FIRAT UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
TÜRKİYE

INVESTIGATION OF ANTIMICROBIAL SENSITIVITY AND ANTIBIOTIC RESISTANCE GENES OF E.COLI STRAINS ISOLATED FROM DUHOK (IRAQ) REGION BY MOLECULAR METHODS

Manar Mohammed Ghazal JIRJEES

Master's Thesis

DEPARTMENT OF BIOLOGY

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THESIS APPROVAL

This thesis, which was prepared according to the thesis writing rules of the Graduate School of Natural and Applied Sciences, Fırat University, was evaluated by the committee members who have signed the following signatures and was unanimously approved after the defense exam made open to the academic audience.

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DECLARATION

I hereby declare that I wrote this Master's Thesis titled “Investigation of Antimicrobial Sensitivity and Antibiotic Resistance Genes of E.coli Strains Isolated from Duhok (Iraq) Region by Molecular Methods” in consistent with the thesis-writing guide of the Graduate School of Natural and Applied Sciences, Firat University. I also declare that all information in it is correct, that I acted according to scientific ethics in producing and presenting the findings, cited all the references I used, express all institutions or organizations or persons who supported the thesis financially. I have never used the data and information I provide here in order to get a degree in any way.

22 April 2022

Manar Mohammed Ghazal JIRJEES

PREFACE

After thanks to Allah, this clinical molecular microbiology thesis was about E.coli was taken from urine samples from all hospitals in Dohuk and Zakho city . I investigate to find the suitable antibiotics to treat this infection by E. coli and find in molecular level the genes which responsible for non-curable infection. The difficulties of this research was some patients do not agree to give me the samples for this reason some time I delayed to get samples. Also I have special thanks to my dear supervisor Prof. Dr. Ökkeş YILMAZ in Firat University for his endless support, great help, and fruitful and useful suggestions, without his helps and guidance this study could not be done. I have thanks to Assist. Prof. Dr. Nermin Saeed Merza in Biology Department in University of Duhok, which helped me in start to end of this study, and I show my appreciation to assistant Prof. Dr. Marwan Khalil QADER for their efforts for my thesis to genetic studies.

Sincere appreciation and thanks to my dear mother and my late father that help me in all filed of my life. Special thanks to my dear husband that aid me and always with me….

Manar Mohammed Ghazal JIRJEES

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ABSTRACT

Investigation of Antimicrobial Sensitivity and Antibiotic Resistance Genes of E.coli Strains Isolated from Duhok (Iraq) Region by Molecular Methods

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In this study, 365 E. coli UTI infection isolates were collected from clinical cases from three major hospitals in Duhok and Zakho / Iraq during the period from March 2021 to September 2021.

43 biochemical tests were performed to identify the species of E. coli isolates collected. 205 (56.16%) of these were isolated from male patients and 165 (43.83%) from female patients. 100 E. coli isolates were randomly selected and subjected to antibiotic susceptibility testing. Many of those tested showed high levels of resistance to the antibiotic. Of all the antimicrobial agents, imipenem was detected to be the strongest, while Ampcillin, Gentamicin, Amikacin, and Nalidixic acid had the least effect on these isolates. Genomic DNA was extracted from these selected isolates using two methods. One hundred isolates were extracted with a commercial kit, with an average of the concentration of genomic DNA extracted using the commercial kit at 115.25ng/µl with a purity of 1.8.

To confirm that the genome of all these isolates is E. coli, all strains with a molecular weight of approximately 657 bp were successfully amplified, producing a single band of uidA as the species-specific gene. The results revealed that Pai and hyl genes related to virulence and antibiotic resistance were absent from any of the tested markers in 10 (28%) of these isolates. As a marker for the presence of a pathogenicity island, the Pai gene is the most dominant marker among all other virulence markers with 75%, followed by the hyl gene with 69 percent.

Keywords: *E.coli,* UTIs, Antibiotic*, uid*A genes*, hyl* gene, *Pai* gene

ÖZET

Duhok (Irak) Bölgesinden İzole Edilen E.koli Suşlarının Antimikrobiyal Duyarlılık ve Antibiyotik Direnç Genlerinin Moleküler Yöntemlerle Araştırılması

Manar Mohammed Ghazal JIRJEES

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Bu çalışmada, Mart 2021'den Eylül 2021'e tarihine kadar olan bir süre içinde Irak'taki Duhok ve Zakho adlı şehirlerdeki üç büyük hastaneden 365 adet E. koli IYE (İdrar Yolları Enfeksiyonu) izolatı klinik vakalardan bir araya getirilmiştir.

Toplanan E. koli izolatlarının türlerini tespit etmek amacıyla 43 tane biyokimyasal test uygulamaya koyuldu. Bunlardan 205 adedi (%56.16'sı) erkek hastalardan, 165 adedi (%43.83'ü) kadın hastalardan izole edildi. 100 adet E. coli izolatı (Duhok ve Zakho ilçelerinin her ikisi de dâhil olmak üzere) rastgele seçildi ve bu izolatlar antibiyotik duyarlılık testine tabi tutuldu. Test edilen izolatların birçoğu antibiyotiğe yüksek düzeyde direnç gösterdi. Mevcut olan tüm antimikrobiyal ajanlar arasında en güçlü olanın İmipenem olduğu tespit edilirken, Ampcillin, Gentamicin, Amikacin ve Nalidiksik asitin bu izolatlar üzerinde en az etkiye sahip olduğu gözlemlendi. Bu seçilen izolatlardan iki yöntem kullanılarak genomik DNA ekstrakte edildi. 100 adet izolat, ticari bir kit aracılığı ile ekstrakte edildi; ticari kit kullanılarak ekstrakte edilen genomik DNA konsantrasyonunun ortalaması 1.8 saflık ile 115.25ng/µl düzeyindeydi.

Bu izolatların tümünün genomunun E. koli olduğunu doğrulamak için yaklaşık olarak 657 baz çifti moleküler ağırlığa sahip tüm suşlarda türe özgü gen olarak uidA'nın tek bir bandını üreterek başarılı bir şekilde amplifiye edildi. Elde edilen sonuçlara göre, virülans ve antibiyotik direnci ile ilgili Pai ve hyl genlerin bu izolatlardan 10 tanesinde (%28) test edilmiş herhangi bir belirteçten yoksun olduğunu ortaya çıkardı. Pai geni, patojenite adasının varlığı için belirteç olarak %75 oran ile diğer tüm virülans belirteçleri arasında en baskın olan belirteçtir ve bunu yüzde 69'luk bir oran ile hyl geni takip etmektedir.

Anahtar Kelimeler: *E. coli,* IYE, Antibiyotik*, uidA* geni, *hyl* geni, *Pai* geni

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ABBREVIATIONS

Abbreviations

CD : Collecting duct
CGA : Clonal group A
Cnf1 : Cytotoxic Necrotizing Factor1
CPS : Capsular polysaccharides
ECOR : Escherichia coli Reference
EMB : Eosin methylene blue
ESBLs : Extended spectrum beta lactamases
GC : Guanine Cytosine
GIs : Genomic islands
hyl : a-Haemolysin
IBC : Intracellular bacterial communities
ICU : Intensive care unit
IS : Insertion sequence
LPS : Lipopolysaccharide
Mbp : Million base pair
MLEE : Multilocus enzyme electrophoresis
MLST : Multilocus sequence typing
NCBI : National center for biotechnology information
PAIs : Pathogenicity islands
PCR : Polymerase Chain Reaction
UidA : β- glucuronidase
UPEC : Uropathogenic Escherichia coli
UTIs : UrinaryTract Infections

1. INTRODUCTION

Escherichia coli (E. coli) is a very diversified bacterial species that can be located in the intestines of humans and different other animals [1]. Despite the fact that *E. coli* is a typical element regarding the gut microbiota, a few strains are pathogenic, producing various extraintestinal and intestinal diseases [2]. The existence of different subsets related to virulence-associated genes in specific path types that are lacking in commensal isolates explains the wide range of pathogenic characteristics and clinical symptoms induced through *E. coli* path types [3]. The presence of various virulence-related genes on Mobil genetic components like plasmids, bacteriophages, and pathogenicity islands (PAIs) suggests that the horizontal gene transfer is crucial in the development of bacterial pathotypes [4]. Those bacteria are the leading cause regarding community-acquired UTIs and significant fraction of nosocomial UTIs, responsible for considerable medical expenses and morbidity around the world [5]. Multi-drug-resistant *E. coli* strains have become more common recently, producing invasive infections and UTIs. Because of the limited therapeutic options available, such organisms are of significant clinical importance. A lack of understanding regarding the population biology of uropathogenic *E. coli* infections has complicated the control and prevention of spread of such infections [6]. In addition, the antimicrobial resistance is a known health problem around the world. Primary care makes a significant contribution because it is here that nearly 80% of all antibiotics administered in the health system are prescribed. The application and development of molecular diagnostic tools has initiated a new era in infectious disease monitoring and diagnosis. Molecular tools were established and have proven to be extremely useful in accurately detecting diseases with higher specificity and sensitivity compared to traditional approaches [7]. Using these tools, particularly PCR-based methods, had a significant effect on the detection, characterization, diagnosis, and taxonomy of infectious disease agents [8]. Over the previous quarter-century, numerous DNA markers depending on PCR techniques were produced in such context [9].

As a result, the study's key objectives were as follows;

- *E. coli* characterization collected from Duhok/Iraq's hospitals.
- The antimicrobial sensitivity profile of *E. coli* was evaluated.
- The use of a specific-species PCR approach for molecular validation of *E. coli* isolates.
- Using PCR-method to investigate *E. coli* phylogenetic analysis.
- Detecting the prevalence related to virulence and antibiotic resistance genes in *E. coli*, as well as their distribution within phylogenetic groupings.
- Examine the patterns and distribution of antibiotic resistance in such *E. coli.*

2. LITERATURE REVIEW

2.1. *Escherichia coli* and its Characteristics

Theodor Escherichia initially described *E. coli* in the year 1885 as bacteria *coli* commune obtained from newborns' feces [10]. It might be very flexible and majorly deadly pathogen along with its significance as a model organism for biochemistry, molecular biology, and genetics. Many strains of such species use virulence factors to produce a variety of extraintestinal and intestinal diseases [11,12]. *E. coli* belongs to the Enterobacteriaceae family, which is considered as a small group of Gram-negative bacteria. They are non-sporing, oxidase-negative, rod-shaped bacteria that are facultative anaerobes which can ferment many carbohydrates while producing both gas and acid, and they are frequently motile thanks to peritrichously organized flagella [10]. The majority of *E. coli* strains can grow at temperatures ranging from 15 to 48 degrees Celsius. It might grow in a range of the pH values of around 5.50–8, with best growth happening at neutrality, and it could also thrive within a restricted range of 37–42°C [13]. The genomic size regarding such bacteria is evaluated to be between $2.3x10^9$ and $3x10^9$ daltons, with a Guanine and Cytosine (G+C) concentration in range of (49-52) % [14].

