Antimicrobial Resistance in Relation to Virulence Determinants and Phylogenetic Background among Uropathogenic *Escherichia coli* in Lebanon

by

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ABSTRACT

Antimicrobial Resistance in Relation to Virulence Determinants and Phylogenetic Background among Uropathogenic *Escherichia coli* in Lebanon

by Georgine Sawma-Aouad

Escherichia coli is the most common causative agent of recurrent, uncomplicated urinary tract infections. Screening of antimicrobial resistance among urinary tract pathogens is very crucial and useful for proper empirical treatment. The aim of this study was to assess how resistance to antimicrobial agents relates to virulence potential and phylogenetic background of clinical *E. coli* isolates in Lebanon. Triplex PCR assays and the disc diffusion method were used to determine and correlate *E. coli* phylogenetic group, three virulence-associated genes and antimicrobial resistance among 100 uropathogens collected from two major hospitals in Lebanon. Group B2 represented 58% of the tested isolates, followed and in equal proportions by group A and D, while none of the isolates belonged to group B1. Isolates with highest percentage of susceptibility to all used antimicrobial agents were within B2 phylogenetic group, whereas resistant isolates mainly exhibited shifts to non-B2 groups. The majority of the isolates resistant to SXT were from group B2 (52.7%) and 45% of them were positive for the three tested virulence determinant genes (*efa*, *fbp* and *picU*) encoded by the PAI II CFT073. However, ciprofloxacin resistance mainly occurred in the low-virulence *E. coli* group A (41.4% resistant). These findings suggest that virulence and antimicrobial resistance are not always mutually exclusive in *E. coli* clinical isolates. The relationship between virulence and antimicrobial varies according to the particular resistance phenotype.

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LIST OF ABBREVIATIONS

CD: Cluster of differentiation

E. coli: *Escherichia coli*

ECOR: Escherichia coli reference collection

efu: *Escherichia coli* fucose metabolism

ExPEC: Extraintestinal pathogenic *Escherichia coli*

fbp: ferric binding protein

IBC: Intracellular bacterial communities

IL: Interleukin

LPS: Lipopolysaccharide

NF-κB: Nuclear factor kappa B

PAIs: Pathogenecity islands

pap: pilus associated pyelonephritis

PMN: Polymorphonuclear leukocytes

SPATE: Serine protease autotransporters of the *Enterobacteriacaea*

TLR: Toll-like receptor

UPEC: uropathogenic *Escherichia coli*

UTI: Urinary tract infection

QIR: Quiescent intracellular reservoir

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I dedicate this thesis to my father, mother, and brother.

Chapter 1

INTRODUCTION

Escherichia coli, the head of the large bacterial family *Enterobacteriaceae* (Todar, 2002), is a normal inhabitant of the intestine of most animals and humans (Swenson et al., 1996), where it can act as a mutualist and serves crucial functions (Hoyle, 2007). Nevertheless, *E. coli* is more than just a laboratory harmless intestinal resident; it can be a highly versatile and commonly lethal pathogen. By means of virulence factors, some *E. coli* strains can cause a wide range of extraintestinal diseases, notably urinary tract infections (Bingen-Bidois et al., 2002).

Urinary tract infection is one of the most important causes of mortality (Raksha et al., 2003), with *E. coli* being the most frequent pathogen isolated in complicated and uncomplicated cases (Karlowsky et al., 2001; Nicolle, 2001; Petrovska et al., 2004) and in both community and hospital settings (Drews et al., 2005). Therefore, *E. coli* represents an attractive organism to study, and learn how environment impacts the microbial genome and structure (Chen et al., 2006).

E. coli can be classified into four main phylogenetic groups termed A, B1, B2, and D. Strains carrying the most robust virulence factors responsible for urinary tract infections and other extraintestinal diseases mainly belong to group B2, and to a lesser extent group D, as opposed to commensal strains that primarily belong to group A and B1 (Goullet & Picard, 1990 ; Lecointre et al., 1998; Picard et al., 1999 ; Duriez et al., 2001; Johnson, 2002; Zhang et al., 2002).

From a therapeutical point of view, antibiotic resistance presents a big challenge in the empirical treatment of uropathogenic *E. coli* due to emerging resistance to most first line antimicrobials such as trimethoprim/sulfamethoxazole and ciprofloxacin (Chen et al., 2001). Therefore, antimicrobial resistance profiles should be known in order to prescribe the appropriate treatment.

To the extent of our knowledge, this study is unique in Lebanon since it is the only one that targets the relationship between phylogenetic groups, virulence and antimicrobial resistance of uropathogenic *E. coli*.

The objective of this study is to determine the phylogenetic groups of uropathogenic *E. coli* collected from two major hospitals in Lebanon and assess the prevalence of three virulence-associated loci (*fbp*, *efa*, and *picU*) by triplex PCR. Antibiotic resistance profiles will also be done using the disk diffusion assay with eight antibiotics, to verify the current situation in Lebanon and provide data that might help in the direct empirical treatment of urinary tract infections. Moreover, a comparison between drug resistance and susceptible isolates will be conducted to better understand the yet unexplained relationship between resistance, phylogenetic groups, and virulence.

Chapter 2

LITTERATURE REVIEW

2.1. Overview of *Escherichia coli*

Escherichia coli is a model laboratory bacterium, a Gram-negative straight rod, facultatively anaerobic chemoorganotroph, that either uses peritrichous flagella for mobility or is nonmotile. Due to its rapid growth rate and simple nutritional requirements, *E. coli* has become a model organism for studying many life's essential processes and is now considered the "heavy lifter" of biotechnology as it is used to produce human insulin and other medicines (Todar, 2006).

E. coli, the head of the large bacterial family *Enterobacteriaceae* (Todar, 2002), is a normal inhabitant of the intestine of most animals and humans (Swenson et al., 1996), where it serves important functions such as participating in the digestion of food, producing vitamins and suppressing the growth of harmful bacterial species (Hoyle, 2007). Colonization of the gastrointestinal tract begins within hours or few days after birth with the bacterium being ingested in foods or water or acquired from individuals handling the infant (Todar, 2002). *E. coli* colonizes the lower gut of animals and survives when released to the natural environment, allowing dissemination to new hosts (Blattner et al., 1997).

E. coli is a species that is extensively distributed in the environment, as well as a mutualist and pathogen in its human hosts (Chen et al., 2006). *E. coli* is more than just a laboratory harmless intestinal inhabitant; it can be a highly versatile and frequently deadly pathogen

(Kaper et al., 2004). Some *E. coli* strains can cause a wide variety of intestinal diseases such as diarrhea while others termed extraintestinal pathogenic *E.coli* (ExPEC) strains (Russo & Johnson, 2000) have the potential to cause a wide variety of extraintestinal diseases, including septicemia, neonatal meningitis, prostatitis, bacteremia and urinary tract infections (Orskov & Orskov, 1992 ; Bingen-Bidois et al., 2002).

E. coli represents a desirable organism to study and learn how environment affects the microbial genome structure and function (Chen et al., 2006). Commensal enteric *E. coli* may be the natural reservoir for pathogenic strains as several studies have shown that pathogenic *E. coli* strains may be derived from commensal strains by the acquisition of chromosomal virulence operons (Ochman et al., 2000), genomic deletions that favors pathogenecity (Maurelli et al., 1998), or random functional point mutations (Sokurenko et al., 1998).

2.2 Urinary Tract Function

The kidneys function in filtering and removing waste and excess water from the blood to produce urine with one and a half to two quarts of urine being eliminated per day in an adult and less than this amount in a child, depending on age. The urine then travels from each kidney through two thin tubes called the ureters and is then stored in a balloon-like organ called the bladder (Figure 1). When the bladder empties, a muscle called the sphincter relaxes and urine flows out of the body through the urethra, a tube at the bottom of the bladder in the lowest part of the urinary tract. The opening of the urethra is at the end of the penis in men (Figure 2) and in front of the vagina in women (NIDDK, 2005) (Figure 3).

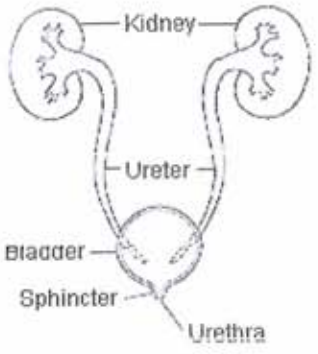

Figure 1: Front View of urinary tract

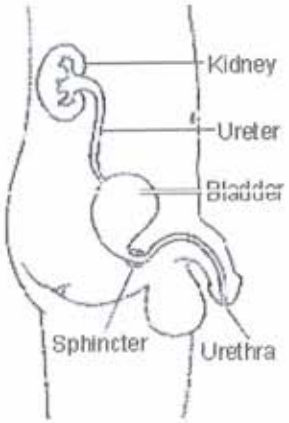

Figure 2: Side view of male urinary tract

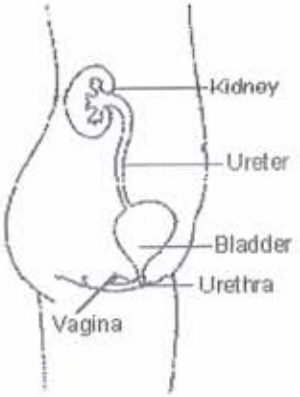

Figure 3: Side view of female urinary tract

(NIDDK, 2005).

2.3. Urinary Tract Infection

Urinary tract infection (UTI), one of the most important causes of morbidity and mortality (Raksha et al., 2003), is a common infection with an estimated 150 million UTIs occurring yearly worldwide (Stamm & Norrby, 2001). The aetiology of UTIs is predictable, with *E. coli* being the most common infecting agent at this site (Karlowsky et al., 2001; Petrovska et al., 2004) responsible for up to 90.6% of the UTIs (Stratchounski et al., 2001), although other pathogens such as *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* infrequently cause the disease (Ronald, 2002).

Complicated in contrast to uncomplicated UTIs occur in patient having a genitourinary tract that does not function normally due to structural or functional abnormalities. However, in the case of complicated UTIs, *E. coli* remains the principal pathogen responsible for 50-60% of infections (Nicolle, 2001).

The majority of community-acquired UTIs occur in women. One in every three women by the age of 24 years will experience at least one episode of UTI that requires antimicrobial therapy, and almost one half of all women will experience at least one episode of UTI during their lifetime (Foxman, 2002).

Uropathogenic *E. coli* (UPEC) are also responsible for ~50% of all nosocomial UTIs (Parham et al., 2005). In North America, *E.coli* was found to be the principle cause of UTIs in both community and hospital settings (Drews et al., 2005). As such, UPEC symbolize one of the most frequently isolated nosocomial pathogens (Struelens et al., 2004).

Urinary infections involve the ascent of bacteria from the urethra to the bladder and in a few patients to the kidney (Santo et al., 2006). In many cases, the bacteria first travel to the urethra where they multiply and cause urethritis. If they reach the bladder and multiply, cystitis occurs. If the infection proceeds to the kidneys, pyelonephritis results (NIDDK, 2005). The factors responsible in the ascent of UPEC from the urethral opening to the bladder are not yet known (Kau et al., 2005). During the ascent and colonization, UPEC must bind a variety of differentiated cell surfaces, obtain nutrients that may be limited, invade the urinary tract epithelial cells, defend against the host response, and contend with other competitors (Haugen et al., 2007).

In the United States, UPEC are responsible for 70 to 90% of the seven million cases of acute cystitis and 250,000 of pyelonephritis reported annually (Foxman et al., 2000). The most common UTI in women is cystitis; prostatitis in men and pyelonephritis in both sexes are more severe but less frequent complaints (Parham et al., 2005). A

serious and life threatening complication of pyelonephritis happens when the bacteria invade the bloodstream producing a systemic response (Lloyd et al., 2007).

2.4. Sources of Uropathogenic *Escherichia coli*

Commensals *E. coli* present in the gastrointestinal tract provide the pool for initiation of UTI (Raksha et al., 2003). The UPEC strains, also known as ExPEC, differ from commensal strains (Buckles et al., 2004). They possess the requisite arsenal of virulence factors (Russo & Johnson, 2000; Johnson et al., 2003) that enable them to colonize host mucosal surfaces, to overcome or circumvent host defenses, and to invade the normally sterile urinary tract (Russo & Johnson, 2000; Johnson et al., 2003; Buckles et al., 2004). The commensalism-versus-virulence duality of *E. coli* depends on the variation in genetic backgrounds and the presence or absence of specialized virulence factors (Zhang et al., 2002).

