre daha yüksek prevalansa sahip olduğu belirlenmiştir; Sideroforla ilişkili genlerin, *E.coli* klinik suşlarının büyümesi, hayatta kalması ve demir sınırlayıcı çevreye adaptasyonu için ana faktörlerden olduğu bu çalışmada da gösterilmiştir.

Anahtar Kelimeler: *E.coli*, siderofor, demir, PCR, PFGE

GENİŞLETİLMİŞ ÖZET

Genel olarak tüm dünyada gıda kökenli enfeksiyonlardan klasik olarak bilinen bir kaç bakteri, virus ve parazit sorumlu tutulurdu.Gıda üretimine bağlı son 30 yılda her aşamada etkili olan değişiklikler yeni veya yeni gen kazanımları ile yeniden önem kazanan birçok mikroorganizmanın da bu tür hastalıklar ile ilişkili olduğunu ortaya koydu. Yeniden önem kazanan mikroorganizmalar içerisinde hiç şüphesiz ki en önemlisi farklı mikroorganizmalardan yeni virülans genleri kazanabilecek transport sistemleri gibi güçlü donanımlara sahip olan *E coli*dir. Örneğin 1970'li yıllarda tanımlanmamış, bilinmeyen tür sero/biyotip olan *E. coli* O157:H7'nin insanlarda gıda kaynaklı enfeksiyonlara sebep olduğu ilk olarak 1982 yılında gösterildi. Bu mikroorganizma gıda üretim laboratuvarlarında duyarlı tekniklerin kullanıldığı batı dünyasında 1990'lı yıllarda major gıda kökenli patojen olarak kayıtlara geçti.Almanya'da popülasyonda EHEC enfeksiyon riskinin 13/100.000'e kadar çıktığı bildirildi. Bu suş gıda ile bulaşma ve hastalık oluşturma yeteneğini plazmidler, bakteriofajlar ve transpozanlar gibi mobil genetik elemanlarda kodlanan toksinler, adezinler, invazinler, proteazlar, serum dirençli proteinler ve demir kazanım sistemleri (sidereforlar) gibi genler ile kazandı. Bu kazanımlar ve dirençten sorumlu mutasyonlardan insanoğlunun yaptığı irrasyonel uygulamalar kadar tarımda antibiyotik kullanımı gibi uygulamaların da sorumlu olduğu düşünülmektedir. Nitekim gıda kökenli enfeksiyonlarda *E.coli* sorumluluğu bu serotiple sınırlı kalmayıp O157/H7 dışındaki O7:H10, O22:H16, O111:H(-), O119:H(-), O174:H21,O26:H11, O113:H21, O118:H16 O123:H11 ve O177:H11 gibi şigatoksijenik *E coli* suşları ile diarejenik *E coli* suşları da gıda kökenli hastalıklarda artan oranda sorumluluk kazanarak, insan beslenme zicirinin vazgeçilmezleri olan, başta az pişmiş sığır eti olmak üzere, çiğ süt, peynir, meyve suyu, sebze, su kaynaklı enfeksiyonlardan izole edildiler. Klinik kökenli ve gıda kökenli *E.coli*'ler yukarıda sayılan etkenlerle beraber konakta; konak mukozal yüzeyine kolonizasyon, lokal ve sistemik konak defans mekanizmalarından kaçış,

demir gibi temel besin maddelerini alma ve oksidasyon redüksiyon potansiyelinin düzenlenmesi, epitel hücrelerde hasar oluşturma ve inflamatuar cevabın uyarılması gibi dokuda patolojilere yol açabilecek potent virülans faktörlerini kazanmaktadır.

Escherichia coli (*E.coli*) insan ve hayvanların intestinal florasında yer alan majör gram negatif, fermentatif, oksidaz negatif basildir. Bu sebeple *E.coli,* gaita kontaminasyonunun önemli olduğu sanitasyon uygulamalarında indikatör mikroorganizmadır ve gaita ile bulaşması mümkün olan bir çok patojen mikroorganizma için prediktif değer taşır. Bununla birlikte özel şartlarda bazı özel *E coli* suşları yüksek morbidite ve mortalitenin de sebebidir.Çoğu barsaklarda kommensal veya simbiotik olarak yaşayan yaklaşık olarak 700 den fazla serotipi olan *E.coli* suşları, antijenik özelliklerinin yanı sıra klinik-patolojik özellikleri ile de sınıflandırılmıştır. Pato-tipleme olarak tanımlanan bu sınıflandırmada *E.coli* suşları kommensalller, intestinal patojenler (InPEC) ve intestinal sistem dışı patojenler (ExPEC) olmak üzere 3 grupta toplanmıştır. Gıda kökenli enfeksiyonların en önemli etkenleri olan EPEC ve EHEC gibi InPEC ve ExPEC suşlarının insanlardaki kolonizasyonu ile ilişkili moleküler mekanizmalar tam olarak aydınlatılamamıştır. Son yıllarda özellikle ExPEC'in karakteristik virülans faktörleri arasında yer alan; konak hücreden demiri kazanmak için düşük molekül ağırlıklı demir şelatorlarının sentezi ve transportu gibi demir alım mekanizmasında görev alan sideroforlar önemli rol oynamaktadır.

Demir, konak hücrelerinde ve dış ortamda *Escherichia coli* büyümesi ve hayatta kalması için gereklidir,ancak ferrik formunun sulu ortamlarda zayıf çözünürlüğü ve konakta demir tutan proteinlerin varlığına bağlı olarak kullanılabilirliği genellikle düşüktür. Çoğu *E. coli*, Fe^{3+}'ya karşı güçlü bir afiniteye sahip olan enterobactin gibi sideroforları oluşturarak demir erişimini artırabilir. İzolatların daha küçük bir kısmı, patogenezle bağlantılı olarak 3 ek siderofor oluşturabilir; Bunlar aerobaktin, salmochelin ve yersiniabaktindir. *E.coli* insan intestinal mikrobiyotasındaki en yaygın mikroorganizmalardan biridir. Kalın bağırsağın normal florasında bulunan bu bakteri virulans faktörlerini kodlayan

genlerin kazanılmasıyla intestinal ve intestinal sistem dışında patojen hale gelmekte, yarattığı morbiditenin yanı sıra ciddi tedavi maliyetlerine sebep olur. *E.coli*'nin patojenik tipleri diyarejenik *E.coli* ve ekstraintestinal *E.coli* olarak ikiye ayrılır. ExPEC suşları, konak mukozal yüzeyine kolonizasyonu, lokal ve sistemik konak defans mekanizmalarından kaçışı, demir gibi temel besin maddelerini alma ve oksidasyon redüksiyon potansiyelinin düzenlenmesi, epitel hücrelerde hasar oluşturma ve inflamatuar cevabın uyarması gibi dokuda patolojilere yol açabilecek potent virülans faktörlerini bünyesinde barındırırlar (Johnson and Russo 2002). ExPEC'in karakteristik virülans faktörleri; polisakkarit kılıflar (lipopolisakkaritler ve kapsül gibi), toksinler, adezinler, invazinler, proteazlar, serum dirençli proteinler ve demir kazanım sistemleri (sidereforlar) gibi çeşitlilik gösterir (Dobrindt, 2005, Jaureguy ***et al***, 2008). Bu virülans faktörleri ExPEC suşlarına lokal enfeksiyonlar kadar sistemik yayılımla genel enfeksiyonlar oluşturabilme yeteneği kazandırır. ExPEC suşları sahip olduğu bu virülans faktörleri ile; üriner sistem enfeksiyonu, yeni doğan menenjiti, sepsis, cerrahi alan enfeksiyonu, nozokomiyal pnömoni, kolesistit, peritonit, selülit, osteomiyelit ve artrit gibi birçok ekstraintestinal hastalığa sebep olabilirler. Bu hastalıklara yol açan etkenler arasında konak hücreden demiri kazanmak için düşük molekül ağırlıklı demir şelatorlarının sentezi ve transportu gibi demir alım mekanizmasında görev alan sideroforlar önemli rol oynamaktadır (Crosa, 1989; Neilands, 1976). ExPEC suşları, intraselüler olarak ***hem*** veya ***hemoglobine*** bağlı demiri şelatlayan siderefor sistemi ve siderofordan bağımsız sistem (hemin reseptörü) içeren birkaç farklı demir alım sistemine sahiptir (Braun, 2003). Son yapılan çalışmalarda siderofor sistemine sahip ExPEC suşlarının kommensal suşlardan daha prevalan olduğu (Johnson et al, 2005 ; Schubert et al, 1998) ve bu özelliğin üriner sistem enfeksiyonların patogenezinde önemli bir rol oynadığı gösterilmiştir (Johnson et al, 2005; Russo et al., 2003).

Demir; DNA replikasyonu, enerji üretimi ve oksitadif stresten korunma gibi pekçok hücresel süreçte rol alan temel elementlerden biridir. Oksijen mevcut olduğunda, serbest ferröz demir (Fe^{2+}) çözülmeyen ferrik (Fe^{3+}) demire hızlı bir

şekilde okside edilir. ExPEC, indirgenmiş demir iyonunun biyoyararlanımını sağlamak için, hücre içi demir konsantrasyonunu 10^{-7} ve 10^{-5} M arasında sabit tutmak amacıyla demir moleküllerini toplama mekanizması geliştirmiştir (Garenaux A, Caza M, Dozois CM, 2011). ExPEC suşlarının farklı çevre şartlarında hayatta kalmak ve çoğalmak için jenerasyon başına 10^{5}-10^{6} Fe^{3+} iyonu gerektiği tahmin edilmektedir (Braun V, Killmann H-1999). *E.coli*'de ferrik demir alımının temel mekanizmasını demire çok yüksek afinite gösteren 4 farklı siderofor molekülünün sentezi oluşturmaktadır.

Bunlar; yersiniabactin siderophore sistem (*irp-fyuA*), (ii) salmochelin sistem (*iroA*), (iii) aerobactin siderophore sistem (*iuc-iutA*) ve (iv) the hemin uptake (*chu*) sistemler olarak sıralanabilir. Bu siderofor moleküllerinin sentezinden sonra, sideroforlar dış ortama sekrete edilir. Siderofor-demir kompleksi spesifik taşıyıcılar aracılığıyla alınır ve bakteriyel sitozelde demiri serbest bırakmak için degrade edilir (Garenaux A, Caza M, Dozois CM, 2011). Bu farklı siderofor sistemlerinin eşzamanlı varlığı, ExPEC'e çoklu demir kaynaklarından faydalanması için imkan sağlayarak konak içinde büyümenin verimliliğini arttırmaktadır. Konak-patojen etkileşiminde patojene üstünlük sağlayarak virülansı arttıran etkenler arasında yukarıda saymış olduğumuz IreA, Iha, fyuA, chuA gibi siderefor reseptörleri önemli rol oynamaktadır.

IreA (iron-responsive element) üriner sistem enfeksiyonlarında; siderofor reseptörü ve adezin fonksiyonuna sahiptir. Dış membrane protein Iha ExPEC suşlarında hem şelat siderofor reseptörü hem de aderans faktörü olarak karakterize edilmiştir (Leveille et al.,2006).

Yersiniabactin siderofor sistemi ilk olarak *Y. pestis*'de tanımlanmış olup yüksek patojenite adasında (HPI) yer alan yüksek derecede korunmuş kromozomal DNA bölgesinde kodlanan *irp* gen ailesi ve dış membran reseptörü FyuA ile karakterizedir. HPI'nın virülan suşlardaki dağılımı özellikle üriner sistem enfeksiyonu, septisemi ve yeni doğan memnejiti etkeni olan ExPEC'teki dağılımı oldukça yaygındır. Yapılan birkaç çalışmada yersiniabactin siderofor sistemini

kodlayan HPI'nın tüm virülansın ekspresyonu için gerekli olduğu bildirilmiştir. Katekolat reseptörü IroN'ı kapsayan salmochelin siderofor sistemi esas olarak *Salmonella enterica*'da bulunan ve sonra UPEC suşu CP9'da gösterilen iroA lokusunda (iroBCDEN gen ailesi) kodlanmaktadır (Baumler et al., 1998).

Dış membran reseptörü IroN sadece salmochelin'in taşınmasıyla sınırlı değil, ayrıca enterobaktin ve dihidrobenzoik asit gibi diğer birkaç katekolat sideroforların taşınmasından da sorumludur. IroN, virülan ExPEC suşlarında oldukça prevalandır ve idrarda bulunduğunda ekspresyon seviyesinde bir artış vardır. IroN'nın ürovirülans için önemli olan aderans gibi diğer virülans özelliklerinin kazanımına katkı sağladığı düşünülmektedir.

Aerobactin biosynthetic gene cluster (iuc) ve ferri-aerobactin receptor (iutA) ilk kez EIEC suşunda virülans plazmidi pCoIV üzerinde identifiye edilmiştir. Bir hidroksimat sideroforu olan aerobactin, üriner sistem enfeksiyonu, bakteriyemi veya diğer ekstraintestinal enfeksiyonlarından müzdarip hastalardan izole edilen pekçok *E.coli* suşu tarafından üretilmektedir.

Reseptör ChuA (*E. coli* heme-utilization protein) dahil olduğu hemin alım sistemi aslında ilk olarak *E.coli* O157:H7'de tanımlanmıştır. Dış membrane protein ChuA'yı kodlayan genler filogenetik grup B2'deki birkaç ExPEC suşunda bulunmuştur; fakat patogenezdeki fonksiyonu ve önemi belirsiz olarak kalmıştır.

ExPEC suşlarında yukarıda belirttiğimiz farklı demir alım sistemlerinin eş zamanlı varlığı ExPEC patogenezinde virülansı arttırarak ek görevlerin yerine getirilmesinde bir ipucu olabilmektedir. Bu bağlamda yapılan son çalışmalar ExPEC siderofor reseptörlerinin aderans gibi diğer virülans özelliklerine katkıda bulunduğuna dair kanıt sağlamaktadır (Russo et al., 2001; Russo et al., 2002). İlgi çekici olan siderofor sistemlerine sahip olan ExPEC'in fare mesane epitel hücresinde biyofilm benzeri yapılar oluşturabildiğinin gösterilmesidir (Anderson et al., 2003). Bu çalışmada, klinik ve gıda örneklerinden izole edilen *E.coli* suşlarında siderofor genlerinin varlığı araştırılmış ve *Escherichia coli* suşlarının genotipik ilişkisi incelemiştir. Çalışmamızda, klinik örneklerden izole edilmiş altmış beş ve

gıda örneklerinden izole edilen 35 *E.coli* suşu kullanılmıştır. *E.coli* suşlarında Sideroforla ilişkili genlerin prevalansı, enterobaktin reseptörü (Fep A),% 18; Enterobactin biyosentezi (Ent A),% 18; yersiniabactin reseptörü (Fyu A),% 17; heme reseptörü (Chu A)% 16; aerobaktin reseptörü (iut A),% 12; aerobaktin biyosentez (iuc A),% 15; ve salmochelin reseptörü (iro N),% 3 ve demir cevabı veren element (ire A),% 1 olarak PCR yöntemi ile belirlenmiştir. *E.coli* suşları arasındaki genotipik ilişki PFGE metodu kullanılarak incelenmiş ve PFGE analizinin değerlendirilmesinde, insan ve gıda kaynaklı izolatların 89'undan 6'sının (%6.8) klonal bir ilişki sergilediği, diğerleri arasında anlamlı bir ilişki bulunmadığı görülmüştür. Sonuç olarak; klinik örneklerden izole edilen *E.coli* suşlarının gıda örneklerinden elde edilen suşlara göre daha yüksek prevalansa sahip olduğu belirlenmiştir; Sideroforla ilişkili genlerin, *E.coli* klinik suşlarının büyümesi, hayatta kalması ve demir sınırlayıcı çevreye adaptasyonu için ana faktörlerden olduğu bu çalışmada da gösterilmiştir.

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DAEC	: Diffuse-adhering *Escherichia coli* (*E. coli)*
DNA	: Deoxyribonucleic acid
E. coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetraacetic acid
EHEC	: Enterohemorrhagic *E. coli*
EIEC	: Enteroinvasive *E. coli*
EMB	: Eosin Methylene Blue
EPEC	: Enteropathogenic *E. coli*
ETEC	: Enterotoxigenic *E. coli*
ExPEC	: Extra-intestinal pathogenic *E. coli*
IMVIC	: Indole, Methyl red, Voges-Proskauer and Citrate
MLST	: Multilocus Sequence Typing
PCR	: Polymerase Chain Reaction
TBE	: Trismabase, Boric Acid, EDTA
TE	: Tris-Hcl, EDTA
UPEC	: Uropathogenic *E. coli*
FLSB	: Fluorocult Lauryl Slfate Broth
MPN	: Most probable number
Fep A	: Enterobactin receptor
Ent A	: Enterobactin biosynthesis
Fyu A	: Yersiniabactin receptor
Chu A	: Heme receptor
Iut A	: Aerobactin receptor
Iuc A	: Aerobactin biosynthesis
Iro N	: Salmochelin receptor
Ire A	: Iron-responsive element

1. INTRODUCTION

Outbreaks and Foodborne pathogens pose a significant threat to human public health, leading to a substantial economic burden both in developed and less developed countries (Chen and Tang, 2012). More than 250 known foodborne diseases could be caused by food contaminated with bacteria, viruses, parasites, and toxins, which continue to be a public health problem in the world (Ahmed and Hall, 2014). Of these, bacteria cause a large proportion (approximately 90%) of all foodborne illnesses. The bacterial pathogens are mostly likely to be found in commonly slaughtered livestock (cattle, sheep, and swine) and poultry (chicken and turkey). Chicken meats comprise about the two-thirds of the total production in the world (Mead, 2000). Meat and poultry carcasses and their parts are frequently contaminated with pathogens which reach the carcasses from intestinal tract or from fecal material on feet and feathers (Dinçer and Baysa, 2004). It is well accepted that bacteria such as *E. coli* can move passively to humans through a variety of routes, including the food chain .Because of the similarity between some human and animal *E.coli*. Similarities in strains isolated from different host species should be expected, particularly with respect to specific virulence factors (VF). These VF are important to ExPEC survival in these extra intestinal environments. Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are the cause of most community and hospital acquired extraintestinal *E. coli* infections, including infections of the urinary tract, bloodstream, and other anatomical sites [Russo and Johnson, 2003]. In this study the prevalence of siderophore genes in *E.coli* isolated from food samples and in ExPEC isolated from clinical samples were determined and then the phylogenetic relationships between these *E.coli* strains were studied by PFGE method to constitute evidence of transmission of pathogenic *E-coli* strains to human from food.