2.2. *E.* coli Diseases

Various common bacterial infections, like bacteremia, cholecystitis, UTI, cholangitis, and traveler's diarrhea, are caused by *E. coli*, as well as other clinical diseases like neonatal meningitis and pneumonia. In addition, the genus *Escherichia* is named after the type species of genus, Theodor Escherich. *E. coli* organisms are gram-negative bacilli which can live alone or in groups [15].

2.3. Pathotypes of Escherichia coli

E. coli strains with biological significance to humans could be divided into three types based on clinical and genetic criteria: extraintestinal pathogenic strains, intestine pathogenic (diarrheagenic or enteric) strains, and commensal strains [16]. Even though commensal *E. coli* strains are generally harmless and play an important role in gut health [17], they might play a role in extraintestinal infections in the case when the host is medically or immunologically impaired [18].

2.4. Virulence Factors of *Escherichia coli*

For overcoming host defenses and establish infection, *E. coli* strains have different virulence characteristics. Toxins, adhesins, host defense avoidance strategies, and various iron acquisition systems are among such factors [19]. In spite of the discovery of many virulence-associated genes in *E. coli*, there is no single urovirulence profile. It was discovered that half of all *E. coli* isolates had either only one of none of the urovirulence determinants known thus far [20].

2.4.1. Adhesins

The strains of *E. coli* have a variety of adhesins that allow them attaching to urinary tract epithelium, allowing them to withstand the hydro-dynamic urine flow forces as well as stimulating UPEC entry to the epithelial cells of the host, allowing *E. coli* to establishment and survival within the urinary tract [21]. Fimbrial adhesins (afa) and fimbrial adhesins (type 1, P, S, Dr) are two types of adhesins that are regarded distinctive virulence features of *E. coli* [22].

The majority of UPEC isolates, as well as various other commensal and pathogenic strains, encode type-1 fimbriae [21]. Intestinal cells, erythrocytes, uroepithelial cells, and vaginal cells all have receptors for such fimbriae [23]. FimH is a small subunit protein which mediates the binding to such receptors and is encoded by the fimH gene. Generally, the strains of the UPEC express the variants of the FimH with a high affinity for monomannose-containing glycoprotein receptors that are found only on the vaginal, urethral, and bladder epithelium [24], whereas commensal isolates of *E. coli* majorly demonstrate high affinity binding to the residues of the trimannose [21]. This form of fimbriae was a mediator of adhesion to the vaginal mucus and to be more prevalent in patients with recurrent UTI [25]. Type P fimbriae are the 2nd most frequent virulence factor in *E. coli* [26], expressed by pap (pyelonephritis-associated pilli) genes, and is of high importance in the pathogenesis regarding ascending UTI and pyelonephritis [25]. They were found in 80% of pyelonephritic *E. coli* isolates. In addition, F1C fimbriae (encoded via foc gene cluster) and S fimbriae (encoded via sfa gene cluster) were shown to bind to endothelial and epithelial cell lines generated from the lower urinary tract and kidney with high efficiency [27]. The S fimbriae are frequently related to *E. coli* strains which result in meningitis, sepsis, and ascending UTIs [17]. They might aid bacterial spread within host tissues. Dr fimbriae shares a fimbrial afa adhesion for Dra blood group antigen recognition receptor that is present on the decay-acceleration factor [21]. The majority of mammalian cells have a complement regulating protein called decay-accelerating factor [28]. The afa/Dr family might potentially play a role as invasins, stimulating bacterium internalization via target cells [21], which has been linked to gestational pyelonephritis and recurring cystitis [17].

2.4.2. Toxins

Toxins can be defined as a factor of virulence that has been found in the strains of the UPEC that trigger inflammatory response and may be a path-way of the symptoms of UTI. A lipoprotein termed a-haemolysin (HlyA) (encoded via the *hy1*A gene) is considered as a poor forming cytolysin that lyses erythrocytes and is the most significant secreted virulence component of the strains of the UPEC [29]. As a result, nutrients and other factors, such as iron, that are important for bacterial growth can be released more easily [1]. Also, it mediated inflammation, weakened host defenses, and tissue injury, all of which could contribute to pyelonephritis-related kidney damage [19]. It was discovered that a-haemolysin is encoded by 50% of UPEC strains that cause pyelonephritis [29]. Another significant toxin produced via 1/3 of all of the strains of the pyelonephritis, Cytotoxic Necrotizing Factor 1 (CNF1) (which is encoded by the cnf gene), might also be implicated in kidney invasion [17]. CNF1 was discovered to increase apoptosis in

bladder epithelial cells, perhaps promoting exfoliation and bacterial access to underlying tissue, as well as to facilitate *E. coli* dissemination and persistence in the urinary tract [1].

2.4.3. Extracellular Polysaccharides

Capsular polysaccharides (CPS) and Lipopolysaccharides (LPS) are virulence factors which protect *E. coli* against several host defenses [30]. LPS are a type of glycolipid present in Gram-negative bacteria's [31] outer membrane that triggers innate immune responses and enhances inflammatory responses [32]. A core oligosaccharide region, Lipid A and a serotype-specific O antigen make up the antigen [30]. The O-antigen serotypes O2, O1, O6, O4, O18, O16, O25, O22, and O75 are more possible to be present in the majority of UPEC strains [33]. Due to the substantial antigenic heterogeneity, capsular polysaccharides coat the cell, interfering with O antigen detection and protecting the cell from host defense mechanisms [34], and it's regarded as well as a crucial IBC matrix component [35]. Furthermore, specific capsular types, like K5 and K1, have been discovered to imitate tissue components, preventing humoral immune response via the infected host [32]. Almost all UPEC strains feature a K type polysaccharide capsule, with the antigens K5, K1, K92, and K30 being the most common [23].

2.4.4. Formation of the Biofilm

The Biofilms can be described as groupings of bacteria which are linked to a surface or to one another [36]. They are encased in a matrix of mostly polysaccharide material, which allows their growth and survival in a variety of target conditions [37]. The capability of UPEC strains to form biofilms on the urothelium regarding the upper urinary tract and bladder is a significant strategy for their persistence [38]. By giving antimicrobial tolerance and enhancing resistance to phagocytosis as well as other mechanisms of host defenses, those biofilms allow the bacteria to stay within genitourinary tract and obstruct bacterial elimination [39]. The capability of *E coli* to form biofilm has been linked to chronic and recurring UTIs [40].

2.4.5. Flagellar motility

The interactions regarding different pathogenic *E. coli* strains with epithelial cells is mediated by flagella, a bacterial motility organelle [17]. Flagella that are functional have been observed to persist throughout the urinary tract, perhaps enhancing the pathogenesis of UPEC [38]. Flagellated UPEC strains have been determined to be accountable for 70–90% of all UTIs, and their pathogenesis includes bacteria contacting the epithelial cell surface related to the urinary tract [17]. Flagellin was shown to allow pyelonephritis-related *E. coli* strains to invade renal collecting duct (CD) cells, also flagellin is acting as an invasin in such process [41]. Other research has revealed that *E. coli* flagella is important in allowing bacteria to rise from the bladder and infect the kidneys in humans [17]. Flagella are also thought to be important adhesive organs in the formation of *E. coli* intracellular and extracellular biofilms [42].

2.5. Genomic of Escherichia coli

Despite the fact that *E. coli* is the most researched bacterial species worldwide, there is still much to learn regarding such organism's genetic potential [43]. On NCBI database, there are now 246 draft and 61 complete genome sequences for *E. coli* [44]. The very mosaic nature of such genomes reveals the enormous variety of these species [43]. The *E. coli* genome is mostly made up of a conserved core of genes that provide the genetic information needed for vital cellular functions. Furthermore, there is a flexible gene pool that isn't shared by all strains and includes an individual of strain-specific genetic information that might offer extra features that allow such strains to adapt to different environmental situations [4]. *E. coli* genomes were reported to be between 4.5 and 5.5 (Mbp) in length [46, 45, 43]. As a result, genome sizes within the species might vary by more than 1 Mb. In the majority of cases, such genetic information was obtained horizontally and is part of the *E. coli* genome's flexible structure, like bacteriophages, plasmids, and genomic islands [4]. Many types of such elements could be laterally transferred and exist in possible all of the major bacterial phylogenetic groupings, leading to intra- and inter-species variabilities in the genome content [4, 48, 49]. As a result of frequent rearrangements, transfer, and excision, along with the acquisition of extra DNA, such genomic regions could be contributing to the quick progression of *E. coli* variations, resulting in the development of new (pathogenic) variants [4].

2.6. Genomic Islands and Pathogenicity Islands

Pathogenicity islands (*PAIs*) have been initially discovered in *E coli* strain, and they were lately been identified in the genomes regarding different pathogens of animals, humans, and plants [47], yet they are rarely found or absent in nonpathogenic members of the related or same species. *PAIs* are large genomic regions with content of GC which differs from the rest of genome [48]. Their size is between (10 and 200) kb. They are also frequently connected with transfer RNA (t-RNA) genes, which operate as sites of integration for the foreign DNA, typically containing portions of other mobile as well as accessory genetic elements including bacteriophages, insertion sequence (IS) and plasmids [50,51]. The link between PAIS and tRNA loci could thus be reflecting formation of PAIs via horizontal gene transfer, a process which contributes to the development of distinct bacteria pathotypes [48] and play a key role in the emergence of new virulent clones [52]. PAIS encodes the majority of the virulence genes in the strains of the UPEC, including adherences factors, toxins, and the system of Iron uptake. PAIs are sub-classes of Genomic islands (GIs), which are found in almost all nonpathogenic and pathogenic bacteria's genomes and might encode accessory activities that have previously spread among bacterial populations [48].

Quinolones have been used for a long time for the treatment of urinary tract infections. When being in a complex with DNA, these antibacterial agents bind DNA gyrase and topoisomerase IV, inhibiting chromosome re-ligation following enzyme-mediated cleavage [53]. Ciprofloxacin is presently suggested to treat complicated and upper UTIs [54]. Fluoroquinolones were utilized more and more in both uncomplicated as well as complicated UTIs because of the increased resistance to the majority of routinely used antibiotics, resulting in a quick increase in fluoroquinolone resistance [55]. In addition, fluoroquinolone resistance is usually caused by the

mutations in target enzymes (topoisomerase IV and DNA gyrase) as well as efflux and drug entry changes. Aminoglycosides are broad-spectrum bactericidal drugs group, including the amikacin, gentamicin, and streptomycin, among others. They work by binding to ribosomes and inhibiting protein synthesis in bacteria. Due to their high toxicity, such antibiotics have only been used to treat serious infections [56].