UPEC emerges from the distal gut microbiota and is incorporated into the vaginal/periurethral microbiota (Foxman, 2002). The introduction of UPEC into the bladder is favored by the proximity of the anus to the urethral opening in the human female, sexual intercourse that facilitates human infection from fecal material, as well as other factors (Hooton et al., 1996). Bladder infections are 14-times more common in females than males due to the shorthened urethra associated with females (Todar, 2002). Moreover, since avian *E. coli* and their plasmids may be transmitted to humans, they could serve as possible reservoirs of urovirulence genes for human UPEC following

the ingestion of contaminated food, such as poultry (Skyberg et al., 2006).

2.5. Uropathogenesis in *Escherichia coli*

A dynamic cross-talk which determines the outcome of the infectious disease is initiated by the attachment of the pathogen to the host epithelium by way of an interaction between an adhesion on the pathogen and a receptor on the host tissue (Kau et al., 2005).

UTIs have been thought to be due to simple extracellular colonization (Justice et al., 2004). However, in the murine model of cystitis, it has been recently shown that UPEC invades the urothelium as a way to circumvent the host innate defenses (Justice et al., 2004). Once UPEC reaches the bladder, at least two adhesins, type 1 and P pili, are known to play crucial roles in the development of cystitis and pyelonephritis, respectively (Kau et al., 2005).

UPEC enters the bladder, where it attaches to lipid rafts and mannosylated uroplakins via the FimH adhesion on the tip of type 1 pili (Martinez et al., 2000; Duncan et al., 2004), and subsequently triggers a signal transduction cascade that results in the uptake of the attached bacteria by superficial umbrella cells (Martinez et al., 2000). Once inside umbrella cells, UPEC speedily multiply and replicate to form intracellular bacterial communities (IBCs). A surge in the number of infectious organisms within the bladder occurs due to the fast bacterial doubling time, thus increasing the likelihood of persistence and dissemination (Justice et al., 2004).

A loose collection of fast-growing, rod-shaped bacteria mature into slower growing, tightly packed, highly organized bacterial community made up of coccoid -shaped organisms that ultimately fill most of the umbrella cell cytoplasm, causing luminal protrusions termed pods (Justice et al., 2004).

In response to environmental signals, UPEC switch to a motile rod-shaped phenotype, allowing bacteria to detach from the IBC and flux out of the umbrella host cell. This phenomenon facilitates invasion of other urothelial cells as part of a process that can lead to persistent or recurrent UTI. Filamentation may occur within the IBC as filamentous bacteria were observed in the late IBC (Mulvey et al., 2001; Justice et al., 2004). Differentiation into the filamentous form is critical for surviving the host innate immune responses as filamentous bacteria in contrast to rod-shaped bacteria are able to escape the polymorphonuclear leukocytes. Thus IBC maturation progresses through different developmental stages that vary in growth rate, bacterial length, colony organization, motility and dispersal (Justice et al., 2004). As a result of bacterial invasion, a quiescent intracellular reservoir (QIR) in the bladder is formed that can persist and cause recurrent infections (Mulvey et al., 2000; Garofalo et al., 2007). Studies are in progress to determine whether UPEC produces IBCs in humans as intracellular bacteria were observed in the human bladder (Garofalo et al., 2007).

2.6. Host Responses

Defense mechanisms such as neutrophil efflux and epithelial exfoliation of the infected cells into the urine are initiated upon UPEC infection (Mulvey et al., 1998). Lipopolysaccharide (LPS) and other

bacterial products are recognized by members of the Toll-like receptor (TLR) family expressed on epithelial cells and immune cells, such as macrophages TLR4, with its co-receptors CD14 and MD2 (Hoshino et al., 1999). TLR11, another member of the TLR family, was shown to be expressed primarily in the murine kidneys, where it binds specifically to UPEC (Zhang et al., 2004). Interaction between the cytoplasmic domains of the TLRs and adapter proteins like MyD88 and Mal takes place to initiate signaling pathways that lead in the activation of the NF-κB (Akira & takeda, 2004), which in turn activates the transcription of anti-apoptotic and pro-inflammatory genes such as interleukin (IL)-6 and IL-8 (Jantausch et al., 2000). The exact role of IL-6 during UTI is unknown, it might play a role in facilitating the transition from a neutrophilic to a predominantly monocytic response (Hurst et al., 2001), while IL-8 attract the circulating polymorphonuclear leukocytes (PMN) to bladder tissue to engulf UPEC (Kau et al., 2005). The cleansing flow of urine, free iron limitation, exfoliation of host cells, and attack by phagocytic and inflammatory mediators are all mechanisms that disfavor the ascent of UPEC (Haugen et al., 2007).

However, several mechanisms are used by the UPEC to evade the innate defenses, to persist and cause disease (Justice et al., 2004). In addition to its ability to invade into superficial umbrella cells and escape the host immune response, UPEC might suppress NF-κB signaling (Klumpp et al., 2001) in the murine bladder by up-regulating the expression of the suppressor of cytokine signaling 3 (*socs3*) gene (Mysorekar et al., 2002). This phenomenon might represent another strategy used by UPEC to evade the host defenses (Mysorekar et al., 2002). Moreover, as the IBC developmental cascade provides a means to increase the number of bacteria, significant numbers will escape the

PMN activity to establish residency in the bladder and disseminate into the environment (Justice et al., 2004).

2.7. Virulence Factors

By means of virulence factors that affect a wide range of cellular processes, *E. coli* strains cause diverse intestinal and extraintestinal diseases (Kaper et al., 2004). Pathogenic *E. coli* strains are marked by the presence of special virulence determinants that are absent in non pathogenic *E. coli* (Guyer et al., 2002). Both the virulence of the infecting agent and the susceptibility of the host influence the severity of the infection (Santo et al., 2006). Several virulence factors are associated with UPEC such as type 1 fimbriae (Bahrani-Mougeot et al., 2002), the mannose-resistant P fimbriae (Drews et al., 2005), S fimbrial adhesion (Daigle et al., 1994), afimbrial adhesion (Daigle et al., 1994), the iron-transporting outer membrane protein TonB (Tores et al., 2001), the transcriptional regulator RfaH (Nagy et al., 2002), the toxin cytotoxic necrotizing factor 1 (Rippere-Lampe et al., 2001), hemolysin (Drews et al., 2005), host defense avoidance such as capsule (Parham et al., 2005), the iron acquisition system aerobactin (Parham et al., 2005), and secreted autotransporter toxin (Sat) (Guyer et al., 2002).

Gunther et al. (2001) found that during bladder colonization in the early stage of infection, type 1 fimbriae are more critical for cystitis-causing strains than for pyelonephritis-causing strains. P fimbriae however, important in upper UTI (Johnson, 1991), are encoded by a "pilus associated pyelonephritis" *pap* operon (Rasko et al., 2001; Drews et al., 2005) and binds to glycoreceptors containing the αGal(1-

4)βGal moiety on uroepithelial cells of the kidney (Lindberg et al., 1984; Drews et al., 2005). This binding protects the washout of bacteria, breaks the mucosal barrier, and initiates the host immune response probably by association with TLR-4 (Frendeus et al., 2001; Bergsten et al., 2004).

In the uropathogenic *E. coli* CFT073 genome, the coding capacity for no less than 12 distinct fimbriae was identified (Welch et al., 2002). A fimbrial usher gene that is part of a novel fimbrial gene cluster in UPEC strain CFT073 termed *auf* was recently identified (Buckles et al., 2004). The *auf* gene cluster was specifically associated with UPEC isolates and joins the lists of potential UTI virulence factors (Buckles et al., 2004). Moreover, the recently described Sat of uropathogenic *E. coli* CFT073, belonging to SPATE (serine protease autotransporters of *Enterobacteriaceae*) subgroup of autotransporters, elicits cytopathic effects on human bladder and kidney epithelial cells (Guyer et al., 2002). The function of the known SPATE proteins is associated with bacterial infection and implies the involvement of these proteins in bacterial pathogenesis by enabling the bacteria to damage the host or avoid an immune response (Guyer et al., 2002). PicU is another serine protease autotransporter of the *Enterobacteriaceae* (SPATE) with a high level of homology (95%) to the Pic mucinase of enteroaggregative *E. coli* and *Shigella flexneri* 2a (Henderson et al., 1999) and like its enteric counterpart can digest mucin and human complement (Henderson & Nataro, 2001; Parham et al., 2005). PicU is more frequently found in UPEC strains than fecal strains, suggestion a role for picU in virulence (Heimer et al., 2004).

Iron uptake systems represent another functional group in UPEC virulence (Xie et al., 2006). UPEC strains usually produce

siderophores that probably play a role in iron acquisition for the bacteria (Todar, 2002). The *fbp* locus (for "ferric binding protein") is involved in the acquisition of iron (Parham et al., 2005). The locus encoding the FbpA-D iron siderophore system was shown to be associated with UPEC and to be more present in these pathogens than in intestinal isolates of *E. coli* (Rasko et al., 2001). In silico analysis revealed that FbpA belongs to the TonB-dependent family of outer membrane receptor proteins associated with iron transport, whereas FbpB, FbpD, and FbpC belong to the ferric enterobactin family of iron sequestering proteins (Parham et al., 2005). Finally, the *efu* locus ("for *E. coli* fucose metabolism") is associated with sugar metabolism, particularly with the ability to degrade the specific carbohydrates fucose and pectin (Parham et al., 2005). The presence of fucosylated structure on uroepithelial cells has a protective effect from UTI (Schaeffer et al., 2001). The virulence factor of *efu* locus is probably linked to the ability to degrade these fucosylated structures (Parham et al., 2005).

2.8. Pathogenicity Islands

One of the driving forces in the emergence of new bacterial variants is by acquisition of new traits by horizontal gene transfer. However, point mutations, gene rearrangements, or loss of genetic information also play crucial roles (Middendorf et al., 2004).

UPEC typically carry large sets of genes coding for multiple virulence factors that tend to be clustered together on large blocks of chromosomal DNA termed pathogenecity islands (PAIs) (Guyer et al., 2002). These PAIs contribute to the evolution of bacterial pathogens

(Hacker & Kaper, 2000). Characteristically, PAIs are >30 kb, inserted at the 3' end of tRNA genes, horizontally acquired, their G+C content is different from that of the rest of the bacterial chromosome, they are flanked by insertion elements or direct repeats, and are associated with mobile genetic elements such as integrase genes or transposase (Middendorf et al., 2004).

Pathogenic and nonpathogenic strains of a given species tend to differ by the presence or absence of such PAIs (Parham et al., 2005). PAIs are found in pathogenic strains but are absent or rarely found in non-pathogenic strains (Hacker & Kaper, 2000). Several of the genes associated with UTIs, such as P fimbriae, hemolysin, are encoded on PAIs (Blum et al., 1995).

PAIs have been defined in uropathogenic strains 536, J96, and CFT073 (Bingen-Bidois et al., 2002). In the UPEC *E. coli* strain 536 (O6:K15:H31), a model organism of the extraintestinal pathogenic *E. coli* (ExPEC) isolated from a patient with acute pyelonephritis, five well characterized PAIs (PAI I536 to PAI V536) and a newly identified sixth island are present (Middendorf et al., 2004). These PAIs encode many virulent genes such as P-related fimbriae (PAI II536), two alpha hemolysin gene clusters (PAI I536 and PAI II536), S-fimbriae (PAI III536), and the salmochelin and yersiniabactin siderophore systems (PAI III536 and PAI IV536, respectively) (Middendorf et al., 2004). PAI I536 to PAI V536 are associated with the tRNA genes *selC*, *leuX*, *thrW*, *asnT*, and *pheV*, respectively with these tRNA genes considered as hotspots for the integration of foreign DNA since they have been described as insertion sites for bacteriophages, conjugative transposons, and several PAIs in other bacterial species (Hacker & Kaper, 2000; Middendorf et al., 2004).