1.1. *E.coli*

Theodor Escherich (1857 - 1911) have discovered *E.coli* as "*bacterium coli commune*" at the end of the 19 the century, Theodor Escherich was German bacteriologist and pediatrician. *E. coli* has a rod shaped and it is a gram-negative and facultative anaerobic bacterium which belongs to the Enterobacteriaceae family of gamma-proteobacteria. The family of Enterobacteriaceae consists of more than forty different genera (amongst others *Citrobacter*, *Salmonella*, *Klebsiella*, *Shigella*, *Erwinia*, *Proteus*,*Yersinia*, *Enterobacter* and *Escherichia*) containing commensal or apathogenic as well as pathogenic strains. The species *E.coli* consists of pathogenic and commensal strains and is exceedingly heterogeneous (Rolhion, 2007). Most strains of *E. coli* are harmless and colonize as normal flora of the gut of warm-blooded humans and animals and these strains can benefit their hosts by producing vitamin K2, and preventing colonization of the intestine with pathogenic bacteria (Reid and Howard, 2001 ; Bentley and Meganathan,1982). However some of *E.coli* strains have several factors and serotype virulence, which make *E.coli* as cause of serious and various diseases. The infection with inherently pathogenic *E. coli* strains led to three general clinical syndromes: (i) sepsis/meningitis, (ii) urinary tract infection and (iii) enteric/diarrheal disease.

1.1.1. *Escherichia coli* Morphology, Growth and Biotyping Characteristics

E. coli is a *gram* negative, straight, non-spore forming rod (1.1- 1.5 µm x 2.0- 6.0µm) (Quim and Carter, 1994). *E. coli* is facultatively anaerobic and grows at an optimal temperature of 37°C. *E.coil* colonies usually grow rapidly 24 hours incubation and are relatively large on blood agar. On blood agar the colonies are shiny, grey, round, smooth, rarely mucoid and some strains are hemolytic. *E.coli* coloies are bright pink on MacConkey agar, because of *E.coli* can ferment lactose in agar and produce acidic metabolic products which decrease the pH in agar to be bright pink. A characteristic 'coliform' (fecal) smell can be noted. The term

'coliform' refers to members of Enterobacteriaceae which can ferment lactose such as, Klebsiella, *E. coli* and *Enterobacter*. *E.coli* colonies are metallic sheen on EMB (Eosin methylene blue) agar and this characteristic is unique for *E.coli* colonies, so EMB is usual used for identification of *E.coli* (Bettelheim, 1994 ; Quim, 1994). The biochemical and fermentation tests are used to identification of *E.coli* (Gyles, 1993). Characteristically, *E.coli* are positive in catalase test, negative in cytochrome oxidase test and ferment glucose (carbohydrates) to produce gas and acid (Bergey, 1994).The production of indole, fermentation of lactose, negative KCN, gelatin and malonate reactions are important tests which differentiate *E.coli* from other genera (Bnkov,1984). The IMVIC (indole, methyl red, Voges – Proskauer, citrate) is a quick and conventional method to identification of *E.coli* because almost no other lactose positive member of the family *Emerobacreriaceae* produces this combination of results (Quim, 1994).

1.1.2. Genomic Diversity of *Escherichia coli*

Horizontal gene transfer (HGT) occurs in *E.coli* and it is made possible by the existence of mobile genetic elements as plasmids which contributing to a noteworthy genome plasticity in the species (Dobrindt, 2005;Pitout, 2012). According to this mechanism the *E.coli* has evolved the ability to survive and colonize in many different locations (Dobrindt, 2005).The genome is consisted of the conserved core of gene and flexible gene pool, the genetic information for most essential cellular processes are constituted by the conserved core of genes, while genetic information which provide the abilities to adjust to new environmental conditions are constituted by the flexible gene pool (Pitout, 2012).The rapid evolution of *E.coli* strains is as a result of the acquisition of new genomic material. Thus, *E.coli* has a highly versatile species with a noticeable adaptive potential, which are the ability to adapt rapidly to new and complex habitats and ecosystems (Bleibtreu, 2013; Barroso, 2014).

1.1.3. Antibiotic resistance in *E.coli*

Generally gram-negative organisms have five main methods to develop antibiotic resistance (Ho, 2010 ;Mcdermott, 2003), first, bacteria can carry genes coding for enzymes, such as beta-lactamases, hydrolysing and inactivating beta-lactam antibiotics. Second, the mutations in the genes cause changes of binding sites for antibiotics, and these changes include the specific target or its function. Third, the permeability is reduced as a result of alterations of the membrane porins. Fourth, bacteria can actively transport antibiotics out of the cell by efflux pumps and finally, alternative metabolic pathways that bypass the action of antibiotics (Ho and Tambyah, 2010;Mcdermott and Walker, 2003). Resistance in gram negative bacteria is often consisted of collection of resistance mechanisms like porin deletions, efflux pumps and beta-lactamases, and can be intrinsic, arise or be acquired (Sommer, 2009; Llarrull, 2010). The hydrolysis of the antibiotic via beta-lactamases is the dominant mechanism of resistance (ECDC, 2012). The production of β-lactamases, including ESBL (Extended-spectrum beta-lactamases) is through large plasmids, which carry many different genes that code resistance against other antibiotic classes, contributing to Multiple drug resistance(MDR) (Falagas, 2009). *E.coli* is one of the organisms most frequently found harbouring ESBL-genes and MDR in ESBL-producing *Enterobacteriaceae* is rapidly becoming a threat to the medical community (Peirano and Pitout, 2010).

1.1.4. Commensal *E. coli*

Commensal *E. coli* are part of the normal flora of the gastrointestinal tract. The commensal *E. coli* and its host organisms co-exist in harmony for long periods of time with mutual benefits for each other (Kaper and Nataro, 2004). The mucous layer is the typical niche of commensal *E.coli* in the vertebrate intestinal tract. These commensal strains provide health benefits to the host during colonise specific intestinal compartments (Vejborg and Friis, 2010). Furthermore, few studies have demonstrated that the commensal *E. coli* colonise different

gastrointestinal compartments of mammals (Dixit, 2004), and they benefit their hosts e.g. by preventing the establishment of pathogenic bacteria within the intestine or by producing vitamin K (Bentley and Meganathan,1982). Some groups of *E. coli* have ability to cause a broad range of diseases by the acquisition of specific virulence factors (Nataro and Kaper, 1998).

1.1.5. *E. coli* as a pathogen

These pathogens have been widely divided into two main categories: the extraintestinal pathogens and the enteric pathogens. Enteric pathogens are the cause of most severe acute diarrhea in humans and animals. The extraintestinal pathogenic *E. coli* that are the cause of the urinary tract infections in all age categories or meningitis and sepsis in small children and young animals(Nataro and Kaper, 1998) and (Levine, 1987). The major subcategories of these pathogens are described below.

1.1.6. Enteric pathogenic *E. coli*

Enteric pathogenic *E. coli* are the well characterized and common pathogenic *E. coli* found in the gastrointestinal tract (GIT). The major intestinal pathotypes are enterohaeomorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998)..

1.1.6.1. Enterotoxigenic *E. coli* (ETEC)

The ETEC infect both human and animals , and are a major cause of diarrhea in young children and adults human and in newborn animals, including mainly piglets (Alexander, 1994), lambs (Hodgson, 1994) and calves (Butler and Clarke, 1994).The contamination of drinking water and food by fecal is the major route of infection for humans. After ingestion, ETEC colonise the small bowel and

contact to its mucosa, They are main cause of dysfunction of electrolyte and water transport in enterocytes which lead to the typical signs of ETEC infections, but they usually do not cause any remarkable local inflammation or histological change in the intestinal mucosa. Some signs of ETEC infection include cramps, vomiting, low grade fever, watery diarrhea, and nausea and are closer to those of cholera, but generally milder (Nataro and Kaper, 1998).

1.1.6.2. Enteropathogenic *E. coli* (EPEC)

EPEC are another main cause of diarrhea in third world countries, which have affected only on the small children below age two and they do not represent a source of traveler's diarrhea. EPEC have several symptoms which are relatively severe and children present with symptoms of fever, profuse watery diarrhea and vomiting. EPEC have a limited number of serogroups (mainly O14, O55, O86, O111, O119, O125, O126, O127, O128, and O142) (Kaper, 1994).The contamination of food is major route to transmit the EPEC, which have ability to colonize the small intestine where they join firmly to the epithelial cells of the villus tips and are a cause of typical lesions called attaching and effacing lesions (Kaper, 1994).These lesions have a major feature which is the reorganization of the enterocyte structure which includes destruction of the brush border and formation of pedestal –like structures on top of which bacteria sit and remain in intimate attach with the cell. EPEC usually neither invade the intestinal mucosa nor produce toxin similar to ETEC toxins and the exact mechanisms which are cause of EPEC-associated diarrhea are still under investigation (Donnenberg and Kaper, 1997). There are strains similar to EPEC in rabbits, which are responsible for severe diarrhea and histological lesions which are similar to those observed in humans (Peeters, 1994).

1.1.6.3. Enterohaeomorrhagic *E. coli* (EHEC)

Shiga-toxin producing *E. coli (*STEC)*, also known as* verotoxin-producing *E. coli* (VTEC), is a cause of a wide range of symptoms in humans (Griffin, 1999) which include uncomplicated diarrhea, and many severe diseases as hemorrhagic colitis and the often deadly hemorrhagic uremic syndrome. STEC are responsible for severe forms of infection which are observed mainly in the elderly and young children. The major reservoir of STEC are cattle, STEC usually are transmitted to human by fecal contamination of food, and the infections can be also transmitted from human to human in outbreaks. STEC have been also demonstrated to be a cause of diarrhea in calves (Butler and Clarke, 1994), and some species serotypes have represented edema disease in pigs (Bertschinger and Gyles, 1994). STEC belong to a very large variety of serotypes, but the majority of clinical infections registered in humans and particularly in food-borne outbreaks are related with the serotypes and serogroups O157:H7, O157:H-, O26, O103, O111, O113, and O145. The severe diseases and outbreaks are caused by these serotypes, which also called enterohemorrhagic *E. coli* (EHEC) (Levine, 1987).The colon is usually colonized by STEC where they are responsible for necrosis of villus tips, in contrast the intestinal mucosa is not invaded by them.

1.1.6.4. Enteroinvasive *E. coli* (EIEC)

EIEC closely resemble Shigella, which are a cause of watery diarrhea and dysentery in severe cases. They cause strong inflammation due to their ability to invade and destroy enterocytes.Their epidemiology remains poorly understood, however infections which are occurred by EIEC may find both in the form of sporadic and in outbreaks cases. The EIEC are mainly transmitted by contaminated food and water (Levine, 1997).

1.1.6.5. Enteroaggregative *E. coli* (EAEC)

EAEC present a typical adherence pattern on cell cultures, with bacteria aligning in parallel clusters like bricks in a wall. EAEC infections occur as sporadic cases or outbreaks mainly in developing countries, but they have also been observed in industrialized countries. The clinical signs of EAEC infections are usually watery mucoid (sometimes bloody) diarrhea with low-grade fever and little or no vomiting. Typical is the persistence of diarrhea for often more than 14 days. The pathogenic potential of EAEC has been demonstrated during applying many experiments on volunteers and on animal models. EAEC are responsible for pediatric diarrhea with lesions which are same to those observed in animal models that has subsequently confirmed their virulence. EAEC infections that occur either in the form of sporadic or in outbreaks cases and they are observed in both developing and industrialized countries (Nataro, 1998).

1.1.6.6. Diffusely adherent *E. coli* (DAEC)

In worldwide DAEC are a mainly responsible for urinary tract infections, but they have a role as a causative agent of diarrhea which are controversial. Sources implicated in outbreaks of DAEC include contaminated food, especially undercooked ground beef, contaminated water and contact with livestock and other animals (Gióon,1991).

1.1.7. Extraintestinal pathogenic *E. coli* (ExPEC)

The majority of community –acquired infections outside the gastrointestinal tract are caused by ExPEC strains. These infections can cause either threatening diseases of life such as sepsis and pyelonephritis or asymptomatic urinary tract infections (UTIs) (Johnson, 2000). The major subtype of ExPEC is uropathogenic *E.coli* (UPEC) strains which are a cause of Urinary Tract Infections (UTIs), that represent a heterogeneous group of disorders which are collectively responsible for significant morbidity, increased health-care costs

and lost productivity. The severity of UTIs can range between asymptomatic UTI and harmless cystitis to systemic infections like severe pyelonephritis. Further the sepsis-associated pathogenic *E. coli* (SPEC) are pathotypes of ExPEC which are the most common cause of community-acquired bacteremia and sepsis and also the newborn meningitis-associated *E. coli* (NMEC) strains are pathotypes of ExPEC, that are cause of neonatal meningitis and neonatal sepsis, which regular cause serious sequelae and to death (Russo and Johnson, 2003). Other infections such as cellulitis, osteomyelitis and wound infections are occasionally caused by ExPEC strains, and ExPEC strains also are responsible for intraabdominal infections and nosocomial pneumonia (Johnson and Russo, 2002). Phylogenetic analyses has shown that the natural *E.coli* isolated are mainly clonal and constitute four major phylogenetic groups: A, B1, B2 and D. ExPEC strains, which are cause of most ExPEC infections in non- compromised hosts, are as different from commensal *E. coli* as the intestinal pathogenic *E. coli* types (Russo and Johnson, 2003). The phylogenetic distribution of ExPEC virulence genes in relation to clinical sources is depicted in Figure 1.1. The commensal *E.coli* strains which lack most virulence factors (VFs), they typically derive from phylogenetic groups A or B1 (Johnson and Russo, 2002),whereas the various intestinal pathogenic *E.coli* strains derive from phylogenetic groups A, B1, or D, or from ungrouped lineages, and these strains rarely cause extraintestinal disease (Pupo and Karaolis, 1997). The intestinal pathogenic strains have distinctive virulence factors (e.g. shigatoxin, ST, LT or intimin) which are responsible for their particular diarrhoeal syndromes. ExPEC strains are different from commensal and intestinal pathogenic *E.coli,* because they derive from group B2 and, to a lesser extent, group D (Johnson and Russo, 2002). Further these phylogenetic groups contain particular virulence genes (papA, P fimbriae; kpsMT, group II capsule synthesis; sfa/foc, S and F1C fimbriae; *iutA*, aerobactin receptor) and these indicate a high association of these VFs to ExPEC strains. Recent studied have demonstrated that almost all ExPEC strains possess virulence factors by a stepwise accumulation through horizontal gene transfer, they

have evolved from an ancestral strain (Pupo,1997). Acquisition and expression of these virulence factors may require the specific genetic background (Dobrindt and Hacker, 2001). However, the sole association of an *E. coli* strain with a certain phylogenetic group does not necessarily render the strain pathogenic or non-pathogenic as the adaptation of a strain to a host also contributes significantly in determining its virulence (Souza and Rocha, 1999).

1.1.7.1. Virulence factors of ExPEC

ExPEC have a high ability to effectively colonize the gastrointestinal tract (Johnson and Russo, 2002), and this ability depends upon 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 and Eto, 2005).The characteristic virulence factors of ExPEC include adhesions, toxins, diverse polysaccharide coatings (e.g. capsules), proteases, invasions, siderophores and serum resistance proteins (Figure 1.2.). The majority of these VFs are encoded on pathogenicity islands (PAIs) (Groisman, 1996). The definition of PAIs is genetic elements which insert chromosomally with a distinct G + C content, harbouring virulence associated genes that are absent from commensal *E. coli* (Hacker, 1990). PAIs are characteristically obtained by horizontal gene transfer that is probable basic step in the evolution of the pathogen. In any case, particular transmissibility of PAIs has not yet been showed and their evolutionary origin stays unknown. The term siderophore (of Greek origin, translating as iron bearer) was popularized in the mid-1970s for description of iron-binding agents which secreted under iron deficient conditions by fungi and bacteria. The siderophores are the small molecules that are secreted by some microorganisms to get iron from the environment, siderophores are used to get iron from the host in pathogenic interactions, and are sometimes necessary for the expression of full virulence.

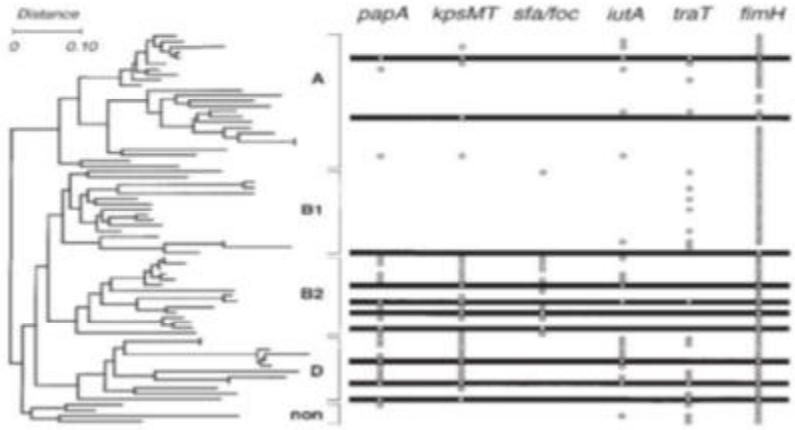

Figure 1.1. Phylogenetic distribution of *E. coli* extraintestinal virulence genes in relation to clinical sources.