Trimethoprim can be defined as one of the bacteriostatic antibiotics which was majorly utilized in the prophylaxis and treatment of urinary tract infections in combination with STX or alone [57]. Despite the recent increases in resistance to trimethoprim in combination with SXT or alone [58], it remains suggested for treating uncomplicated UTIs [54]. In addition, SXT-resistant UPEC isolates found that 66% of them encoded a dfr allele encoding a trimethoprim resistant dihydrate folate reductase, and 96% of them had a sul gene encoding a sulfamethoxazole-resistant dihydropteroate synthetase [59]. The inclusion of such genes on plasmids and integrons makes it easier for them to spread among bacteria [60]. Drug resistance, on the other hand, is not limited to pathogenic bacteria. It also includes commensal bacterial flora, which has the potential to become a large reservoir of resistant strains [61]. *E. coli* is recognized for its mobile genome and ability for exchanging genetic materials [62]. This could play a role in the emergence and propagation of different patterns of resistance in the UPEC. The spread of 'clonal' organisms harboring resistance has been well established as well [63]. It's been evaluated that a limited number of clonal groups are responsible for (10-20) % of all *E. coli* UTIs [64].

2.7. Epidemiology

UTI is considered as a very frequent bacterial infection and the second most infectious illness in the world, responsible for over 25% of all infections and affecting up to 150 million people yearly, costing more than $6 billion in direct health care costs [5, 65]. There is mounting evidence that *E. coli* which results in UTIs is to blame for community-wide outbreaks. For instance, epidemics caused by certain strains were observed. Clonal Group A (CGA) was identified by [66] as one of the clonal outbreaks and was found to be responsible for about 50% of community-acquired UTIs in women caused by *E. coli* strains resistant to trimethoprim-sulfamethoxazole [63]. Two study groups have researched the biology of the population regarding *E. coli* ESBL-producing strains in the year 2008 and characterized a specific CTX-M 15, 025b:H4-ST131, which was documented as a 'pandemic' appearing among ESBL-producing and other antimicrobial-resistant clinical isolates in the middle of this decade [64]. The emergence of UPEC lineages with abnormal traits, like a high level of virulence or antibiotic resistance, provides evidence of sporadic epidemics or breakouts that might have gone unnoticed otherwise. The advancement of molecular epidemiology methods allows researchers to examine more closely at microbial features that explain pathogen virulence and host predilections, as well as gain insight into the origins and spread of bacterial diseases [66, 67].

2.8. Phylogenetic grouping of *Escherichia coli*

Selander and Ochman classified the strains of *E. coli* into 4 main groups B1, A, D, and B2, in the year 1984, with some unclassified strains indicated as the E group, with the use of a

standard reference collection which consists of 72 *E. coli* isolated from humans as well as 16 other mammalian species, indicated as ECOR (*E coli* reference) for representing the species' full diversity, host range, an geographical distribution [68]. The life-history features, ecological niches, and propensity to cause disease of such phylo-groups differed [69]. Aside from genomic size, it was discovered that the strains of groups (B1 and A) have small genomes compared with the strains of group (D or B2) [70]. Groups B1 and A are regarded sister groups, while group B2 is thought to represent *E. coli's* 'ancestral lineage' by others [71]. Furthermore, it has been discovered that the majority of extraintestinal *E. coli* isolates are D or B2 strains rather than B1 or A strains [72]. Through specifying the phylo-group membership of an unknown strain, a lot can be learned about its properties [69]. This classification is based on allelic variation observed via Multilocus Enzyme Electrophoresis (MLEE) at enzyme encoding genes [68]. Yet, such method is time-consuming, difficult, and necessitates a collection of strains that have been typed [9]. Clermont and colleagues (2000) devised a quick and easy approach for *E. coli* phylogenetic grouping depending on a triplex PCR approach and the absence or presence of 2 genes (yjaA & chuA) as well as anonymous fragment of DNA (TSPE4.C2). The chuA gene, which codes for a 69-kDa outer membrane protein, is the first genetic marker. It was discovered to be accountable for heme take in O157:H7 *E. coli* and was lately detected in other pathogenic *E. coli* strains [73, 74]. Although the yjaA gene (2nd genetic marker) has been discovered in the most recent complete genome sequencing of *E. coli* K12, its function remains unknown. The third genetic marker was discovered by Bonacorsi, who discovered that an anonymous 14.9-kb fragment (TSPE4.C2) is closely related with neonatal defined *E. coli* strains [73].

2.9. Typing methods

In both the community and health care environment, strain typing is an important aspect of epidemiological investigations of bacterial diseases. Phenotypic methods, which detect traits displayed by micro-organisms, and genotyping approaches, which entail direct nucleic acid-base examination of extrachromosomal or chromosomal genetic elements, are the two types of typing methods [75].

2.9.1. Phenotypic methods

Susceptibility to bacteriophages, bio-chemical profiles, cell surface antigens, whole protein analysis, and anti-microbial patterns of susceptibility are examples of the phenotypic approaches, characterizing gene expression products for the purpose of identifying the species level or differentiate strains [76]. As inadequate methods for bacterial comparison, because such attributes involve gene expressions, they tend to vary depending on environmental factors [75].

2.10. Biotyping

One of the most extensively utilized approaches is biotyping. It is determined through strain differentiation based on characteristics like as morphology, biochemical reactions, and environmental tolerances. Automated systems developed for species identification are currently

commonly used in laboratories [77]. The system's major strength is its capacity to identify between strains within a species [78]. The majority of procedures are typically technically straightforward and inexpensive, along with the fact that the data obtained is simple to interpret and score, and all tests may be done on large numbers of isolates, even in the smallest facilities [79]. Biotyping has little discriminatory power and is unable to distinguish between several of the current nosocomial problem pathogens, which have little biochemical variation [77]. Furthermore, the results of individual strains have been unpredictable, making biotyping an unreliable typing approach [80].

2.10.1. Antibiogram-based typing

Antibiogram typing entails comparing isolates' susceptibilities to a variety of antibiotics [81]. It might be done utilizing a number of measurement tools to accomplish drug dilution in liquid media or drug diffusion in solid growth media [79]. This approach is affordable, simple to use, easily available in a typical microbiology lab, and produces quick results; it has been effectively utilized for epidemic strain screening. Antibiogram typing, on the other hand, cannot be employed as the sole way of type in the majority of cases due to its low discriminatory power. The selective antibiotic pressure, environment, acquisition and loss of plasmids containing resistance genes, and a variety of additional genetic mechanisms all impact antibiotic resistance patterns [81].

2.10.2. Serotyping

Despite the fact that serotyping has long been regarded as one of the most significant phenotypic approaches that are utilized in the epidemiological researches to trace the infection source and define the different *E. coli* pathogenic strains, typeability and discrimination could be low since the current serotyping methods do not fully cover genetic variation, as well as the labor and cost involved [82, 83, 79].

2.10.3. Genotyping methods

Different genetic approaches for genotyping bacteria were established since the late 1980s and are now widely utilized in bacterial identification [84]. The extensive use and development of such approaches, which depend on direct study of genomic polymorphisms, has substantially enhanced our understanding of bacteria's population dynamics, evolutionary history, and distribution patterns [85]. The main criteria regarding the DNA molecule making it one of the most significant tools for molecular species identification are: DNA can be defined as a long-lived and extremely stable biological molecule; it could be identified in all of the biological tissues or fluids with non-nucleated or nucleated cells with mitochondria and/or plastids. Lastly, due to degradation of genetic code and existence of extensive non-coding stretches, DNA might offer more information compared to proteins [86]. Polymerase chain reaction-based methods, restriction-based methods, and DNA sequence-based methods are the three primary types of genotyping methods [87].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Instruments

The instruments used in the diagnosis of this pathogen.

Table 3.1. Instrument, producer &country

No.	Instrument	Producer	Country
1.	Disposable Petri dish	JOYLA	China
2.	Reusable Inoculating Loop	FISHER	Canada
3.	Wood Stick	KEY	USA
4.	Microscope Slide	AMBALA	India
5.	Cover Glass	AMBALA	India
6.	Pipette Dropper	Bulk Apothecary	United states
7.	Khan Tube	FIRATMED	Turkey
8.	Disposable Plastic Urine Tube 10mL with white screw	International Scientific Supplies Ltd	United Kingdom
9.	Wooden stick with cotton head in a plain tube	CITOTEST	China
10.	Straight Spatulas with Handles	eBAY	UK
11.	Low-Nitrogen Weighing Paper	FISHER	Canada
12.	Plastic Ruler	Cardinal Health	Ireland
13.	Measuring Cylinder	MON SCIENTIFIC	Nigeria
14.	Media Bottle	SIMAX	USA
15.	Parafilm	BEMIS	USA
16.	Test Tube Rack	FISHER	Canada
17.	Diamond Point Marker	FISHER	Canada
18.	Forceps	World Precision Instrument	US
19.	Plastic Eppendorf	AVANTOR	US
20.	Paper Medical Waste Container	TICARE	China

3.1.2. Apparatuses

The apparatuses utilized in the process of this thesis.

Table 3.2. Apparatuses the study used in

No.	Apparatus Name	Producer	Country
1.	Refrigerator and freezer	LG	South Korea
2.	Digital Scale	GlassCast	England
3.	Filter for deionized water	Mes	Turkey
4.	Oven	Memmert	Germany
5.	Centrifuge	KOKUSAN	Japan
6.	Autoclave	LMS	Germany
7.	Vortex	Labnique	USA
8.	Light Microscope	OLYMPUS	Japan
9.	Hot Plate	STUART SCIENTIFIC	UK
10.	Laminar flow hood with uv	Nuaire	USA
11.	Bunsen Burner	Westlab	Canada
12.	Incubator	electro-mag	Indonesia
13.	Filter for Distilled Water	Dogu	Turkey

3.1.3. Chemicals

These chemicals were used in this research.

Table 3.3. Chemical Substances

No.	Chemicals	Producer	Country
1.	Pure Glycerine	Natural Medical	Turkey
2.	Sodium Chloride 0.9% w/v	PIONEER	Iraq
3.	Immersion oil	Scharlau	Spain
4.	Ethyl Alcohol 100 %	SHAGUL MED	Turkey
5.	Liquefied petroleum gas	Basrah Gas Company	Iraq
6.	K2EDTA 5.4mg Tube	Sanli Medical	China
7.	Hydrogen Peroxide	Scharlau	Spain

3.1.4. Gram's Stain

These kits are used as to stain this pathogen.

Table 3.4. Gram's Stain Kits

No.	Content	Producer	Country
1.	Crystal Violet	BIOGNOST	Croatia
2.	Polyvinylpyrrolidone iodine	BIOGNOST	Croatia
3.	Decolorizer	BIOGNOST	Croatia
4.	Safranine	BIOGNOST	Croatia

3.1.5. Antibiotics

Sixteen antibiotic disks have been used in this study, supplied by (Bioanalyse/ Turkey). Table (3.5) illustrates these antibiotics, their codes, and potency (µg).