In the uropathogenic strain J96 (O4:K6:H5), isolated from a human pyelonephritis patient, virulence factors were identified such as pap, alpha-hemolysin, and cytotoxic necrotizing factor type 1 (Blum et al., 1995). Several of these virulence genes are present on chromosomal regions termed PAI I and II (Swenson et al., 1996).

In the CFT073 strain (O6:K2:H1) isolated from the blood and urine of a hospitalized patient with acute pyelonephritis (Mobley et al., 1990), *PicU* locus is present on a PAI II CFT073 inserted at the *aspV* tRNA locus (Parham et al., 2005). PAI II CFT073 possesses other loci involved in virulence such as the two-partner secretion system (*etp*), a type I secretion system (*tos*), iron acquisition genes (*fbp*), and a carbohydrate metabolism locus (*efu*) (Parham et al., 2005).

2.9. Phylogenetic Grouping

Phylogenetic studies have shown that *E. coli* can be divided into four main phylogenetic groups, designated A, B1, B2, and D (Clermont et al., 2000). Duriez et al. (2001) assessed the distribution of the human commensal and pathogenic strains among *E. coli* phylogenetic groups. A and B1 group were the most common (40% and 34%, respectively), followed by the D group (15%) and B2 group (11%). In the extraintestinal pathogenic strains however, 9% belonged to group A, 3% group B1, 16% group D, and 72% group B2. *E. coli* strains responsible for UTIs and other extraintestinal infections, including those with the most robust virulence factor repertoires and those that are able to infect noncompromised hosts, derive from *E. coli* phylogenetic group B2, and to a lesser extent from group D (the second largest group), as opposed to commensal *E. coli* strains which

generally belong to phylogenetic groups A and B1 (Goullet & Picard, 1990 ; Lecointre et al., 1998; Picard et al., 1999 ; Duriez et al., 2001; Zhang et al., 2002). Strains of phylogenetic groups B2 and D often carry virulence determinants that are absent in group A and B1 strains (Picard et al., 1999; Johnson & Stell, 2000). Although group B2 is uncommon among commensal, they are highly virulent when present (Sabate et al., 2006), which additionally supports the notion that the intestinal microbiota may act as a reservoir for UPEC (Sabate et al., 2006). B2 strains account for about two-third of all extraintestinal *E. coli* infections (Zhang et al., 2002), representing 72% of *E. coli* strains isolated from patients with meningitis (Duriez et al., 2001), and 65% of strains isolated from patients with bacteremia (Johnson et al., 2001).

Phylogenetic grouping can be achieved by multilocus enzyme electrophoresis or ribotyping with both methods being time consuming and complex (Clermont et al., 2000). PCR detection of specific DNA fragments known as phylogenetic group markers represents a rapid technique for the determination of the phylogenetic groups of *E. coli* (Clermont et al., 2000). Three markers are used: *chuA*, a gene required for heme transport in enterohemorrhagic O157:H7 *E. coli* (Torres & Payne, 1997; Bonacorsi et al., 2000, Clermont et al., 2000), *yjaA*, a gene identified in the genome sequence of *E. coli* K12 (Blattner et al., 1997), and an anonymous DNA fragment designated TSPE4.C2 (Bonacorsi et al., 2000). The *chuA* gene is present in all B2 and D strains, but absent in groups B1 and A. *yjaA* gene permits perfect discrimination between groups B2 (all having the *yjaA*) and D (absent in all) (Clermont et al., 2000) (Figure 4).

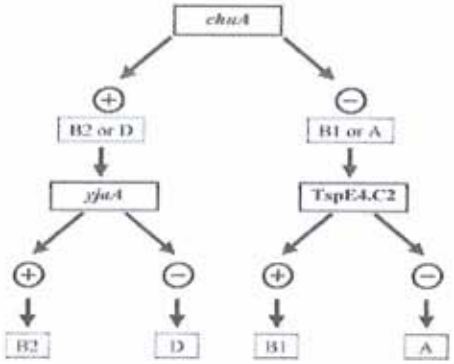

Figure 4. The phylogenetic grouping of *E. coli* strains by the PCR amplification of the *chuA* and *yjaA* genes and DNA fragment TSPE4.C2 (Clermont et al., 2000).

2.10. Antimicrobial Testing

Antibiotic resistance presents an ongoing challenge in the effective treatment of patients (Steinke et al., 2001; Critchley & Karlowsky, 2004) due to the emerging resistance to most first-line antimicrobial agents (Chen et al., 2001). Antibiotic resistance increases in incremental steps and could be the result of genetic mutation, the acquisition, incorporation and expression of exogenous genetic material, or clonal dissemination (Critchley & Karlowsky, 2004).

The most commonly used antibacterial drugs in the treatment of community-acquired UTIs are the following: Trimethoprim/sulfamethoxazole, ciprofloxacin, cephalosporins, semi-synthetic penicillins with or without inhibitors, nitrofurantoin and fosfomycin (Hryniewicz et al., 2001). However, the Infectious Diseases Society of America (IDSA) recommends using

trimethoprim/sulfamethoxazole as the treatment of choice, where the prevalence of resistance is <10-20% (Warren et al., 1999). If the trimethoprim/sulfamethoxazole resistance rate is higher than 20%, it should be replaced by fluoroquinolones for both complicated and uncomplicated UTIs (Warren et al., 1999). Resistance to trimethoprim-sulfamethoxazole in many locales surpasses the 10 to 20% threshold at which authorities suggest to use alternative empirical regimens such as fluoroquinolone, nitrofurantoin, or fosfomycin (Johnson et al., 2005a). For the last two decades, fluoroquinolones have been in clinical use as the first-line antibiotics for the treatment of UTI (Zervos et al., 2003), and accordingly emergence of resistance to fluoroquinolones threatens the use of these drugs as a "fall-back" option for empiric treatment of UTIs (Gupta et al., 2001; Steinke et al., 2001). Nevertheless, resistance to fluoroquinolones has emerged to a concerning extent in several areas such as in Latin America and Costa Rica (Gales et al., 2000 ; Sannes et al., 2003), while a slow but steady increase was observed in other locales such as United States (Karlowsky et al., 2002; Zervos et al., 2003). Resistance to fluoroquinolones is mainly mediated by point mutations in gyrase and topoisomerase but may be due to efflux and plasmid-mediated mechanisms (Drews et al., 2005). There is a great concern that as the use of the alternative empirical regimens increases, resistance to the agents will arise and limit their use also (Johnson et al., 2005a; Sannes et al., 2003).

Several studies tested the sensitivity or resistance of UPEC to different antimicrobial agents. In Russia, the cumulative percentage of both resistant and intermediate strains to ciprofloxacin (fluoroquinolone) was 2.6% (Stratchounski et al., 2001). Ciprofloxacin was found to be superior to all other tested drugs including gentamicin, nitrofurantoin,

ampicillin, and trimethoprim-sulfamethoxazole, the resistance percentages of which were 5.9%, 2.9%, 33.3%, and 18.4%, respectively (Stratchounski et al., 2001). On the other hand, Gulsun et al. (2005) reported that 85% of UPEC were sensitive to ciprofloxacin, while in Turkey the percentage of resistance to ciprofloxacin and trimethoprim/sulfamethoxazole was 17% and 36% in uncomplicated UTI strains and 38% and 42% for complicated UTI strains (Arslan et al., 2005). Accordingly, nitrofurantoin and fosfomycin were considered as reasonable alternatives for the treatment of UTIs given that the resistance rate was 4% and 0.3%, respectively (Arslan et al., 2005). Moreover, in pregnant women and in community-acquired infections, simple antimicrobial drugs like nitrofurantoin might still be useful (Christiaens, 2002).

Chapter 3

MATERIALS AND METHODS

3.1. Reference strains

Reference strains used in this study were the ECOR collection, ECOR10, ECOR28, ECOR55, ECOR59, ECOR41, thankfully donated by Dr. Thomas Whittam from the National Food Safety and Technology Center (Table 2), and the uropathogenic *E. coli* CFT073 obtained from ATCC.

Table 1: ECOR collection reference strains

Isolate	Group	Host	Local, year of isolation	Clinical
ECOR-10	A	Human(Female)	Sweden	Healthy
ECOR-28	B1	Human(Female,4yr)	USA(Iowa)	Healthy
ECOR-55	B2	Human(Female)	Sweden	UTI(P)
ECOR-59	B2	Human(Male)	USA(Mass.) 1979	Healthy
ECOR-41	D	Human(Female,22yr)	Tonga 1982	Healthy

P=acute pyelonephritis (Whittam, 2003)

3.2. Clinical isolates

Isolates of *E. coli* isolated from urine samples were thankfully donated by Dr. George Abdelnour from Notre Dame de Secours in Jbeil and Dr. Ziad Daoud from St. George Hospital in Achrafieh.
In this study, 68 *E.* coli isolates were obtained from Notre Dame de secours and were designated by EM (for *E. coli* Mahounet), and 32 isolates from St George Hospital designated by ER (for *E. coli* Roum). All the isolates were from year 2006, with ages and clinical cases being undisclosed.

3.3. Bacterial storage and culture conditions

All clinical isolates were stored in cryobank tubes at -80°C. The reference strains and clinical isolates were first cultivated on tryptone soy agar plates (Mast Group Ltd., Merseyside, U.K.) and incubated at 37°C for 24h. Identification of *E. coli* isolates was performed using API20E Strips (BioMérieux, Marcy-L'Etoile, France).

3.4. API20E strips

The API20E strips (BioMérieux, Marcy-L'Etoile, France) consists of a series of microtubes containing dehydrated substrates that are reconstituted by adding the microorganism suspended in 0.85% saline. The strips were inoculated and incubated at 37°C. Results were reported after 24h of incubation. Positive reactions were converted to a seven-digit profile number. These profiles were then compared with the index supplied by the manufacturer (API 20E System: publication no. 20190) and with computer data base for the API20E system (APILAB PLUS, version 3.3.3).

3.5. DNA extraction

Bacterial colonies were cultured on tryptone soy agar plates prior to extraction. DNA extraction was done using the NucleoSpin DNA extraction kit (Macherey-Nagel, Germany), according to the manufacturer's instructions.

3.6. PCR amplification

All PCR assays were performed on PerkinElmer GeneAmp 9700 (PerkinElmer, Wellesly, Massachusetts).

3.6.1. 16S rDNA amplification

16S rDNA amplification was used as a positive PCR control ensuring the integrity of all sample DNA used for phylogenetic grouping and detection of virulence genes.

PCR assays were performed directly on 1.5 µl of DNA, which was added to 18.5 µl of PCR mixture containing 0.4 mM dNTP from 2 mM stock, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.25 µM 27F primer from a stock of 50 pmol/µl (5'-AGAGTTTGATCCTGGCTCAG-3') (Thermo Hybaid, Germany), 0.25 µM 1492R primer from a stock of 50 pmol/µl (5'-GGTTACCTTGTTACGACTT-3') (Thermo Hybaid, Germany), 1U Gold Taq Polymerase (Fermentus), with an initial denaturation of 95°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 2 min, and a final soak at 4°C (Delong 1992; Suzuki & Giovannoni 1996). All PCR assays incorporated a negative reagent control. The PCR amplicons were visualized using a UV bioimaging system after electrophoresis on a 1% agarose gel containing 0.5 µg/ml ethidium bromide.

3.6.2. Phylogenetic group determination

Each reaction was carried out by using a 20-µl mixture containing 2.5 mM $MgCl_2$, 1× PCR buffer, 20 pmol of each primer (Table 2), each deoxynucleoside triphosphate at a concentration of 2 µM, 2.5 units of Platinum *Taq* DNA polymerase (Invitrogen Inc., Carlsbad, CA), and 3 µl DNA. The thermal cycling conditions were: denaturation for 4 min at 94°C, 30 cycles of 5 s at 94°C and 10 s at 59°C, and a final extension step of 5 min at 72°C (Clermont et al., 2000). ECOR 10, 28, 55, 59, and 41 were used as positive controls. Negative controls include a negative reagent control, and a negative PCR control with *Staphylococcus aureus* DNA. Products were analyzed on a 2.5% gel containing 0.5 µg/ml ethidium bromide.