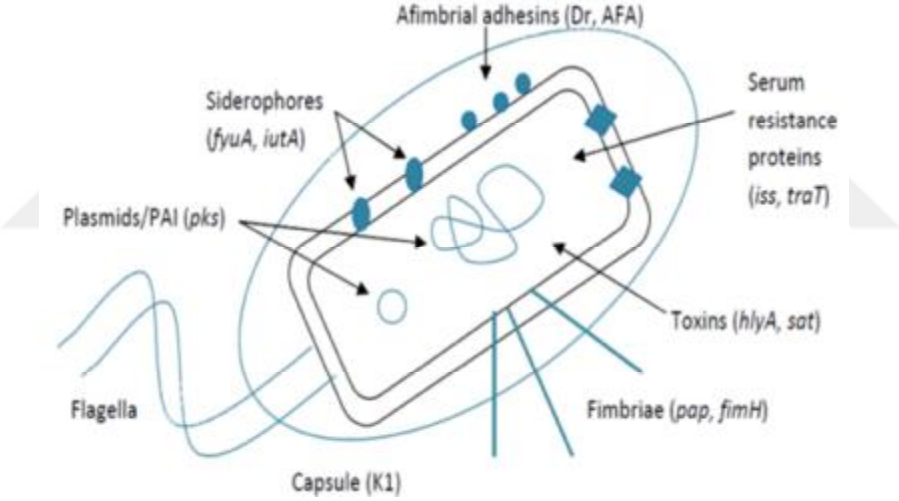

Figure 1.2. Schematic representation of an *E. coli* cell interacting with host tissue, highlighting features relevant to bacterial pathogenicity. Membrane proteins involved in transport, serum resistance, etc., are indicated by solid black circles, triangles, and rectangles. OM, Outer membrane; CM , cytoplasmic membrane; LPS, lipopolysaccharide (Johnson, 1991).

1.1.7.1. (1). Siderophore

Iron is the fourth most abundant element in Earth's crust (Huber, 2005). It has ability to exist in two oxidation states , Fe (III) and Fe (II), so it is considered a

transition metal. Iron is able to play a basic role in the oxidation-reduction reactions and this is because of variable valence of iron. Iron is needed in several metabolic processes which include electron transport chain, tricarboxylic acid cycle, photosynthesis and oxidative phosphorylation (Fardeau and Mullie, 2001). It also controls the biosynthesis of antibiotics, cytochromes, toxins, porphyrins, siderophores, vitamins, aromatic compounds, and pigments, and nucleic acid synthesis (Messenger and Barclay, 1983). Recently it has also been shown that iron contributes to the formation of microbial biofilm as it controls the surface motility of microorganism (Glick and Gilmour, 2010). At physiological pH (7.35–7.40), Iron (as Fe^{2+}, ferrous ion) is soluble while iron (as Fe^{3+}, ferric ion) is insoluble (Bou-Abdallah, 2010). Fe (III) is very insoluble and biologically inaccessible such that the concentration of free ferric iron available to pathogens in the human host ranges in estimation from 10^{-15} to 10^{-24} M , whereas a typical pathogenic bacterium requires ~1 μM iron for optimal growth (Vasil, 1999). Figure 1.3.

Figure 1.3. The difference between the Ferrous and Ferric (Poole and McKay, 2003)

Some microorganisms to survive under iron deficient conditions they produce specific organic compounds which have low molecular masses called

siderophores (Ahmed and Holmstrom, 2014). Siderophores (of Greek origin, translating as iron bearer) play key role as the metal-chelating agents which capture the insoluble ferric iron (Fe^{3+}) from different habitats (Nagoba and Vedpathak, 2011). Existing literature demonstrated that Siderophores are produced by both gram positive and gram negative bacteria under iron deficient conditions (Tian and Ding, 2009).

Siderophore binds with iron (Fe^{+3}) firmly as first step , then the specific siderophore receptors on the cell membrane allow the siderophore iron complex to move into the cell, Permeases, siderophore binding proteins, and ATPases participate in the transport of siderophore -iron complex through the cell membrane in gram-positive bacteria (Ahmed and Holmstrom, 2014). The gram negative bacteria possess the membrane network which is noticeably different from that of gram-positive bacteria , therefore the transport of siderophore iron (Fe^{+3}) complex through gram negative bacteria membranes includes an outer membrane receptor, a periplasmic binding protein and cytoplasmic membrane protein belonging to Adenosine triphosphate (ATP)-binding cassette transporter (ABC-transporter) (Ahmed and Holmstrom, 2014) .When siderophore iron (Fe^{+3}) complex enters to cytosol, the ferric iron will be reduced to ferrous form which becomes free from the siderophore. After release of iron, siderophores either get degraded or recycled by excretion through efflux pump system. Almost all ***E. coli*** strains synthesize the catecholate siderophore enterochelin (also called enterobactin). In addition to enterochelin, ExPEC strains also produce three additional types of siderophores: yersiniabactin (a phenolate), salmochelins (catecholates), and aerobactin (a mixed-type, citrate-hydroxamate) (Figure 1.4) (Johnson and Kariyawasam, 2007).

Catecholates type of siderophore is found only in bacteria. It consists of catecholate and hydroxyl groups and binds Fe3+ with adjacent hy- droxyls or catechol ends (Paul and Dubey, 2015). It has dihydrox- ybenzoic acid (DHBA) coupled to an amino acid. Lipophilicity, complex stability and resistance to

environmental pH are its unique properties. Catecholate siderophore group also forms hexadentate octahedral complex by providing two oxygen atoms for iron chelation (Saha ,2016).

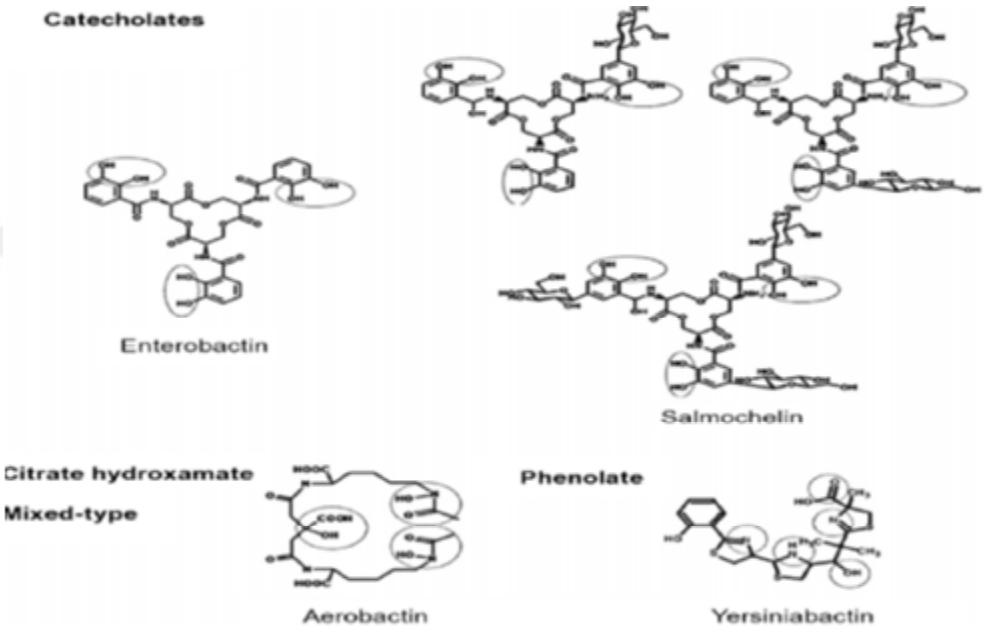

Figure 1.4. Chemical structure of known siderophores

1.1.8. Molecular typing of *E.coli*

The typing methodologies are used to understand intraspecific interaction and exact characterization of strains, moreover, these methods are very important in screening and characterising probiotic strains. *E. coli* community is diversity which needs identification and a systematic categorization of individual isolates. The *E. coli* isolates were differentiate by used different typing methodologies. Some of the most important typing methods used for *E. coli* are: pulse field gel electrophoresis (PFGE), serotyping, phylogenetic typing, random amplified polymorphic DNA (RAPD) and multi locus sequence typing (MLST).

1.1.8.1. Pulse field agarose gel electrophoresis (PFGE)

Pulse field agarose gel electrophoresis is used in typing bacterial isolates and it cosideres as the gold standard (Riley, 2004). It includes the extraction of DNA in a plug then restriction enzyme as *Xba*I and *Not*I will be used to cut the DNA and this process is called restriction digestion (Goering, 2007). The large DNA fragments 30-50 Kb which produced after restriction enzyme digestion are separated by alternating field direction (current) on agarose gel. The large fragments move slower than the small fragments on the agarose gel which results in a DNA pattern specific to each clone (Goering, 2007). The PFGE is used in different laboratories around the world, because of the use of international data bases by Center for Disease control (CDC), therefore the comparison of new outbreak strains and their clonal origin are very easy. Studies have demonstrated that discriminatory powers of PFGE are very high compared to other typing methodologies as RAPD and MLST. However, PFGE is labour intensive and needs specialized equipment; training and time compared to other genotyping methods (Goering, 2007).

2. LITERATURE REVIEW

2.1. İsolation of *E.coli* from food

There are several new studies which have documented the prevalence of *E. coli* in food samples, Badri et al. (2009) have performed bacteriological analysis of *E.coli* in different types of sample and they found that the prevalence of *E. coli* was 48%, 45%, 35.5%, 30%, 8.3% and 0% in well water, ground beef, turkey, sausage, sea food and domestic water, respectively.

Iyer et al. (2013) they have studied the prevalence of both pathogens *Salmonella* spp. and *E. coli* in meat samples and PCR confirmed these findings. They found that the rate of incidence of *E. coli* in open butcher shops was a high (65%) when compared to groceries and hypermarkets which were (40%) and (20%) respectively. With respect to *Salmonella*, the rate of incidence was 45%, 25%, and 5% in butcher shops , groceries, and hypermarkets respectively .

Abdaslam et al. (2014) they have studied the prevalence of four types of bacteria including *E.coli*, *Salmonella, Klebsiella*, and *S. aureus* in 90 samples of fish, chicken, beef and prawn. They have found that the *E.coli* was found in 45 (50%) of total samples while the prevalence of *Salmonella, Klebsiella* and *S. aureus* were 21 (23.3%), 2 (2.2%) and 27 (30%) respectively. 5 out of 45 of identified *E.coli* strains were *E.coli* O157. Sorbitol Mac Conkey Agar was used as selective medium to identified non -sorbitol fermenters of *E.coli* after that rapid latex agglutination test was performed on typical colourless colonies to identify the *E.coli* O157.

Adwan et al. (2105) have reported that *E.coli* was found in 95% of meat samples. The prevalence of *E. coli* was in beef 100%, chicken 93.3% and turkey meat 90%. In this study the results demonstrated that 89.5% of meat samples which contaminated with *E. coli* belonged to *E. coli* pathotypes tested. The total prevalence of uni-infected samples with ETEC was 60% and with EAEC was 5%. The prevalence of more than one pathotype was found in 21% of the tested

samples. Tanih et al. (2015) have studied the prevalence of bacteria pathogens in various abattoir and animal types. They found that, the *E.coli* was the most prevalent bacteria was found in 67.5% (104/154) of samples, while *S. aureus* was found in 32.5% (50/154) of samples. No *Salmonella* was found in this study. Both *S. aureus* and *E. coli* were more found in pork with percentages of 40% (20/50) and 48.1% (50/104), respectively, than in cattle across the different abattoirs . Jabur et al. (2016) found that 34.4% (21) of food samples was positive for *E. coli*. Macconkey agar with sorbitol (McA and SMCA) was used to isolate *E. coli* and the biochemical tests were used to confirm *E. coli* strains. Seven isolates of out 21 *E.coli* isolates were confirmed as *E. coli* O157:H7 on HiCrome EC O157:H7 selective agar. Wang et al. (2016) have done other study which reported that, the *E. coli* O157 was found in 5 of the 375 chicken samples and *E. coli* O157:H7 was detected in none of the samples. Al-jobor et al. (2016) used the multiplex PCR method for detection of three food-borne pathogens after enrichment. The amplifications of the mPCR products were visualized in agarose gel and invA gene (a 284 base pair fragment) was identified for the samples belonging to the *S. spp*., uid R gene (153 base pair fragment) was used in the positive samples for the genus *E. co*li; and *LIS* gene (a 217 base pair fragment) was identified for the samples which belong to the *L. monocytogenes.* One or more of the bacterial pathogens were found in 58% (87 of 150) of samples. Uid R gene was found in 67 samples, the inv A gene was found in 26 samples and LIS gene was present in eleven samples in this study.

2.2. The prevalence of siderophore genes in *E.coli*

Several studies have shown that the distribution of virulence factors as siderophores in *E.coli*, Zhao et al. (2009) have done comparison of virulence factors and expression of specific genes between avian pathogenic *E. coli* and uropathogenic *Escherichia coli*, they have found that nearly all isolates (≥ 90 %) had feoB and fimH. All the pTJ100-related genes (iroN, iucC, iutA, iss, sitA and

traT) occurred in the majority of APEC isolates, which occurred in ≥71% of isolates, except for cvaC, occurring in 53% of APEC isolates. Four of the pTJ100-related genes (iutA, sitA, traT, and iss) also found in > 50% of UPEC isolates. Other pTJ100-related genes (iuc C, iro N, and cva C) were also occurred in UPEC isolates: 37 %, 30 % and 13 % respectively. Also, other genes not linked to pTJ100 were frequently occurred in both UPEC and APEC isolates. fyuA and irp-2 genes, which included in the yersinabactin operon, and ompT occurred in ≥ 60% of both UPEC and APEC isolates. Various pap elements, papC, papA, and papEF, founded commonly in ≥ 54 % of UPEC isolates, and papC and papEF aslo founded in APEC isolates (≥ 37%). kpsMT (K1) and kpsMTII, that included in the synthesis of capsules, occurred in both the APEC and UPEC isolates(≥ 45%).

Park et al. (2009) have reported that the single kps gene was found in both non-UTI cases (45%) and UTI cases (82%). The uropathogenic gene (kps) was detected statistically significantly more often in the UTI cases than in the non-UTI cases (p=0.03). The usp gene was found in 55% of non-UTI cases and in 73% of UTI cases. The ireA gene was found in 55% of the non-UTI cases; while it was found in 5% of UTI cases. Both the usp and kps genes were found in 50.0% of UTI cases, and both the ireA and kps genes were found in 5% of UTI cases. Both the usp and kps genes were found in 9% of the non- UTI cases; both the ireA and kps genes were found in 18% of the non-UTI cases, and both the ireA and usp genes were found in 27% of the non-UTI cases. The uropathogenic usp and kps genes were found statistically significantly more frequently in the UTI cases than in the non-UTI cases (p=0.01).

Yun et al. (2014) have documented the frequently of virulence genes in UPEC isolates which were as that, papA (n = 29, 45.3%), fyuA (n = 29, 45.3%), iutA (n = 34, 53.1%), feoB (n = 43, 67.2%),(n = 54, 84.4%), and fimH (n = 62, 96.9%). The, cdtB, kpsMTIII and papGI genes were not detected. Isolates from the UTI group expressed higher virulence factors than those from the ABU group (5.93 ±1.44 vs 4.98 ± 1.81 respectively, $p < 0.05$). There was no difference between the

prevalence of virulence genes in patients < 6 months of age and in patients over 6 months of age (p >0.05).The pap EF gene frequency was in females more than in man (p <0.05), while the other virulence genes were similar in both men and women. The fimH gene was the most frequent in patients with ABU and UTI (ABU group: n = 47, 95.9%; UTI group: n = 15, 100.0%). The detected genes in the UTI group, in descending order of prevalence, were kpsMTII (n = 14, 93.3%), fyuA (n = 12, 80.0%), iutA (n = 9, 60.0%), and papA (n = 9, 60.0%). In in the ABU group, kpsMTII (n = 14, 93.3%), fyuA (n = 12, 80.0%), iutA (n = 9, 60.0%), and papA (n = 9, 60.0%) were commonly identified in that order.

Koga et al. (2014) they found through molecular screening of virulence genes in ExPEC isolated from human blood culture that the most common virulence gene was iut A was detected in 65% of the isolates tested, while iut A wan not detected in commensal isolated. PAI I_{CFT073} included aerobactin genes, and 5 of the 13 strains containing the *iut*A gene also contained sequences similar to PAI I_{CFT073}. Genes of other iron uptake systems were also detected: the salmochelin receptor *iro*N, was detected in 55% of isolates; and the yersiniabactin receptor *fyu*A was detected in 45% of isolates [24, 25]. The yersiniabactin receptor *fyu*A was detected in 9 isolates and 8 of this isolates contained PAI IV_{536} related sequences. Thus, the presence of this island and the yersiniabactin receptor *fyu*A have good relationship. There was a good relationship between the commensal strains too and of the 24 isolates that contained the *fyu*A gene, 22 isolates had also PAI IV_{536}.

