An-Najah National University

Faculty of Graduate Studies

Virulence Factors and Phylogenetic Grouping in Uropathogenic Isolates of *Escherichia coli* Recovered from Thabet Hospital-Tulkarm, Palestine

By

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Dedication

To my parents, teachers, husband, sisters, brothers, to my angels (Mohammad and Yomna), students and all Palestinians, with love and appreciation.

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Thank you all ……

الإقرار

أنا الموقعة أدناه، مقدم الرسالة التي تحمل العنوان:

Virulence Factors and Phylogenetic Grouping in Uropathogenic Isolates of *Escherichia coli* Recovered from Thabet Hospital-Tulkarm, Palestine

أقر بأن ما شملت عليه هذه الرسالة إنّما هو نتاج جهدي الخاص، باستثناء ما تمّت الإشارة إليه حيثما ورد، وأنّ هذه الرسالة ككل، أو أيّ جزء منها لم يقدّم من قبل لنيل أيّ درجة أو لقب علميّ لدى أيّ مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researchers own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ :**

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List of Abbreviations

Abbreviation: Description/Function

Different Abbreviations :

ABU: Asymptomatic bacteriuria

CLSI: Clinical and Laboratory Standard Institute

DNA: Deoxyribonucleic acid

E.coli: Escherichia coli

ESBL: Extended Spectrum Beta Lactamases

EXPEC: Extraintestinal Pathogenic Escherichia coli

IBD: Inflammatory Bowel Disease

MDR: Multidrug Resistance

MLST: Multilocus sequence type

PAIs: pathogenicity associated islands

PCR: Polymerase chain reaction

UPEC: Uropathogenic Escherichia coli

UPGMA: Unweighed Pair Group Method With Arithmetic Mean

UTI: Urinary Tract Infection

VF: Virulence Factors

VFGs: Virulence Factor Genes

Chemical Abbreviations:

dNTPs: deoxyribonucleotides

EDTA: Ethelendiaminetetraacetic acid

Taq DNA polymerase: Thermous aquaticus DNA polymerase

Tris: Common pH buffer

H_2S: Hydrogen Sulfide

$MgCl_2$: Magnesium chloride

Media Abbreviations:

EMB: Eosin Methylene Blue

SIM: Sulfied Indole Medium

MR-VP: Methyl Red and Voges-Proskauer

Tests Abbreviations:

χ^2: Chi square test

IMViC: Indole production, Methyl red test, Voges-Proskauer test and Citrate utilization

TSI: Triple Sugar Iron

Virulence genes:

afa: a fimbrial adhesins

Bla-CTX-M: An extended-spectrum beta-lactamase with greater activity against Cefotaxime, CTX for cefotaximase and M for Munich

bmaE :M-agglutinin subunit

chuA: *gene required for heme transport in EHEC O157:H7*

Cnf1: Cytotoxic necrotizing factor type 1

cvaC: colicin V

dra: Dr-antigen binding adhesins

fimH: Type one fimbria adhesion

fyuA: Yersiniabactin receptor

gafD: G fimbria

hlyA: α- hemolysin

hlyD: δ-hemolysin

ibeA: invasive of brain endothelium

iha: iron regulated A homologue adhesion

ireA: Iron regulated element, a siderophor receptor

iroN: Catecholate siderophore receptor

iutA: aerobactin receptor

kpsMTII: Group 2 capsule synthesis gene

malX: pathogenic island marker ,PAI2 of the CFT073

ompT: Outer membrane protease T

papA: P-fimbria

papC: P-fimbria assembly

papG1: P-fimbria adhesion variant 1

papGII: p-fimbria adhesion variant 2

Sat: Secreted autotransporter toxin

sfaS: S fimbrial adhesion

sfa / foc: *sanel /F1C fimbriae subunits*

traT: Serum resistance-associated outer membrane protein

TspE4.C2: Anonymous DNA fragment

usp: Uropathogenic specific protein

yjaA: gene identified in *E.coli* K_{12} with unknown function

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Abstract

Escherichia coli (E. coli) is one of the most common bacteria that cause urinary tract infection (UTI). This pathogen is reported in 50-90% of all uncomplicated urinary tract infections.

This study aimed to analyze phylogenetic groups, virulence factors and antibiotic resistance phenotype in a collection of fifty *E. coli* strains, isolated from urine specimens obtained from suspected cases of UTIs, of inpatients and outpatients at Thabet Hospital during May-December 2012.

Phylogenetic analysis was detected by multiplex polymerase chain reaction (PCR). Results showed that the majority of the studied isolates, namely 36 (72%) strains, belonged to group D. Thirteen (26%) strains were assigned to group A, while 1 (2%) strain was belonged to group B1. There was a significant difference between the prevalence of these groups at $P < 0.001$.

The rate of resistance of these isolates to different antibiotics was detected by disk diffusion method, and has ranged from 24% for Gentamicin to 100% for Cefazoline and Erythromycin. These results showed that the mean antibiotic resistance scores were 8.4 and 6.5 for strains belonged to group D and group A, respectively, and statically significant difference at *P*

= 6.2 x 10. The prevalence of antibiotic resistance tested in each strain ranged from 14.3% to 100%. It was found that 61.5% (8/13) of strains belonged to group A were resistant to 5 or less antibiotics, while 66.7% (24/36) of strains belonged to group D were resistant to 6 or more antibiotics. Results showed association of Trimethoprim/Sulphamethoxazole resistance with the D group and was statistically significant difference at $P < 0.05$. Among fluoroquinolones and/or quinolones-resistant strains, in fact, the frequencies of groups D and A, were, respectively, 75.0% (27/36) and 61.5% (8/13).

Virulence genes were detected by PCR. Results showed that the prevalence of virulence genes ranged from 0% P-fimbria adhesion variant 1 (*papG I*) allele, and α- hemolysin (*hlyA*)) to 86% Type1 fimbria adhesion (f*imH)* and Serum resistance-associated outer membrane protein (*traT*) in strains tested. Virulence genes aerobactin receptor (*iutA*), Iron regulated A homologue adhesion (*iha*), Group 2 capsule synthesis gene (*kpsMTII*), Uropathogenic specific protein (*usp*), and Yersiniabactin receptor (*fyuA*) have the following prevalence 74%, 64%, 64%, 58% and 52%, respectively. Distribution of *fimH* gene was 86.1% and 84.6% in group D and A, respectively. The prevalence of genes tested in each strain was ranged from 5.6% to 83.3%. The results showed that the mean virulence scores for group D was 8.2 and ranged from 2 to15, while for group A was 6.2 and ranged from 1 to14. The mean virulence scores between the 2 groups was statically significant difference at $P = 6.2 \times 10^{-4}$. Also it was found that 69.2% (9/13) of strains belonged to group A carried 6 or less

virulence factors, while 66.7% (24/36) of strains belonged to group D carried 7 or more virulence factors. The quinolones and/or fluoquinolones sensitive strains related to group D showed an increase in prevalence of Catecholate siderophore receptor (*iron*) virulence gene than resistant. In group A, average of prevalence of P-fimbria adhesion variant 2 (*papGII*) *was* increased and *fimH* decreased in quinolones and fluoquinolones sensitive strains. Comparison between resistant and sensitive strains to quinolones and fluoquinolones regardless to phylogenetic groups, sensitive strains showed increase in Outer membrane protease T (*ompT*) virulence genes. It seems that there is no single virulence factor nor virulence profile that is entirely specific to UTI. It also was found that *traT* gene was the most common prevalence among strains resistant to Nalidixic acid, fluoroquinolones and Trimethoprim/Sulphamethoxazole and it was 90.1% (30/33), 95.8 (23/24) and 90.6% (29/32), respectively.

In conclusion, the molecular analysis of 50 *E. coli* urine isolates exhibited a greater prevalence of phylogenetic group D, high virulence scores and high resistance scores than group A. Urinary tract infections caused by such strains represent a clinical problem because of limited therapeutic options. Even though additional, in vivo studies by using mutant strains or the product of these genes should be performed to confirm the significance of the detected VFs. Further analysis of VF profiles with regard to specific clinical symptoms and defined severity is recommended.

Chapter One

Introduction

Escherichia coli (E. coli) is the most abundant facultative anaerobic bacteria of the human and of many animals intestinal microflora. It is one of the most common isolates in clinical microbiology laboratories and is classified into three major groups: harmless commensal strains, intestinal pathogenic strains, and extraintestinal pathogenic strains, according to their biological significance to humans (Russo and Johnson, 2000; Dobrindt, 2005). *E. coli*, the most frequent cause of urinary tract infections (UTI), intra-abdominal and soft tissue infections, neonatal sepsis, osteomyelitis often associated to bacteremia and meningitis, pneumonia, and bacterial infectious diarrhea, it is responsible for an enormous burden of morbidity, mortality, and health care costs (Russo and Johnson, 2000; Johnson and O'Bryan, 2000).

E. coli is one of the most common bacterial causes of UTI, including acute cystitis, pyelonephritis, and prostatitis. The basis for the occurrence of these distinctive disorders, which presumably arise from localized infection and inflammation within the bladder, kidney, and prostate gland, respectively, is undefined (Johnson *et al*., 2005a). The pathogen is reported 50 – 90% of all uncomplicated urinary tract infections (Steadman *et al*., 1998; Struelens *et al*., 2004). The clinical management of UTIs has become very complicated due to the emergence of resistance to most commonly used antimicrobial agents, particularly among uropathogenic *E. coli* (UPEC) strains (Karaca *et al*., 2005). A noticeable increase in the occurrence of strains of *E. coli* causing UTIs that are resistant to

cephalosporins, quinolones, fluoroquinolones, and trimethoprim, which has special clinical importance because of the limited therapeutic options available was reported (Paterson and Bonomo, 2005; Piatti *et al*., 2008).