Table 2: Primers used for phylogenetic grouping (Clermont et al., 2000)

Primer	Primer sequence (5'-3')	Size (bp)
ChuA.1	5'-GACGAACCAACGGTCAGGAT-3'	279
ChuA.2	5'-TGCCGCCAGTACCAAAGACA-3'	
YjaA.1	5'-TGAAGTGTCAGGAGACGCTG-3'	211
YjaA.2	5'-ATGGAGAATGCGTTCCTCAAC-3'	
TspE4C2.1	5'-GAGTAATGTCGGGGCATTCA-3'	152
TspE4C2.2	5'-CGCGCCAACAAAGTATTACG-3'	

3.6.3. Detection of virulence genes

Genomic DNA preparations were tested for three virulence genes: *fbp*, *efu*, and *picU* in 50-µl PCR mixtures containing 15 pmol of each of the forward and reverse primers (Table 3), 10 nmol of each deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase (Invitrogen Inc., Carlsbad, CA), and 2 mM $MgCl_2$ in 1x PCR buffer. The PCR conditions were as follows: initial incubation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30s, and extension at 72°C for 45 s, and a final extension step of 72°C for 10 min (Parham et al., 2005). Products were analyzed by electrophoresis on 2.5% gels containing 0.5 µg/ml ethidium bromide. Uropathogenic *E. coli* CFT073 was used as a positive control. Negative controls included a negative reagent control (without template DNA), and a negative PCR control with *Staphylococcus aureus* DNA.

Table 3. Primers used for detection of virulent genes (Parham et al., 2005).

Target (orientation)[a]	Primer sequence (5'-3')	Genomic position[b]
fbp (F)	TTGCTCGTCGGGCTGAAAAAGTTGT	278466-278490
fbp (R)	TTGCACCATCCACTATCACCATCGA	279008-278984
efu (F)	TATCGTGTTTATTCCCGCTATGG	302752-302774
efu (R)	CCCCTCGGAGAAAACCAGATATT	303203-303181
picU (F)	TCAGGCCGGTAAGAACAGCAAAAT	327844-327821
picU (R)	ACGGTAAGAGTGTGGATGGCGGAGTC	327473-327498

[a] F, forward; R, reverse.

[b] Relative to the UPEC strain CFT073 genome.

3.7. Antibiotic susceptibility using the disk diffusion assay

All 100 isolates were subjected to a disk diffusion test. Bacterial colonies were first suspended in a Mueller-Hinton broth at a density equivalent to a 0.5 McFarland standard. They were then spread using a swab on a Mueller-Hinton agar plate. Each plate was then stamped with 8 different antibiotic diffusion disks (Oxoid, Basingstoke, Hampshire, England): Gentamicin (CN, 10μg), Trimethoprim-sulfamethoxazole 1:19 (SXT, 25 μg), Chloramphenicol (C, 30 μg), Ampicillin (AMP, 10 μg), Ciprofloxacin (CIP, 5 μg), Netilmicin (NET, 30 μg), Nitrofurantoin (F, 300 μg) and Tetracycline (TE, 30 μg). Plates were then incubated for 18-24h at 37°C. Following incubation, diameter of the zone of inhibition around each disk was measured. Resistance or susceptibility profiles were established according to the Clinical and Laboratory Standards Institute (NCCLS, 2006).

RESULTS

4.1. Gender

From the 68 isolates obtained from Notre Dame de Secours hospital in Jbeil, 69.1% (n=47) of patients were females, while 30.9% (n=21) were males. Group A represented 27.7% (n=13) of isolates collected from females, 25.5% (n=12) group D, and 46.8% (n=22) group B2. Isolates from males mainly belonged to group B2 (71.4%, n=15), followed and with equal proportions by group A (14.3%, n=3) and group D (14.3%, n=3).

4.2. Identification using API 20E system

Identification of all isolates (n=89) collected from Notre Dame de Secours hospital revealed that only 68 were actually *E. coli*, while the remaining 21 were *Enterobacter cloacae, Kluyvera* or *Citrobacter freundii*. All isolates from Saint Georges hospital (n=32) were confirmed to be *E. coli* by the API 20E system.

Identification using API 20E strips gave 25 different numerical profiles (Annex I). Around 30% (n=30) of the *E. coli* isolates belonged to the numerical profile 19 (Figures 5 and 6).

The difference between the numerical profiles was mainly within the following tests: β-Galactosidase, arginine dihydrolase, lysine

decarboxylase, ornithine decarboxylase, oxidation/fermentation of rhamnose, saccharose, melibiose, and sorbitol (Table 3). Isolates positive for those tests and having the numerical profile 25 belonged to group B2 (Figure 7). However, those with the numerical profile 19, mainly belonged to group B2 (73.3%, n=22), followed by group D (16.7%, n=5), and group A (10%, n=3) (Figure 7).

4.3.PCR assays

4.3.1.16S rDNA Amplification

Amplification of the 16S rDNA was considered as a positive PCR control to ensure the integrity of all DNA samples used for phylogenetic grouping and detection of virulence genes. All the extracted DNA products yielded a band with the expected position size (1500 bp) (Figure 8).

4.3.2. Phylogenetic Group

Phylogenetic grouping was based on the incorporation of reference strains along with all the PCR assays conducted (Figures 9a and 9b). Overall, the results obtained revealed that among the 100 uropathogenic *E. coli* isolates, 21% (n=21) belonged to group A, 21% (n=21) to group D, and 58% (n=58) to group B2. None of the isolates belonged to group B1 (Figure 10).

ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP
+	-	+	+	-	-	-	-	+	-
GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
-	+	+	-	+	+	+	+	-	+

Figure 5: The API 20E numerical profile 19

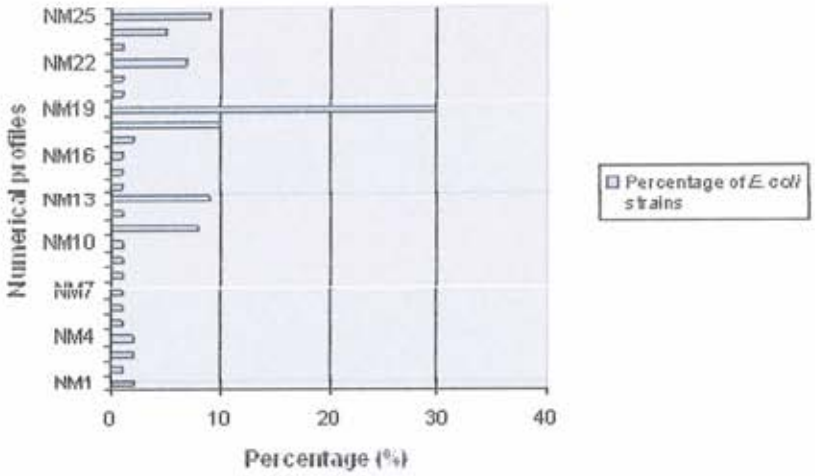

Figure 6: The distribution of the different obtained API 20E numerical profiles among the tested *E. coli* population. Details of the numerical profiles are shown in Annex I

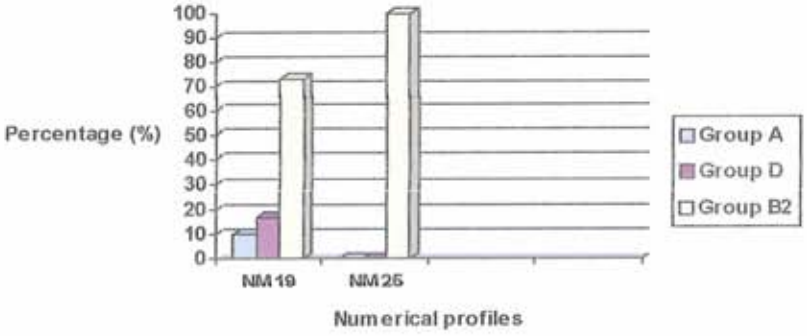

Figure 7: Distribution of *E. coli* API 20E numerical profiles 19 and 25 among the three detected phylogenetic groups

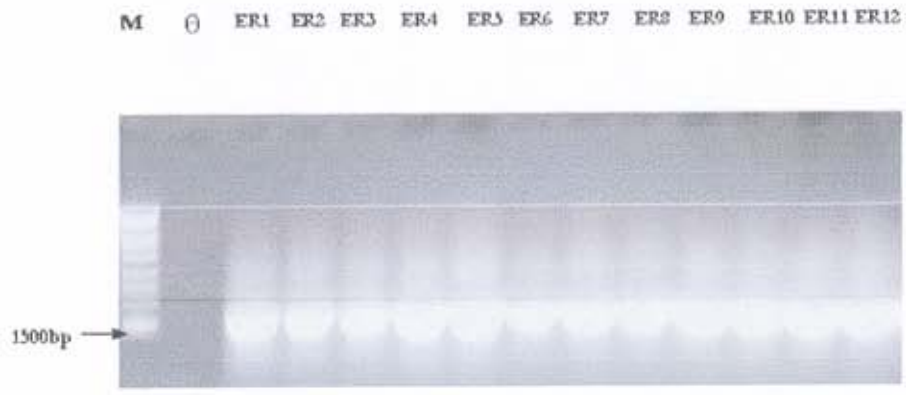

Figure 8: Amplification of 16S rDNA gene as a positive PCR control. Lane 1: "M" for marker, high range DNA ladder. Lane 2: negative control. Lane 3 through 13 had ER1 to ER12 (ER for *E. coli* Roum, isolates obtained from Saint Georges hospital). They all gave a band at the expected position (1500bp).

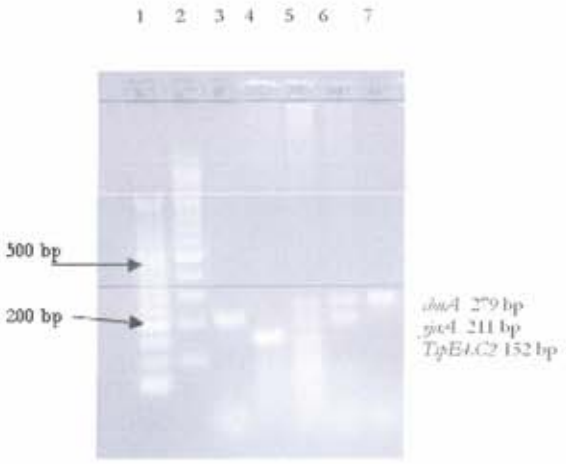

Figure 9a: Triplex PCR for the ECOR collection reference strains. Lane 1: O'RangeRuler 50bp DNA ladder. Lane 2: O'RangeRuler 100bp ladder. Lane 3 through lane 7 had: ECOR10 (group A), ECOR 28 (group B1), ECOR 55 (group B2), ECOR 59 (group B2), and ECOR 41 (group D). Isolates negative for both *chuA* and *tspE4.C2* represents the group A whereas isolates negative for *chuA* but positive for *tspE4.C2* represents group B1. Isolates positive for *chuA* and *yjaA* represents group B2. Isolates positive for *chuA* but negative for *yjaA* represents group D.

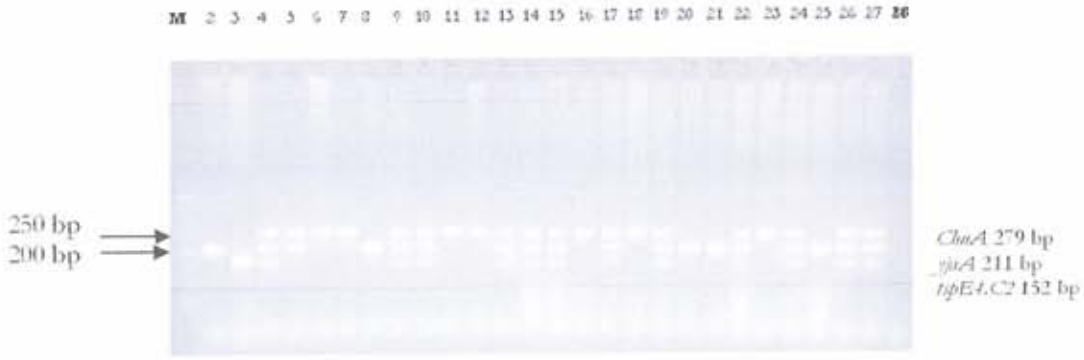

Figure 9b: Triplex PCR showing the three detected phylogenetic groups among the studied *E. coli* clinical isolates. Lane 1: "M" for marker, O'range 50 bp DNA ladder. Lane 2 through lane 6 had ECOR reference strains: ECOR 10 reference strain (group A), ECOR 28 (group B1), ECOR 55 (group B2), ECOR 59 (group B2), and ECOR 41(group D). Lane 7 through lane 28 had: EM1 (group D), EM7 (group A), EM19 (group B2), EM21 (group B2), EM26 (group D), EM29 (group D), EM43 (group B2), EM44 (group B2), EM45 (group B2), EM47 (group D), EM48 (group B2), EM50 (group D), EM55 (group B2), EM61 (group A), EM 65 (group A), EM66 (group B2), EM74 (group D), EM75 (group B2), EM79 (group A), EM80 (group B2), EM81 (group B2), and a negative PCR control (using *Staphylococcus aureus*). EM stands for *E. coli* Mahounet (isolates obtained from Notre Dame de Secours hospital).