Khan el al. (2015) have shown the distribution of virulence genes in *Escherichia coli* along the production and supply chain of pork (PSCP), the most prevalent gene in group B2 isolates was kpsmII (74.5%) followed by iutA and fimH (70.4%), papC (47.3%), cnf (39.6%), and hlyD (31.9%). Among the group D isolates, the most prevalent virulence genes were sfaS & focG (76.9%), fimH (46.2%), afa (38.5%), and cnf (15.4%). Similarly, B1 isolates were higher in ireA (92.5%), fyu (77.5%), and vat (57.5%) genes. Palma et al. (2016) they have

reported the prevalence of virulence factors in the 65 *E. coli* isolates: fimA(type I fimbriae) was detected in 89.2% of isolates, iutA (aerobactin receptor) was detected in 83.1% of isolates, and *agn43*(antigen 43) was detected in 72.3% of isolates. The siderophore-encoding genes iron acquisition aerobactin (iucA) and yersiniabactin receptor (fyuA) were found in 67.7% and 49.2% of isolates, respectively, however the remaining VGs were detected in less than 30% of isolates. The toxin-related genes, pet, sepA sen and espC as well as fimbriae encoding genes (aafC and papGIII) were not found. Additionally, *fimA* gene expression was observed in 27 (41.5%) out of the 58 strains that possess the gene. Biofilm formation was not observed in any isolate. In total 47.7% (N=31) of isolates belonged to phylogroup D, 26.1% (N=17) of isolates belonged to phylogroups A, 21.5% (N=14) of isolated belonged to B1, while only 4.6% (N=3) of isolates belonged to phylogroup B2. The number of VGs per strain was variable, with a maximum of 11. Forty-five (69.2%) isolates, presented between 4 and 7 VGs, with an average of 4.9 genes per isolate. The strains which belonged to phylogroups B2 and D possessed more virulence genes than those belonged to phylogroups A and B1.

Safi et al. (2016) have done new study; this study was to determine the relative distribution of virulence associated traits among urine *Escherichia coli* isolates from patients in onco-hematology. They found that among the 61 urine isolates, the prevalence of individual VF genes ranged from 2% (sfaS) to 97% (entF). All 19 VF encoding genes were detected in at least one isolate. Four VF encoding genes (fimH, entF, ompT and malX) detected in more than 75% of strains. Five genes (chuA, fyuA, iron, usp, and iutA) detected in more than 50% of the strains. Five virulence genes (pap AH, papC, papG alle II, papEF, and hlyA) occurred in more than 25% of the strains. The five remaining genes (focG, cnf1, papG allele III , cvaC, and sfaS) were found in less than 25% of strains. All the virulence factor genes detected, significantly, more frequently among UTI strains than among fecal strains except for entF and fimH which were present in nearly all

the strains and sfaS that was rare in both groups. All urine strains possessed at least one gene significantly associated with UTIs. The F1C fimbriae focG, the P fimbriae papC, papAH, and the toxin genes hlyA and cnf1 were significantly related to pyelonephritis. Virulence factor scores ranged from 2 to 16 among UTI isolates while they ranged from 0 to 15 in stool isolates. Virulence scores which were more than seven (>7) significantly related with UPEC ($p < 0.001$).

3. METHOD AND MATERIAL

3.1. Material

3.1.1. Food samples

A total of 48 samples were collected from September to October 2016 from retail vegetable stores and markets in which included vegetables (16 samples), chicken (16) and meat (16). All samples were randomly collected in plastic bags, labeled and transported to the Microbiology laboratory/ department of food engineering of Cukurove University for analysis.

3.1.2. Clinical samples

On the other hand clinical samples were collected from urinary tract infections, surgical site infections, pneumonia, meningitis strains between 2014-2016 in Cukurova university Balcali Hospital (Onler, 2016).. The isolates obtained (N=65) were our material in this study (Onler, 2016). A total of 65 ExPEC isolates were used in this study, including 54 (83%) isolates from urine, 5 (8%) isolates from wound, 3 (4%) isolates from blood and 3(4%) isolates from aspirtation. The majority of ExPEC strains were isolated from urine samples as Figure 3.1.

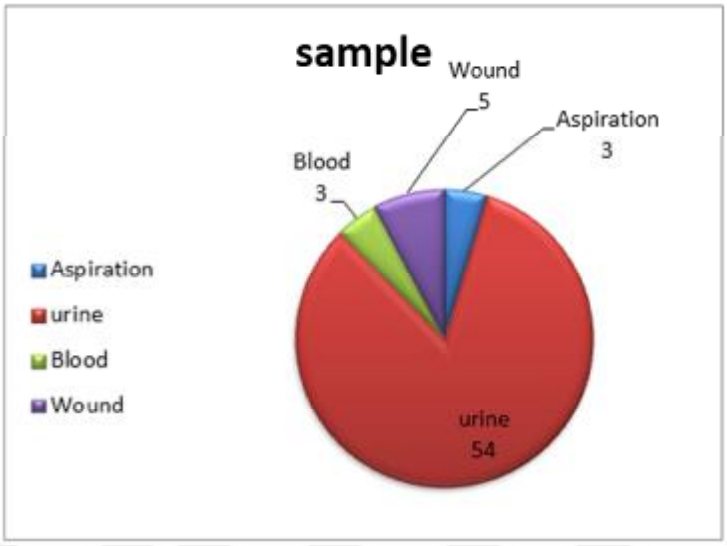

Figure 3.1. Clinical Material

There were 46 (71%) women and 19 (29%) men (Figure 3.2), 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 (N=28,43.04%) were between 50-90 years of age (Figure 3.3).

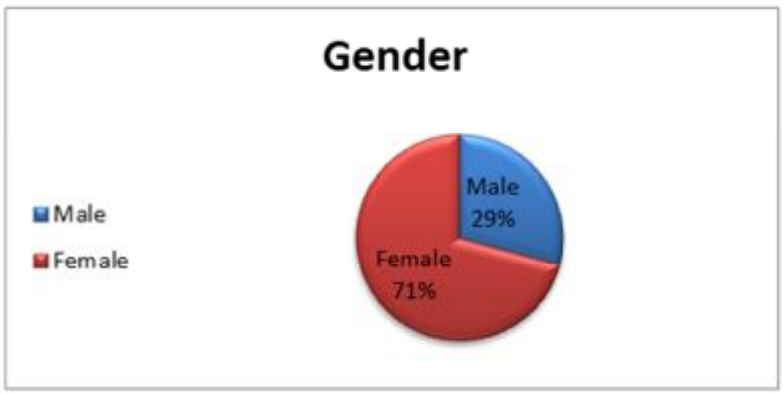

Figure 3.2.The percentage of males and females

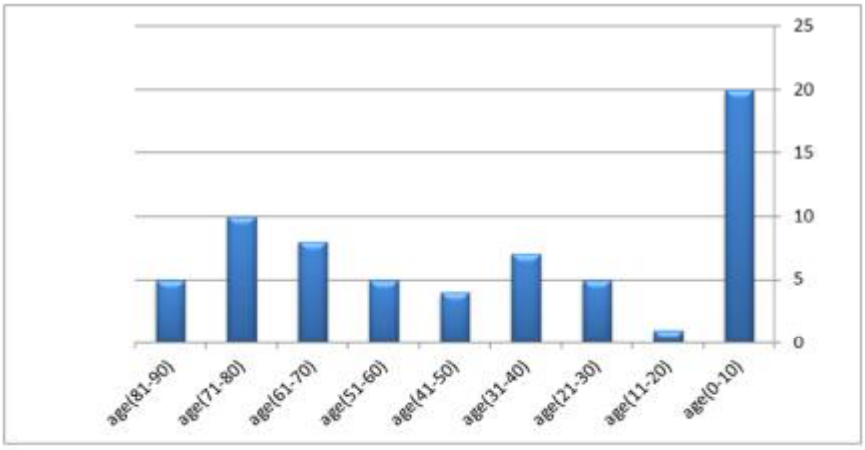

Figure 3.3.Sample Age Composition

3.2. Method

3.2.1. Isolation and identification of *E.coli* strains from the food by classic method

3.2.1.1. Most probable number (MPN) method

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*. In the MPN test, the sample to be tested was prepared in 3-fold dilution series, and then 1mL of each dilution are inoculated into triplicate broth culture tubes for incubation. we incubated the culture medium for 24 to 48 hours at 35 °C aerobically. We checked the tubes after 24 hours of incubation if gas formation was negative, we did not check indole reaction because if we added KOVACS reagent it destroyed the growth conditions in the medium. We continued incubation for another 24 hours, and then we checked the gas formation again. The presence of *E. coli* was indicated by gas formation (fermentation of lactose) and a positive indole reaction. We checked the tubes under UV light (366 nm) too. Light blue fluorescence indicated to the presence of *E. coli* Table 3.1.

Figure 3.4. Gas and indol test

Figure 3.5. MPN Test

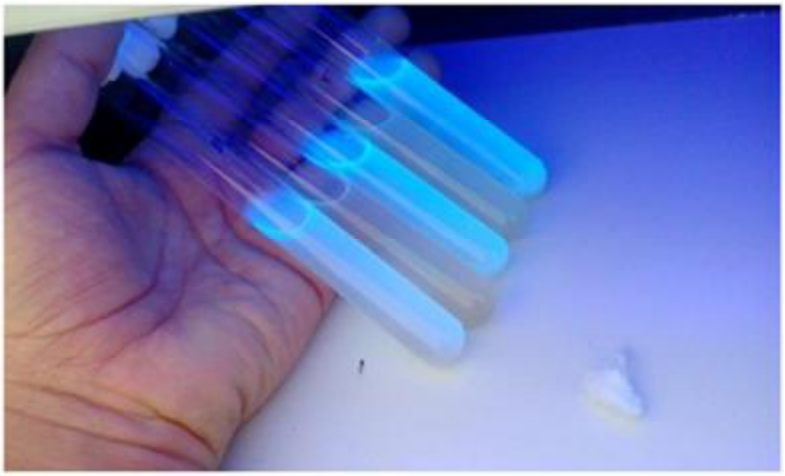

Figure 3.6. Fluorescence test

Table 3.1. Isolation and identification of *E.coli* strains by fluorocult laurly sulfate broth

Test strains	**Growth**	**Gas**	**Fluorescence**	**Indole**
Escherichia coli	Very good	+	+	+
Escherichia colio157:H7	Very good	+	-	+

Fluorocult *E. coli* O157:H7 Agar was used to culture the positive strains of *E. coil*, the mode of action is as that Sodium deoxycholate inhibits the growth of the Gram-positive accompanying flora for the greater part. Sorbitol serves, together with the pH indicator bromothymol blue, to determine the degradation of sorbitol which, in the case of sorbitol-positive microorganisms, results in the colonies turning yellow in colour. Sorbitol-negative strains, on the other hand, do not lead to any change in the colour of the culture medium and thus proliferate as greenish colonies. 4-methylumbelliferyl-β-D-glucuronide (MUG) is converted into 4-methylumbelliferone by β–Dglucuronidase- forming pathogens; 4-methylumbelliferone fluoresces under UV light. The activity of β-D-glucuronidase

is a highly specific characteristic of *E. coli*. In contrast to most *E. coli* strains, *E. coli* 0157:H7 is not capable of forming β D glucuronidase. When irradiated with long-wave UV light, no fluorescence is formed (Schindler, 1991) Table 3.2.

Table3.2. Isolation and identification of *E.coli* strains by Fluorocult *E. coli* O157:H7 Agar

Test strains	Growth	Colony color	MUG	Sorbitol
Escherichia coli	Very good	yellow	+	+
Escherichia colio157:H7	Very good	colorless	-	-

3.2.1.2. Media and biochemical tests

All strains were grown on EMB agar. The culture incubated overnight for 37°C at 18-24 hours. *Escherichia coli* is gram negative bacteria that ferment the lactose produce acid which turns the colonies **dark purple** as the acid acts upon the dyes. In addition, certain lactose-fermenting bacteria produce flat, dark colonies with a green metallic sheen. we have also used phenotipic identification method IMVIC (Indol, Metil Red-Voges Proskauer, citrat) and it was performed according to methods described by (Barrow and Feltham, 1993;Barrow and Feltham, 1993).

1- Indol test: *Escherichia coli* produce indole from amino acid tryptophan using the enzyme typtophanase. Production of indole was detected using Kovac's reagent. Indole reacted with the aldehyde in the reagent to give a red color. An alcoholic layer concentrated the red color as a ring at the top.
2- Methyl red test: the bacterium to be tested in inoculated into glucose phosphate broth, which contained glucose and a phosphate buffer and incubated at 37^{o}C for 48 hours. Over the 48 hours the mixed-acid producing organism must produce sufficient acid to overcome the

phosphate buffer and remain acid. The pH of the medium was tested by the addition of 5 drops of MR reagent. Development of red color was taken as positive (*E.coli*). MR negative organism produced yellow color.

3- Voges proskauer test : Bacterium to be tested was inoculated into glucose phosphate broth and incubated for at least 48 hours. 0.6 ml of alpha-naphthol was added to the test broth and shaken. 0.2 ml of 40% KOH was added to the broth and shaken. The tube was allowed to stand for 15 minutes. Appearance of red color was taken as a positive test. The negative tubes must be held for one hour, since maximum color development occured within one hour after addition of reagents, *Escherichia coli* was negative fort this test.

4- Citrate utilization test: Bacterial colonies were picked up from a straight wire and inoculated into slope of Simmon's citrate agar and incubated overnight at 37^{o}C. If the organism had the ability to utilize citrate, the medium changes its color from green to blue. *Escherichia coli* was negative fort this test.

Figure 3.7. *E.coli* showed on EMB agar as

Figure 3.8. Blood agar green metallic sheen

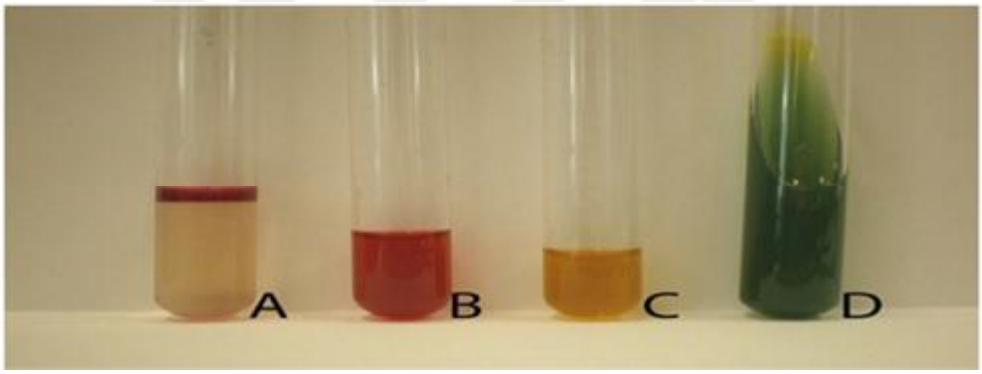

Figure 3.9. IMVIC test

3.2.2. Genotypic identification of *E.coli* strains

3.2.2.1. Chemical Solutions and Enzymes which used in DNA Extraction and PCR

TE Buffer

Tris HCl (SIGMA T-3253): 0,1576gr

EDTA (AMRESCO 0105-500): 0,0372gr

Distile Su: 100 ml

(pH 8.0)

dNTP Mixture

(25µmol each one) (Fermentas R0181)

(2,5 mM each one, total 10mM)

dATP (100 mM): 25 µl

dCTP (100 mM): 25 µl

dGTP (100 mM): 25 µl

dTTP (100 mM): 25 µl

Distilled water: 900µl.

Stored in -20°C.

Marker

Gene Ruler 200bp DNA Ladder Plus (Fermentas SM0323)

Gene Ruler 100bp DNA Ladder Plus (Fermentas SM0323)

Gene Ruler 50bp DNA Ladder (Fermentas SM0323)

Taq DNA polymerase enzyme

(Fermantas EP0402) 5U/ µl

10X PCR Buffer solution

25 mM $MgCl_2$

10xTBE Solution

Tris-base (SIGMA T-1503): 108gr

Boric Acid (MERCK 1.00165.0500): 55gr

EDTA (AMRESCO 0105-500): 7,44gr

(PH 8.4) and Completed to 1000ml with ultra-pure water..

Ethidium bromide

Ethidium bromide (Sigma E8751) (1000X, 5mg/ml): 0,5g

Distilled water: 100ml

To protect it from light we put it in aluminum foil then it was stored at + 4 ° C

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

We have used *uid A* gene, encoded beta-D-glucuronidase, for molecular identification of *E. coli*. *UidA* gene has used as positive control in our study for amplification Table 3.4 (AL- Henrissat, 1999). Firstly for genomic DNA extraction we extracted it according to thaw and freeze protocol (Squirsr, 1955) as follow:

1. To obtain pure colonie of *E.coli* strain it was cultured in Blood agar medium (figure 3.8).
2. The one pure colony of *E.coli* strain was taken and it was again cultured for one night at 37 °C.
3. The pure colony was taken and mixed with 2 ml of TE and the density was adjusted by MC Farland to be between 5-6.
4. One ml of the suspension was taken and put into freezer at –20°C for 1.30 hours
5. Then the suspension was put in dry heater at 95 °C for 15 min.
6. After that the suspension was again put into freezer at –20°C for 1.30 hours
7. Then it was put into dry heater at 95 °C for 15 min.
8. Finially the suspension was centrifuged 13000 g x3 minutes then supernatant was taken and put in new tube,and it was used in the PCR.