Pathogenic and commensal strains of *E. coli* to a large extent derive from separate evolutionary groups within the highly clonal *E. coli* population (Caugant *et al*., 1983; Selander *et al*., 1987; Picard *et al*., 1999). The genetic structure of *E. coli* can be considered clonal, several intestinal or extraintestinal *E. coli* infections have been linked to specific clones or related clones (Selander *et al*., 1986; Whittam *et al*., 1993; Maslow *et al*., 1995). Strains from lineages have ability to cause disease in intact hosts, this might be due to possess specific virulence traits, which associated with pathogenicity. These virulence traits are transmitted vertically within the resulting virulent clones but also can be transmitted horizontally to other lineages sometimes as part of pathogenicity-associated islands (*PAIs*) (Johnson *et al*., 2000). Determination of the prevalence of certain virulence factors in UPEC strains, suggests that they might make useful targets for vaccines and other protective interventions (Johnson *et al*., 2005a).

Phylogenetic studies have shown that *E. coli* clones can be allocated to 4 main phylogenetic groups, designated A, B1, B2 and D. A method was developed to type pathogenic *E coli* based on certain genes or DNA fragments, which might be specific phylogenetic group markers. These

include a gene required for heme transport in EHEC O157:H7 (*chuA)*, a gene initially identified in the recent complete genome sequence of *E. coli* K-12, the function of which is unknown (*yjaA*) and an anonymous DNA fragment designated *TspE4.C2* (Clermont *et al*., 2000).

1.1. Objectives of the present study

The relationship among virulence factors of *E. coli*, phylogenetic background, and antibiotic resistance is a complex phenomenon, resulting from their different interactions (Piatti *et al*., 2008). To clarify whether the virulence factors is directly associated with resistance or, instead, depends on a phylogenetic distribution, our study aims to analyze phylogenetic groups, virulence factors and resistance phenotype in a collection of *E. coli* strains isolated from UTIs. This study has not been investigated previously in Palestine.

Chapter Two

Literature review

Strains that cause extraintestinal infections mostly belonged to groups B2 and/or D (Picard *et al*., 1999; Russo *et al*., 2000; Duriez *et al*., 2001; Johnson *et al*., 2005b; Piatti *et al*., 2008; Petkovsek *et al*., 2009; Lee *et al*., 2010; Bashir *et al*., 2011; 2012), where most commensal and less virulent strains belong mostly to group A or B1 (Picard *et al*., 1999; Russo *et al*., 2000; Duriez *et al*., 2001). Other studies reported a high proportion of group B2 or D strains in the intestinal microflora of humans (Zhang *et al*., 2002; Nowrouzian *et al*., 2005; Lee *et al*., 2010). Some authors suggested that group B2 *E. coli* strains have evolved characteristics that allow them to survive in the complex ecosystem of the human intestine. Type 1 fimbriae (*fimA)* and P fimbriae assembly (*papC*), and especially the combination of these two adhesins, were significantly associated with intestinal persistence (Nowrouzian *et al*., 2005). A strong correlation was found between isolation of *E. coli* of the phylogenetic group B2 from Inflammatory Bowel Disease (IBD) patients with left-sided colitis. It was found that these isolates belonged to a specific cluster based on multilocus sequence type (MLST), confirming a common ancestry of these IBD associated B2 *E. coli*, also B2 strains with extraintestinal pathogenic *E. coli* (ExPEC) genes were found more frequently among IBD patients with active disease compared to patients with inactive disease (Petersen *et al*., 2009). In another study, a strong correlation was found between phylogenetic groups B2 and D from IBD patients with Crohn's disease and ulcerative colitis. It was shown that serine protease autotransporters and adherence factors in

B2 and D phylogenetic groups may have a significant role in disease aetiology (Kotlowski *et al*., 2007).

It was reported that group A strains to be the most common of *E. coli* isolates from feces of asymptomatic people and approximately 50% of the strains carried PAI markers (Li *et al*., 2010). Strains of *E. coli* groups B2 and D often carry virulence-factor genes that are lacking in other groups (Picard *et al*., 1999; Johnson *et al*., 2001; Duriez *et al*., 2001). This difference can probably be attributed to the bacterial characteristics in different geographic regions under the influence of antibiotics usage or host genetic factors (Lee *et al*., 2010). In another study on skin and soft tissue infections, the majority of *E. coli* isolates belonged to the B2 phylogenetic group. The most prevalent virulence factor among tested strains was outer membrane protease T *(ompT*) followed by group II capsule (*kpsMT II*). This study revealed that *E. coli* isolates from skin and soft tissue infections exhibited a remarkable virulence potential that is comparable to that of *E. coli* isolates recovered from urinary tract infections and bacteremia. In general, strains belonging to the B2 phylogenetic group exhibited the highest prevalence of virulence factors, and the most prevalent adhesin sequences among B2 group strains was P fimbriae (*papA*). (Petkovsek *et al*., 2009). The prevalence of all virulence factors except S fimbrial adhesion (*sfaS*) was significantly higher in pathogenic strains than in

commensal strains and they were most frequent in phylogenetic group B2. α-Haemolysin (*hlyA*), yersiniabactin receptor (*fyuA*), serum resistance-associated outer membrane protein (*traT*), and aerobactin receptor (*iutA*) were found to be independent predictors for pathogenicity. This finding suggests that the combinatorial approach of virulence factors, including a toxin that can modulate the host signaling pathway (*hlyA*), two iron acquisition systems (*iutA* and *fyuA*) that allow bacteria to multiply in an environment of limited concentration of free iron such as in tissues and fluids of the host, and *traT,* may enable ExPEC to enter the primary infection sites, spread to secondary internal organs, and survive in the blood, causing septicemia. Of these virulent factors, *traT* and *iutA* were significantly more common in *bla*CTX-M-9 group and *bla*CTX-M-1, respectively (Lee *et al*., 2010). The prevalence of *fyuA, traT,* and *PAI* was significantly higher in *E. coli* isolates from urine than in isolates from blood, as previously reported in *E. coli* strains from urosepsis (Lee *et al*., 2010). Group D UPEC isolates showed highest presence of virulence genes. The prevalence of hlyA was highest among tested virulence factors and these isolates showed highest level of cytotoxicity (Bashir *et al*., 2012). In regard to the association of antimicrobial resistance genes and virulence factors, there are controversial reports. There were positive associations between several antimicrobial resistance genes *iutA* and *traT* (Bagger-Skjot

et al., 2007). In contrast, there were negative associations between quinolone-resistance and *hlyA* and cytotoxic necrotizing factor type 1 (*cnf1*) in UPEC (Vila *et al*., 2002). Ten virulence factors were less prevalent in the Extended Spectrum Beta Lactamases (ESBL) *bla*CTX-M-producing *E. coli* strains isolated from UTIs than the susceptible *E. coli* , while *iutA* and *traT* were more prevalent in these isolates (Lavigne *et al*., 2006). Molina-López et al. revealed that more than half of the Multidrug resistance (MDR) of *E. coli* isolates belonged to virulent phylogenetic group B2, suggesting that antimicrobial resistance could be compatible with certain strains that carry virulence-factor genes, such as the pathogen O25-ST131 clone (Molina-López *et al*., 2011). It was shown that carboxylesterase type B2 UPEC strains (phylogenetic group B2) were more susceptible to antibiotics than carboxylesterase type B1 strains (phylogenetic groups A, B1, and D) (Picard *et al*., 1999). Other studies on *E. coli* from human isolates showed negative association between resistances and virulence factors (Cherifi *et al*., 1990; Johnson *et al*., 2004). Data reported by Bashir *et al*., (2011), showed that emergence of multiple drug resistant isolates among UPEC with dominance of phylogenetic group B2. However, group D isolates were found to be highly resistant to all drugs as compared with group B2 but were less frequent. A lower prevalence of B2 group was detected in resistant than in fluoroquinolones-

susceptible UPEC strains, and the combination of certain virulence determinants with group B2 and with susceptible isolates was confirmed (Johnson *et al*., 2004; 2005c; Horcajada *et al*., 2005; Moreno *et al*., 2006; Piatti *et al*., 2008; Kawamura-Sato *et al*., 2010). Within group B2, fluoroquinolone-susceptible strains showed higher prevalence of *papC, hlyA*, and *cnf1* than their resistant counterparts. In contrast, the incidence of *iutA* appeared higher for refractory isolates, including group B2, than for susceptible isolates. Mutations conferring quinolone and fluoroquinolone resistance may thus require a particular genetic background, not strictly correlated with phylogenetic groups (Piatti *et al*., 2008). Results obtained by Saeed *et al*., (2009), showed that most MDR *E. coli* infecting wounds belonged to phylogenetic group A. *E. coli* under high antibiotic selective pressure showed a high prevalence of integrons regardless of the phylogenetic group. It was shown that *E. coli* isolated from urine samples and under very low selective pressure, resistance emerges without integrons. As the antibiotic pressure increases, quinolone and integron-mediated resistance occurs outside phylogenetic group B2 (Skurnik *et al*., 2009).