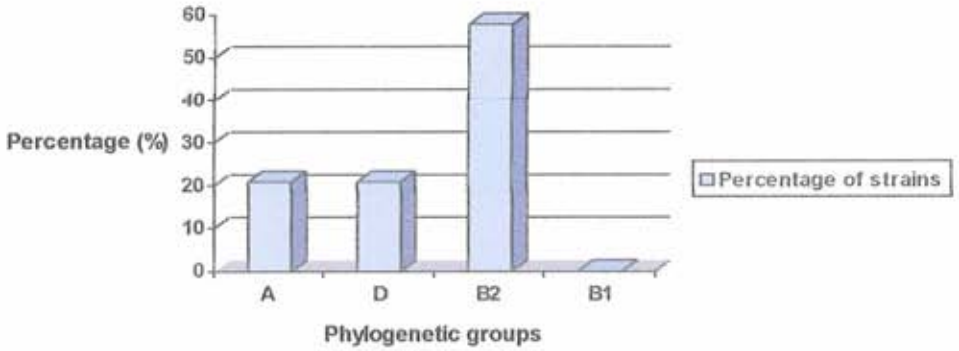

Figure 10: The percentage of the different *E. coli* phylogenetic groups within the tested population.

4.3.3. Virulence genes

The presence of three virulence-associated loci (*fbp*, *efa*, and *picU*) was determined using a triplex PCR assay (Figure 11b) along with the CFT073 reference strain (Figure 11a). At least one virulence gene was in all tested isolates, while 52% (n=52) of the isolates were positive for all three tested genes (Figure 12a). The distribution of the three virulence determinants among *E. coli* isolates was the following: 77% (n=77) had the *fbp* gene, 95% (n=95) the *efa*, and 61% (n=61) the *picU* gene. Correlating between the phylogenetic groups and virulence genes revealed that 47.6% (n=10) possessed the locus *fbp* in group A, 81% (n=17) in group D, and 86.2% (n=50) in group B2 (Figure 12b). For the *efa* locus, 90.5% (n=19) of group A isolates were positive to this gene, 94.8% (n=55) of group B2, and all isolates within group D (100%, n=21) (Figure 12b). However, within group A, 52.4% (n=11) possessed the locus *picU*, 81% (n=17) in group D, and 56.9% (n=33) in group B2 (Figure 12b). Within group A, 38.1% (n=8) had the three tested virulence genes, while the percentage was higher in group D (71.5%; n=15) and in group B2 (50%, n=29) (Figure 12c). None of the tested isolates had a combination of *fbp* and *picU* genes in the absence of the *efa* locus (Figure 12a).

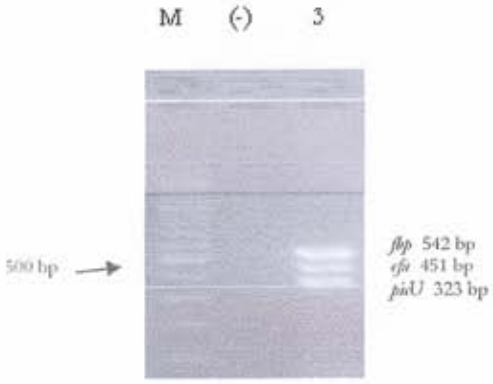

Figure 11a: Triplex PCR showing the PCR amplification of the three tested virulence genes (*fbp*, *efu*, and *picU*) in the CFT073 UPEC reference strain. Lane 1: "M" for marker, O'Range Ruler 100 bp DNA marker. Lane 2: negative control. Lane 3 had the CFT073 reference strain with all the three virulence- associated loci.

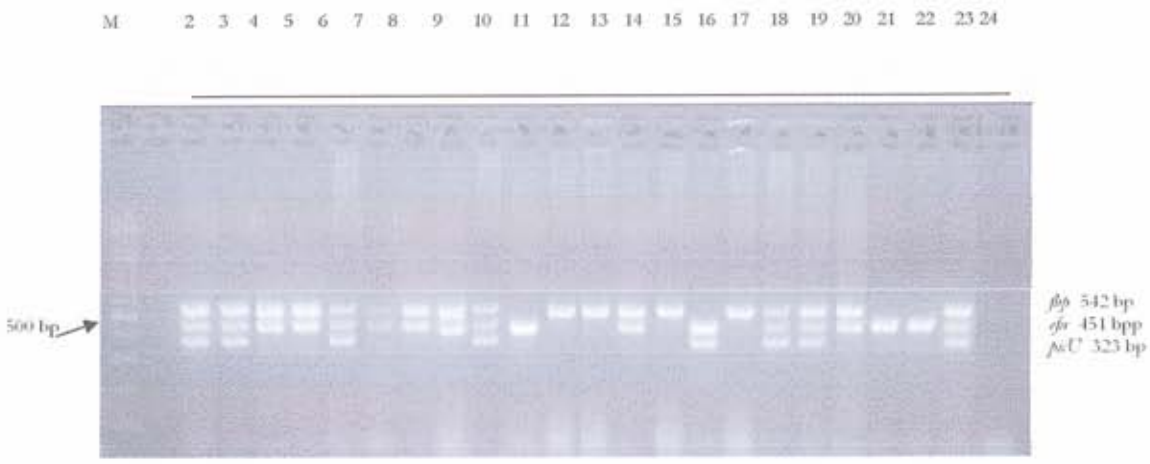

Figure 11b: Triplex PCR showing PCR amplification of the three studied virulence genes (*fbp*, *efa*, and *picU*) among *E. coli* isolates.

Lane 1: (M) for marker, O'Range Ruler 100 bp DNA ladder. Lane 2 through 24: CFT073 reference strain, EM56, EM57, EM58, EM66, EM68, EM70, EM76, EM82, EM88, EM6, EM37, EM39, EM81, EM80, EM81, ER1, ER2, ER3, ER10, ER15, ER16, and a negative PCR control (using *Staphylcoccus aureus*). EM stands for *E. coli* Mahounet (isolates collected from Notre Dame de Secours hospital), whereas ER stands for *E. coli* Roum (isolates obtained from Saint Georges Hospital).

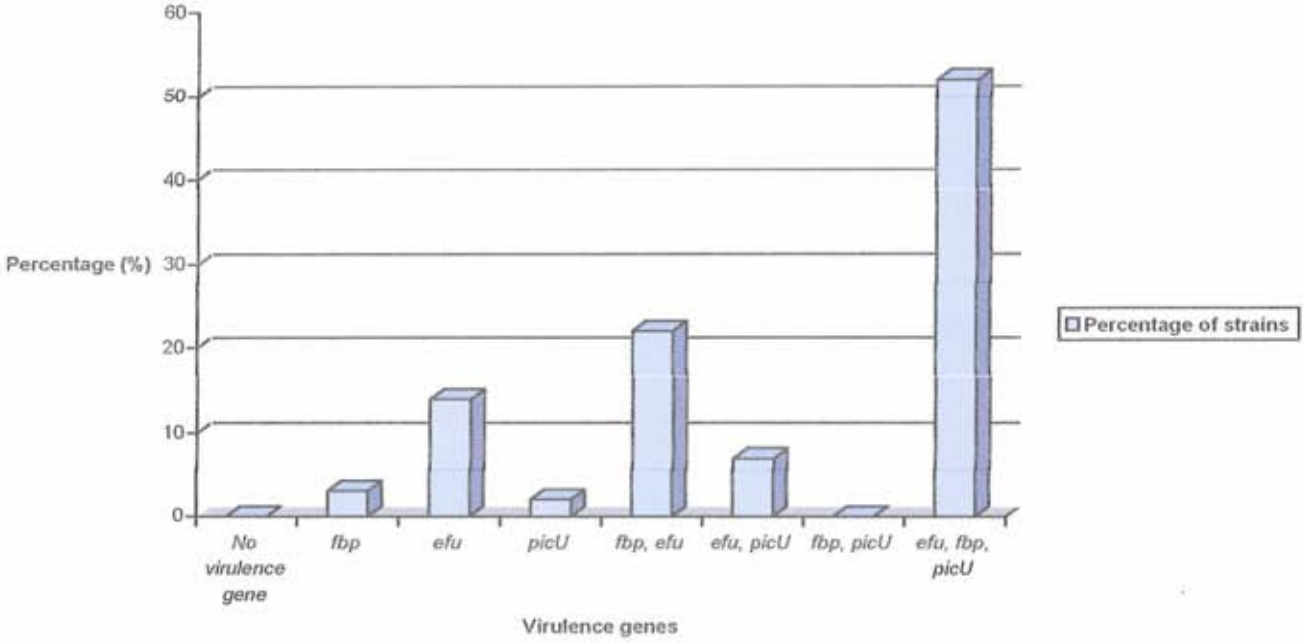

Figure 12a: Distribution of the possible combinations of virulence genes among *E. coli* isolates.

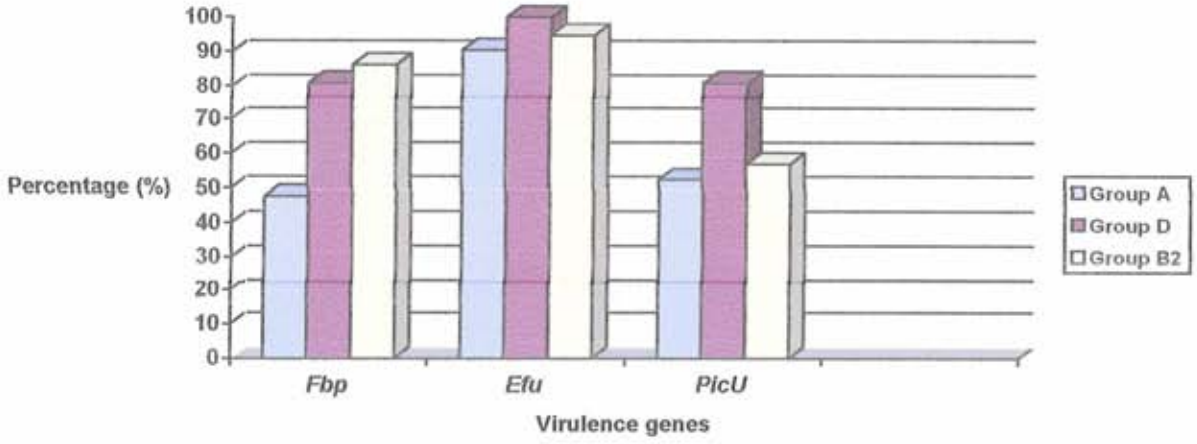

Figure 12b: Relation between virulence genes and phylogenetic groups among the studied *E. coli* isolates.