After that polymerase chain reaction (PCR) to detect *uid A* gene 5 µl of DNA is 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,2012). The product of PCR loaded in 2% gel of agarose in electrophoresis device and the bands evaluated in imaging device Figure 3.10. (Muller and Greune, 2007)**.**

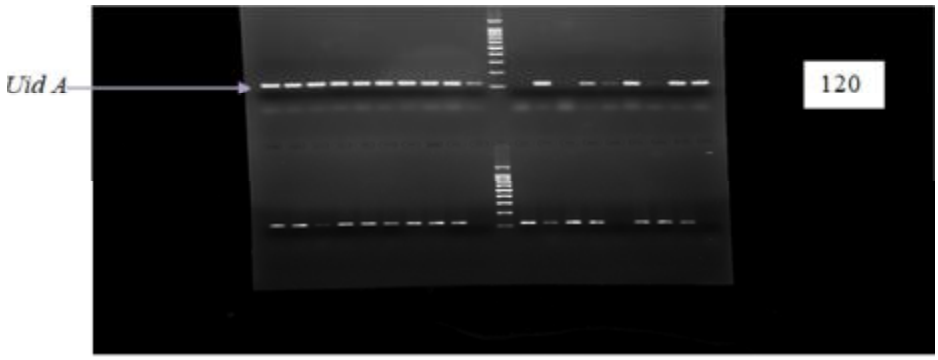

Figure 3.10. Using of *uid A* gene for molecular identification of *E.coli*, Lane M, The 100 bp DNA Ladder (100 – 1500 bp range).

3.2.2.3. Identification of siderophore genes in *E.coli* strains by using polymerase chain reaction (PCR)

This method, first described by Saiki et al. (1988) allows the exponential amplification of DNA regions in vitro by using a heatstable DNA polymerase from Thermophilus aquaticus (Taq). This way, even small amounts of template DNA can be amplified to high copy numbers and easily visualized. Polymerase chain reaction (PCR) used to detect fyuA, entA, iroN, iucA, iutA, chuA, FepA and ireA genes. We used 5 µl of DNA for each PCR in a 25 µl reaction volume containing 12.5µl master mix, 0.5µl primer-F, 0.5µl primer-R, 6.5 µl distilled water. Primers were designed to target conserved regions of biosynthetic and receptor genes for all siderophore systems (Table 3.3). PCR amplification programs which we used them they are shown in Table 3.4. The product of PCR loaded in 2% gel of agarose in electrophoresis device and the bands of siderophore genes evaluated in imaging device and the positive genes are shown (figure 3.11; figure 3.12; figure 3.13;figure 3.14; figure 3.15;figure 3.16; figure 3.17; figure 3.18).

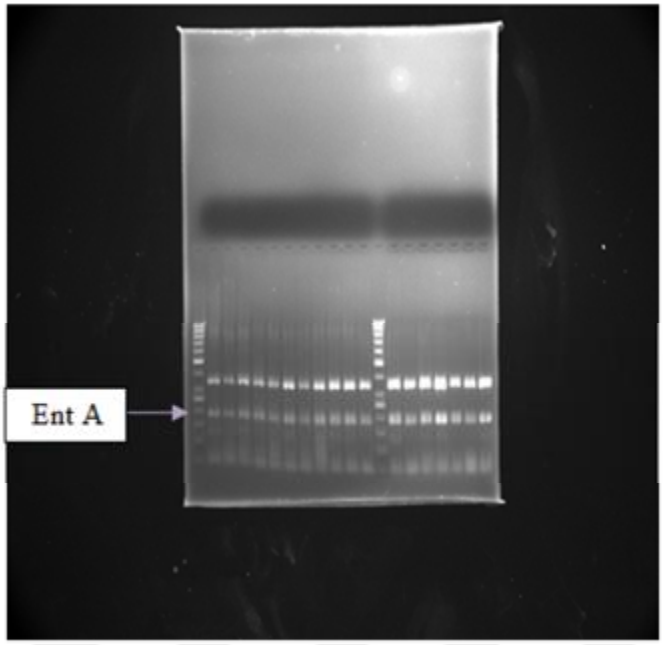

Figure 3.11. PCR amplification of Ent A(184bp) gene from *E.coli*. PCR product was visualized on 2% agarose

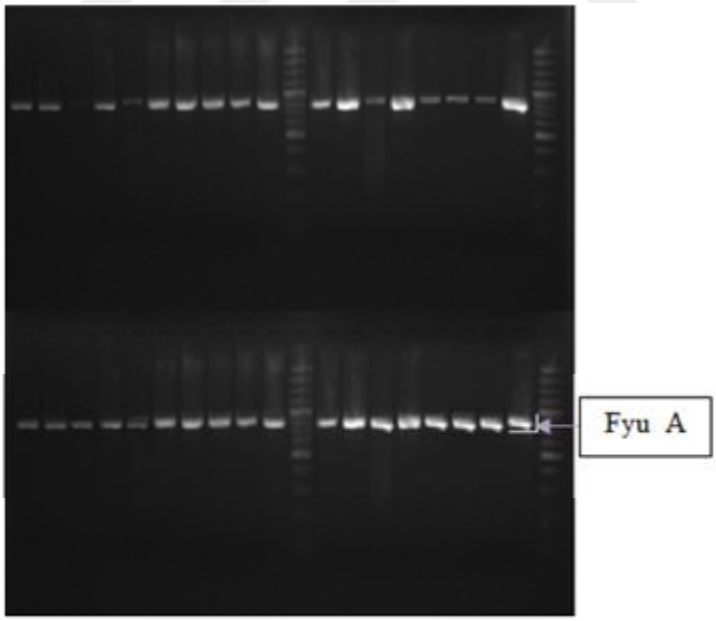

Figure 3.12. PCR amplification of Fyu A (791bp) gene from *E.coli*. PCR product was visualized on 2% agarose

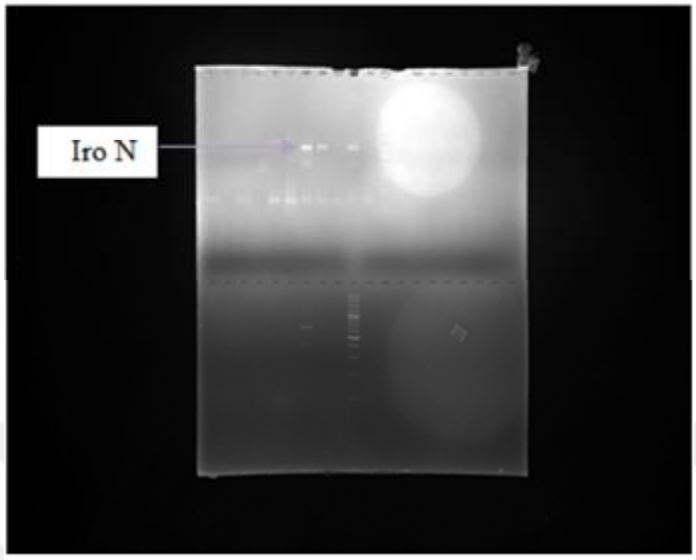

Figure 3.13. PCR amplification of Iro N 648bp) gene from *E. coli.* PCR product was visualized on 2% agarose

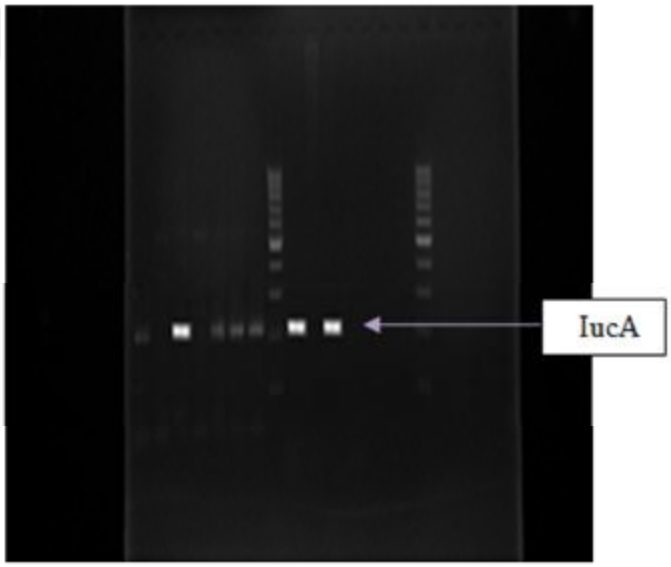

Figure 3.14. PCR amplification of IucA (212bp) gene from *E.coli*. PCR product was visualized on 2% agarose

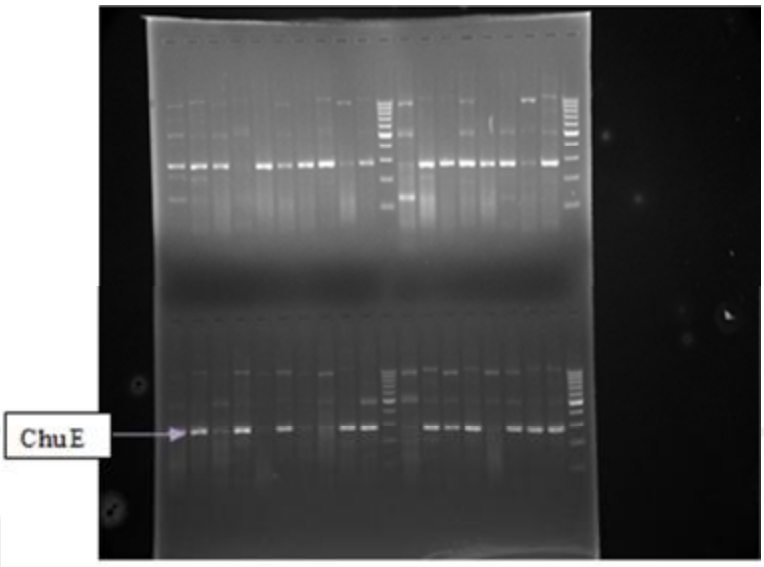

Figure 3.15. PCR amplification of Chu A (279bp) gene from *E.coli*. PCR product was visualized on 2% agarose

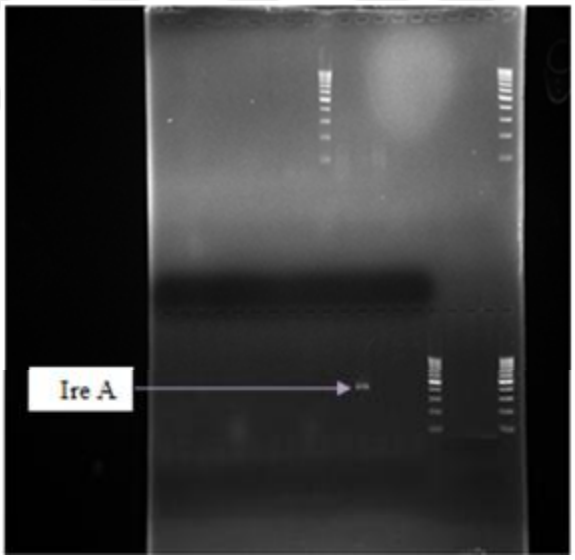

Figure 3.16. PCR amplification of Ire A (415bp) gene from *E coli*. PCR product was visualized on 2% agarose

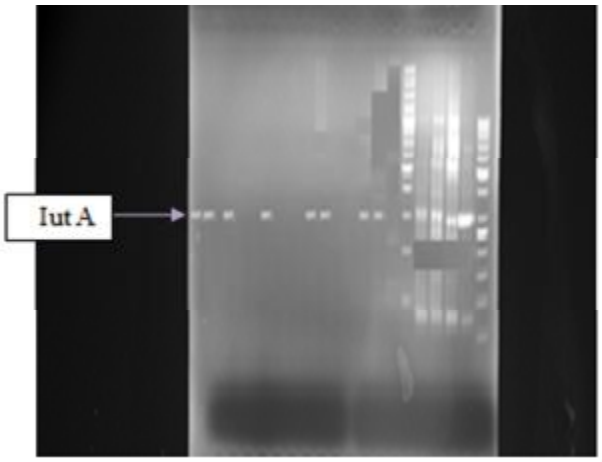

Figure 3.17. PCR amplification of Iut A (300bp) gene from *E.coli*. PCR product was visualized on 2% agarose

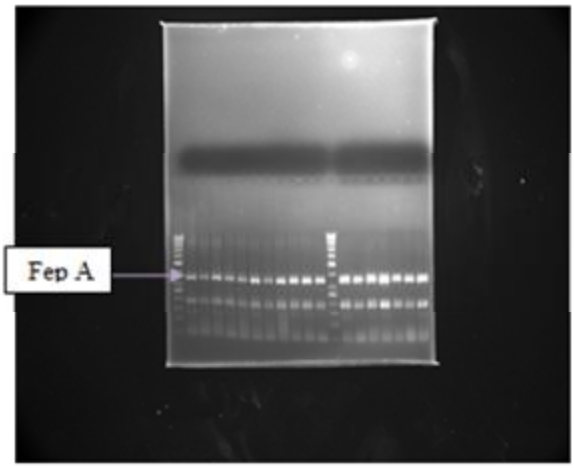

Figure 3.18. PCR amplification of Fep A (349bp) gene from *E.coli*. PCR product was visualized on 2% agarose

Table 3.3. Primers used in this study for PCR

primer	Sequence (Forward)	Sequence (Reverse)	Size (bp)	R
FyuA	GGGAATGTGAAACTGCGTCT	CGGGTGCCAAGTTCATAGTT	791	Searle,2015
IroN	CTTCCTCTACCAGCCTGACG	GCTCCGAAGTGATCATCCAT	648	Searle,2015
IucA	ATAAGGGAAATAGCGCAGCA	TTACGGCTGAAGCGGATTAC	212	Searle,2015
IutA	GGCTGGACATCATGGGAACTGG	CGTCGGGAACGGGTAGAATCG	300	Yun and Ki, 2014
ChuA	GACGAACCAACGGTCAGGAT	TGCCGCCAGTACCAAAGACA	279	Koga and Vanessa, 2014
FepA	TTTGTCGAGGTTGCCATACA	CACGCTGATTTTTGATTGACG	349	Searle,2015
EntA	GTGCGCTGTGTAATGTGGTTTC	CAGAGGCGAGGAACAAAATC	184	Searle,2015
IreA	TGGTCTTCAGCTATATGG	ATCTATGATTGTGTTGGT	415	Russo and Thomas, 2001
UidA	ATGCCAGTCCAGCGTTTTTGT	AAAGTGTGGGTCAATAATCAGGAAGTG	120	AL-zuwainy,2012

Table 3.4. PCR program of Siderophors genes (Searle, 2015).

Name of Gene	Name of Gene	PCR program
Aerobactin	IucA	1 cycle 95 °C 5 min 95°C for 30s, 35 cycles 55°C for 30s, 72°C for 40s 1 cycle 72°C for 5 min
	IutA	1 cycle 94 °C 5 min 94°C for 1min, 35 cycles 55°C for 1min, 72°C for 2 min 1 cycle 72°C for 10 min
Yersiniabactin	fyuA	1 cycle 95 °C 5 min 95°C for 30s, 35 cycles 60 °C for 30s, 72°C for 1 min 1 cycle 72°C for 5 min
The receptor ChuA	ChuA	1 cycle 94 °C 5 min 94°C for 1 min , 30 cycles 55 °C for 1min, 72°C for 1 min 1 cycle 72°C for 10 min
Iron-responsive element	IRE A	1 cycle 95 °C 10 min 95°C for 15s , 25 cycles 55 °C for 1min, 72°C for 30s 1 cycle 72°C for 7 min

Table 3.4. Continue

Salmochelin	IroN	1 cycle 95 °C for 1min. 35 cycles 95°C for 30s, 55°C for 30s, 72°C for 1 min 1 cycle 72°C for 5 min
Enterobactin	FepA	1 cycle 95 °C for 1min. 35 cycles 95°C for 30s, 55°C for 30s, 72°C for 1 min 1 cycle 72°C for 5 min
	EntA	
Beta-D-glucuronidase enzyme activity	Uid A	1 cycle 94 °C 5 min 35 cycles 94°C for 1min, 55°C for 1min, 72°C for 1min 1 cycle 72°C for 10 min

3.2.2.4. Determination of phylogenetic relationship among *E.coli* strains between food and clinical samples by pulse field gel electrophoresis (PFGE) method

3.2.2.4.(1). preparation of isolates

1. One of *E.coli* colonies was cultured overnight and the purity of culture was tested.
2. Then pure isolate was inoculated onto an appropriate agar medium, and the plate was incubated overnight to ensure adequate growth.
3. Pure colony was suspended in 2 ml of Cell Suspension Buffer (pH8, 0.01M), then the density of suspension was adjusted to be between 5-6 by McFarland.

4. 1 ml of the bacterial suspension was taken, centrufuge (13000g x 3dk) was done, the supernatant part was dispensed and finally 1 ml of Cell Suspension Buffer was added to pellet.

3.2.2.4.(2). Preparation of Agarose Plugs

1. Agarose plugs were prepared in Cell Suspension buffer with 2% low melting agar. For this purpose 9 ml of Cell Suspension buffer was added to 0.2 gr low melting agarose in 10 ml screw-cap flask and shaked gently to disperse agarose. After that it was placed in microwave for 10 seconds, then mixed gently and placed again in microwave until agarose was completely dissolved. After agarose was fully dissolved, placed in a water bath at 45-50°C. Then it was gently mixed with 1 ml of 10% sodium dodecyl sulfate solution. At least 200 µl of this mixture distributed in ependorf tubes and was placed in a water bath at 45-50 ° C until bacterial suspension added.
2. The same volume (200 µl) of bacterial suspension was mixed with 200 µl of low melting agarose at 50 °C.
3. Then 100 µl of bacteria-agarose mixture were filled to each plug molds with absence of air bubbles.
4. Then plug molds were stored for 20-30 min at +4°C.

In this stage it is very important to keep the agarose plug molds in the cold to get quality DNA because of the homogenous solidification of agarose plug molds, reduced the edonuclease activity and early cell disruption.