Table 3.5. The antibiotic disks used in this study

No.	Antibiotics	Code	Disc Potency
1.	Amikacin	AK	10 µg
2.	Gentamicin	CN	30 µg
3.	Cefotaxime	CTX	10 µg
4.	Ceftriaxone	CRO	10 µg
5.	Cefexime	CFM	5 µg
6.	Chloramphenicol	C	30 µg
7.	Amoxicillin/ Clavulanic acid	AMC	(20/10) µg
8.	Ampicillin	Am	10 µg
9.	Amoxicillin	AX	25 µg
10.	Nalidixic acid	NA	30 µg
11.	Ciprofloxacin	CIP	10 µg
12.	Norfloxacin	NOR	10 µg
13.	Trimethoprim	TMP	10 µg
14.	Trimethoprim/sulfamethoxazole	STX	75 µg
15.	Tetracycline	TE	10 µg
16.	Imipenem	IMP	10 µg

3.1.6. VITEK® 2

VITEK® 2 is an automated identification system produced by BIOMERIEUX, France: this system utilized to confirm our diagnosis.

Table 3.6. Vitek 2 Properties

No.	Name	Producer	Country
1.	Rack	bioMérieux	France
2.	Blue Pipette Tip	Thermo Fisher Scientific	American
3.	Blue Pipette Tip Rack	Thermo Fisher Scientific	American
4.	The pipette of gram-Negative bacteria	bioMérieux	France
5.	Spectrophotometer	bioMérieux	France
6.	The dispenser of saline solution	bioMérieux	France
7.	Saline solution For VITEK	bioMérieux	France
8.	Gram-Negative Identification Card	bioMérieux	France
9.	Antimicrobial Susceptibility Test Card for Gram-Negative Bacteria	bioMérieux	France

3.1.7. Culture Media That Used in This Research

These media used to support growth of microorganisms

Table 3.7. These media used in the study

No.	Medium Name	Producer	Country
1.	MacConkey Agar	CONDA	Spain
2.	Nutrient Broth	NEOGEN	UK
3.	Mueller Hinton Agar	CONDA	Spain
4.	Brain Heart Infusion Broth	Neogen	USA
5.	Brain Heart Infusion Agar	Neogen	USA

3.1.8. Bacterial Genomic DNA Extraction

Materials of Bacterial Genomic DNA Extraction Kit

Table 3.8. Components of DNA Extraction Kit

No.	Solution and Material	Producer	Country
1.	Spin Column	Add Bio	Korea
2.	Collection Tube	Add Bio	Korea
3.	Lysis buffer	Add Bio	Korea
4.	Binding buffer	Add Bio	Korea
5.	Washing buffer 1	Add Bio	Korea
6.	Washing buffer 2	Add Bio	Korea
7.	Elution	Add Bio	Korea
8.	Proteinase K Solution	Add Bio	Korea

3.1.9. Other requirements

These requirements also require for genetic tests that not provide by kit.

Table 3.9. Some other requirements

No.	Requirement	Producer	Country
1.	Ethanol	Merck	Germany
2.	TGL-16 High-Speed Centrifuge	PIOWAY	China
3.	Eppendorf Tube 2ml	Thermo Fisher scientific	United States of America
4.	Vortex Mixer	Thermo Fisher scientific	United States of America
5.	Water Bath	witeg	Germany
6.	Shaker Incubator Vortemp 56	Marshall	North America
7.	Different Size Tips	Thermo Fisher Scientific	United States of America
8.	Different Size Pipettes	Thermo Fisher Scientific	United States of America

3.1.10. Materials of PCR

Polymerase Chain Reaction (PCR) requirements used for this work.

Table 3.10. PCR materials

No.	Material	Producer	Country
1.	AddStart Taq Master	Add Bio	Korea
2.	Distilled water	Indiamart	India
3.	Primers	Macrogen	Korea
4.	Multigene Thermal Cycler	Labnet	United States
5.	PCR Eppendorf tube	Thermo Fisher scientific	United States of America
6.	Genomic DNA which we isolated		

3.1.11. Primers

A primer is a short nucleic acid sequence that provides a starting point for DNA synthesis.

Table 3.11. Displays the nucleotide sequences of PCR primers and their amplified outputs

Method and primer	Nucleotide sequence (5'-3') Forward and Reverse	Size of amplifications	Annealing temperature	Reference or source
Species-Specific PCR *uidA*	F - CATTACGGCAAAGTGTGGGTCAAT R - CCATCAGCACGTTATCGAATCCTT	657 bp	63C	[88]
Virulence Gene *Pai*	F - GGACATCCTGGTACAGCGCGCA R - TCGCCACCAATCACAGCCGAAC	930 bp	63C	[89]
Hyl	F - AGATTCTTGGGCATGTATCCT R - TTGCTTTGCAGACTGTAGTGT	556 bp	65C	[90]

3.1.12. Gel Electrophoresis Materials

Gel electrophoresis is a technique used to separate DNA fragments according to their size.

Table 3.12. Materials of Gel Electrophoresis

No.	Material	Producer	Country
1.	Agarose	GeneON	Germany
2.	Distilled water	Indiamart	India
3.	Tris-acetate- EDTA Buffer	Add Bio	Korea
4.	Aluminum foil	Diamond	United Kingdom
5.	Sensitive Balance	Zhengzhou	China
6.	Microwave Oven	Thermo Fisher scientific	USA
7.	Safe Gel Stain Dye	Add Bio	Korea
8.	100pb Plus DNA Marker	Add Bio	Korea
9.	Horizontal Electrophoresis Chamber	Labnet	UK
10.	UV Transliminator	Major Science	Taiwan

3.2. Methods

3.2.1. The collection of samples

A total of 350 samples were collected from Three hospitals (Emergency Teaching Hospital, Azadi Teaching Hospital, zakho General Hospital,) in Duhok City, Iraq, from March 2021 to September 2021. Data were also collected including the age and sex of the patient.

3.2.2. Sterilization Methods

All media, solutions and other autoclavable instruments were sterilized using autoclave at 121C (15 pound/in) for 15 min. Table (3.2.)

3.2.3. Culture Media Preparation

All media were manufactured in the laboratory according to guidance from the manufacturer and the media involved MacConkey Agar (CONDA, Spain) Nutrient Broth (UK), Mueller Hinton agar (CONDA, Spain), Brain Heart agar (Neogen, USA), Brain Heart broth (Neogen, USA), and Mannitol salt agar (CONDA, Spain). Table (3.7.)

3.2.4. Culture Media Characterization of E. coli

All collected clinical isolates of suspected UPEC were recultured on selective media including; MacConkey agar, Eosin Methylene Blue agar (EMB) by streak plate method incubated at 37C° for 24 hours. Table (3.7.) . To see the bacterial shape under the light microscope, Gram stain must be made and the materials shown in Table (3.4) should be used and Gram-negative bacteria are shown in red and Gram-positive in purple.

3.2.5. Preservation of Bacterial Strains

All of the *E. coli* isolates have been kept in 10.0ml slants of brain heart infusion agar that have been produced in the screw-capped bottles and kept under 4^{o}C. All confirmed isolates of the *Escherichia coli* have been stored for a long duration of the storage within 50% broth of the glycerol under a temperature degree of -20^{o}C [91]. It has been prepared through the addition of 500.0µl of the bacterial growth to 500µl of 50% of the sterilized glycerol in 1.50ml micro-centrifuge tube, 24hrs later, the organism's purity and viability has been checked through re-culturing it in the broth of the brain heart infusion as media of enrichment, after that, it has been cultured on the Mac-Conkey agar. Table (3.7.)

3.2.6. Test of Antibiotic Susceptibility

Isolates have been subjected to the test of the antibiotic sensitivity by using the approach of the disc diffusion on the Mueller-Hinton agar based on National Committee for Clinical Lab Standards and the Manual of the Antimicrobial Susceptibility Test guide-lines [92, 93]. Inoculums have been made through taking a minimum of 4 of the colonies that have morphological similarity with sterile loop to 10.0ml of the broth of the brain heart infusion Table (3.1), which has been incubated under a temperature of 37^{o}C for 16hr., a sterilized cotton-wool swab has been dipped in that broth and broth excesses have been removed though turning swab against the container's inside. Inoculum has been evenly distributed over the Muller-Hinton agar plate in 3 directions, rotating plates through a 60^{0}C temperature for the purpose of ensuring the even dispersion and ultimately, the swab has been passed around the agar surface edge, after that, inoculum has been left for a few minutes, to dry at the temperature of the room. Then, the antibiotic discs have been placed on inoculated plate with the use of the sterile forceps. The discs have been placed readily at a distance of 30mm-36mm for the purpose of avoiding the overlap of the zone of inhibition and those plates have been incubated under a temperature of 37^{0}C for 24hrs. Every inhibition zone's diameter has been evaluated and interpreted as sensitive or resistant, with the use of interpretative zone size chart. Table (3.5.)

3.2.7. Test of Catalase

One colony of every one of the bacterial isolates has been taken then smeared upon a clean glass slide, after that, a hydrogen peroxide drop (3 drops have been flooded with 1ml of 3% of the hydrogen peroxide). The presence of the gaseous bubbles is an indication of positive results. Table (3.3.)

3.2.8. Vitek Test

Each sample was *E.coli* by previous tests, confirmed by Vitek 2.

Procedure:

For the identifying the microorganism we must use Vitek and following:

1. If culture rules are followed, select isolated colonies from a predominant plate.
2. Subculture and incubate the organism to be examined to an appropriate agar medium.
3. Transfer 3.0 mL of sterile saline to a clear (polystyrene) tube (12 mm x 75mm) of (aquatic 0.45% to 0.50% NaCl, pH 4.5 to 7.0%).
4. Use a sterile stick or swab to transfer a sufficient number of morphologically similar colonies to the saline tube prepared in step 2. Prepare a homogeneous organism suspension with a density equivalent to a McFarland No. 0.50 to 0.63 using a calibrated VITEK® 2 DensiCHEK TM Plus or VITEK® 2 DensiCHEK TM. Note: Age of suspension must not exceed 30 minutes before inoculating card.
5. Place the suspension tube and GP card in the cassette.
6. Refer to the appropriate Instrument User Manual for instructions on data entry and how to load the cassette into the instrument. Table (3.6.).

3.2.9. Genomic DNA Extraction

A total of 100 UPEC isolates have been chosen, then subjected to the molecular approaches, 50 isolates have been randomly chosen from every one of the provinces (Duhok & Zakho). The extraction of the genomic DNA has been performed with the use of the conventional approach through the commercial kit (DNP^{TM} High yield Purification of the DNA). On the other hand, before the extraction of the DNA in every one of the methods, the strains have been cultured on a MacConkey agar plate at a temperature of 37^{o}C for a period of 24hrs and pure a few single colonies from the Mac-Conkey agar plate have been utilized for the extractions. The DNA that has been extracted has been stored in a freezer under a temperature of -20^{o}C, prepared for later use in the PCR process. Table (3.3.)

Extracting* Genomic *DNA with the use of the Commercial Kit

The genomic DNA has been obtained from 100 strains of the UPEC with the use of the high yield Kit of DNA Purification based on the instructions of the manufacturer (**Korea**). Table (3.8.) and Table (3.9.).

Concentration and Purity Determinations

The genomic DNA purity and concentration for every one of the samples have been determined with the use of the NanoDrop Spectrophotometer through the recording of every sample's purity and concentration. This process was carried out at central lab. of Duhok.