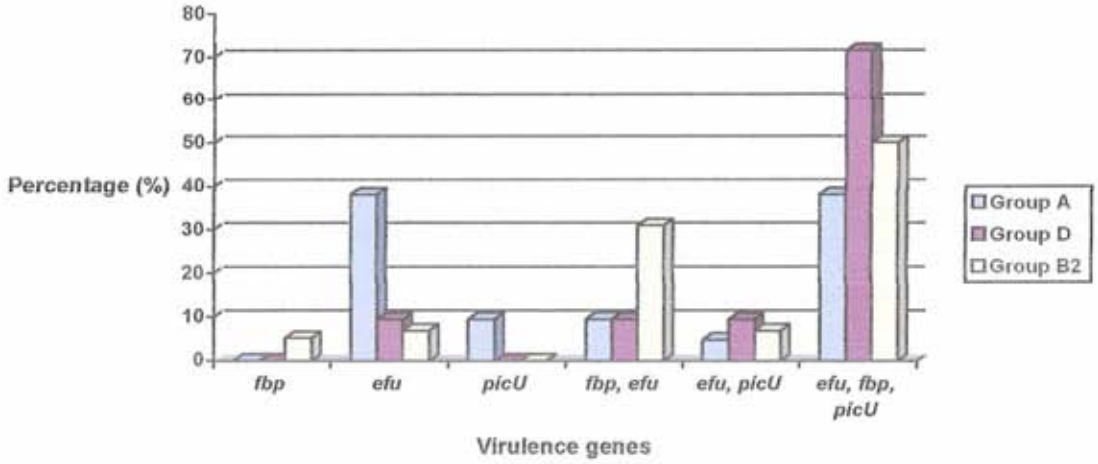

Figure 12c: The possible combinations of virulence genes among the the obtained phylogenetic groups.

4.4. Antibiotic susceptibility using the disk diffusion assay

Antibiotic susceptibility was determined using eight antibiotics: Ampicillin (10 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Netilmicin (30 µg), Trimethoprim/sulfamethoxazole 1:19 (25 µg), Nitrofurantoin (300 µg), Chloramphenicol (30 µg) and Tetracycline (30 µg) (Figures 13a, 13b, 13c, 13d, 13e, and 13f). Majority of the isolates (68%, n=68) were resistant to ampicillin, 43% (n=43) to tetracycline, 38% (n=38) to trimethoprim/sulphamethoxazole, 29% (n=29) to ciprofloxacin, 16% (n=16) to chloramphenicol, 15% (n=15) to gentamicin, 8% (n=8) to nitrofurantoin, and 3% (n=3) to netilmicin (Figure 13g). Within the different detected phylogenetic groups, 19% (n=4) of the A group, 9.5% (n=2) of the D, and 25.9% (n=15) of B2 were susceptible to all of the eight used drugs (Figure 14a). On the other hand, 61.9 % (n=13) of the A group isolates, 52.5% (n=11) of group D, and 31% (n=18) of group B2 were resistant to at least 3 drugs (Figure 14b). In group A, 19.1% (n=4) were resistant to one or two drugs, 38% (n=8) in group D, and 37.9% (n=22) in group B2 (Figure 14b). Among the group A that were multiple drug resistant (at least to 3 drugs) (n=13), 7.7% (n=1) possessed the three virulent genes. Moreover, isolates that were susceptible to all tested drugs within the A phylogenetic group had the three virulence genes. In group D, the virulence genes were detected in 72.7% (n=8) of the multiple resistant isolates (n=11) and in all susceptible ones. Finally, in group B2, 22.2% (n=4) of the multiple resistant isolates (n=18) versus 53.3% (n=8) of the susceptible ones (n=15) had all the studied virulence genes. Susceptible isolates exhibited a higher prevalence of *fbp*, *efa*, and *picU* (90.5%, n=19; 100%, n=21; and 76.2%, n=16, respectively) compared to multiple drug resistant isolates (64.3%, n=27; 95.2%, n=40; 43%, n=18, respectively).

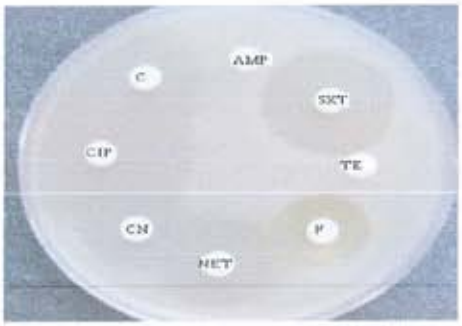

Figure 13a: *E. coli* isolate resistant to AMP and TE using the disk diffusion assay. This isolate is susceptible to C, CIP, CN, NET, F, and SXT. Ampicillin (AMP, 10μg), Chloramphenicol (C, 30 μg), Ciprofloxacin (CIP, 5μg), Gentamicin (CN, 10μg), Netilmicin (NET, 30μg), Nitrofurantoin (F, 300μg), Tetracycline (TE, 30μg), Trimethoprim/sulfamethoxazole (SXT, 25 μg).

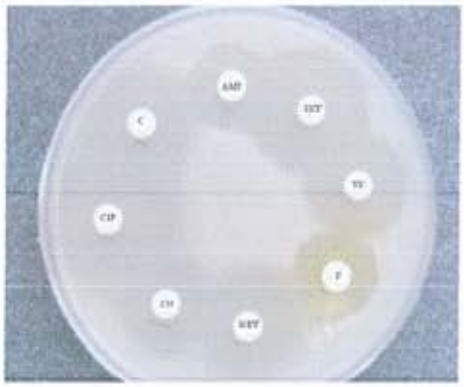

Figure 13b: *E. coli* isolate susceptible to all eight tested drugs. Refer to figure 13a.

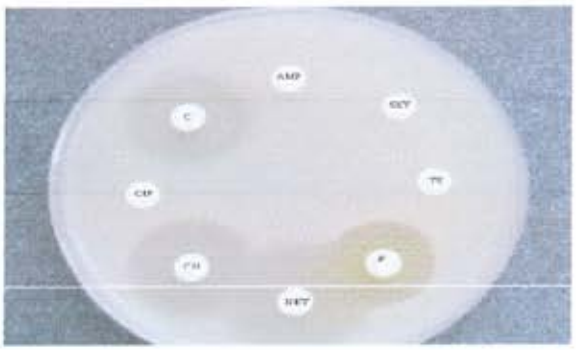

Figure 13c: *E. coli* isolate resistant to AMP, SXT, TE, and CIP. Refer to figure 13a.

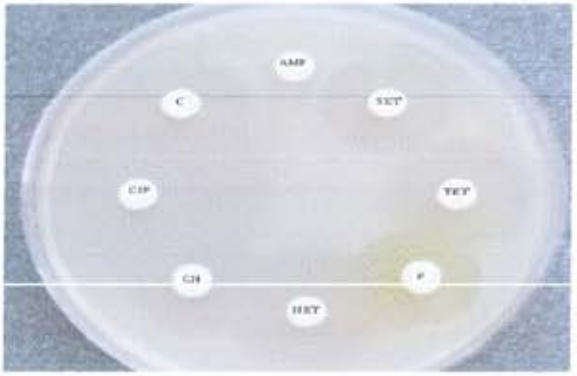

Figure 13d: *E. coli* isolate resistant to AMP. Refer to figure 13a.

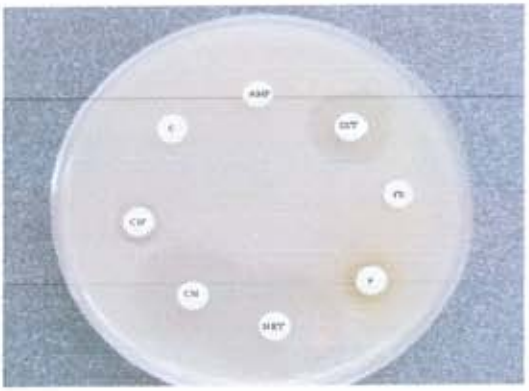

Figure 13e: *E. coli* isolate resistant to AMP, C, TE, F, and CIP. Refer to figure 13a.

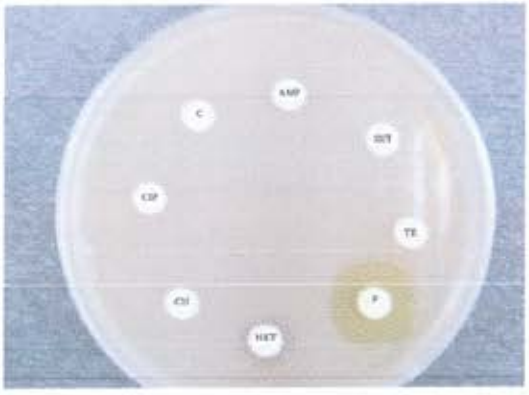

Figure 13f: *E. coli* isolate resistant to AMP, SXT, TE, NET, C, CN, and CIP. Refer to figure 13a.

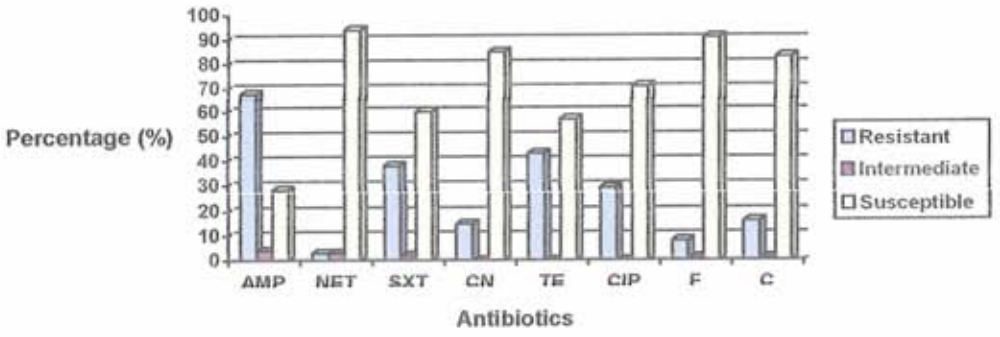

Figure 13g: Percentage of isolates resistant, intermediate, or susceptible to each of the tested drugs.
Ampicillin (AMP), netilmicin (NET), trimethoprim/sulfamethoxazole (SXT), gentamicin (CN), tetracycline (TE), ciprofloxacin (CIP), nitrofurantoin (F), and chloramphenicol (C).

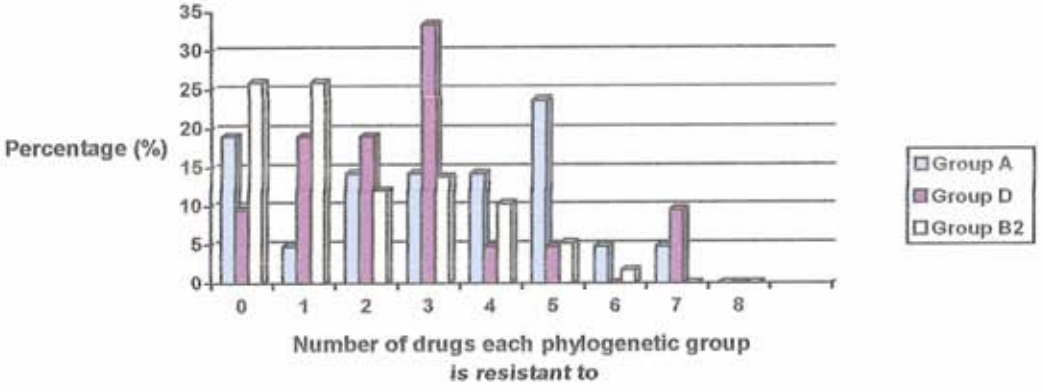

Figure 14a: Distribution of resistance among phylogenetic groups

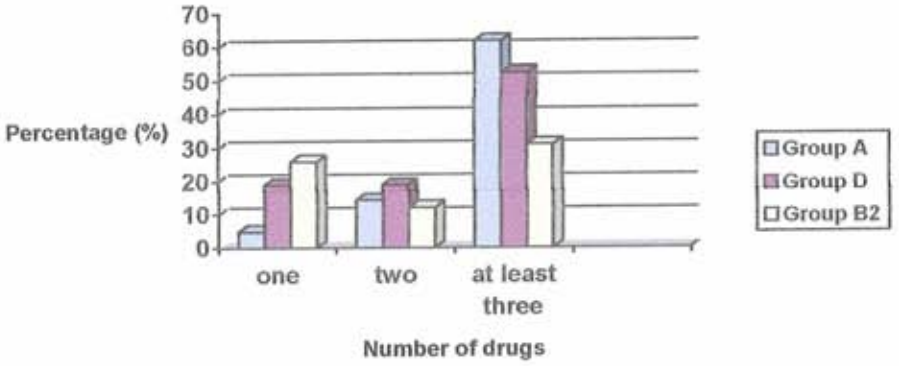

Figure 14b: Percentage of resistance to one, two or at least three antimicrobial agent among the different phylogenetic groups.