3.2.2.4.(3). Lysis of Cells in Agarose Plugs

1. For the lysis step, 0.5 ml of Lysis I solution, (1.5 mg / ml proteinase K and 2.5 mg / ml lysozyme) were added in 5 ml sterile short-lid tubes and agarose plugs were removed from molds and placed in each tubes.

2. The agarose plugs in lysis solution were incubated at 37 °C for 1 hour in a slightly inclined position on the shaking water bath.
3. After that, it was placed into Lizis II buffer containing 400 µg / ml Protinase K and incubated at 55 ° C for 2 hours in the shaking water bath.

3.2.2.4.(4). Washing of Agarose plugs

1- After the lysis phase, the tubes were placed in ice for at least 15 minutes for solidification of agarose plate and the lysis solution aspirated carefully.
2- The agarose plugs were washed three times with 4 ml of ultrapure water at 50 °C for 15 minutes and then the agarose plugs were washed three times with 4 ml at 50 ° 1X TE C for 15 minutes.
3- Finally, the agarose plugs were stored in 1X TE buffer at + 4 °C until restriction enzyme phase.

3.2.2.4.(5). Restriction Digestion of DNA in Agarose Plugs

1- The agarose plugs were placed on a slide and cut by a scalpel to 1/3 of their size, each of the fractions was placed in 100 µl of 1X enzyme buffer and incubated at 37 °C for 10-15 minutes in a shaking water bath, after 30 minutes the liquid was dispensed (The other parts of agarose plug are stored in TE buffer for + 4 °C for later use).

Table 3.5. The ratio of enzyme buffer and sterile water in agarose plugs.

	P.aeruginosa	***Acinetobacter***	***Klebsiella***	***E.coli***
Sterile water	**90ul**	**90ul**	**90ul**	**90ul**
Enzyme buffer	**10ul**	**10ul**	**10ul**	**10ul**
∑	**100ul**	**100ul**	**100ul**	**100ul**

2- The following mixture was prepared for each agarose plug

Table 3.6.The ratio of enzyme, enzyme buffer and sterile water in agarose plugs

	P.aeruginosa	*Acinetobacter*	*Klebsiella*	*E.coli*
Sterile water		87ul	87ul	87ul
Enzyme buffer	10ul	10ul	10ul	10ul
Enzyme	SpeI:	(ApaI:30U) 3ul	(XbaI: 30U) 3ul	(XbaI: 30U) 3ul
∑	100ul	100ul	100ul	100ul

3- Agarose plugs which were washed by enzyme buffer were added to this mixture and then incubated in a shaking water bath for 4 hours at 37 ° C.
4- At the end of the incubation, the tubes were placed in the refrigerator for 15 minutes.
5- The agarose plugs were ready for electrophoresis

3.2.2.4.(6). Gel Preparation and Casting

İt was done as following steps :

1. The required components to cast a PFGE gel were assembled: casting stand and backplane, leveling table, bubble level, and comb. The casting stand and backplane were assembled, and placed it on the levelling table. The bubble level was placed in the center of the backplane, and the levelling table was adjusted until the bubble was centered. The comb was positioned in the empty tray, and ensured that it sat with the teeth completely level, in contact with the backplane. To prepare for sample loading, the comb was layed down across the casting stand, so that the teeth were horizontal and facing the user.
2. Totally 100mL of a 1% (w/v) agarose gel was prepared by weighing 1.1g of Agarose, and adding 100mL of 0.5X TBE (44.5 mM Trisma base, 44.5 mM Borik asit, 1 mM EDTA, pH 8.0) in a clean, dry 500mL Erlenmeyer flask and swirled gently to dislodge clumps.

3. The cap was placed loosely on the Erlenmeyer flask, and microwaved for 1 to 1.5 minutes, swirling occasionally until agarose was completely dissolved but not over boiled because agarose may superheat and be prone to bumping. Molten agarose was removed from microwave, and transferred to a 45-50°C water bath until it became ready for use.
4. Restricted sample plug slices, carefully were applied to the teeth 2, 3, 4, 5, 6, 7,8, 9, 10, 11, 12, 13 and 14 of the comb by using an a metal spatula and gently positioned against the edge. The molecular markers were inserted to the 1^{st} and 15^{th} teeth.
5. The comb was carefully rotated until it was upright, and set into position in the notches on either side of the casting stand. A metal spatula was used to adjust the plug slices so that they made contact with both the tooth of the comb, and the backplane of the gel casting stand. We ensured that there were no air bubbles, and that the plug slices made complete contact. `
6. Molten agarose was removed from 55°C water bath, and allowed to cool until it could be comfortably held. The agarose was poured into the casting stand, pouring slowly and steadily from a point near the center of the backplane. We took care to avoid introducing air bubbles or dislodging the plug slices from the comb. Air bubbles should be removed or moved to the margins using a metal spatula.
7. Allow the agarose to set for at least 15minutes

3.2.2.3.(7). Gel Loading and Electrophoresis

1. Using a bubble level, we ensured that the electrophoresis chamber was in level, that all hoses are properly attached, and that the system was in good working order.
2. The black gel frame was placed in the electrophoresis chamber, and we ensured that it was properly seated.

1. Two litters of 0.5X TBE by adding 100mL of 10X TBE to 1900mL in a 2000mL graduated cylinder was prepared and gently mixed.
2. All 1900 mL of 0.5X TBE were poured into the electrophoresis chamber.
3. We carefully removed the comb from the gel in a fluid motion, and unscrewed the side panels on the casting stand. Then we gently lifted the backplane from one side and removed the gel from the casting stand, taking care not to dislodge it or compromise its adherence to the backplane. a Kim Wipe was used, the excess agarose was removed from the bottom surface of the backplane, and the gel was carried over to the waiting PFGE system.
1. The gel carefully placed into the chamber, sliding the backplane securely into the frame in the center of the electrophoresis chamber. The gel slightly angled when submerging it to avoid trapping air bubbles underneath the gel.
2. The gel was allowed to equilibrate for several minutes and come down to the ambient temperature of the surrounding buffer.
3. We pressed “START” to begin the run.
4. We placed the completed PFGE worksheet on top of the electrophoresis chamber for reference and recorded the starting current. For a standard gel with 2L of 0.5X TBE buffer, the starting current should register between 110 and 150mA.

Table 3.7 Electrophoresis Conditions

Electrophoresis Conditions	***E.coli ,Acinetobacter, Klebsiella***
Initial time	5sn
Final time	30sn
Voltage	6v/cm
Time	20 saat
Current	150-160 mA

3.2.2.4.(8). Post Staining and Imaging

1. We verified that the electrophoresis run had completed successfully and documented any errors that had registered on the controller display.
2. We opened the electrophoresis chamber, and carefully removed the gel. We gently slided the gel off of the backplane and into a 25cm x 25cm gel staining tray.
3. Ethidium bromide poststain was poured over the gel to submerge it completely. Then it was placed on a rocker platform on low speed for 10 to 15 minutes.

1. **Note:** Ethidium bromide post stain consisted of 500µL of Ethidium bromide in 1L of water, and could be reused up to 10 times. It must be stored away from ambient light to prevent photo bleaching.

4. The gel was resubmerged in reverse osmosis water and returned it to the rocker platform for 30 minutes to an hour to allow for complete destaining.
5. The gel was imaged according to the most current instructions for our gel documentation system software. We ensured that the gel was properly aligned and framed in the camera's field of view before activating the UV trans illuminator. When taking our picture, we took care not to over or under expose the image, and avoided prolonged UV exposure of the gel.

The image was saved in both the editable, native format for the gel documentation system, as well as a tagged image file format (TIFF). For Bio Numerics (Applied Maths, Austin TX), the TIFF should be saved as a black and white image, with a color depth of 8 bits per pixel (8bpp).

3.2.2.4.(9). The solutions used in PFGE method

The solutions which used in the pulsed field gel electrophoresis method were prepared in our laboratory as described below.

a- Cell Suspension Buffer (1x/100ml) pH:8,0

100mM Tris-HCl 1,576gr

100mM EDTA 3,722gr

Complete with 100 ml of ultra-pure water. Adjust pH with NaOH pellet.

b- Lysis I solution (1x/100ml) pH:8,0

50mM Tris-HCl0,788gr

50mM EDTA 1,861gr

Complete with 100 ml of ultra-pure water. Adjust pH with NaOH pellet.

c- Lysis II solution (1x/100ml) pH:8,5

500mM EDTA 18,61gr

Complete with 100 ml of ultra-pure water.

However, NaOH pellets were added to dissolve EDTA. When the pH was adjusted to 8, 22, 1gr N-lauryl sarcosine was added.

**** 400 µg / ml proteinase K should be added to this solution.

d- TE solution (1x/1000ml) pH:7,6

10mMTris-HCl 1,576gr

0,1mM EDTA 0,0372gr

Complete with 100 ml of ultra-pure water. Adjust pH with NaOH pellet.

e- TBE solution (10x/1L)

Tris base 107,8gr

Boric Acid 55gr

EDTA 7,44gr

Complete with 1 L of ultra-pure water.

f- %10 SDS solution

4. RESULT AND DISCUSSION

4.1. Isolation of *E.coli* of food samples

A Total of 34 (70.8%) of *E.coli* were isolated from 48 samples of chicken, raw vegetables, and meat. The prevalence of *E. coli* was 93.75%, 62.5%, and 56,25 in chicken, vegetables, and meat samples, respectively (Table 4.1). The prevalence of *E.coli* O157:H7 was 31.25% in chicken and 18.75% in meat, and vegetables samples, as shown in Table 4.1 and Figure 4.1. The number of *E. coli* and *E. coli* O 157:H7 which isolated from chicken, raw vegetables and meat samples was shown in Figure 4.2.

Table 4.1. Number and percentages of *E.coli* isolated from raw vegetables, chicken and meat using MPN test.

	Sample	**Number of sample**	**No. and % of isolated *E.coli***	**No. of isolated *E.coli* O157** :H7
1	Vegetables	16	10(62.5%)	3(18.75%)
2	Meat	16	9(56.25%)	3(18.75)
3	Chicken	16	15(93.75)	5 (31.5%)
Total		48	34 (70.8)	11(22.9 %)

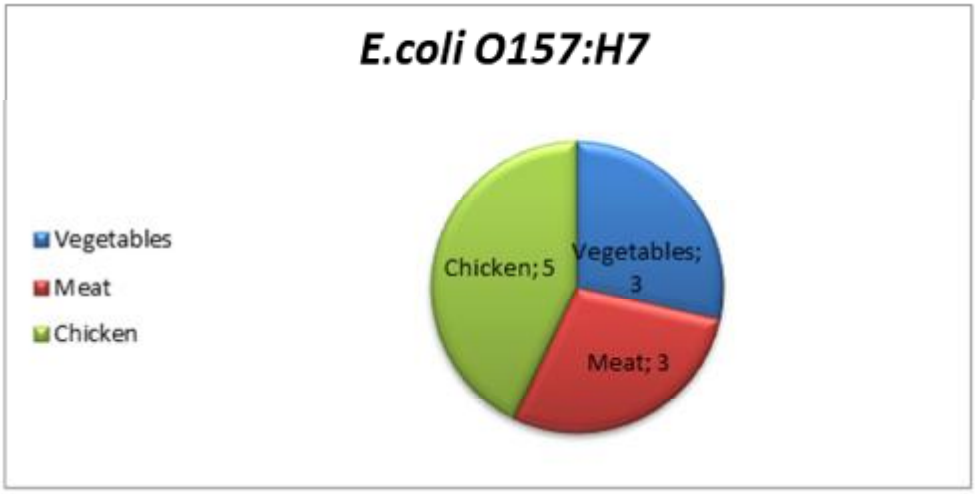

Figure 4.1. The frequency and distribution of *E. coli* O157:H7 among meat, chicken and vegetables samples

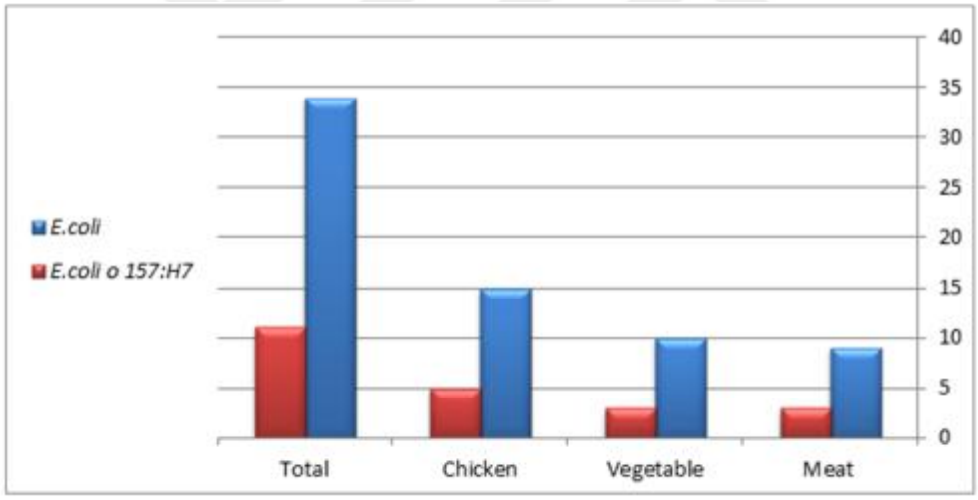

Figure 4.2. The number of *E.coli* and *E.coli* O 157:H7 isolated from raw vegetables, chicken and meat

E.coli O157:H7 is the most studied strains among all other pathogenic strains of *E.coli* because it has been recognized as the leading causes of human food born infections throughout the world with fatal complications such as hemolytic uremic syndrome that ends in renal failure. The food such as

contaminated beef ground meat, water, raw milk, fruit juice and vegetables can be transmitted this strain to humans by direct and indirect ways. It has been recognized that vegetables can be an important vehicles for transmitting *E.coli* to humans.

Our study has shown that *E. coli* was detected in 70.8 % of samples which is higher than those conducted in Iraq by Saeed et al. (2013) who found that *E. coli* was found in 19.3% of unwashed raw vegetables. On other hand our result is lower than those conducted in Nigeria by Enabulele et al. (2009) who found that *E. coli* was detected in 85.65 %, while *E. coli* O157: H7 was detected in 4.63% of fresh meat samples .Tanih et al. (2015) in their study found that *E.coli* was the most prevalent bacteria in meat sample 67.5% (104/154). Iyer et al. (2013), who showed that a high incidence of *E. coli* was in open butcher shops (65%) and these studies are close to our study .Our finding have also reported the prevalence of *E.coli* O157:H which ranged from 18.75 % to 31.25 % in meat, vegetables, and chicken, this finding is close to that of Sharif et al. (2014) who also found the prevalence of *E.coli* O157:H7 ranged from 0% to 33% in onions and cabbage respectively. However, our study is contrary to other studies which did not find *E.coli* O157:H7 in their study on unwashed raw vegetables (Saeed, 2013). Enabulele et al. (2009) found *E.coli* O157:H in meat samples but it was lower of our finding. Several studies have also reported the presence of *E. coli* O157:H7 in chicken samples, Karadal et al. (2013) found that *E. coli* O157:H7 was detected in 1% (1/100) in processed chicken samples, and Momtaz et al. (2013) who also detected *E. coli* O157:H7 in chicken samples which was 7.3% (31/422). Doyle et al. (1987) who found *E. coli* O157:H7 in 4/263 (1.5%) of fresh and uncooked complete chicken samples. Nkanga et al.(1981) who reported that meat is frequently found to be contaminated because of poor sanitary environment through slaughter of animals and during transportation, usage and handling. *E.coli* is the normal flora in the digestive tract of both humans and animals and could contaminate the vegetables

from different source either from animal manures used as soil fertilizer or through contaminated transportation vehicles. In Thailand, Suthienkul et al.(1990) assayed 107 chicken carcasses samples and they isolated *E. coli* from 9 (1 %) samples. The other study was performed in the UK by Adams et al who detected *E. coli* O157:H7 in 1-2% of lamb, chicken, and pork meat samples. In Egypt, Abdul-Raouf et al. (1996) assayed 50 chicken meat samples, and detected *E. coli* O157:H7 in 2 samples. In France Rozand et al. (1997) examined 250 samples 160 chicken drumstick samples and 160 chicken breast samples for *E. coli* O157:H7, the researchers documented that *E. coli* O157:H7 was detected in 2.5% (4) of drumstick samples (Ahmed and Shimamoto, 2014). Many factors such as geographical area samples size, and agricultural methods for vegetable production and different conditions of sanitary environments during slaughter and transportation of animals could contribute Nkanga et al. (1981) who reported that meat is frequently found to be contaminated because of poor sanitary environment through slaughter of animals and during transportation, usage and handling. *E.coli* is to these differences in the results. the normal flora in the digestive tract of both humans and animals and could contaminate the vegetables from different source either from animal manures used as soil fertilizer or through contaminated transportation of meat products and meat, and they reported that 4 (1.6%) of samples were contaminated with non verotoxin producing-*E. coli* O157:H7. A study in Egypt assayed vehicles. Var et al .(2014) in their study, the total number of coliform bacteria and *E coli* counts in a total of 28 chicken meat specimens which including 22 chicken carcasses, 2 drumstick, 2 breast, 1 wing and 1 wrap were analyzed by the Most Probable Method. They reported that all of the samples examined in this study were found to be contaminated with coliform bacteria and *E coli*.

In conclusion, the presence of *E.coli* TypeI and *E.coli* O157:H7 in chicken, vegetable, and meat determined in the present study, suggests that they may be a main cause of human infections because of this organism. In order to protect public

health, a series of control strategies should be developed and implemented at all stages of food production, system “from farm to table”, to eliminate contamination with this important human pathogen. More epidemiological studies are needed in order to determine the possible role of poultry and cattle as a source and/or reservoir of *E. coli* O157:H7. In addition, the exact mode of entry of this bacterium into food chain should be determined in order to develop and implement control measures.