Molecular analysis of *E. coli* urine isolates showed that pyelonephritis and prostatitis isolates exhibited more virulence factors than did cystitis isolates. The phylogenetic groups B1 and B2 showed highest virulence

factor scores while groups A and D exhibiting the lowest. The distribution of phylogenetic groups did not differ significantly among the 3 UTI syndromes (Johnson *et al*., 2005a). It was shown that *bmaE, gafD*, K2 *kpsM* variant, *ireA, cvaC, ibeA*, *pap* and *papG* allele II as novel pyelonephritis-associated virulence factors and *pap, hlyD,* and *cnf1* as prostatitis-associated virulence factors (Terai *et al*., 1997; Johnson *et al*., 2005a). The severity of the UTI depends both on the virulence of the pathogen and on the susceptibility of the host. *E. coli* strains in some patients establish significant bacteriuria without causing symptoms of UTI. Several studies suggested that asymptomatic-bacteriuria (ABU) was caused by certain strains that have been shown to express fewer virulence factors than the UPEC strains, which do not provoke a host response and therefore cause no symptoms (Leffler and Svanborg-Eden, 1981; Caugant *et al*., 1983). Genotypic analyses contradicted this notion as many ABU strains carry virulence genes but fail to express them (Plos *et al*., 1995; Zdziarski *et al*., 2008). These studies suggest that these strains may have evolved from virulent UPEC strains by genome loss and virulence gene attenuation (Zdziarski *et al*., 2008; Wiles *et al*., 2008).

Chapter Three
Materials and Methods

3.1. Sample collection and *E. coli* identification

3.1.1. Sample collection:

Fifty isolates of *E. coli* were isolated from urine specimens obtained from suspected cases of urinary tract infections of inpatients and outpatients at Thabet Hospital during May-December 2012. These isolates were identified in laboratory of Thabet Hospital and also were confirmed in Microbiology laboratories at An-Najah National University-Nablus, Palestine. The isolates were cultured on MacConkey and/or EMB agars, Gram stain and biochemical tests were used as IMViC Tests (Indole production, Methyl red test, Voges-Proskauer test and Citrate utilization), H_2S production and motility test.

3.2. Media preparation

3.2.1 MacConkey Agar:

MacConkey agar was prepared according to the manufacturer's instructions labeled on the bottle. A 1L bottle containing 500 ml deionized water and 25 g MacConkey agar was heated and stirred until the agar dissolved. The solution was allowed to boil for 1min, and then was autoclaved at 121 °C for 15 min. After that it was allowed to cool, and the agar was poured into sterile Petri dishes to have 20 ml that covered and left overnight. The following morning the Petri dishes were turned upside down and stored at 4°C.

3.2.2. Eosin Methylene Blue (EMB) Agar:

EMB medium was prepared according to the manufacturer's instructions labeled on the bottle. A 1L bottle containing 500 ml deionized water and 18.75 g of EMB agar was heated and stirred until the agar dissolved. The solution was allowed to boil for 1 min, and then was autoclaved at 121 °C for 15 min. After that it was allowed to cool, and the agar was poured into sterile Petri dishes to have 20 ml that covered and left overnight. The following morning the Petri dishes were turned upside down and stored at 4°C.

3.2.3. Sulfied Indole Motility (SIM) Medium:

SIM medium was prepared according to the manufacturer's instructions labeled on the bottle. A 0.5L bottle containing 250 ml deionized water and 7.5 g of SIM agar was heated and stirred until dissolved. Medium was dispensed into tubes to a give depth of about 4-5 cm. Then the medium was autoclaved at 121 °C for 15 min, allowed to cool and then stored at 4°C.

3.2.4. Methyl Red and Voges Proskauer (MR-VP) medium:

MR-VP medium was prepared according to the manufacturer's instructions labeled on the bottle. A 0.5L bottle containing 250 ml deionized water and 4.25 g of MR-VP medium was mixed thoroughly, heated to dissolve if necessary. Five ml of MR-VP broth was dispensed into tubes, autoclaved at 121 °C for 15 min, allowed to cool and then stored at 4°C.

3.2.5. Simmons Citrate Agar:

Simmons citrate agar was prepared according to the manufacturer's instructions labeled on the bottle. A 1L bottle containing 500 ml deionized water and 11.25 g of Simmons citrate agar was heated and stirred until dissolved. Ten ml of Simmons citrate agar was dispensed into tubes, autoclaved at 121 °C for 15 min. The medium was prepared as slant agar tubes and then stored at 4°C.

3.2.6. Triple Sugar Iron (TSI) Agar:

TSI agar was prepared according to the manufacturer's instructions labeled on the bottle. A 1L bottle containing 500 ml deionized water and 32.5g of Triple sugar Iron agar was mixed thoroughly, heated to dissolve if necessary. Ten ml of Triple sugar Iron medium was dispensed into tubes, autoclaved at 121°C for 15min. The medium was prepared as slant agar tubes and then stored at 4°C..

3.3. Sample Identification

3.3.1. Gram staininig:

Gram staining of bacteria was performed from nutrient broth as described previously (Cappiccino and Sherman 1996).

3.3.2. Indole test:

Indole test was carried out by adding 10 drops of Kovac's reagent into SIM agar deep tube inoculated by means of stab method (Cappiccino and Sherman 1996).

3.3.3. MR-VP tests:

MR test was carried out by adding 5 drops of methyl red indicator, while for VP test was carried out by adding 10 drops of Barritt's reagent A, the culture was shaken, then immediately 10 drops of Barritt's reagent B was added, then culture was shaken and re-shaken after every 3-4 min (Cappiccino and Sherman 1996).

3.3.4. Citrate utilization test:

Citrate utilization test was carried out by stabbing and streaking inoculation of Simmons citrate agar (Cappiccino and Sherman 1996).

3.3.5. TSI test:

TSI test was carried out by inoculation the TSI agar slants by means of stab-and-streak (Cappiccino and Sherman 1996).

3.4. Antibiotic resistance

Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method (CLSI, 2010). All *E. coli* isolates were examined for resistance to

tetracycline (30µg), streptomycin (10µg), gentamicin (10µg), kanamycin (30µg), nalidixic acid (30µg), norfloxacin (10µg), ciprofloxacin (10µg), ofloxacin (5µg), Levofloxacin (5µg), ceftriaxone (30µg), ceftazidime (30µg), cefazolin (30 µg), Trimethoprim/Sulfamethoxazole (1.25 /23.75µg) and erythromycin (15 µg). Zones of inhibition were determined in accordance with procedures of the Clinical and Laboratory Standard Institute (CLSI, 2010).

3.5. DNA extraction

E. coli DNA was prepared for PCR according to the method described previously with some modifications (Adwan *et al*., 2013). Briefly, cells were scraped off an overnight nutrient agar plate with a sterile loop, washed with 1 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), then the pellet was resuspended in 0.5 ml of sterile distilled water, and boiled for 10-15 min. The cells then were incubated on ice for 10 min. The debris was pelleted by centrifugation at 11,500 X *g* for 5 min. DNA concentration was determined using a spectrophotometer and the samples were stored at -20ºC until use for further DNA analysis.

3.6. Polymerase chain reaction

3.6.1. Phylogenetic classification:

Strains were assigned to one of the four *E. coli* phylogenetic groups (A, B1, B2 and D) using a triplex PCR based on the presence or absence of three DNA fragments: chuA, yjaA, and TspE4C2 (Clermont *et al*., 2000). The

primer pairs used were chuA.1 (5′-GAC GAA CCA ACG GTC AGG AT-3′) and chuA.2 (5′-TGC CGC CAG TAC CAA AGA CA-3′), yjaA.1 (5′-TGA AGT GTC AGG AGA CGC TG-3′) and yjaA.2 (5′-ATG GAG AAT GCG TTC CTC AAC-3′), and TspE4.C2.1 (5′-GAG TAA TGT CGG GGC ATT CA-3′) and TspE4.C2.2 (5′-CGC GCC AAC AAA GTA TTA CG-3′) giving amplification products of 279, 211 and 152 bp respectively. The combination of PCR products allowed phylogenetic group determination of *E. coli* isolates (Table 3.1)

Each PCR reaction mix (25 μL) was performed using 12.5 μL of PCR premix with $MgCl_2$ (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4 μM of each primer, and 3 μL DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 4min at 94ºC followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 30 s, ending with a final extension step at 72ºC for 5 min. The PCR products (10 μL) were analyzed by electrophoresis on 2% agarose gel.

Table 3.1: Phylo-types resulting from the application of the Clermont method (Clermont *et al*., 2000).

ChuA	yjaA	TspE4.C2	Phylo-group assignment
-	-	-	A
-	+	-	A
-	-	+	B1
+	+	-	B2
+	+	+	B2
+	-	-	D
+	-	+	D

3.6.2. Virulence factors:

The presence of 18 virulence genes was investigated using multiplex PCR divided into seven pools and their amplicon sizes are listed in Table 3.2. The genes detected were f*imH*, *afa*, *sfa/foc*, *papG* (three alleles), *cnf1*, *sat*, *hly*, *iutA*, *iroN*, *fyuA*, *iha*, *kpsMTII*, *ompT*, *malX*, *traT* and *usp*.

Each PCR reaction mix (25 µL) was performed using 12.5 µL of PCR premix with $MgCl_2$ (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4 µM of each primer, and 3 µL DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 4 min at 94ºC followed by 25 cycles of denaturation at 94ºC for 30 s, annealing at 60ºC for 30 s and extension at 72ºC for 1 min, ending with a final extension step at 72ºC for 5 min. The PCR products (10 µL) were analyzed by electrophoresis on 2% agarose gel. The sizes of the amplicons were determined by comparing them with a 100-bp DNA ladder.

Table 3.2: Virulence gene primers used in this study (Ferjani et al., 2012).