4.5. Inhibitor of folic acid synthesis

4.5.1. Trimethoprim/ sulphamethoxazole drug (SXT)

In isolates resistant to SXT (n=38), 52.7% (n=20) belonged to group B2, 28.9% (n=11) to group A, and 18.4% (n=7) to group D (Figure 15a). Resistant isolates with three virulence genes represented 45% (n=9) of group B2, 9.1% (n=1) of group A, and 71.4% (n=5) of group D (Figure 16a).

Within the susceptible population (n=60), 60% (n=36) belonged to group B2, 23.3% (n=14) to group D, and 16.7% (n=10) to group A (Figure 15b) with 70% (n=7), 71.4% (n=10), and 50% (n=18) of those susceptible to SXT and belonging to groups A, D, and B2, respectively, possessing the three virulence loci (Figure 16b).

4.6. Inhibitor of DNA synthesis

4.6.1. Ciprofloxacin (CIP)

Highest percentage of ciprofloxacin resistance was detected among isolates within the phylogenetic group A (41.4%, n=12), followed by group B2 (34.6%, n=10), and group D (24%, n=7) (Figure 15a). Resistant isolates with three virulence genes represented 16.7% (n=2) of group A, 57.1% (n=4) of group D, and 20% (n=2) of group B2 (Figure 17a). On the other hand, within the susceptible isolates, 67.6% (n=48) belonged to group B2, 19.7% (n=14) to group D, and 12.7% (n=9) to group A (Figure 15b). Among the isolates susceptible to ciprofloxacin, the percentage of isolates with three virulence genes was 56.2% (n=27) in group B2, 78.7% (n=11) in group D, and 66.7% (n=6) in group A (Figure 17b).

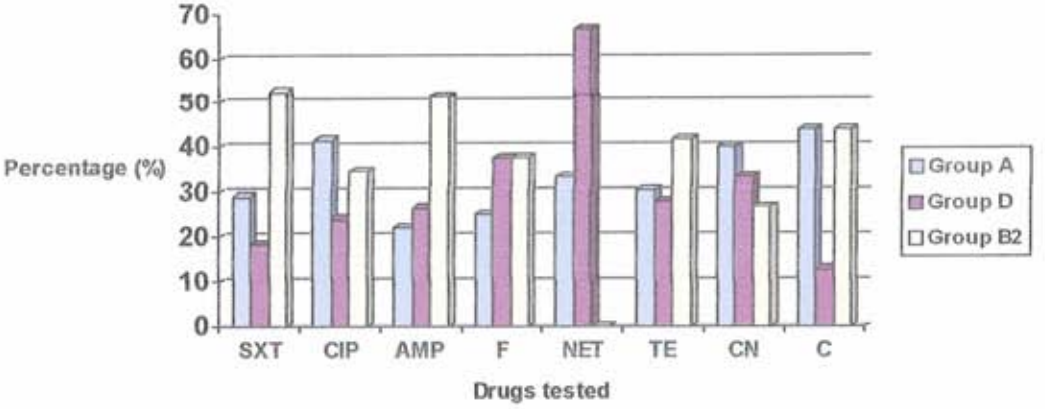

Figure 15a: Distribution of the phylogenetic group among isolates resistant to each of the tested drugs.

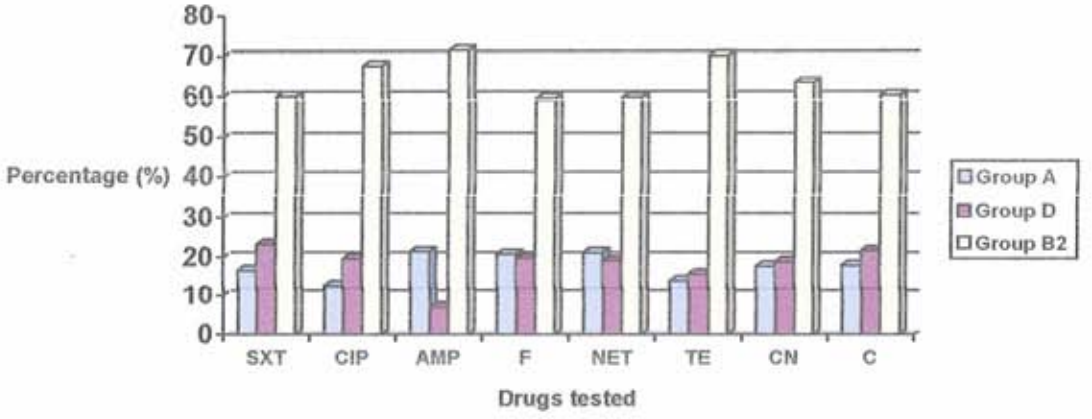

Figure 15b: Distribution of the phylogenetic group among isolates susceptible to each of the tested drugs.

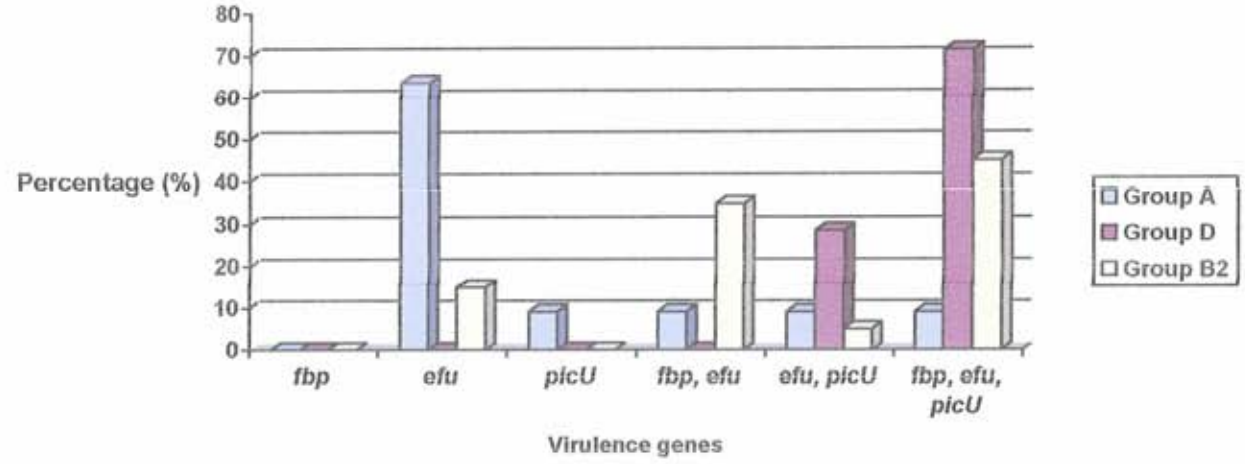

Figure 16a: Relation between the phylogenetic group and virulence genes among SXT resistant isolates.

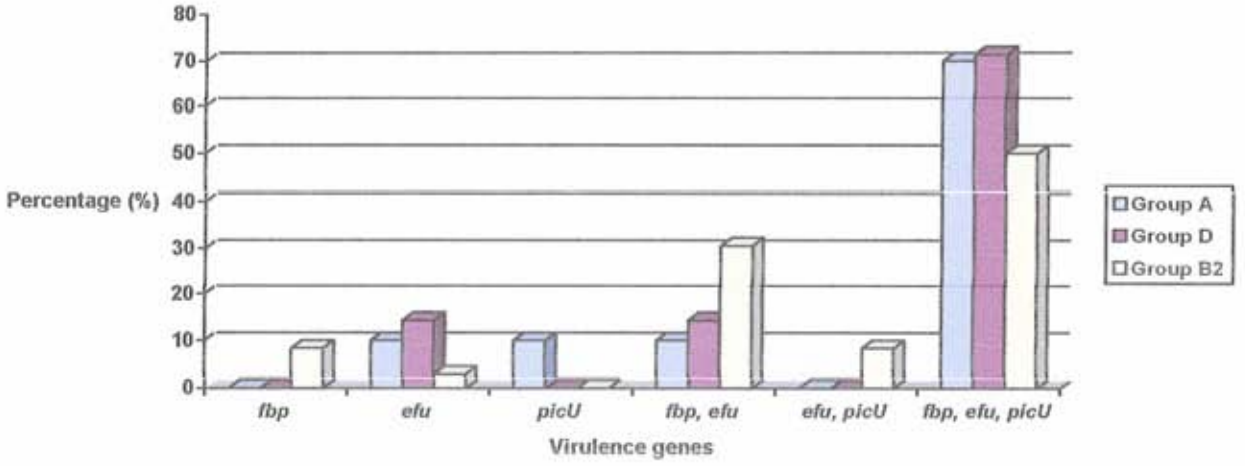

Figure 16b: Relation between virulence genes and the phylogenetic group among SXT susceptible isolates.

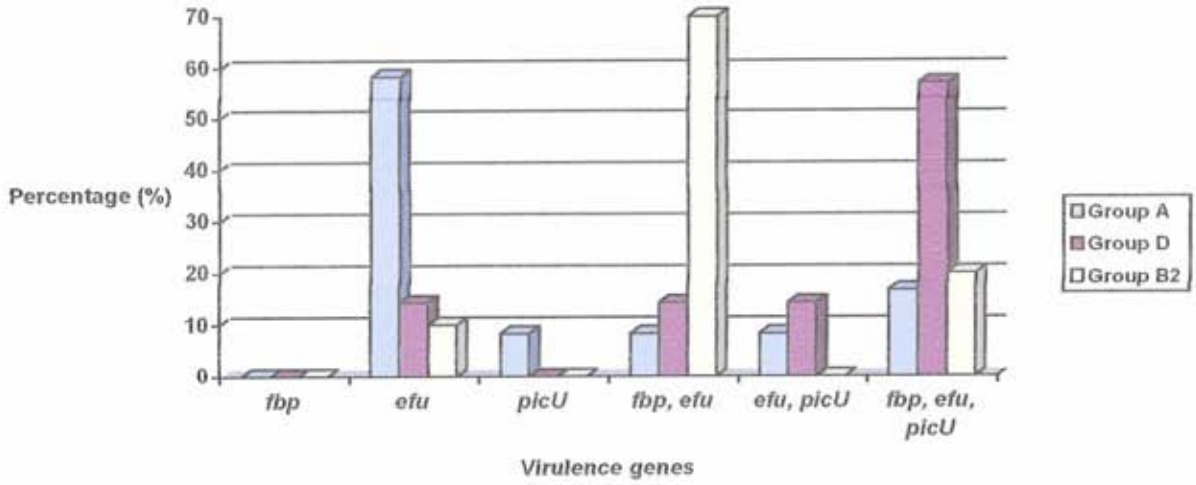

Figure 17a: Relation between the phylogenetic group and virulence genes among ciprofloxacin resistant isolates.

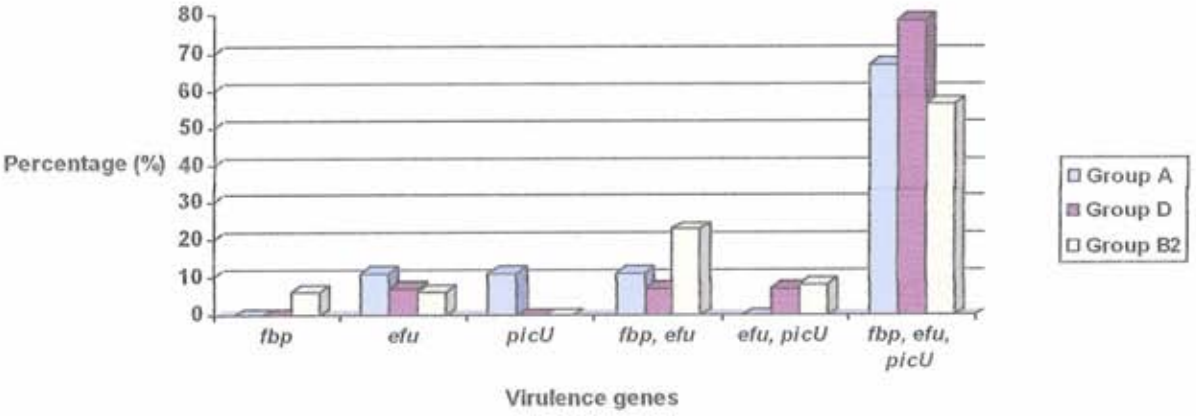

Figure 17b: Relation between the phylogenetic group and virulence genes among ciprofloxacin susceptible isolates.