3.2.10. PCR-Amplification

Species-Specific PCR-Amplifications

The internal *uidA* gene fragment with the molecular weight of 670 bp Table (3.11.) has been amplified with the use of 1 *uidA* primer pair that includes (Reverse & Forward). The master-mixture has been prepared for one hundred of the UPEC isolates plus a control in every one of the tests. The reaction of the amplification includes 25.0µl as the final volume for every one of the samples that contain 12.50µl of the master mixture, 1.0µl of every one of the primers that include

the reverse and forward (10.0pmol/µl), 2.0µl of the genomic DNA (25ng/µl -50 ng/µl) and 8.50µl of the sterile distilled water Table(3.10.). All of the reaction tubes that have been prepared, have been placed in a thermal cycler for a purpose of carrying out amplification . The conditions of the amplification have been listed in the Table (3.13.).

Table 3.13. The amplification conditions of Species-Specific PCR analysis using *uid* primer

Initinal denaturation	Denaturation	Annealing	Extension	Final extension	Storage	Ref.
94 Cº	94 Cº	63 Cº	72 Cº	72 Cº	4 Cº	[88]
4 min	5 sec	20sec	15sec	5 min		
1 cycle		30 cycles		1 cycle		

3.2.11. Screening of Virulence and Antibiotic Resistance Related Genes with the use of the PCR Amplifications

Two primers (*hyl* and *pai*) have been utilized in order to detect the virulence as well as the Antibiotic Resistance related genes, which include; hemolysin, island patogeneicity, respectively amongst a hundred of the chosen strains Table (3.11.). The reactions of the amplification for every one of the genes have been performed in 25.0µl volumes that contain, 1.0µl of every one of the primers, which include the forward and reverse (10.0pmol/ µl except for hly 30.0pmol/ µl), 10.0µl Taq Polymerase (1 unit) and 3.0µl (25ng-50ng) of the genomic DNA .This volume has been completed to 25.0µl through the addition of 10µl of the sterile deionized distilled water (Table 3.10.). The conditions of the amplification of every one of the genes have been presented. Following the amplification, PCR product presence has been electro-phoretically proven with th3e use of 1% (w/v) of the agarose in the TAE Buffer. The conditions of the amplification of the PCR-based analyses for detecting the variety of the virulence and Antibiotic Resistance markers amongst the isolates of E. coli utilizing *Pai* and *hyl* primers. Table (3.14.)

Table 3.14. The amplification conditions of Virulence genes PCR analysis using *hyl* and pai primer

Primer	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Ref
Hyl	94 Cº	94 Cº	55 Cº	72 Cº	72 Cº	[90]
	4min	30 sec	30 sec	1 min	5 min	
	1 cycle		30 cycles		1 cycle	
Pai	94 Cº	94 Cº	63 Cº	72 Cº	72 Cº	[89]
	1min	1min	30 sec	90 sec	5 min	
	1 cycle		30 cycles		1 cycle	

3.2.12. Gel Electrophoresis

The Agarose gel has been produced with a variety of concentration levels, based on required applications. For instance a 1% (w/v) of this gel in the TAE has been utilized to detect

the genomic DNA through the addition of 1.0gm of the agarose to 100ml of 1 X TAE buffer and it's dissolved throughout the heating at the temperature of boiling. After that, agarose has been left to cool at a temperature of 55^{o}C, prior to being poured in casting plate for solidification. A needed comb has been placed near one gel edge, and this gel has been left to cast. 1 XTAE has been poured to gel tank and gel plate has been horizontally placed in a tank of electrophoresis. Samples of the DNA have been produced through the addition of 1.0µl of the loading buffer and mixed with 5.0µl samples of DNA, after that, samples have been carefully added to separate wells. The power has been turned on at 45V for 15min and 85 V for 2 hrs. for the purpose of running the DNA or at 5v/cm-8v/cm. The Agarose gels have been stained by the ethidium bromide the immersion into the distilled water that contains the dye at 0.50µg/ml final concentration for a duration of 15min-30min. After shortly de-staining in the distilled water, the DNA bands have been visualized with the UV lighting at a wave-length of 366nm on UV trans-illuminator. After that, gels have been documented with the use of a digital camera [94]. Table (3.12.)

4. RESULTS AND DISCUSSION

4.1. Biotyping by VITEK 2 identification system

VITEK 2 can be defined as an automatized fungi and bacterial recognition apparatus which produces consistent and repeatable results, as evidenced by clinical studies. With its colorimetric reagent cards and related software and hardware advancements, the VITEK 2 provides a state-of-the-art evolution program for phenotypic discovery procedures The present study revealed that the accuracy of the VITEK 2 system for a direct identification and susceptibility testing in blood cultures of Grampositive rods and Gram-negative cocci varied according to the type and species of bacteria as well as the antimicrobial screened. [95]. The VITEK 2 automated system (bioMe´rieux) is one of the most widely used instruments in clinical microbiology laboratories for the identification and evaluation of the susceptibility profiles of bacteria including the detection of extended-spectrum b-lactamases produced by *Escherichia coli,* Currently, the Clinical and Laboratory Standards Institute recommends the use of ESBL confirmatory tests in addition to standard susceptibility testing. In order to evaluate the accuracy of VITEK 2-positive results regarding clinical isolates of *E. coli* Growth-based technology was identified by this automated method, which had 43 biochemical tests to detect our study illustrates how distinct methods for ESBL detection can provide discordant results, and highlights the need to evaluate carefully and review individual reports, as well as global data. We demonstrated that the VITEK 2 system overdetected the presence of ESBL producing bacteria, and therefore there is a need to confirm such results to avoid false-positives. The development of novel, accurate and speedy methods of ESBL detection is a priority in order to face the challenges raised by rapidly evolving ESBL genotypes. The development of automated susceptibility testing systems and their subsequent introduction into routine diagnostic laboratories sparked an interest in the performance of such systems for the detection of ESBLs and a number of studies on the Vitek 2 were conducted, mostly prior to These studies vary in many key aspects including the use of different Vitek AST cards, which may or may not contain ESBL confirmatory wells with combinations of cephalosporins and clavulanic acid.

Table 4.1. Biochemical Test of *Escherichia coli* (*E. coli*)

Basic Characteristics	**Properties (*E. coli*)**
Capsule	Capsulated
Catalase	Positive (+ve)
Citrate	Negative (-ve)
Coagulase	Negative (-ve)
Flagella	Flagellated
Gas	Positive (+ve)
Gelatin Hydrolysis	Negative (-ve)
Gram Staining	Negative (-ve)
Growth in KCN	Negative (-ve)
H2S	Negative (-ve)
Hemolysis	Negative (-ve)
Indole	Positive (+ve)
Motility	Motile
MR (Methyl Red)	Positive (+ve)
Nitrate Reduction	Positive (+ve)
OF (Oxidative-Fermentative)	Fermentative
Oxidase	Negative (-ve)
Pigment	Negative (-ve)
PYR	Negative (-ve)
Shape	Rod
Spore	Non-Tracking
TSIA (Triple Sugar Iron Agar)	Acid/Acid, Gas +ve
Urease	Negative (-ve)
VP (Voges Proskauer)	Negative (-ve)

Table 4.2. Biochemical Test of Escherichia *coli* (*E. coli*)

Fermentation	
Adonitol	Negative (-ve)
Arabinose	Positive (+ve)
Arabitol	Negative (-ve)
Cellobiose	Negative (-ve)
DNase	Negative (-ve)
Dulcitol	Variable
Erythritol	Negative (-ve)
Glucose	Positive (+ve)
Glycerol	Variable
Lactose	Positive (+ve)
Malonate	Negative (-ve)
Maltose	Positive (+ve)
Mannitol	Positive (+ve)
Mannose	Positive (+ve)
Melibiose	Positive (+ve)
Mucate	Positive (+ve)
Myo-Inositol	Negative (-ve)
Raffinose	Variable
Rhamnose	Positive (+ve)
Salicin	Variable
Sorbitol	Positive (+ve)
Sucrose	Variable
Tartrate	Positive (+ve)
Trehalose	Positive (+ve)
Xylose	Positive (+ve)

Table 4.3. Enzymatic Reaction

Enzymatic Reactions	
Acetate Utilization	Positive (+ve)
Aesculine Hydrolysis	Variable
Arginine Dihydrolase	Negative (-ve)
Beta Lactamase	Positive (+ve)
Esculin Hydrolysis	Variable
Lipase	Negative (-ve)
Lysine Decarboxylase	Positive (+ve)
ONPG (β-galactosidase)	Positive (+ve)
Ornithine Decarboxylase	Variable
Phenylalanine Deaminase	Negative (-ve)

4.2. Microbiological Investigations

The biochemical test for *Escherichia coli* (*E. coli)* appeared as follows as in Table (4.1.) and (4.2.) and in Enzymatic Reaction as in Table (4.3.).

4.2.1. Characterization and Incidence of E. coli

The re-culturing results of all of the isolates on Eosin Methylene Blue agar (EMB) and MacConkey revealed that they were *E .coli* and protected them against contamination throughout travel between Iraqi hospitals. Those isolates have been reported to be capable of fermenting the lactose, resulting in smooth pink colonies on the Mac-Conkey agar and extremely dark purple with the green metallic sheen colonies on the EMB agar as a result of precipitation of the methylene blue in the medium as a sign of formation of a high amount of acid [96]. Table(4.4.) shows that out of 365 strains of the *E. coli* that have been obtained from 3 hospitals in Iraq (Duhok and Zakho), 68 strains were identified from males (18.68%) and 296 were isolated from females (81.3%). Females were more susceptible to UTIs compared to males, according to the findings.

Table 4.4. The distribution of collected E. coli from DUHOK and ZAKHO- Iraq and their frequency among males and female's patients

Provinces	**Patients**	**Female**	**Male**
	No.	**No. (%)**	**No. (%)**
Duhok	220	100 (45.45)	120 (54.54)
Zakho	145	60 (41.37)	85 (58.62)
Total	**365**	**165 (43.83)**	**205(56.16)**

These findings were in line with the findings of some other investigations. For instance, between 40% and 50% of females and just 5% of males are projected to acquire a UTI in their lifetime [97]. UTI caused by *E. coli* was shown to be more common in females (70%) than in males (30%), according to Jadhav and coworkers (2012). Various factors, including anatomical

variations and the hormonal milieu regarding the urinary tract, could make females more susceptible to UTI than males [5]. Other factors, including contraception and sexual activity, could be considered [98].

4.2.2. Antibiotic Sensitivity Test

Table (4.5.) shows the results of antibiotic sensitivity tests for *E. coli* isolates (50 randomly-selected isolates) from 3 main hospitals. Although none of the isolates were sensitive to all antibiotics, they showed a wide range of resistance to the majority of those tested, ranging from resistance to only two antibiotics (12.5%) to resistance to 15 of the 16 antibiotics examined (93.75%). With a resistance rate of 4.6%, the imipenem antibiotic, which is one of the carbapenems agents, has been shown as the most potent of all of the rest of the antimicrobial agents utilized. Comparable rates were recorded, with the suggestion that this rate be taken into account because such agents have been recommended in empiric therapy for serious bacterial infections resulting from β-lactam resistant bacteria [99].