Pool	Virulence Gene	Primer forward/reverse	Forward sequence 5→3	Reverse sequence 5→3	Product size (bp)
Pool I	*fimH*	FimHf/FimHr	TGCAGAACGGATAAGCCGTGG	GCAGTCACCTGCCCTCCGGTA	508
	malX	malXf/malXr	GGACATCCTGTTACAGCGCGCA	TCGCCACCAATCACAGCCGAAC	930
Pool II	*sfa/foc*	sfa1/sfa2	CTCCGGAGAACTGGGTGCATCTTAC	CGGAGGAGTAATTACAAACCTGGCA	410
	iutA	AerJf/AerJr	GGCTGGACATCATGGGAACTGG	CGTCGGGAACGGGTAGAATCG	300
	papGIII	allele IIIf/allele IIIr	GGCCTGCAATGGATTTACCTGG	CCACCAAATGACCATGCCAGAC	258
	fyuA	fyuaf/fyuar	TGATTAACCCCGCGACGGGAA	CGCAGTAGGCACGATGTTGTA	880
Pool III	*hlyA*	hlyf/hlyr	AACAAGGATAAGCACTGTTCTGGCT	ACCATATAAGCGGTCATTCCCGTCA	1117
	KpsT II	kpsIIf/kpsIIr	GCGCATTTGCTGATACTGTTG	CATCCAGACGATAAGCATGAGCA	272
	papGI	allele If/allele Ir	TCGTGCTCAGGTCCGGAATTT	TGGCATCCCCCAACATTATCG	461
Pool IV	*traT*	TraTf/TraTr	GGTGTGGTGCGATGAGCACAG	CACGGTTCAGCCATCCCTGAG	290
	papGII	allelele If/allele IIr	GGGATGAGCGGGCCTTTGAT	CGGGCCCCCAAGTAACTCG	190
Pool V	*afa/dra*	Afaf/Afar	GGCAGAGGGCCGGCAACAGGC	CCCGTAACGCGCCAGCATCTC	559
	cnf1	cnf1/cnf2	AAGATGGAGTTTCCTATGCAGGAG	CATTCAGAGTCCTGCCCTCATTATT	498
Pool VI	*Iha*	IHAf/IHAr	CTGGCGGAGGCTCTGAGATCA	TCCTTAAGCTCCCGCGGCTGA	827
	Usp	USP81f/USP695r	CGGCTCTTACATCGGTGCGTTG	GACATATCCAGCCAGCGAGTTC	615
Pool VII	*ompT*	ompTf/ompTr	ATCTAGCCGAAGAAGGAGGC	CCCGGGTCATAGTGTTCATC	559
	sat	sat1/sat2	ACTGGCGGACTCATGCTGT	AACCCTGTAAGAAGACTGAGC	387
	iroN	IRONECf/IRONECr	AAGTCAAAGCAGGGGTTGCCCG	GACGCCGACATTAAGACGCAG	665

3.7. Statistical analysis

The virulence factor scores (VF score) or antibiotic resistance scores was calculated for each isolate as the sum of all virulence factor genes or antibiotic resistance present in each strain (Johnson *et al*., 2005c).

Different tests were used to analyze data in this research. These tests were Mann-Whitney U-Test (Two-tailed), Fisher exact test and/or Chi-square (χ^2) test. $P < 0.05$ values were considered statistically significant.

Chapter Four
Results

4.1 Identification of *E. coli* isolates

The results of gross colony morphology were green metallic sheen on EMB agar, bright pink or red colonies on MacConkey agar, Gram stain negative, short rod, single, pair or in short chain. All isolates were lactose fermenter with gas production, Methyl Red positive, Voges-Proskauer test negative, Indole test positive, H_2S production negative, motility test positive and citrate utilization negative.

4.2 Phylogenetic groups

The majority 36 (72%) of *E. coli* isolated from suspected cases of urinary tract infections of inpatients and outpatients at Thabet Hospital were belonged to group D. Thirteen strains (26%) were assigned to group A while 1 strain (2%) was belonged to group B1 (Figure 4.1). There was a significant difference between the prevalence of these phylogenetic groups at $P < 0.001$.

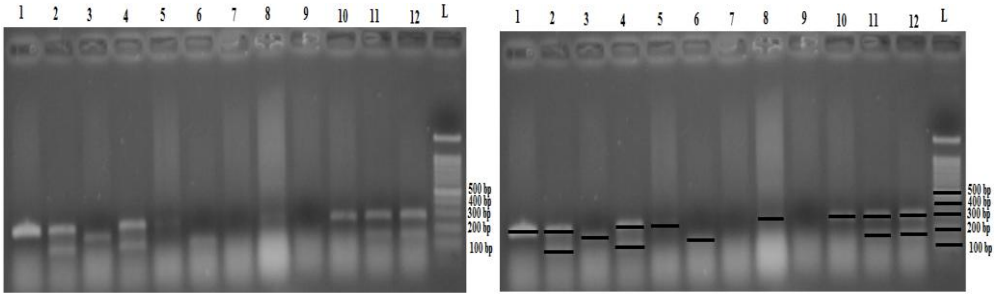

Figure 4.1: Triplex PCR profiles specific for *E. coli* phylogenetic groups. Lanes 1, 2, 4, 5, 8, 10, 11 and 12 belonged to phylogenetic group D; lanes 3, 7 and 9 belonged to phylogenetic group A; lane 6 phylogenetic group B1, lane L contained Ladder.

4.3 Antibiotic sensitivity test

All tested antibiotics were classified into 7 different classes according to their chemical structure: Quinolones (nalidixic acid), Cephalosporines (ceftriaxone, ceftazidime, cefazolin), Tetracyclines (tetracycline), Fluoroquinolones (ofloxacin, ciprofloxacin, levoflxacin, norfloxacin), Aminoglycosides (streptomycin, kanamycin, gentamicin), Sulfonamides (Trimethoprim/Sulphamethoxazole) and Macrolides (erythromycin). The rates of resistance of *E. coli* isolates to different antibiotics tested are presented in Table 4.1. Antibiotic resistance has ranged from 24% for Gentamicin to 100% for Cefazolin and Erythromycin. Forty resistance patterns were observed in the UPEC isolates with profile Streptomycin-Kanamycin-Cefazolin-Erythromycin, being the most predominant indicating a striking diversity of resistance patterns among uropathogenic strains in this hospital (Table 4.2). Also results showed that 98% of strains were multidrug resistant. Gentamicin, Norfloxacin and Levoflxacin were the most effective drugs in general. Group D isolates were more drug resistant as compared with group A. The correlation between the antibiotic

resistance scores and the phylogenetic group was examined. The mean antibiotic resistance scores were 8.4 and 6.5 for strains belonged to group D and group A, respectively, and significant difference at $P = 6.2 \times 10^{-4}$. The prevalence of antibiotic resistance tested in each strain was ranged from 14.3% to 100%. It was found that 61.5% (8/13) of strains belonged to phylogenetic group A were resistant to 5 or less antibiotics, while 66.7% (24/36) of strains belonged to phylogenetic group D were resistant to 6 or more antibiotics. Results showed that 2 large clusters depend on resistance/sensitive of strains to fluoroquinolones, but clustering is independent to phylogenetic groups (Figure 4.2). Results showed association of Trimethoprim/ Sulphamethoxazole resistance with the D group and there was statistically significant difference at $P < 0.05$ (Table 4.1). Among fluoroquinolones and quinolones susceptible strains, in fact, the frequencies of groups D and A, were, respectively, 25% (9/36), and 38.5% (5/13), while among fluoroquinolones and/or quinolones-resistant strains these frequencies were 75.0% (27/36) and 61.5% (8/13).

Table 4.1: Antibiotic resistance of 50 *E. coli* isolates recovered from urine samples in Thabet Hospital, Tulkarm, Palestine.

Group	Antibiotic	Resistant strains		[a] Prevalence of AR between groups		AR scores (mean)	
		No. of samples	%	D n=36 (%)	A n=13 (%)	D AR score (%) 304 (8.4)	A AR score (%) 84 (6.5)****
Tetracycline	**Tetracycline**	31	62	24 (66.7%)	6 (46.2%)	237 (6.6)	56 (4.3)
Quinolones	**Nalidixic Acid**	34	68	26 (72.2 %)	7 (53.8%)	261 (7.3)	59 (4.5)****
Cephalosporines	**Ceftriaxone**	23	46	19 (52.8%)	4 (30.8%)	257 (7.1)	38 (2.9)****
	Ceftazidime	17	34	14 (53.8%)	3 (23.1%)	163 (4.5)	28 (2.2)****
	Cefazolin	50	100	36 (100%)	13 (100%)	304 (8.4)	84 (6.5)
Fluoroquinolones	**Ciprofloxacin**	21	42	17 (47.2%)	4 (30.8%)	200 (5.5)	38 (2.9)****
	Levoflxacin	19	38	16 (44.4%)	3 (23.1%)	206 (5.7)	33 (2.5)****
	Norfloxacin	18	36	15 (41.7%)	3 (23.1%)	184 (5.1)	33 (2.5)****
	Ofloxacin	22	44	18 (50%)	4 (30.8%)	216 (6.0)	44 (3.4)***
Aminoglycosides	**Kanamycin**	29	58	19 (52.8%)	10 (76.9%)	191 (5.3)	67 (5.2)**
	Gentamicin	12	24	9 (25%)	2 (15.4%)	108 (3.0)	24 (1.8)
	Streptomycin	37	74	28 (77.8%)	8 (61.5%)	241 (6.7)	59 (4.5)
Macrolides	**Erythromycin**	50	100	36 (100%)	13 (100%)	304 (8.4)	8.4 (6.5)
Sulfonamides	**Trimethoprim/Sulphamethoxazole**	32	64	27 (75%)	4 (30.8%)**	259 (7.2)	41 (3.2)***

** significant difference at $P < 0.05$, *** significant difference at $P < 0.01$, **** significant difference at value $P < 0.001$.