4.2. Prevalence of siderophore genes

Eight of siderophore-related genes were determined in the *E.coli* strains (100) by the PCR method which were as follow: 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 % (Figure 4.3) . Among the 66 ExPEC isolates, the prevalence of individual siderophores genes rangeed from 15% salmochelin receptor (Iro N) to 95% enterobactin receptor (FepA) Table 4.2. Seven of genes encoding siderophores were occurred in at least one isolate of ExPEC**,** the prevalence of the siderophore-related genes was as follows: enterobactin receptor (Fep A), 97%**;** enterobactin biosynthesis (Ent A), 97%**;** yersiniabactin receptor (Fyu A), 91%; heme receptor (Chu A), 86%; aerobactin receptor (Iut A), 72%; aerobactin biosynthesis (Iuc A), 71%; and salmochelin receptor (Iro N),15%, while Iron-responsive element (IreA) was not detected in ExPEC isolates (Figure 10.2)**.**

On the other hand the prevalence of the siderophore-related genes among 35 *E.coli* strains which isolated of 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*, which are 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%) (Figure 4.4). All the the

siderophore-related genes occurred , significantly, more frequently among ExPEC strains than among food strains except for ire A which was detected only in food strains (Table 4.3; Figure 4.5).

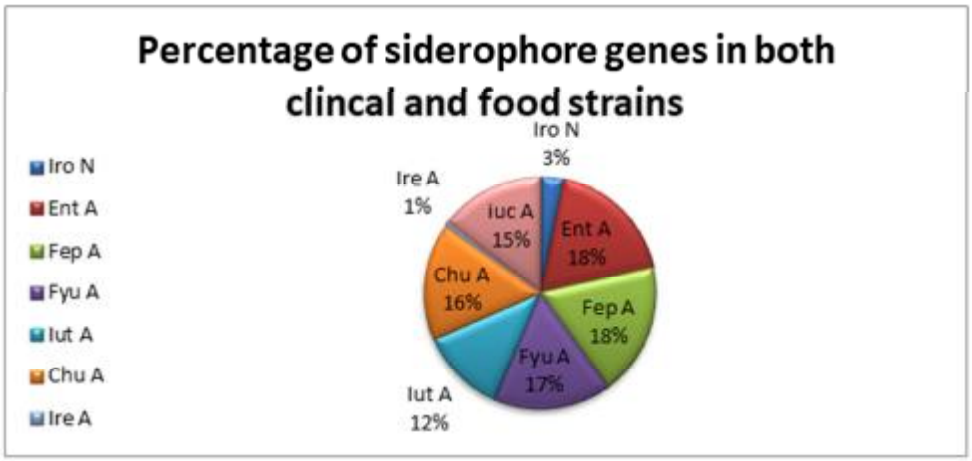

Figure 4.3. The Frequency of siderophore-related genes among *E.coli* strains

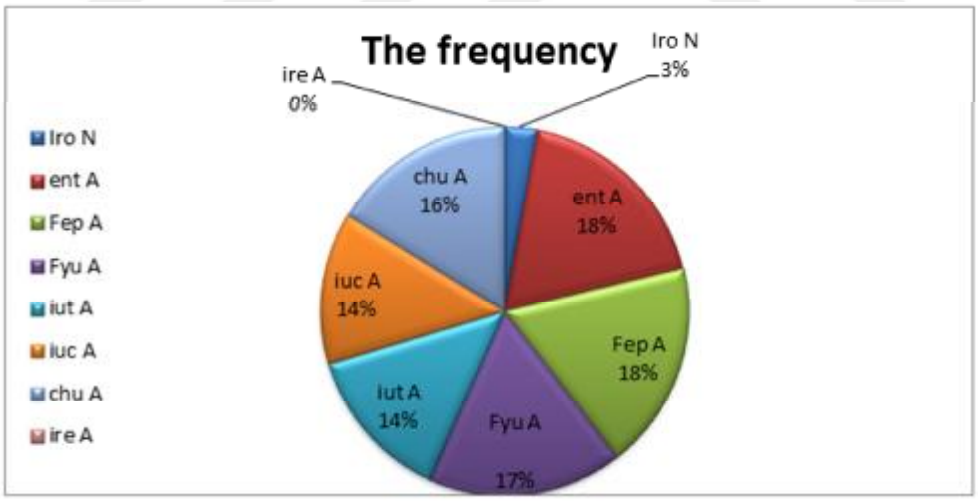

Figure 4.4. The frequencey of siderophore-related genes among ExPEC isolates

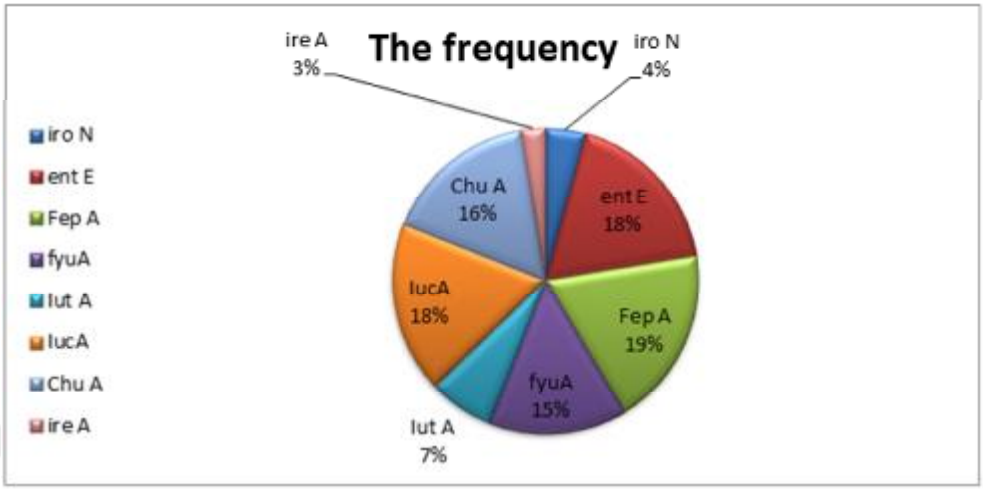

Figure 4.5. The frequency of siderophore-related genes among *E.coli* strains of food.

The components of the siderophore genes were higher in females than males, except for iut A, which was found more frequently in males than in females and ire A, which was similar in both sexes (Table 4.2; figure 4.6) .

Table 4.2. Distribution of siderophore-associated genes in Urine, Blood ,Wound, and Aspiration sample among 66 *Escherchia coli* isolates of clinical samples.

	Urine N=54	Blood N=3	Wound N=5	Aspiration N=3	Total N=65	Woman 46(100%)	Man 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

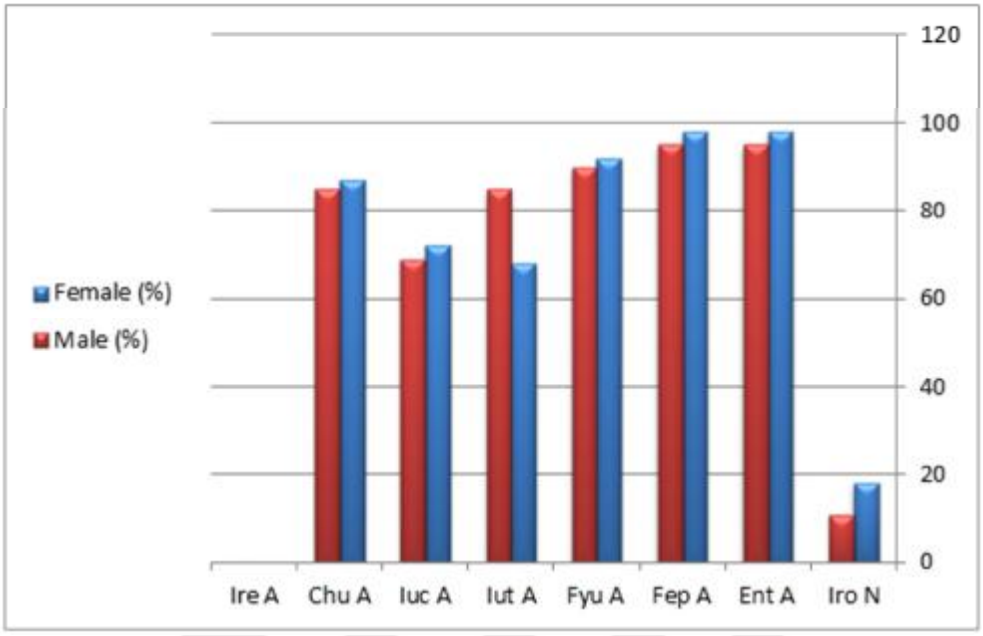

Figure.4.6. The comparison of distribution the siderophore-related genes in women and men.

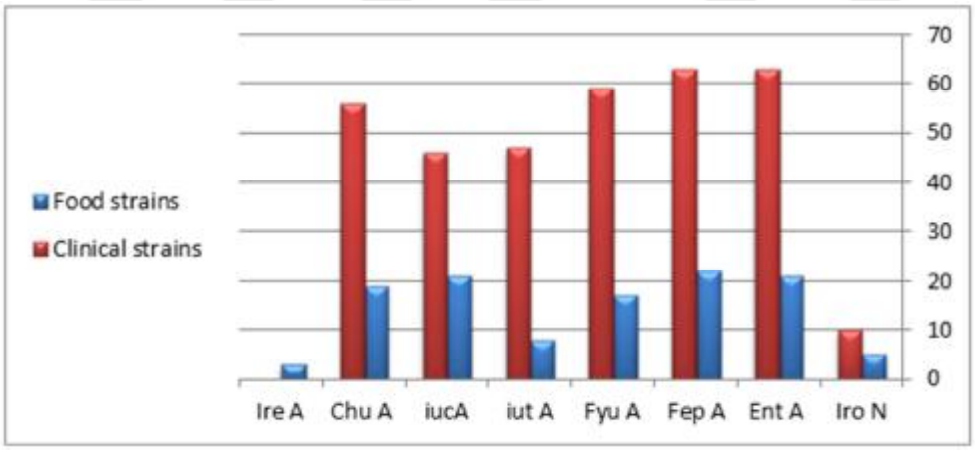

Figure 4.7. Comparison between the distributions of siderophore related genes in clincal strains and food strains.

Table.4.3. Comparison between the distributions of siderophore related genes in clincal strains and food strains.

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

Extraintestinal *Escherichia coli* (ExPEC) are a diverse group of pathogenic *E. coli* strains, which have high ability to colonize several extraintestinal sites, as the blood stream, the meninges and the urinary tract, and this ability is dependent upon specific virulence factor genes. The great differences have been showed through comparison of whole-genome of *E.coli* strains which cause different diseases (i.e. intestinal and extraintestinal infections) (Welch and Burland, 2002). These differences are also present within ExPEC strains which are a cause of the same disease and infect the same host tissues (Dobrindt and Blum, 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 and Eto, 2005). 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 has shown that, the prevalence of siderophore genes were as follow: fyuA (n=59, 91%), iutA (n=47, 72%), and chuA (n=56, 86%) in ExPEC strains which are higher than those recorded by Safi et al. (2106) in Tunis who found that fyuA (74%), iut A (56%), and chuA (65%) in urine *E.coli* isolates, while iro N was 59% which is higher than our result iro N (10%).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. Our finding has also shown that Ire A gene was not detected in ExPEC strains while it was detected in 9% of food strains and this 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; while , it was detected in 55% (6/11) of the non-UTI cases. In our study the fyu A and iut A genes were present in the ExPEC strains as follow: 91 %(n=59), 72% (n=47), which are of the bacterial iron acquisition systems. However, the prevalence of these genes were much less in Food strains, which was as follow: fyuA 49% (n=17), and iut A 23% (n= 8). This finding supports the importance of fyuA in virulence, particularly in the establishment of symptomatic UTI. The relationship between fyuA and invasive UTI had been suspected in previous studies (Cheng and Tsau, 2011), and our result is 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). 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 (Gar and enaux, 2011).The aerobactin receptor (IutA) is commonly associated with ExPEC (Moreno,2005).

Koga et al. (2014) they found through molecular screening of virulence genes in ExPEC isolated from human blood culture that the most common virulence gene was iut A was detected in 65% of the isolates tested, while iut A was not detected in commensal isolated and this result is close to our result. They have also reported the presence of iroN and fyuA genes which were found in 55% and 45% of isolates, respectively, while our results showed that iro N was in 15% of isolates and fyu A was in 91% of isolates. Our study have also showed that the prevelence of Iuc A gene was present in 71% of ExPEC strains and it is close to those documented by Palma et al. (2016), who also found that iuc A was present in 67.7% of isolates, while they found that iut A and fyu A were found in 83.1% and 49.2% of isolates, respectively. On the other study, Khan el al. (2015) they found that iutA was present in 70.4% of isolates, ireA was present in 92.5% and fyu A was present in 77.5% of isolate. The high level of siderophore-related genes among our strains is in accordance with other descriptions (Burdet and Clermont, 2014), highlighting the relevance of this kind of virulence factor in the development of bacteremia. This is related to the iron needs of bacteria, which contains approximately 10^5-10^6 Fe atoms, and to the difficulty to access iron within human fluids due to binding to transport molecules such as ferritin or lactoferrin (Bidet and Bonarcorsi, 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 and Pramanik, 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 results which showed that siderophore genes are higher prevalence in ExPEC isolates than food isolates. ExPEC strains expressed different combinations of these genes, with some strains expressing all siderophores.

4.3. Determination of the phylogenetic relationship among E coli strains, which were isolated from food and human

In this study, the phylogenetic relationships between *E.coli* strains isolated from food and human were studied by PFGE method (PFGE) using restriction enzyme XbaI, to constitute evidence of transmission of pathogenic *E-coli* strains to human from food, and the distribution of siderophore genes have also studied by PCR method. Gel-compar II program was used to evaluate and interpret the DNA fragment profiles obtained from PFGE method. The relationship between strains was determined by similarity coefficient of DNA profiles and cluster analysis was performed on the basis of ≥ 80% of similarity ratio and the similarity ratios were shown in dendograms (Figure 4.10). The genotype analysis of *E.coli* strains by XbaI-PFGE method show that:

- Only 13 (20.96%) out of the 62 clinical strains were clonally related to each other and these strains were distributed in a total of 5 clusters (Ah, Ch, Eh, Fh and Gh), the cluster F included two of subgroup with 4 members (hu34-hu35-hu43-hu42), the remaining strains were distributed in unrelated clusters with one member.
- Only 4 (14.81%) out of 27 the food strains, were clonally related to each other, and they were in two clusters (A and D), the remaining strains form single member clusters.
- Three strains of clinical samples and 3 strains of food borne samples were collected in 2 clusters, 2 members in B cluster and 4 members in A cluster and there was no clonal relationship between other clinical and food isolates (Table 4.4).

As a result, the evaluation of PFGE analysis showed that 6 (6.8%) out of 89 human and foodborne isolates had a clonal relationship, while there was no

significant relationship between the other isolates, this number of evaluated isolates was not sufficient to produce the statistical significance, and there was no strong evidence of transmission of ExPEC to human from food.

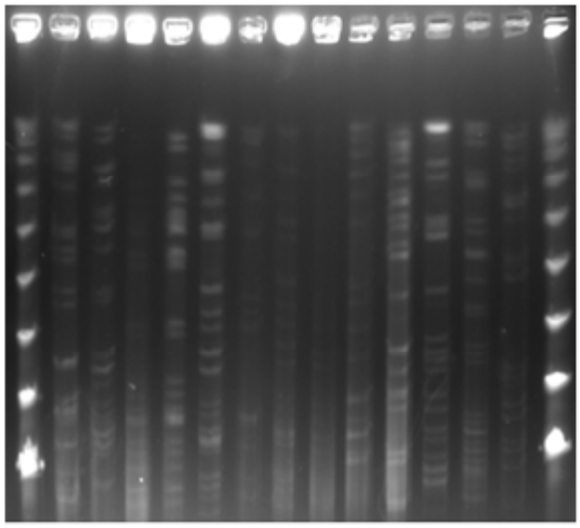

Figure 4.8. Pulsed-field gel electrophoresis (PFGE) profiles of selected *Escherichia coli* isolates from humans

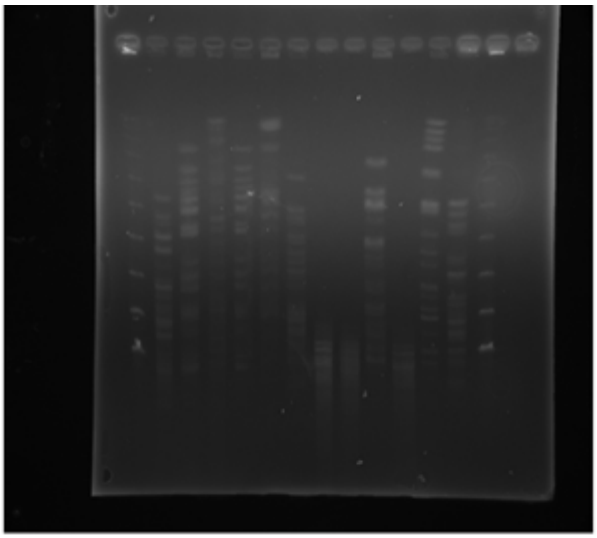

Figure 4.9. Pulsed-field gel electrophoresis (PFGE) profiles of selected *Escherichia coli* isolates from food.