The emergence of Carbapenem resistance due to the production of capabenemase enzyme in Gram-negative organisms is an increasing international public health problem. Detection of carbapenems resistant isolate in a hospital environment poses not only a therapeutic problem, but also a serious concern for infection control management Chloramphenicol may be considered as the second most effective antibiotic against these isolates with a resistant rate of 30%. The tested UPEC isolates were exhibited a high resistant to most tested antibiotics including; Ampicillin, Amoxicillin, Amoxicillin/ Clavulanic acid, and Tetracycline with resistant rate of 92.6%, 90.6%, 90%, and 83.3% respectively. Comparative studies including older antimicrobial agents are limited, but some of these agents remain useful for the treatment of selected patients The advent of the Carbapenem resistance because of Gram-negative organism producing the capabenemase enzyme is becoming a growing global public health issue. The discovery of a carbapenems-resistant isolate in a hospital setting is not just a therapeutic issue, yet also a severe infection control concern [99, 100].

Table 4.5. The distribution of tested antibiotic susceptibilities of E. coli isolates from Duhok and Zakho

No	Antibiotics	code	Duhok Resistance No. (%)	Total Resistance No. (%)	Total Resistance No. (%)
1	Amikacin	AK	39 (78)	25 (50)	64 (64)
2	Gentamicin	CN	37 (74)	43 (86)	80 (80)
3	Cefotaxime	CTX	37 (74)	44 (88)	81 (81)
4	Cefixime	CFM	39 (78)	43 (86)	82 (82)
5	Ceftriaxone	CRO	36 (72)	38 (76)	74 (74)
6	Chloramphenicol	C	12(24)	19 (38)	31 (31)
7	Amoxicillin/ Clavulanic acid	AMC	40 (80)	50 (100)	90 (90)
8	Ampcillin	Am	46 (92)	45 (90)	91 (91)
9	Amoxicillin	AX	44 (88)	44 (88)	88 (88)
10	Nalidixic acid	NA	42 (84)	38 (76)	80 (80)
11	Ciprofloxacin	CIP	34 (68)	24 (48)	58 (58)
12	Norfloxacin	NOR	28 (56)	21 (42)	49 (49)
13	Trimethoprim	TMP	40 (80)	40 (80)	80 (80)

14	Trimethoprim/sulfamethoxazole	SXT	42(84)	31 (62)	73 (73)
15	Tetracycline	TE	47 (94)	37 (74)	84 (84)
16	Imipenem	IMP	4(8)	1 (2)	5 (5)

With a 30% resistance rate, chloramphenicol could be indicated as the second most effective antibiotic against such isolates. The majority of studied antibiotics, such as amoxicillin, ampicillin, tetracycline, and amoxicillin/clavulanic acid, showed a high resistance rate of 90.6%, 92.6%, 83.3%, and 90%, respectively. Although there are few comparative trials including earlier antimicrobial agents, a few of such agents are still effective in treating a few patients [101].

Trimethoprim and TMP/SMX, which are commonly used to treat UTIs, had a minimal impact on such isolates, with resistance rates of 77.3% and 73.3%, respectively. Due to rising resistance, the effectiveness of such antibiotics has decreased in specific locations [59]. Resistance to Ciprofloxacin, Nalidixic acid, and Norfloxacin was detected in the proportions of 52.66%, 78%, and 48.66 percent in the tested isolates, respectively.

Antibiotics that are aminoglycosides, such as Amikacin and Gentamicin, had a limited impact on tested isolates, with resistance rates of 46%, and 70.66% respectively. Cefotaxime, Cefixime, and Ceftriaxone resistance rates were 78%, 78%, and 71.3%, respectively, for the third generation of Cephalosporins. Several investigations have indicated that the extensive utilization of 3rd-generation cephalosporins is the main force behind the rise of ESBL-producing organisms By one means or another comparative rates have been detailed and proposed that this rate ought to be considered since these operators are favored in empiric treatment for serious bacterial infections caused by β-lactam resistant bacteria .The development of Carbapenem resistance due to the generation of Carpabenemase chemical in Gram-negative organisms is an expanding universal open wellbeing issue. Discovery of carbapenems resistance separate in a healing center environment posture not as it were a restorative issue, but moreover a genuine concern for infection control administration [99]. The genes which code for ESBLs are typically discovered on the same plasmids as genes which code for aminoglycoside and TMP-SMX resistance [102]. This means that ESBL-producing are frequently multidrug resistant, which makes treating nosocomial infections more difficult. Inadequate empiric antimicrobial treatment for nosocomial or community-acquired diseases was linked to considerably higher mortality rates in ICUs. In addition, the most significant independent driver of hospital mortality was insufficient antimicrobial treatment of infections [99]. The clonal expansions as well as the introduction competitive, resistant *E. coli* strains in community is another significant mechanism supporting the increase in antimicrobial-resistance UTIs [103].

The findings of this work might corroborate those of other studies on antibiotic resistance published in regional and, particularly, poor nations. In a work published in Turkey by Nazik and collaborators (2011), *E. coli* isolates exhibited high resistance to TMP/SMX (76%), amoxicillin-clavulanic acid (77%), Ciprofloxacin (68%), Norfloxacin (70%), Gentamicin (51%), while all isolates were susceptible to Imipenem. UPEC resistance patterns to Tetracycline 72.8%, TMP/SMX 75%, Norfloxacin 50.7%, Nalidixic acid 60.7%, Gentamicin 33.6%, Ciprofloxacin 47.6%, Amikacin 12.1%, Chloramphenicol 20.7%, and Imipenem 1.4% were discovered in another research conducted in Iran [104]. Recently, a study in India, indicated that *E. coli* isolates have high levels of resistance to Nalidixic acid 95%, Ampicillin 97.5%, TMP/SMX 82.5%,

Amoxicillin 92.5%, Amikacin 70%, and Ciprofloxacin 80 %, sensitivity [105]. For many years, simple UTIs were treated with cephalexin, amoxicillin/clavulanate, TMP/SMX, or fluoroquinolones (for instance, ciprofloxacin) as first-line therapy [44]. Yet, *E. coli* resistance to each of such antibiotics has grown to the point where they can no longer be applied as empiric therapy in various regions of the world [106]. There is a definite need to better describe ways for preventing emergence, and additional research is clearly needed in this area. Clinicians will need to rely on more laboratory assistance, and laboratories will need to give resistance pattern data more quickly for best patient care. Different studies have demonstrated that *E. coli* strains' resistance to routinely applied antimicrobial agents has hampered clinical management of UTIs through increasing the infections' incidence [107]. For many years, amoxicillin/ clavulanate, cephalexin, trimethoprim/ sulfamethoxazole or fluoroquinolones (for example, ciprofloxacin) were used as first line treatment of uncomplicated UTI, However, resistance of *E. coli* to each of these antibiotics is now substantial and in many parts of the world these drugs can no longer be used as empiric therapy The majority of such works agreed that infection control procedures must be improved In almost all UTI cases, empirical antimicrobial treatment is initiated before the laboratory results of the urine culture are available Misuse and self-medication in many countries including ours may be considered a major problem as antibiotics could be purchased without any prescription. Up to 95% of UTI cases are treated without bacteriological investigations There is a need to better define strategies to prevent emergence and more studies in this area are clearly required. Clinicians also must depend on more laboratory guidance, while laboratories must provide resistance pattern data for optimal patient management more rapidly. Many reports suggested that the resistance of *E. coli* strains to commonly used antimicrobial agents has made the clinical management of UTI complicated by increasing incidence of their infections [105].

4.3. Molecular Investigations

4.3.1. Extraction of Genomic DNA

The average concentration of genomic DNA extracted using a commercial kit was 115.25ng/lµl, with an extremely high purity of 1.8 The commercially available kit approach was discovered to be an extremely effective approach for: suitable for PCR amplification, rapid yielding of DNA, high purity, and the extraction of a large number of samples. Yet, owing of the low yield amount, this procedure may not be acceptable in the case when the samples of DNA are to be submitted to other types of molecular investigations like multiple experiments and cloning. As a result, future use regarding such commercial kit may be limited. The number of samples to be extracted, amount of sample available, and the availability of certain equipment all influence the approach of DNA extraction chosen Commercially kit method was found as a very efficient method for; rapid yielding of DNA, suitable for PCR amplification, with high purity and a large number of samples can be extracted. However, this method might be unsuitable if DNA samples are to be subjected to other kinds of molecular investigations such as cloning and or in multiple experiments because of low yield quantity. Thus, it might lead to limit the use of this commercial kit in future. The selection of the appropriate method for DNA extraction depends on the amount

of the sample available, number of samples to be extracted and the availability of specific equipment [108].

4.4. PCR Analysis

4.4.1. E. coli Species-Specific PCR Amplification

All *E. coli* isolates (100) have been amplified effectively in repeated tests, resulting in a single band regarding the *uidA* as species-specific locus in all strains with molecular weight of roughly 657bp, as can be seen In Figure (4.1.). The effective amplification regarding *uidA* amplicon in all of the chosen samples has been validated at the molecular level, confirming that all of the strains have been *E. coli*. This phase, according to several research, is a prerequisite for any subsequent molecular investigation [109]. Yet, with the use of the same primer sequence as Adamus-Bialek et al., (2009), such molecular weight was shown to be different. This variation could be explained by the fact that genes often include multiple coding mini- and microsatellite repeats, which are extremely dynamic genomic components. As a result of recombination events within such tandem repeats, the repeat numbers vary, causing the sequences to change.