[a] AR: Antibiotic Resistance; One isolate belonged to group B1 is not included in prevalence of AR between groups and aggregate AR score (median).

Table 4.2: Antimicrobial resistance patterns of UPEC isolates recovered from urine samples in Thabet Hospital, Tulkarm, Palestine.

	Resistance pattern	No. of multidrug resistant Isolates
1	S,K,CZ, E[a]	5
2	CZ,E	1
3	S,CZ,E	2
4	NA,CZ,E	1
5	CRO,CAZ,CZ,E	1
6	S,K,CZ,E	1
7	NA,S,CZ,E	1
8	TE,S,SXT,CZ,E	1
9	NA,S,SXT,CZ,E	2
10	TE,S,SXT,CZ,E	1
11	TE,CIP,K,CZ,E	1
12	NA,S,K,CZ,E	1
13	TE,S,SXT,CZ,E	1
14	TE,CIP,S,SXT,CZ,E	1
15	TE,NA,S,K,CZ,E	1
16	TE,S,K,SXT,CZ,E	1
17	NA,S,K,SXT,CZ,E	1
18	TE,NA,CRO,S,SXT,CZ,E	1
19	TE,NA,S,K,SXT,CZ,E	1
20	TE,NA,CRO,CAZ,SXT,CZ,E	1
21	TE,NA,S,SXT,CN,CZ,E	2
22	TE,NA,CRO,CAZ,OFX,CIP,S,SXT,CZ,E	1
23	TE,NA,OFX,CIP,LEV,NOR,K,SXT,CZ,E	1
24	NA,CRO,CAZ,OFX,LEV,S,K,CN,CZ,E	1
25	TE,NA,CRO,OFX,CIP,LEV,NOR,S,CZ,E	1
26	NA,CRO,CAZ,OFX,CIP,LEV,NOR,S,K,CZ,E	1
27	TE,NA,CRO,OFX,CIP,LEV,NOR,K,SXT,CZ,E	1
28	NA,CRO,CAZ,OFX,CIP,LEV,NOR,K,SXT,CZ,E	1
29	TE,NA,CRO, OFX,CIP,LEV,NOR,S,SXT,CZ,E	1
30	TE,NA,CRO,CAZ,OFX,S,K,SXT,CN,CZ,E	1
31	TE,NA,OFX,CIP,LEV,NOR,S,K,SXT,CN,CZ,E	1
32	TE,NA,CRO,CAZ,OFX,CIP,LEV,NOR,K,SXT,CZ,E	1
33	TE,NA,CRO,CAZ,OFX,CIP,LEV,NOR,S,SXT,CZ,E	2
34	TE,NA,CRO,CAZ,OFX,CIP,LEV,NOR,K,SXT,CZ,E	1
35	TE,NA,CRO,CAZ,OFX,LEV,S,K,SXT,CN,CZ,E	1
36	TE,NA,CRO,OFX,CIP,LEV,NOR,S,K,SXT,CZ,E	1
37	TE,NA,CRO,CAZ,OFX,CIP,LEV,NOR,K,SXT,CN,CZ,E	2
38	TE,NA,CRO,OFX,CIP,LEV,NOR,S,K,SXT,CN,CZ,E	1
39	TE,NA,CRO,CAZ,OFX,CIP,NOR,S,K,SXT,CN,CZ,E	1
40	TE,NA,CRO,CAZ,OFX,CIP,LEV,NOR,S,K,SXT,CN,CZ,E	2
	Total	50

[a]Tetracycline, TE; Streptomycin, S; Gentamicin, CN; kanamycin, K; Nalidixic acid, NA; Norfloxacin, NOR; Ciprofloxacin, CIP; Ofloxacin, OFX; Levofloxacin, LEV; Ceftriaxone, CRO; Ceftazidime, CAZ; Cefazolin, CZ; Trimethoprim/Sulfamethoxazole, SXT; Erythromycin, E.

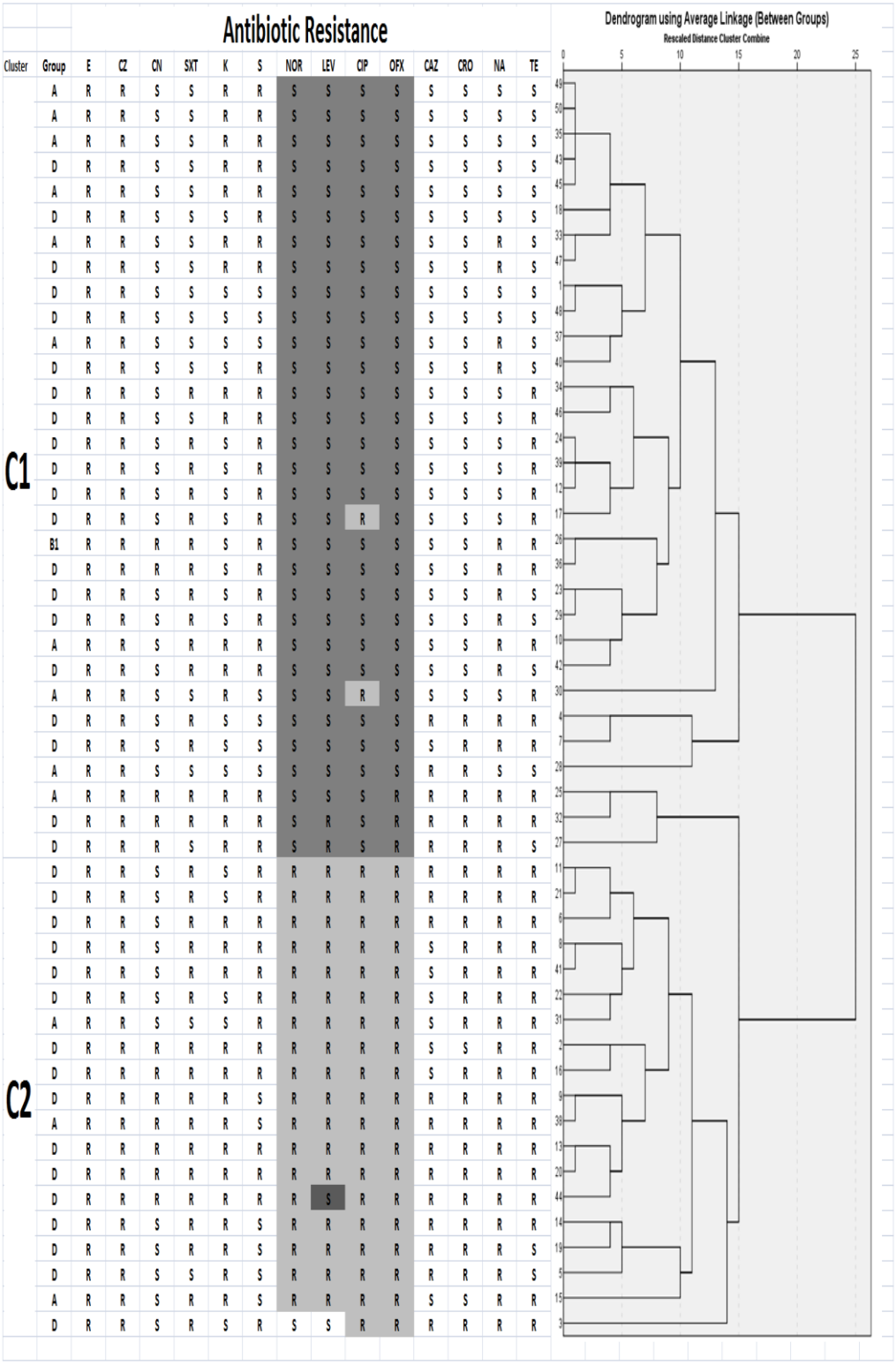

Figure 4.2: Dendrogram of 50 UPEC strains isolated from urine samples based on the UPGMA method derived from analysis of the antibiotic resistance profile and Phylogenetic.

4.4 Virulence genes

Virulence-related UPEC genes were studied by multiplex PCR. Detection determinants coding for different adhesions, toxins, iron uptake system genes, cell protection genes, *malX* (pathogenic island marker, PAI II of the CFT073 reference strain), and *usp* (uropathogenic specific protein). The prevalence of virulence genes ranged from 0% (*papG allele I* and *hlyA*) to 86% (f*imH* and *traT*) in strains tested. Virulence genes *iha*, *kpsMTII*, *usp* and *fyuA* have the following prevalence 74%, 64%, 64%, 58% and 52%, respectively (Figure 4.3 and Table 4.3). Distribution of *fimH* gene was 86.1% and 84.6% in group D and A, respectively. The prevalence of genes tested in each strain was ranged from 5.6% to 83.3%. The strains belonging to the D phylogenetic group exhibited the highest prevalence of VFs. The mean virulence score for group D was 8.2 and ranged from 2 to15, while for group A was 6.2 and ranged from 1 to14. There was statistically significant difference between the mean virulence scores of these groups at $P = 6.2 \times 10^{-4}$. Also it was found that 69.2% (9/13) of strains belonged to phylogenetic group A carried 6 or less virulence factors, while 66.7% (24/36) of strains belonged to phylogenetic group D carried 7 or more virulence factors. The results showed that mean VF scores for strains resistant to fluoroquinolones and /or quinolones was 7.3, while for strains sensitive to both fluoroquinolones and quinolones was 8.1. The quinolones and/or fluoquinolones sensitive strains related to phylogenetic group D showed an increased prevalence of *iroN* virulence gene than resistant. In phylogeneic group A, average of prevalence of *papGII* was increased and

fimH decreased in quinolones and fluoquinolones sensitive strains. Comparison between resistant and sensitive strains to quinolones and fluoquinolones regardless to phylogenetic groups, sensitive strains showed increased prevalence of *ompT* virulence genes (Table 4.4). It seems that there is no single virulence factor nor virulence profile that is entirely specific to UTI in general. Unfortunately cluster analysis did not reveal a specific combination of presence/absence of the tested VFGs that clustered together among the phylogenetic groups. Results showed that 3 large clusters depend on the presence/absence of *cnfI* and *afa/dra* virulence factors, but clustering is independent into phylogenetic groups (Figure 4.4). It also was found that *traT* gene was the most common prevalence among strains resistant to Nalidixic acid, fluoroquinolones and Trimethoprim/Sulphamethoxazole and it was 90.1% (30/33), 95.8 (23/24) and 90.6% (29/32), respectively.