Table 4.4. Phylogenetic relationship among *E.coli* strains

Sample	Cluster	subgroup	isolate	Phylogenetic relation (%)
Clinical samples	A	a_h1	hu52	85
		a_h2	hu22	
	C	c_h1	hu17	87
		c_h2	hu18	
		c_h3	hu26	
	E		hu33/ hu41	80.6
	F	f_h1	hu34/ hu35	88.9
		F_h2	hu43/ hu42	
	G		hu37/ hu45	88.7
Food samples	A	a_f1	f9	85
		a_f2	f11	
	D		f10/f12	85.3
Clinical and food samples	A	a1	hu52/ f 9	85
		a2	hu22/f11	
	B		hu25/f2	87.1

The phylogenetic analysis between the human *E. coli* and Food-borne *E. coli* strains were studied by PFGE method and the prevalence of siderophore genes in PFGE clusters of these strains were examined, and the result was as that :

- Ire A gene was not found in any of the isolates.
- The fep A gene was the most common in 17 isolates.

- The presence of the chu A gene and the absence of iro N and ire A genes were the common features between one isolate of human and one isolate of foodborne which were in subgroups of A1.
- The simlarity of siderophore genes of two strains of foodborne in D group was 100% and the simlarity of siderophore genes of two strains of human in f1 and one strain in f 2 was also 100%.
- PFGE analysis of isolates belonging to human and food samples, which had 93.2 % of similarity and located in subgroup 2, they had same 75 % of siderophore genes.

The prevalence of the siderophore genes in food-borne isolates was less than human-borne isolates, and It was concluded that the ability to produce siderophore was higher in human *E. coli* isolates (Table 4.5; Table 4.6). The prevalence of siderophore genes in human strains (ExPEC) are assoiated with virulence factors and their incidence and diversity are very high in ExPEC strains. As a result the analysis of siderophore genes distribution alone and with PFGE analysis showed that there were not clonal correlations between food-borne strains and ExPEC strains. It has been concluded that ExPEC strains may have been the result of mobile genetic elements or chromosomal mutations in human and animal intestinal systems and this confirmed the hypothesis that individual virulence gene acquisitions can occur concurrently against a background of horizontal gene transfers of pathogenicity islands. Over time, this could enable specific clonotypes to respond to host selection pressure and to evolve into new strains with increased virulence.

Table 4.5.The prevalence of siderophore genes in PFGE clusters of *E.coli* strains

Cluster	Subset	Strain No	IroN	EntA	FepA	FyuA	luA	lucA	chuA	İreA
A	a1	hu52	-	+	+	+	+	+	+	-
		f9	-	-	-	-	-	-	+	-
	a2	hu22	-	+	+	+	-	-	+	-
		f11	+	+	+	+	-	-	-	-
B	b1	hu25	+	+	+	+	-	+	+	-
		f2	-	-	+	+	-	+	-	-
C	c1	hu17	-	+	+	+	-	-	+	-
		hu18	+	+	+	+	-	-	+	-
	c2	hu26	-	+	+	+	+	-	+	-
D	d1	f10	-	+	+	-	-	-	-	-
		f12	-	+	+	-	-	-	-	-
E	e1	hu33	-	-	-	+	+	-	+	-
		hu41	-	+	+	+	+	+	+	-
F	f1	hu34	-	+	+	+	+	+	-	-
		hu35	-	+	+	+	+	+	+	-
	f2	hu43	-	+	+	+	+	+	+	-
		hu42	-	+	+	+	+	+	+	-
G	g1	hu37	-	+	+	+	+	-	+	-
		hu45	-	+	+	+	+	+	+	-

Table4.6. Comparison of similarities between siderophore genes in PFGE clusters of *E.coli* strains.

Cluster	Subset	Strain No	IroN	EntA	FepA	FyuA	luA	lucA	chuA	İreA
A	a1	hu52	-	+	+	+	+	+	+	-
		f9	-	-	-	-	-	-	+	-
	a2	hu22	-	+	+	+	-	-	+	-
		f11	+	+	+	+	-	-	-	-
B	b1	hu25	+	+	+	+	-	+	+	-
		f2	-	-	+	+	-	+	-	-
C	c1	hu17	-	+	+	+	-	-	+	-
		hu18	+	+	+	+	-	-	+	-
	c2	hu26	-	+	+	+	+	-	+	-
D	d1	f10	-	+	+	-	-	-	-	-
		f12	-	+	+	-	-	-	-	-
E	e1	hu33	-	-	-	+	+	-	+	-
		hu41	-	+	+	+	+	+	+	-
F	f1	hu34	-	+	+	+	+	+	-	-
		hu35	-	+	+	+	+	+	+	-
	f2	hu43	-	+	+	+	+	+	+	-
		hu42	-	+	+	+	+	+	+	-
G	g1	hu37	-	+	+	+	+	-	+	-
		hu45	-	+	+	+	+	+	+	-

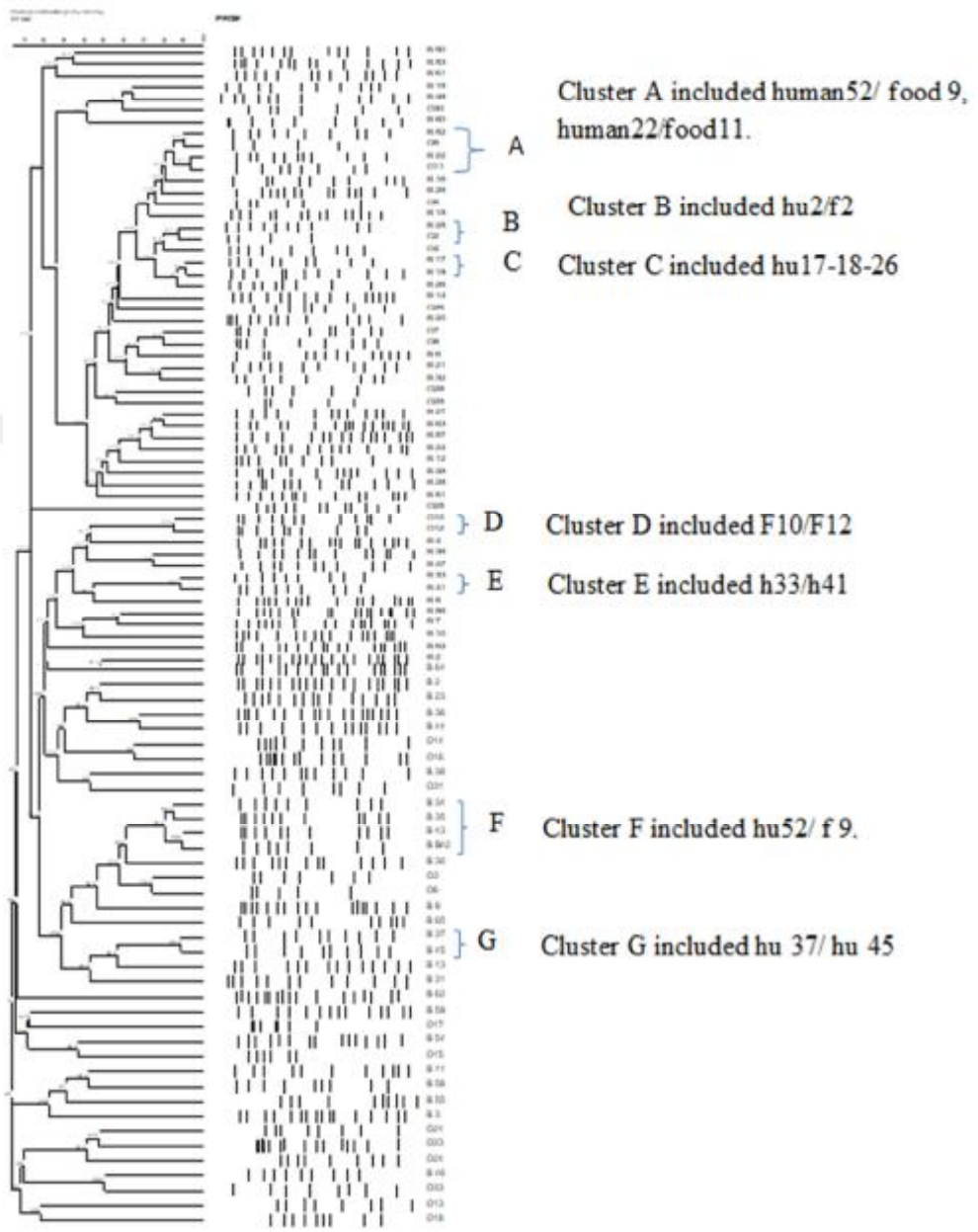

Figure 4.10. Dendrogram depicting inferred phylogeny of 89 isolates using PFGE method.

We have reported that the identification of *E. coli* isolates from retail chicken, meat and other food sources, and then we determinated the genetic relatedness of these strains with *E.coli* strains (Extraintestinal *E.coli*) isolated from human. Several previous studies suggested that *E.coli* strains which were isolated from retail meat (specifically retail chicken meat) were able to cause human extraintestinal infections. Our study has determined genetic similarities between human clinical and food isolates to support the previous studies. Johnson et al. (2006), have documented that there were similar between antimicrobial-resistant and –susceptible *E.coli* from retail chicken meat sources and antimicrobial drug– resistant *E. coli* from human bloodstream infections and human feces . They found that only one instance of a close match between chicken and a human isolate by PFGE analysis. Our study found that three strains of human and three strains of food were collected in 2 clusters, 2 members in B cluster and 4 members in A cluster (table 4.4 ; figure 4.10). Our study was close to those documented by Vincent et al. (2010), who have found that some *E. coli* strains from retail poultry meat sources and other food sources were closely related to *E. coli* strains causing human urinary tract infections (UTIs), they could able to identify of two clonal groups containing isolates from retail poultry meat and human infections (Vincent,2010). Bergeron et al (2012) showed that cecal samples from food animals in abattoirs and human samples could belong to the same clonal groups. The three major clonal groups (groups 1, 2, and 3) with the highest level of similarity included isolates from abattoir and retail meat , which suggested that animal source foods might serve as a reservoir for ExPEC in humans.

There were other several studies in North America which have also documented the role of retail meat isolates in human ExPEC infections. Hannah et al. (2007), they could identify ExPEC in 14% of isolates tested (retail meat isolates, human clinical infections and stool) and virulence characteristics seemed similar, but PCR-based and PFGE patterns were not related. In Canada Bergeron et al

(2012) have also found that there were high levels of ExPEC in poultry versus beef and pork retail and evidence of clusters of *E. coli* from retail poultry meat and human infections that were similar by PCR and PFGE methods and this study was similar to our study. Existing evidence showed that retail poultry meats are closely related to human ExPEC. Overdevest et al. (2011) have documented that poultry-associated *E. coli* could be more extensively antimicrobial resistant than *E.coli* isolated from other meats. Our study have shown that the PFGE analysis of isolates belonging to human and food samples, which had 93.2 % of similarity and located in cluster A (subgroup a_2), they had same 75 % of siderophore genes, this similarity of virulence genes between food-associated *E.coli* and human ExPEC, suggesting the potential to cause human disease. This would underscore the potential threat posed by such strains and their resistance plasmids, some of which also carry other VFs such as iss, traT, and iroN, since aerobactin-encoding resistance plasmids are often conjugally transferable and may provide a vehicle for coordinate horizontal transfer of VFs and resistance elements (Hannah and Johnson, 2009). Johnson et al (2010) they found that the resistant of *E.coli* isolates in human and chicken had extensive overlap by virulence profile analysis, and similar types of *E. coli* could be a cause of fluoroquinolone-resistant infections in humans and contaminate retail poultry products. Hannah et al. (2009) assessed the virulence potential of food-source *E. coli* and examined the extent of commonality between food-source and geographically and temporally matched human-source (commensal and clinical) *E. coli* with respect to genomic background and VF profiles, all in relation to antimicrobial resistance, they found as that first, an appreciable minority (19%) of antimicrobial-resistant retail meat isolates represented ExPEC, suggesting possible human pathogenic potential, and they found that only 14% of meat-source *E. coli* isolates (19% if resistant) qualified as ExPEC, compared with 61% of stool isolates and 74% of clinical isolates, whereas the few that did qualify exhibited VF profiles quite different from the VF profiles of human-source ExPEC isolates. A likely factor underlying the observed emergence of anti- microbial-resistant *E. coli* strains

resembling human ExPEC in food animals, especially poultry, is the disproportionate increase in human consumption of retail chicken during the past 30 years (Lawrence, 2006). This has led to changes in the scale and methods of food animal production, including increased use of antimicrobial agents for growth promotion, feed efficiency, infection prevention, and treatment. Multiple lines of evidence indicate that these changes have helped to select for and amplify multidrug-resistant ExPEC (Manges, 2012). Resistant *E. coli,* once established in food animal reservoirs, can spread among animals, maintaining the circulation of drug-resistant ExPEC in animal source foods. Norman et al. (2015) found that cattle-derived *E.coli* and human *E.coli* had both similar virulence gene profiles and they had the same polymorphism derived genotypes, and this finding had supported that similar strains were found in humans and cattles and transmission between the two species might occur. Similarities between *E. coli* recovered from retail chicken, turkey, and pork products and human ExPEC have suggested a probable role for a food animal reservoir. Transmission of antimicrobial-resistant *E. coli* to humans by food- borne had supported when these strains were found in the intestinal tract of individuals who worked to prepare carcasses of chicken in kitchen. Moreover, volunteers fed a diet of irradiated food exhibited markedly reduced levels of antimicrobial-resistant intestinal *E. coli,* with reversion to baseline after intervention, implying that a conventional diet sustains the baseline prevalence of intestinal resistant *E. coli* through a steady input of food-borne resistant organisms. Even though the size and scope of this study is limited, we are able to detect several instances of groups containing closely related isolates from human and food sources. It is therefore probable that a food reservoir exists and that food-borne transmission of extraintestinal *E. coli* is common.

5. CONCLUSION AND FUTURE WORK

5.1. 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 microorganismssurvive under such iron-limited conditions by secretingsiderophores. 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.The fight for iron represents a robust ‘tug-of-war’, which begins with the host secreting iron-binding proteins such as lactoferrin to sequester any readily available iron. When deprived of a readily available iron source, pathogens upregulate siderophore biosynthesis and iron-trafficking pathways. Siderophores have much higher affinity for iron and can strip iron from lactoferrin and other host iron-binding proteins, restoring access to iron for pathogens. As the molecule with the highest known affinity for ferric iron, Enterobactin (Ent) represents a huge challenge to the host. To oppose the acquisition of iron by Ent, host epithelial cells and neutrophils secrete the protein Lipocalin 2 (Lcn2). Initially discovered as a neutrophil granule protein, it was later shown to bind to and sequester Ent with high specificity and affinity. The affinity of Lcn2 for Ent is similar to that of its bacterial receptor FepA, allowing Lcn2 to effectively compete with bacteria for binding to Ent, thus limiting bacterial growth. Due to the strong bacteriostatic effects of Ent sequestration by Lcn2, bacteria have developed mechanisms to evade Lcn2 in their quest to obtain iron. One mechanism of

evasion is to modify Ent through the addition of glucose groups to produce Salmochelin (Sal), Thus we concluded that the high prevalence and many kind of siderophore genes were found in ExPEC isolates, because there was difficult to get enough iron from host because of its defence systems (Lipocalin 2), while the siderophore-related genes were also found in food strains but it was lower than ExPEC isolates, because food isolate can easily get iron from environment because there is not defence system in its environment. It is also is well accepted that bacteria such as *E. coli* can move passively to humans through a variety of routes, including the food chain. Because of the similarity between some human and animal *E.coli*. Similarities in strains isolated from different host species should be expected, particularly with respect to specific virulence factors (VF). The individual virulence gene acquisitions can occur concurrently against a background of horizontal gene transfers of pathogenicity islands. Over time, this could enable specific clonal types to respond to host selection pressure and to evolve into new strains with increased virulence. Phylogenetic analyses has shown that the natural *E.coli* isolated are mainly clonal and constitute four major phylogenetic groups: A, B1, B2 and D. ExPEC strains, which are cause of most ExPEC infections in non-compromised hosts, are as different from commensal *E. coli* as the intestinal pathogenic *E. coli* type. The intestinal pathogenic strains have distinctive virulence factors (e.g. shigatoxin, ST, LT or intimin) which are responsible for their particular diarrhoeal syndromes. ExPEC strains are different from commensal and intestinal pathogenic *E.coli,* because they derive from group B2 and, to a lesser extent, group D. Further these phylogenetic groups contain particular virulence genes (papA, P fimbriae; kpsMT, group II capsule synthesis; sfa/foc, S and F1C fimbriae; *iutA*, aerobactin receptor) and these indicate a high association of these VFs to ExPEC strains. Recent studied have demonstrated that almost all ExPEC strains possess virulence factors by a stepwise accumulation through horizontal gene transfer, they have evolved from an ancestral strain. Acquisition and

expression of these virulence factors may require the specific genetic background. However, the sole association of an *E. coli* strain with a certain phylogenetic group does not necessarily render the strain pathogenic or non-pathogenic, as the adaptation of a strain to a host also contributes significantly in determining its virulence.

5.2. Future work

Siderophores are extremely versatile molecules that capture iron from the environment to ensure microbial iron homeostasis. Humans have been exploiting these molecules for medical and environmental applications for many years. In the medical field, highly satisfactory progress has been achieved and siderophores are routinely used in the clinic to treat iron overload diseases. Siderophore-conjugated antibiotics are now tested in clinical trials to overcome bacterial resistance and improve antiinfective therapy. Siderophores could also be used to inhibit metalloenzymes, of which many are responsible for disease pathologies, an aspect that still requires a lot of research.

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