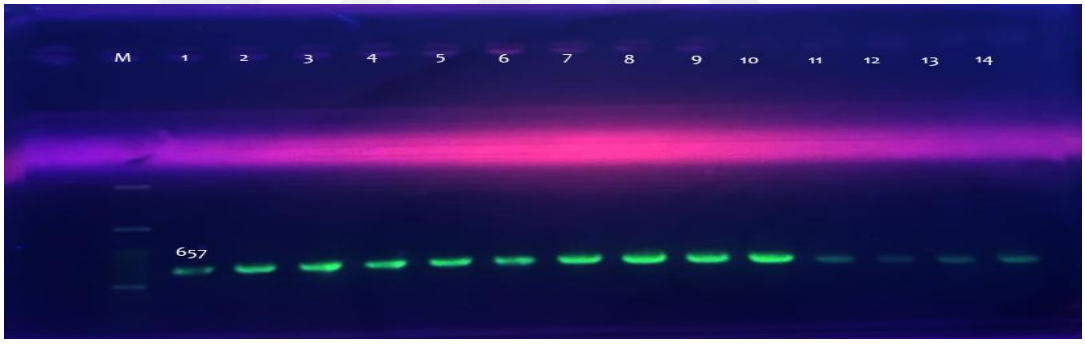

Figure 4.1. Showing Species- Specific PCR amplification for (Table 3.13.) *E. coli* produced with uidA amplicon with molecular weight 657bp. Electrophoresis was performed on 1.2% agarose gel and run with 5v/cm, for 2 hours. Lane M contained DNA molecular weight marker (1500-100bp)

This variety might give functional diversity and/or the illusion of the host immune system, allowing for quick adaptability the obtained result was in agreement with a study conducted in Duhok Province using the same primer (*uidA* gene) for the identification of *E. coli* which produced the same molecular weight [110]. Due to the fact that *uidA* gene is considered as a housekeeping gene, it encodes the B-D-glucuronidase enzyme [111], which is produced by up to 97% of *E. coli* [112]. As a result, numerous investigations have employed the gene as one of the molecular markers for the identification of *E. coli* However, this molecular weight was found different from that obtained by by using the same primer sequence. This variation may be attributed to the fact that genes usually contain multiple coding mini- and microsatellite repeats that are highly dynamic components of genomes. Therefore, recombination events within these tandem repeats lead to changes in repeat numbers, which in turn alters the sequences. [113, 109]. This approach could deliver speedy and reliable findings in a short amount of time for *E. coli*

specific-species identification, as well as pave the path for more detailed molecular applications This variation may provide the functional diversity and allows rapid adaptation to the environment and/or elusion of the host immune system Since *uidA* gene is a housekeeping gene encoded *B*-D-glucuronidase enzyme and up to 97% of *E. coli* produce this enzyme therefore, many studies used this gene as a molecular marker for the identification of *E. coli* This method may provide rapid and robust results in a short time for the confirmation of specific-species identification of *E. coli* and may also pave the way for further and more details molecular applications.

Prevalence and distribution of virulence and Antibiotic Resistance genes among E. coli strain

All *E. coli* isolates (100) were exposed to PCR methods to evaluate the prevalence rates regarding virulence as well as antibiotic resistance-associated genes such as the *hyl* and *pai* markers, as well as their distribution throughout phylogenetic groupings. The findings of such tests are detailed in detail for each city independently.

Figure (4.2.) and Figure (4.3.) shows the results of Duhok isolates that were examined. The pai marker was identified to be fairly common among such isolates, responsible for 76% of them, while the hyl gene was discovered in 70%.

Figure 4.2. Showing the amplification of the PCR of the *E.coli* strains that have been isolated from Duhok produced *pai* amplicon with the 930 bp molecular weight. The electrophoresis has been carried out on 1.5% of the agarose gel and run with 5v/cm, for 2hours. Lane M contained DNA molecular weight marker (1500-100bp)

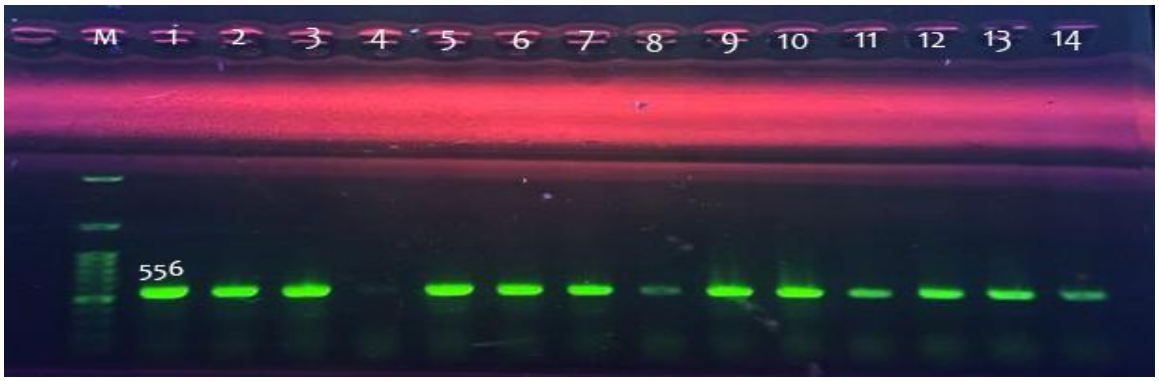

Figure 4.3. Showing PCR amplification of *E.coli* strains isolated from Duhok produced *hyl* amplicon with molecular weight 556 bp. Electrophoresis was performed on 1.5% agarose gel and run with 5v/cm, for 2hours. Lane M contained DNA molecular weight marker (1500-100bp)

The prevalence regarding virulence as well as antibiotic resistance-associated genes among *E. coli* isolates in Zakho is depicted in Figure (4.4.) and Figure (4.5.). These results revealed that such isolates had a pattern that was comparable to those reported in Duhok. *Pai* marker was found in 74% of the isolates, while *hyl* gene was found in 68%.

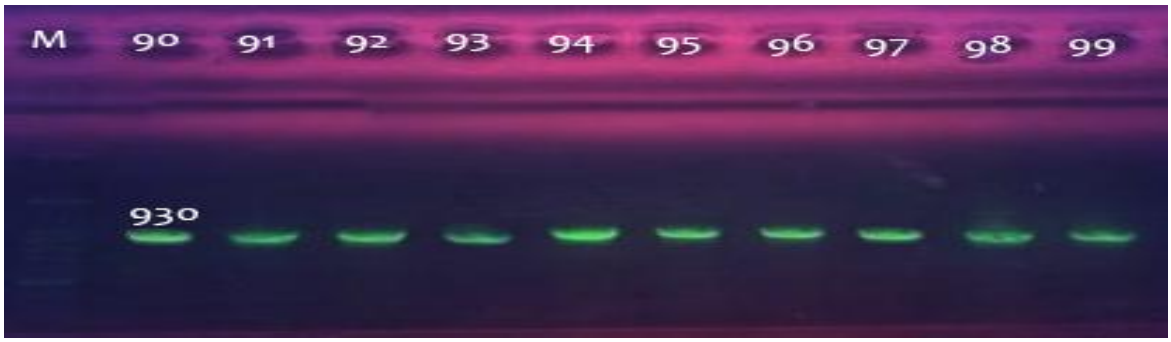

Figure 4.4. Showing PCR amplification of *E.coli* strains isolated from Zakho produced *pai* amplicon with molecular weight 930bp. Electrophoresis was performed on 1.5% agarose gel and run with 5v/cm, for 2hours. Lane M contained DNA molecular weight marker (1500-100bp)

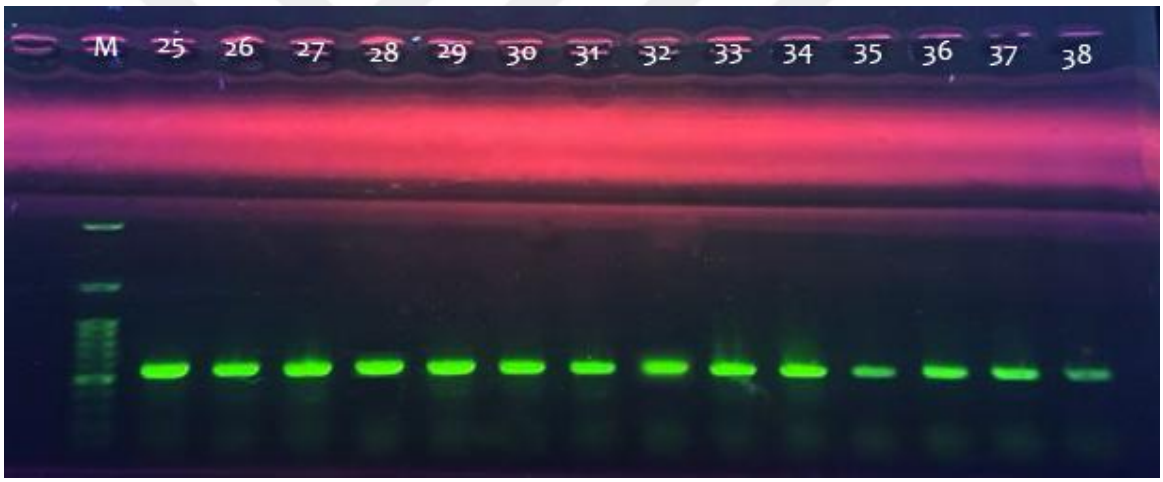

Figure 4.5. Showing PCR amplification of *E. coli* strains isolated from Zakho produced *hyl* amplicon with molecular weight 556 bp. Electrophoresis was performed on 1.5% agarose gel and run with 5v/cm, for 2hours. Lane M contained DNA molecular weight marker (1500-100bp)

Only 28% of *E. coli* isolates collected from Zakho and Duhok lacked any of the assessed virulence indicators, according to the overall results regarding virulence as well as antibiotic resistance associated genes prevalence among *E. coli* isolates. Table (4.6.) summarizes the results of isolates which included such markers.

Table 4.6. The prevalence of virulence and Antibiotic resistance markers among *E. coli* strains collected from Duhok and Zakho

	Virulence and Antibiotic markers	
	Pai	***hyl*(%)**
Duhok	38(76)	35(70)
Zakho	37 (74)	34(68)
Total	75(75)	69(69)

These rates are consistent with a number of other researches. For instance, Oliveira and colleagues (2011) discovered that 28% of *E. coli* strains lacked the virulence and antibiotic resistance genes. Rasol (2013) found that virulence gene prevalence amongst isolated of E. *coli* from the urine was 38% and 67%, respectively, in another work done in Duhok province. Karimian and associates (2012) investigated the virulence factors regarding *E.coli* isolated from urine and discovered that *hyl* and *pai* had varying prevalence rates of 50.4% and 50.4%, respectively.

In the current investigation, the high prevalence of *pai* as a (*PAI*) marker that might carry virulence genes amongst the strains of *E.coli* has been also consistent with other recent researches. Brzuszkiewicz and collaborators (2006), for example, found that genomic differences between the strains of *E. Coli* were mostly limited to large (*PAIs*). In another research, Navidinia and colleagues (2012) found that (*PAIs*) have been enriched among the isolates of *E. coli* and that the prevalence regarding 8 *PAI* markers in the strains of *E. coli* isolated from the urine of children with UTI was confirmed. Furthermore, the high incidence of both hly genes correlating to the incidence of *pai* marker found in this work could indicate a strong association between such genes, which are frequently connected and known to carry *PAIs*.

The results of this study Table (4.6.) have shown as well that there have been differences in rates of prevalence of those genes within this city. Differences in the city of *E. coli* virulence and Antibiotic resistance genes as a result of the geographical area has been reported as well by Rasool., 2013 in Duhok governorage and by Karimian and coworkers (2012) in Iran who had suggested that climate of every region, food, customs, public health levels and hospital hygiene can be considered as factors that are attributed to presence of variations in rates of prevalence of the virulence genes of the strains of *E. coli* amongst various regions. The high incidence of both *hlyA,* gene corresponding with incidence of pai marker obtained in this study may reflect the fact that there is a high association between these genes which are often linked and known to carry PAIs. Similar studies have been reported reflecting this type of association [114]. Furthermore, Hacker and Kaper (2000) suggested that sfa genes are also present on PAI in *E. coli* strains. Thus; PAIs may contribute to urovirulence and may potentially serve as targets for interventions.