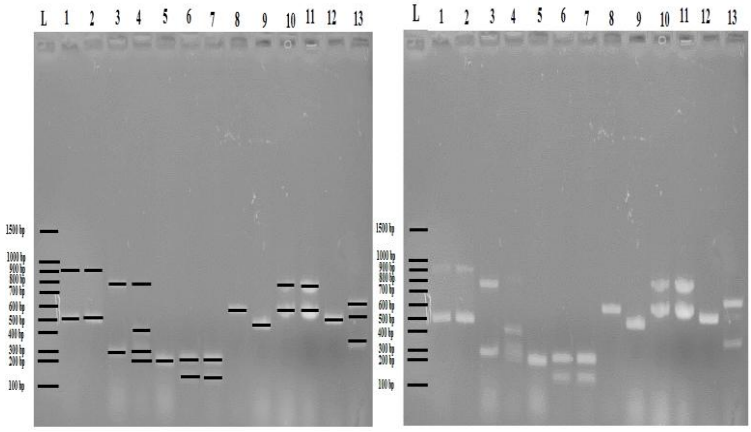

Figure 4.3: Multiplex PCR profiles specific for *E. coli* virulence factors. Lanes 1 and 2 *malx* and *fimH* genes**;** 3 and 4 for *fyuA, papGIII, utaA* and *sfa/foc* genes; 5 for *kpsMTII* gene; 6 and 7 for *traT* and *papGII* genes; 8 for *afa/dra* gene*;* 9 *cnfI* gene; 10 and 11 for *usp* and *iha* genes; 12 for *ompT* and 13 for *ompT, sat* and *iron* genes; Lane L contained Ladder.

Table 4.3: Virulance genes, virulence scores, prevalence of virulence factors and their distribution to the phylogenetic groups A and D.

Virulence genes		Prevalence		Distribution of VF according to phylogenetic group [a]		VF score (mean)	
		No. of isolates	(%)	**D** n=36 (%)	**A** n=13 (%)	**D** VF score (%) 294 (8.2)	**A** VF (%) 80 (6.2)****
Adhesin genes	***papGI***	0	0	0 (0.0%)	0 (0.0%)	0 (0.0)	0 (0.0)
	papGII	14	28	12 (33.3%)	2 (15.4%)	128 (3.6)	19 (1.5)***
	papGIII	2	4	2 (5.6%)	0 (0.0%)	11 (0.3)	0 (0.0)
	sfa/foc	20	40	15 (41.7%)	4 (30.3%)	145 (4.1)	36 (2.8)
	afa/dra	19	38	16 (44.4%)	3 (23.1%)	168 (4.7)	33 (2.5)**
	fimH	43	86	31 (86.1%)	11 (84.6%)	269 (7.5)	74 (5.7)
Toxin genes	***hlyA***	0	0	0 (0.0%)	0 (0.0%)	0 (0.0)	0 (0.0)
	cnfI	19	38	16 (44.4%)	3 (23.1%)	166 (4.6)	33 (2.5)**
	Sat	21	42	18 (50%)	3 (23.1%)	179 (5)	28 (2.2)****
Iron uptake system genes	***fyuA***	26	52	21 (58.3%)	5 (38.5%)	192 (5.5)	41 (3.2)**
	utaA	37	74	27 (75%)	10 (69.2%)	247 (6.9)	64 (4.9)
	iroN	20	40	15 (41.7%)	5 (38.5%)	145 (4.1)	39 (3)
	Iha	32	64	22 (61.1%)	10 (76.9%)	217 (6.2)	69 (5.3)***
Cell protection genes	***kpsMTII***	32	64	26 (72.2%)	6 (46.2%)	235 (6.6)	41 (3.2)***
	traT	43	86	34 (94.4%)	9 (69.2%)	282 (7.9)	64 (4.9)****
	ompT	15	30	12 (33.3%)	3 (23.1%)	118 (3.3)	30 (2.3)
Other genes	***Malx***	4	8	4 (11.1%)	0 (0.0%)	46 (1.3)	0 (0.0)****
	Usp	29	58	23 (63.9%)	6 (46.2%)	225 (6.3)	49 (3.8)***

[a]One isolate belonged to group B1 is not included in distribution of VF and VF score (mean).
** significant difference at $P < 0.05$, *** significant difference at $P < 0.01$, **** significant difference at value $P < 0.001$.
VF: Virulence factor

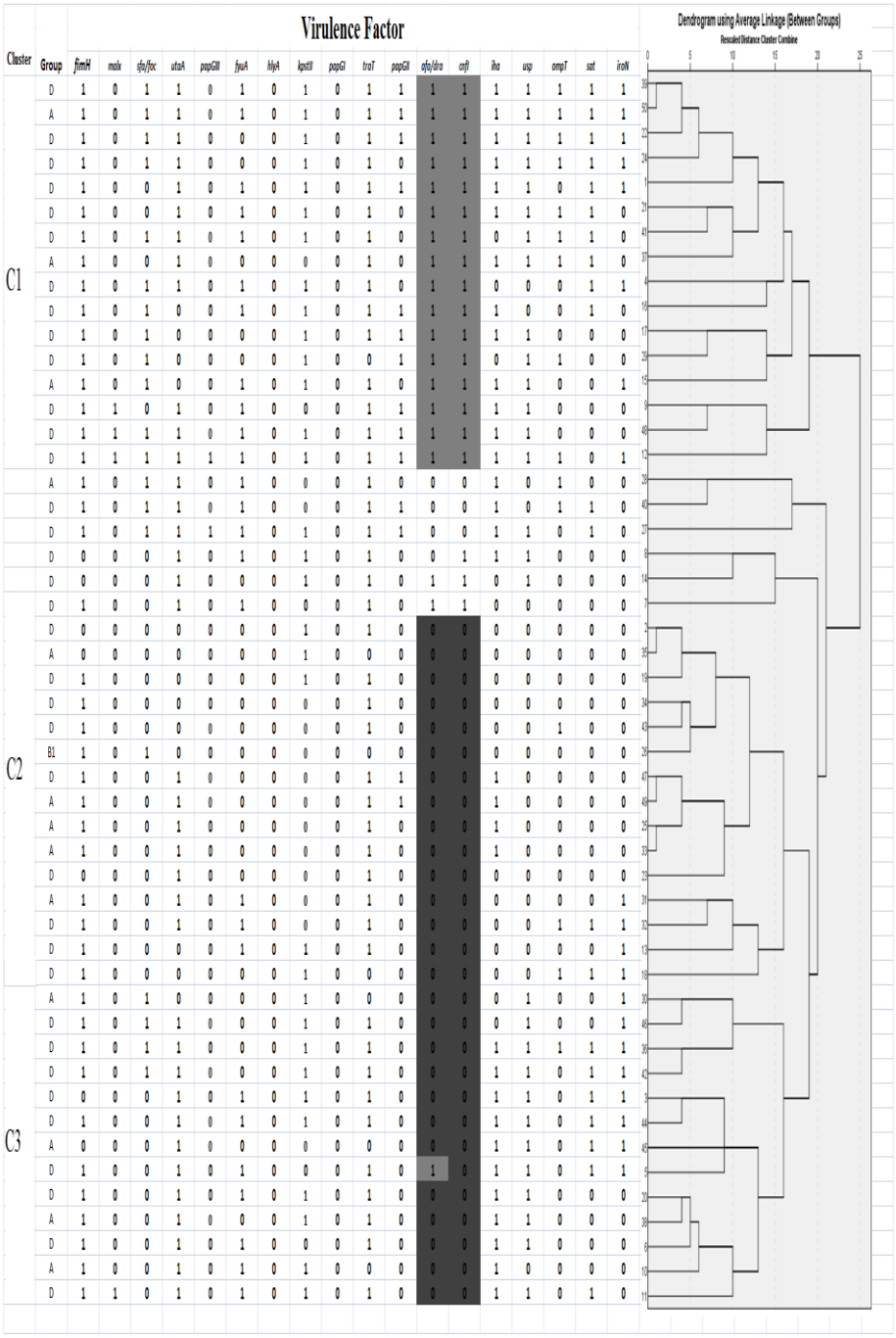

Figure 4.4 : Dendrogram of 50 UPEC strains isolated from urine samples based on the UPGMA method derived from analysis of the virulence factors and Phylogenetic groups.

Table 4.4 : Distribution of phylogenetic groups A and D and VFs among 49 *E. coli* isolates from UTIs according to fluoroquinolones and quinolones and resistance phenotypes.