5. CONCLUSION

- There may be a spill of information for the resistance circumstance exterior the clinic environment, particularly for the predominance of multiresistant microbes in solid individuals
- Males are more affected with UTI than females in Iraq.
- The advancement of the pathogenesis of *E. coli* constitutes a challenge for open wellbeing specialists in both created and creating nations. As reviewed here above, PAIs are widespread in *E. coli* and are major actors in the genome plasticity of this bacterium.
- High prevalence rate of multidrug resistance of *E. coli* strains accounting for 98% against antimicrobial agents tested in this study. Imipenem is found to be the most potent at all other antimicrobial agents.
- The PAIs have shaped the genome of this bacterial species, which further explains the emergence of different pathotypes that result in different intestinal and extra intestinal diseases.

Recommendations

- The Introduction of the atomic points of interest of *E.coli* necessary to create effective techniques for the anticipation of human urinary tract contaminations and urological complications related with UTIs.
- Since PCR innovation is increasingly accessible indeed in healing center research facilities, the utilize of *uidA* quality as a molecular marker is profoundly suggested especially in dealing with expansive number of tests and may give quick and strong comes about in a brief time for affirmation of particular genus-species of *E. coli*.
- More molecular test is required for the location of most *E. coli* strains creating ESBLs chemicals that are dependable for safe to antimicrobial specialists.

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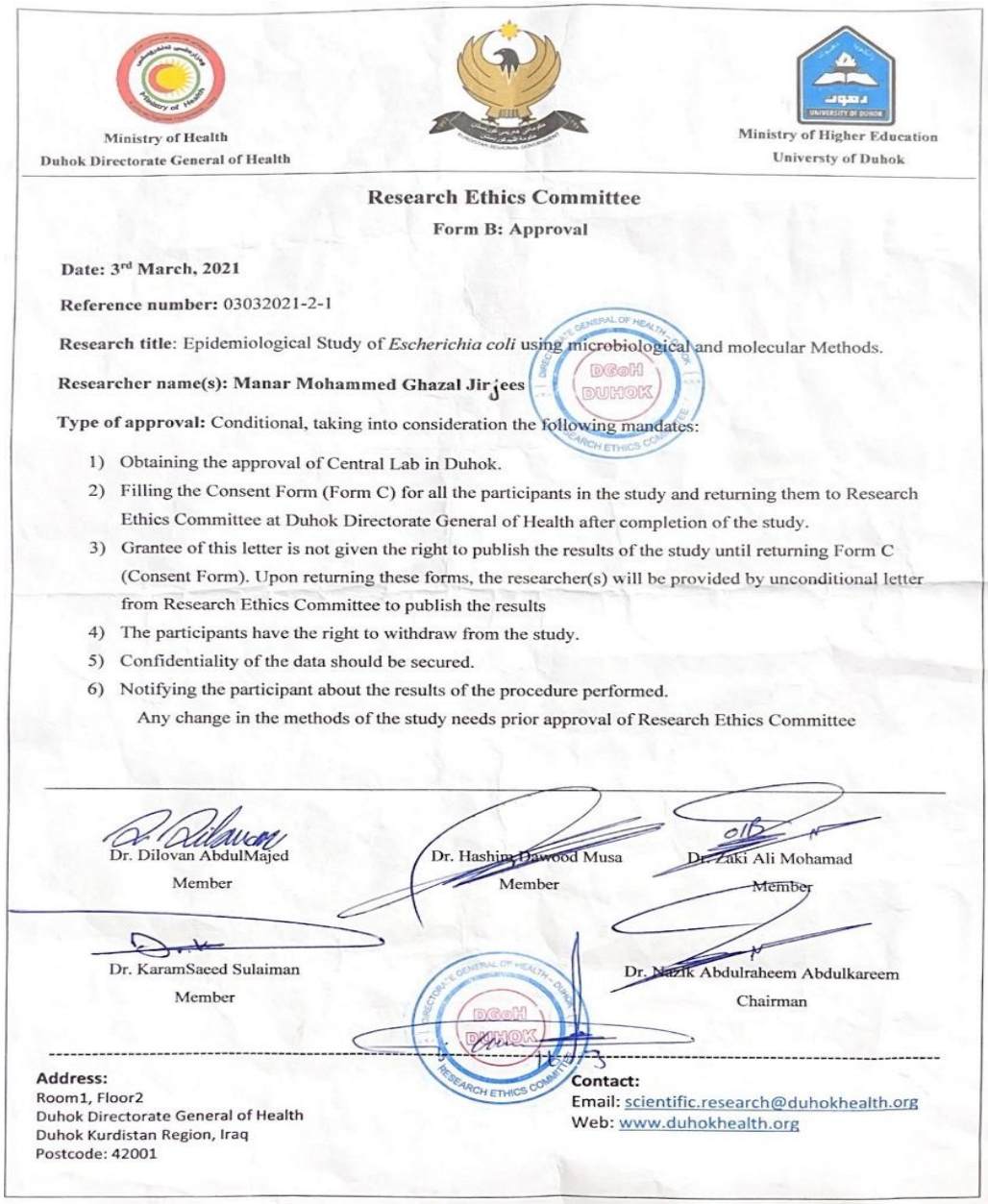
APPENDICES

APPENDIX- 1: APPROVAL OF RESEARCH ETHICS COMMITTEE



Ministry of Health
Duhok Directorate General of Health

Ministry of Higher Education
Universty of Duhok

Research Ethics Committee

Form B: Approval

Date: 3rd March, 2021

Reference number: 03032021-2-1

Research title: Epidemiological Study of *Escherichia coli* using microbiological and molecular Methods.

Researcher name(s): Manar Mohammed Ghazal Jirjees

Type of approval: Conditional, taking into consideration the following mandates:

1) Obtaining the approval of Central Lab in Duhok.
2) Filling the Consent Form (Form C) for all the participants in the study and returning them to Research Ethics Committee at Duhok Directorate General of Health after completion of the study.
3) Grantee of this letter is not given the right to publish the results of the study until returning Form C (Consent Form). Upon returning these forms, the researcher(s) will be provided by unconditional letter from Research Ethics Committee to publish the results
4) The participants have the right to withdraw from the study.
5) Confidentiality of the data should be secured.
6) Notifying the participant about the results of the procedure performed.

Any change in the methods of the study needs prior approval of Research Ethics Committee

Dr. Dilovan AbdulMajed
Member

Dr. Hashim Dawood Musa
Member

Dr. Zaki Ali Mohamad
Member

Dr. KaramSaeed Sulaiman
Member

Dr. Nazik Abdulraheem Abdulkareem
Chairman

DGoH DUHOK RESEARCH ETHICS COMMITTEE

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APPENDIX- 2. SOME PHOTOS

Figure A1.1. VITEK® 2 is automated identification system produced by BIOMERIEUX, France.

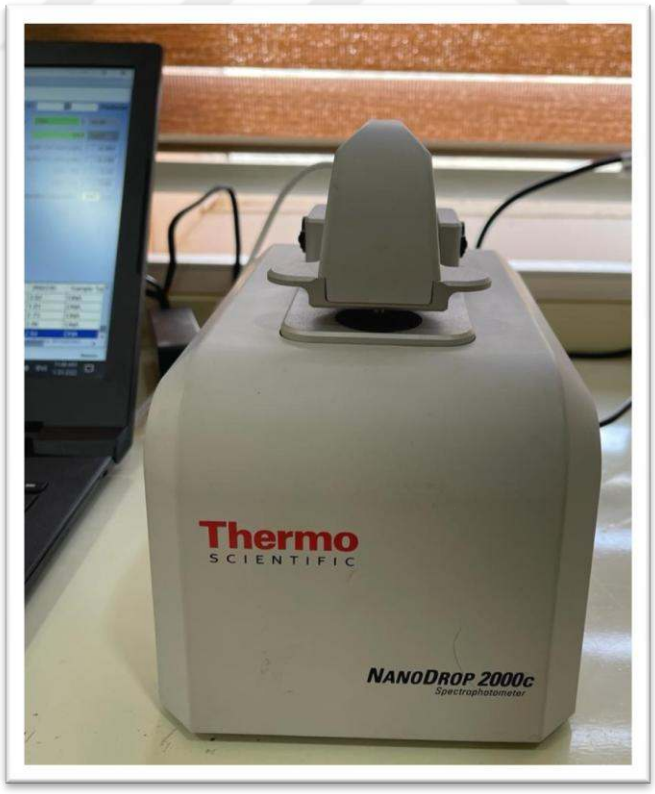

Figure A1.2. Nano Drop

Figure A1.3. Multigene Thermal Cycler

Figure A1.4. Horizontal Electrophoresis Chamber

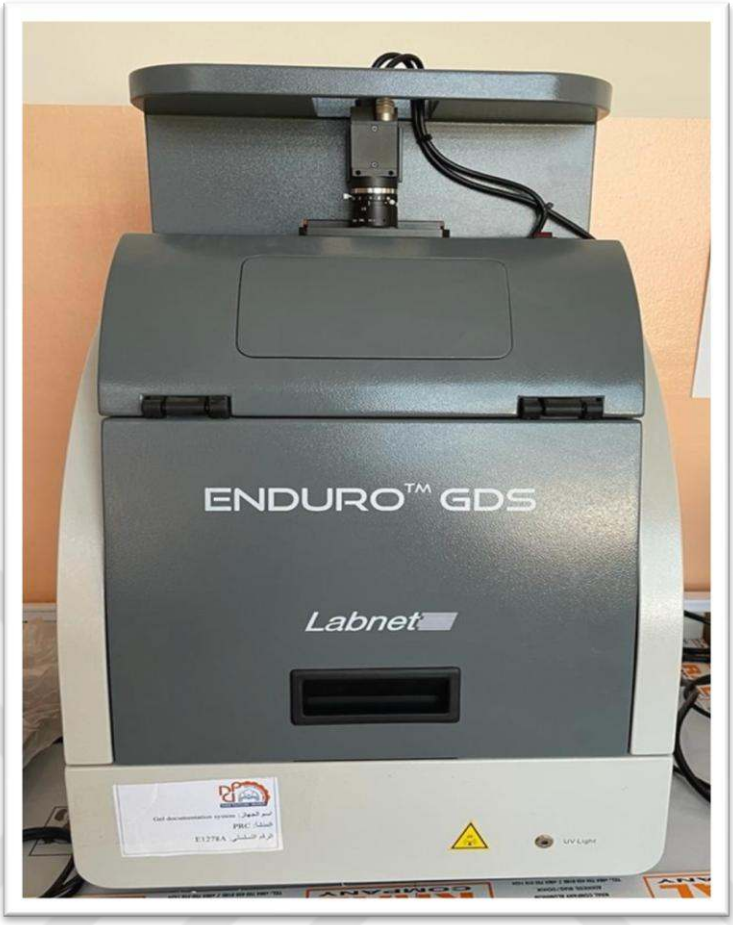

Figure A1.5. UV Transliminator

CURRICULUM VITAE

Manar Mohammed Ghazal Jirjees

[redacted]

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