VF gene	No. (%) of *E. coli* isolates[a]					
	group D n (%)		group A n (%)		groups D and A n (%)	groups D and A n (%)
	resistant (n=27)	susceptible (n=9)	resistant (n=8)	susceptible (n=5)	resistant (n=35)	susceptible (n=14)
papGI	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
papGII	8 (29.6)	4 (44.4)	0 (0.0)	2 (40.0)	8 (22.9)	6 (42.9)
papGIII	1 (3.7)	1 (11.0)	0 (0.0)	0 (0.0)	1 (2.9)	1 (7.1)
sfa/foc	10 (37.0)	5 (55.6)	2 (25.0)	2 (40.0)	12 (34.3)	7 (50.0)
afa/dra	11 (40.7)	5 (55.6)	2 (25.0)	1 (20.0)	13 (37.1)	6 (42.9)
fimH	22 (81.5)	9 (100.0)	8 (100.0)	3 (60.0)	30 (85.3)	12 (85.7)
hlyA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
cnfI	11 (40.7)	5 (55.6)	2 (25.0)	1 (20.0)	13 (37.1)	6 (42.9)
Sat	14 (51.9)	4 (44.4)	1 (12.5)	2 (40.0)	15 (42.9)	6 (42.9)
fyuA	17 (63.0)	4 (44.4)	3 (37.5)	2 (40.0)	20 (57.1)	6 (42.9)
utaA	21 (77.8)	6 (66.7)	6 (75.0)	4 (80.0)	27 (77.1)	10(71.4)
iroN	9 (33.3)	6 (66.7)	3 (37.5)	2 (40.0)	12 (34.3)	8 (57.1)
Iha	17 (63.0)	5 (55.6)	6 (75.0)	4 (80.0)	23 (65.7)	9 (64.3)
kpsMTII	19 (70.4)	7 (77.8)	4 (50.0)	2 (40.0)	23 (65.7)	9 (64.3)
traT	26 (96.3)	8 (88.9)	6 (75.0)	3 (60.0)	32 (91.4)	11 (78.6)
ompT	7 (26.0)	5 (55.6)	1 (12.5)	2 (40.0)	8 (22.9)	7 (50.0)
Malx	2 (7.4)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.7)	0 (0.0)
Usp	17 (63.0)	6 (66.7)	4 (50.0)	2 (40.0)	21 (60.0)	8 (57.1)

[a]All strains were susceptible to fluoroquinolones and quinolones or resistant to fluoroquinolones and or quinolones.
VF: Virulence factor; UTI: Urinary tract infection.

Chapter Five
Discussion

UTI is one of the most common infectious diseases and remain a major cause of morbidity and mortality. Uropathogenic *E. coli* strains cause 70 to 90% of community-acquired UTIs in an estimated 150 million individuals annually and about 40% of all nosocomial UTIs (Struelens *et al*., 2004). Intestinal or ExPEC infections are caused by strains harboring numerous VFs located on plasmids, bacteriophages, or the bacterial chromosome (Branger *et al*., 2005). *E. coli* strains can be assigned to one of the main phylogenetic groups: A, B1, B2 or D. Pathotypes of this pathogen, which can cause extraintestinal infections are collectively called ExPEC. They are phylogenetically and epidemiologically distinct from commensal and diarrheagenic strains. According to human, commensal strains usually derive from phylogenetic groups A and B1, while most of ExPEC strains usually belong to the B2 and D groups and harbor large number of various virulence factors, which allow them to induce diseases in both healthy and compromised hosts. ExPEC were currently defined as *E. coli* isolates containing two or more of the following genes: *papA*, and/or *papC*, *sfa/foc*, *afa/dra*, *kpsMT II*, and *iutA*. (Johnson and Russo, 2003).

This study showed that the majority of *E. coli* strains from patients with UTIs were belonged to phylogenetic group D. The finding results were inconsistent to other studies concerning phylogenetic groups in UPEC (Johnson *et al*., 2005b; Takahashi *et al*., 2006; Moreno *et al*., 2008; Molina-López *et al*., 2011; Kawamura-Sato *et al*., 2010; Ejrnæs *et al*., 2011; Bashir *et al*., 2011; Bashir *et al*., 2012), which reported that group B2 being the predominant phylogenetic group followed by other groups.

These results were consistent with a previous report (Abdallah *et al*., 2011), which showed the 46% of ExPEC including UPEC were belonged to phylogenetic group D, other phylogenetic groups were 25%, 15% and 14% for phylogenetic groups A, B1 and B2, respectively. Recent study published in Palestine (Adwan *et al*., 2014), which showed that 61% of *E. coli* isolated from urine were belonged almost equally to phylogenetic groups B2 and D. These differences in the distribution of the phylogenetic groups among the strains of geographically different populations may be affected by different factors such as geographical, climatic conditions, dietary factors, the use of antibiotics and host genetic factors.

The antimicrobial resistance among UPEC has increased dramatically worldwide (Johnson *et al*., 2003). In our study, the most prevalent resistances were to nalidixic acid, ceftriaxone, cefazolin, tetracycline, ofloxacin, ciprofloxacin, streptomycin, kanamycin, Trimethoprim/Sulphamethoxazole and erythromycin. These antibiotics were, or still are, of high clinical significance, and therefore higher resistance prevalence is not surprising (Petkovsek *et al*., 2009). The emergence of 40 drug resistance patterns showed high variability among local UPEC isolates. The findings of this research showed that 98% of strains were MDR and showed higher resistance rates to all antibiotics. These results were consistent with a previous reports (Bashir *et al*., 2011), which showed 100% of the isolates were MDR. In addition, these results were consistent with new report published from Palestine (Adwan *et al*., 2014), which showed that *E. coli* strains isolated from patients with UTIs

had high resistance to different antibiotics. This high rate of resistance is most likely due to selective pressure resulting from inappropriate use of these antimicrobial agents in hospitals as well as in the country as a whole. This is promoted by the lack of an antibiotic policy and the availability of antibiotics sold over the counter in Palestine or this could be explained by the frequent occurrence of UTI for which antibiotherapy is usually prescribed, which favorite the emergence of resistant strains. UTIs caused by such strains represent a clinical problem because of limited therapeutic options. These results were in contrast to report published recently (Ejrnæs *et al.*, 2011), which showed that *E. coli* strains collected from recurrent UTIs characterized by a low level of resistance. This may be due to that these isolates were belonged to community acquired strains as well as isolated from a region characterized by a low level of resistance. Also these finding results were consistent with previous results (Molina-López *et al*., 2011), which showed UPEC strains expressed highest resistance rates to different antimicrobial agents including norfloxacin, ofloxacin, trimethoprim/sulfamethoxazole and ciprofloxacin. Results of this research were consistent with results published recently, which showed approximately similar rate of resistance for some antibiotics such as nalidixic acid, tetracycline, ciprofloxacin, gentamicin and trimethoprim/ sulphamethoxazole against isolates recovered from cystitis (Ferjani *et al*., 2012). Trimethoprim/sulfamethoxazole has been widely used for the treatment of UTIs (Moreno *et al*., 2006; Walter *et al*., 2001). Data of this research were consistent with previous reports (Bashir *et al*., 2011; Picard

et al., 1988), which showed that phylogenetic group D isolates were more drug resistant as compared with phylogenetic group A.

Among the 18 virulence genes studied, *fimH* and *traT* genes were the most frequently detected. *fimH* gene was equally distributed in isolates of phylogenetic groups A and D. These results confirmed previous knowledge about the genes mostly present in all isolates (*fimH*). The high prevalence of *fimH* gene in uropathogenic *E. coli* has been reported (Ruiz *et al*., 2002; Takahashi *et al*., 2006; Rijavec *et al*., 2008; Abdallah *et al*., 2011; Ferjani *et al*., 2012). This was explained by the determining role of *fimH* adhesin encoded by *fimH* gene in the initiation of infection. In this research, it was found that the 63% of strains carried *usp* gene. This result is consistent with other report (Darko *et al.*, 2013), which showed 72.48% of UPEC carried this gene. Results of this study showed that all isolates were *hlyA* negative which were in contrast to other reports which identified this gene in 34%-37% of UPEC isolates (Bashir *et al*., 2012; Bingen-Bidois *et al.*, 2002). *hlyA* gene plays a major role in the damage of uroepithelium and all *hlyA* gene positive isolates hemolysed human erythrocytes (Bashir *et al*., 2012). Other toxin genes including *cnfI* (38%) and *sat* (42%) were detected in UPEC isolates. These results indicate that *sat*, a vacuolating cytotoxin expressed by UPEC elicits defined damage to kidney epithelium during upper UTI (Guyer *et al*., (2002). It was proposed that *cnfI* production increases the capacity of UPEC strains to resist killing by neutrophils, which in turn permits these bacteria to infect deeper tissue and persist better in the lower urinary tract (Karen *et al*., 2001). *IroN* and *iha* genes

were reported to be putative uropathogenic virulence genes and were frequently associated with UPEC (Bauer *et al.*, 2002; Kanamaru *et al*., 2003).

The association of other virulence factors, such as *sfa/foc*, *iutA*, *fyuA*, *ompT* and *traT* with UPEC isolates has been reported (Johnson *et al*., 2005a; 2005c; Rijavec *et al*., 2008). *OmpT* considered a strong virulence factor predictor of bacteremia (Sannes *et al*. 2004). An outer membrane protease is the product of *ompT* gene that contributes to virulence by inactivating host defense proteins, cleaving host cell-surface peptide to expose them or by hydrolysing protamine, an antimicrobial peptide (Sannes *et al*., 2004; McCarter *et al*., 2004).

No single virulence factor nor virulence profile that is entirely specific to UTI in general, but various combinations of these virulence factors have been found in isolated cultures of UPEC as the cause of pathogenicity (Hull *et al*., 1998; Kanamaru *et al*., 2003; Takahashi *et al*., 2006; Ferjani *et al*., 2012). This observation therefore, suggests synergistic action of virulence factors as the mechanism of UPEC invading host defense system to cause disease (Johnson, 1991). Variation in the distribution of these virulence factors in isolated UPEC has been attributed to geographical differences, strain types, and populations sampled and differences in association with

host characteristics (Kanamaru *et al*., 2003; Santo *et al*., 2006; Rijavec *et al*., 2008).

Results of current study are in line with other studies where they were found that virulent isolates of *E. coli* mainly belong to phylogenetic group D were as more virulent than phylogenetic group A. It was found that virulent isolates of *E. coli* mainly belong to phylogenetic group B2 and D, where as less virulent of *E. coli* belong to phylogenetic groups B1 and A (Bashir *et al*., 2011; Johnson and Stell., 2000). Other studies concerning VFs in UPEC (Johnson *et al*., 2005a; 2005c; Ejrnæs *et al*., 2011), showed that *E. coli* strains belonging to phylogenetic group B2 contained a greater number of VFGs than *E. coli* belonging to a non-B2 phylogenetic group. As reported in other studies, the differences observed between urinary tract syndromes were reflected in virulence scores, which were lowest among cystitis and highest among pyelonephritis isolates (Johnson *et al*., 2005a; 2005b). No information about the origin of these strains whether from pyelonephritis or from cystitis, according virulence score may be strains belongs to phylogenetic group D are responsible for pyelonephritis, while phylogenetic group A are responsible for cystitis (Johnson *et al*., 2005a; 2005b).

Data of current study about VF scores showed that no significant difference between uropathogenic strains resistant to fluoroquinolones and/or quinolones and strains sensitive to both fluoroquinolones and quinolones. These results were in contrast to data reported previously (Velasco *et al.*, 2001; Vila *et al*., 2002; Horcajada *et al*., 2005; Kawamura *et al*., 2010), which suggested that quinolone-resistant ExPEC are less able to cause upper UTI and have fewer VFs than quinolone-susceptible *E. coli*. In addition, these results are not in agreement with previous results (Ferjani *et al.*, 2012), which showed that pyelonephritis isolates exhibited a greater prevalence of phylogenetic group B2, high virulence scores and were associated with susceptibility to fluoroquinolones. This study demonstrated a relationship between phylogenetic origin and high antibiotic resistance and high prevalence of VFs among strains belonged to phylogenetic group D. These results were in contrast to other studies (Johnson *et al.*, 2002; 2003; Horcajada *et al*., 2005), which suggested that quinolone resistance may be directly associated with virulence loss.

Many studies on this subject have been carried out in various parts of the world; such data is not available from Palestine where UTI are very common. This study was designed to characterize local isolates of UPEC with respect to phylogenicity and distribution of most important virulence factors. Although our study is comprised of a relatively small number of samples and therefore faces limitations in statistical analysis, even it provides important information about the phylogenetic background of UPEC isolated from Northern Palestine. In conclusion, the molecular

analysis of 50 *E. coli* urine isolates exhibited a greater prevalence of phylogenetic group D, high virulence scores and high resistance scores than group A. Urinary tract infections caused by such strains represent a clinical problem because of limited therapeutic options. Even though additional, in vivo studies by using mutant strains or the product of these genes should be performed to confirm the significance of the detected VFs. Further analysis of VF profiles with regard to specific clinical symptoms and defined severity is recommended.

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جامعة النجاح الوطنية

كلية الدراسات العليا

العوامل الممرضة والمجموعات التطورية للبكتيريا الإشريكية القولونية لعزلات سريرية من الجهاز البولي في مستشفى ثابت– طولكرم، فلسطين.

إعداد

بثينة أحمد محمود عيسى

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قدمت هذه الأطروحة استكمالاً لمتطلبات نيل درجة الماجستير في برنامج العلوم الحياتية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، في نابلس، فلسطين.

2014

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إعداد

بثينة أحمد محمود عيسى

إشراف

د. غالب عدوان

الملخص

البكتيريا الإشريكية القولونية هي واحدة من أكثر أنواع البكتيريا المسببة لإصابات الجهاز البولي. هذا المسبب المرضي مسجل في 50%–90% من كل إصابات الجهاز البولي غير المعقدة.

تهدف هذه الدراسة لتحليل المجموعات التطورية، والعوامل المسببة للمرض والمقاومة للمضادات البكتيرية في مجموعة من 50 عزلة من البكتيريا الاشريكية القولونية تم الحصول عليها من بول لحالات ربما تكون مصابة بإصابات في الجهاز البولي لمرضى مقيمين و غير مقيمين في المستشفى خلال الفترة ما بين شهر أيار و كانون الاول من عام 2012 م.

تم الكشف عن المجموعات التطورية وذلك باستخدام تقنية سلسلة تفاعل البلمرة المتعدد، أظهرت النتائج أن معظم العزلات التي تم دراستها وبشكل أساسي 36 عزلة (72%) تنتمي الى المجموعة (D)، 13عزلة (26%) تم تحديدها في المجموعة (A) ، بينما وجدت عزلة واحدة (2%) تنتمي للمجموعة (B1) ذات دلالة إحصائية ($P < 0.001$).

تم دراسة مقاومة عزلات بكتيريا الاشريكية القولونية بواسطة طريقة الانتشار القرصي. إن مقاومة المضادات تراوحت من 24% للجنتاميسين الى 100% للاريثرومايسين والسيفازولين. أظهرت هذة النتائج أن معدلات سجل النقاط لمقاومة المضادات الحيوية كانت 8.4 و 6.5 لسلالات المجموعتين D و Aعلى التوالي، ذات دلالة إحصائية ($P = 6.2 \times 10^{-4}$). إن انتشار مقاومة المضادات التي تم اختبارها في كل سلالة تراوحت من 14.3% الى 100%. تم التوصل الى أن 61.5% (13/8) من العزلات المنتمية للمجموعة التطوريةA كانت مقاومة ل 5 أو أقل

من المضادات الحيوية بينما 66.7% (36/24) من العزلات المنتمية للمجموعة التطورية D كانت مقاومة ل 6 أو أكثر من المضادات الحيوية. كما واظهرت ارتباط مقاومة ترايميثوبريم/سلفاميثوكسازول بالمجموعة D التي كانت ذات فروق احصائية على ($P < 0.05$). بين السلالات المقاومة للفلوروكينولونات والكينولونات كانت تكرارات مجموعات A ,D 75.0% (36/27) و 61.5% (13/8) ،على التوالي.

تمت دراسة الجينات الممرضة عن طريق تفاعل البلمرة المتعدد. أظهرت النتائج أن انتشار الجينات الممرضة تراوحت من 0% ل (*papG* allel1 ,*hlyA*) إلى 86% ل (*fimH, traT*) في السلالات التي تم فحصها. وكان انتشار الجينات الممرضة (*utaA, iha, kpsMTII, usp,* *fyuA*) 74%، 64%، 64%، 58%، و52%، على التوالي. وكان توزيع *fimH* 86.1% و84.6% في مجموعات A ,D على التوالي .إن انتشار الجينات التي تم اختبارها في كل سلالة تراوحت من 5.6% إلى 83.3%. أظهرت النتائج ان معدلات سجل النقاط للعوامل الممرضة للمجموعة D كان 8.2% وتراوح بين 2–15 بينما للمجموعة A كان 6.2 وتراوح بين 1–14 وكان ذا دلالة إحصائية ($P = 6.2 \times 10^{-4}$). كما وأظهرت النتائج أن 69.2% (13/9) من السلالات كانت تنتمي للمجموعة التطورية A وحملت 6 أو أقل من العوامل الممرضة، بينما 66.7% (36/24) من السلالات كانت تنتمي للمجموعة التطورية D، وحملت 7 أو أكثر من العوامل الممرضة. إن السلالات الحساسة لكل من الفلوروكينولونات والكينولونات والمتعلقة بالمجموعة التطورية D أظهرت ارتفاعا مهما في انتشار الجين الممرض *iroN* أكثر من المقاومة. بينما في المجموعة التطورية A ارتفع انتشار *papG2* وانخفض انتشار *fimH* للسلالات الحساسة لكل من الفلوروكينولونات والكينولونات. بالمقارنة بين السلالات المقاومة والحساسة لكل من الفلوروكينولونات والكينولونات بغض النظر عن المجموعات التطورية أظهرت السلالات الحساسة ارتفاعا مهما في الجين الممرض*ompT*. يبدو انه ليس هناك عامل ممرض واحد أو نمط محدد لالتهاب المسالك البولية. أظهرت النتائج أن 3 عناقيد كبيرة تعتمد على وجود/غياب العوامل الممرضة *cnf1* و*afa/dra* وليست على المجموعات التطورية. تم التوصل أيضا الى ان جين *traT* كان الأكثر انتشاراً بين السلالات المقاومة لحمض

الناليديكسيك، الفلوروكينولونات وترايميثوبريم/السلفاميثوكسازول وكان 90.1% (33/30) و95.8% (24/23) و90.6% (32/29)، على التوالي.

وفي النهاية اظهر التحليل الجزيئي ل 50 عزلة بكتيريا اشريكية قولونية بولية انتشاراً واسعاً للمجموعات التطورية D، معدلات سجل النقاط للعوامل الممرضة ولمقاومة المضادات مرتفعة أكثر من المجموعة A. إن إصابات الجهاز البولي الناتجة عن هذه السلالات تمثل مشكلة سريرية بسبب الخيارات العلاجية المحدودة . بالإضافة إلى ذلك، مزيد من التحليل يجب إجراؤه خاصة داخل الكائن الحي باستخدام سلالات متحولة أو المنتج من هذه الجينات للكشف عن أهمية وتأثير العوامل الممرضة بخصوص الأعراض السريرية.