4.6.2. Nitrofurantoin (F)

Group B2 and Group A showed the highest percentage of resistance to F (37.5%, n=3) for each group, while it was 25% (n=2) among isolates belonging to group A (Figure 15a). The percentage of isolates resistant to nitrofurantoin in which the three virulence determinants were detected was 50% (n=1) for group A, and 66.7% (n=2) for group D and also for group B2 (Figure 18a). Within the susceptible population, the percentage of isolates having all three virulence genes was 36.8% (n=7) in group A, 72.2% (n=13) in group D, and 50% (n=27) in group B2 (Figure 18b).

4.7. Inhibitor of cell wall synthesis

4.7.1. Ampicillin (AMP)

Resistance to ampicillin was common among B2 isolates (51.5%, n=35), followed by group D (26.5%, n=18) and group A (22%, n=15) (Figure 15a). Among the resistant isolates, 26.7% (n=4) of those classified as group A had three virulence genes, while it was much higher among group B2 (51.5%, n=18) and D (72.2%, n=13) isolates. (Figure 19a). Among isolates susceptible to ampicillin however, the percentage of with three virulence genes was 66.7% (n=4) for group A, 50% (n=10) for group B2 with all the D group possessing the three genes. (Figure 19b).

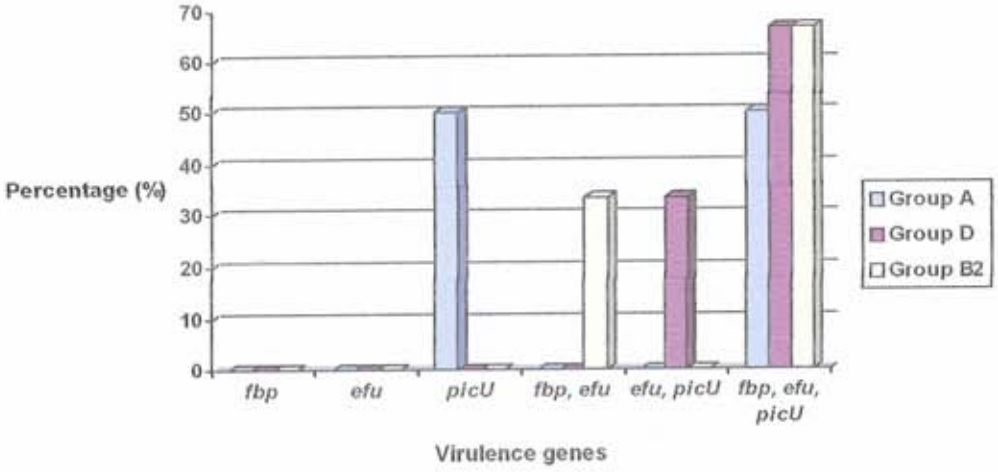

Figure 18a: Relation between phylogenetic group and virulence genes among nitrofurantoin resistant isolates.

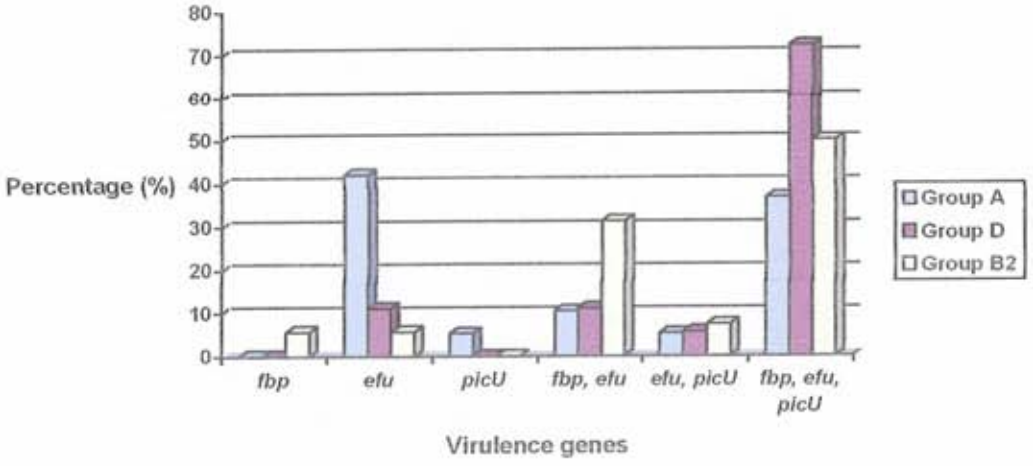

Figure 18b: Relation between the phylogenetic group and virulence genes among nitrofurantoin susceptible isolates.

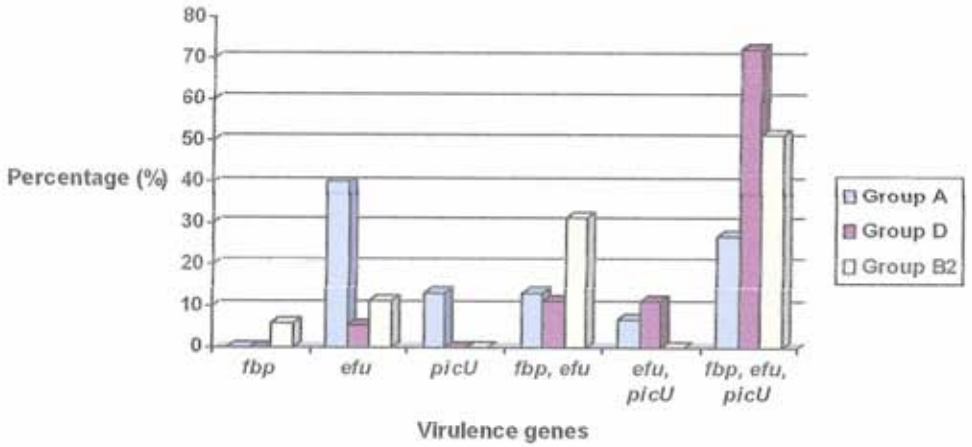

Figure 19a: Relation between phylogenetic group and virulence genes among ampicillin resistant isolates.

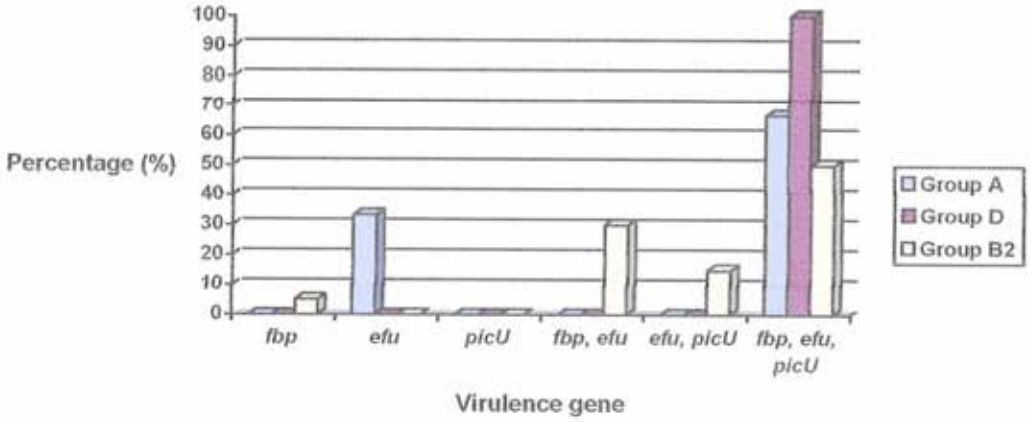

Figure 19b: Relation between the phylogenetic group and virulence genes among ampicillin susceptible isolates.

4.8. Inhibitors of protein synthesis by binding to the 30S ribosomal subunit

4.8.1. Netilmicin (NET)

Netilmicin resistant isolates mainly belonged to group D (66.7%, n=2) and group A (33.3%, n=1) (Figure 15a). None of the isolates resistant to netilmicin belonged to group B2. All those that were resistant to NET and belonging to group D possessed the three virulence genes, whereas none of the isolates resistant to NET and belonging to group A possessed the three virulence genes (Figure 20a). For those that were susceptible to NET, 59.7% (n=56) belonged to group B2, 19.1% (n=18) to group D, and 21.2% (n=20) to group A (Figure 15b). The percentage of susceptible isolates with three virulence genes was 40 % (n=8) for group A, 72.2% (n=13) for group D, and 51.8% (n=29) for group B2 (Figure 20b).

4.8.2. Tetracycline (TE)

Isolates belonging to group B2 had the highest percentage of resistance to tetracycline (41.7%, n=18) followed by group A (30.3%, n=13), and group D (28%, n=12) (Figure 15a). Within those resistant isolates, group D had the highest percentage of isolates possessing the three virulent determinants (58.3%, n=7), followed by group B2 (27.8%, n=5) and group A (7.7%, n=1) (Figure 21a). Among the susceptible isolates, the percentage of isolates having the three virulence determinants was 88.9% (n=8) for group D, 87.5% (n=7) for group A, and 60% (n=24) for group B2 (Figure 21b).

4.8.3. Gentamicin (CN)

The highest percentage of resistance to gentamicin was among group A isolates (40%, n=6), followed by isolates from group D (33.3%, n=5) and group B2 (26.7%, n=4) (Figure 15a). In contrast to group B2 in which none of the isolates had all the three virulence determinants, 80% (n=4) and 16.7% (n=1) of isolates from group D and A respectively had the three virulence genes (Figure 22a). Among the isolates susceptible to gentamicin however, 46.7% (n=7) were from group A, 68.7% (n=11) from group D, and 53.7% (n=29) from group B2 (Figure 22b).

4.9. Inhibitors of protein synthesis by binding to the 50S ribosomal subunit

4.9.1. Choramphenicol (C)

Chloramphenicol resistance was mainly detected in group B2 and group A (43.7% for each group) and to a lesser extent in group D (12.6%, n=2) (Figure 15a). All three virulence determinants were detected in 50% (n=1) of group D, and 42.8% (n=3) of group B2 (Figure 23a). Among the susceptible isolates, the percentage of those having the three virulence determinants was 53.3% (n=8) for group A, 77.9% (n=14) for group D, and 52% (n=26) for group B2 (Figure 23b).

Moreover, regardless of all the possible gene combinations, the prevalence of *fbp*, *efa*, and *picU* virulence determinants was also compared between resistant and susceptible isolates for each of the tested drugs (Annex II).

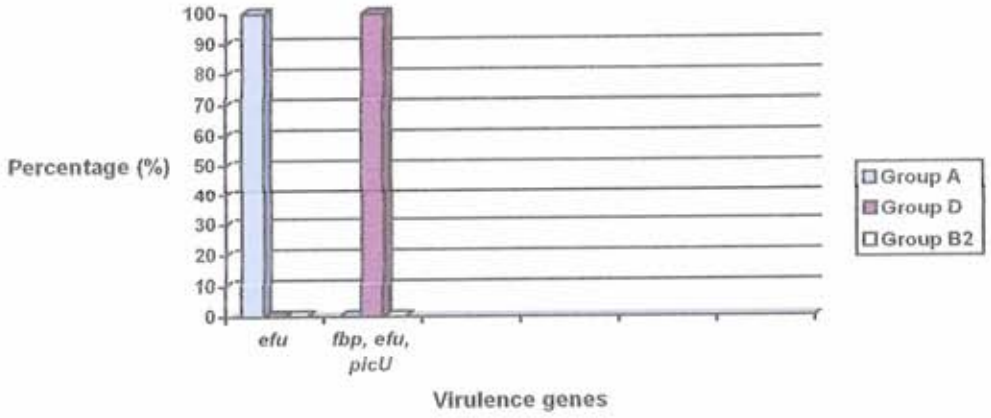

Figure 20a: Relation between the phylogenetic group and virulence genes among netilmicin resistant isolates.

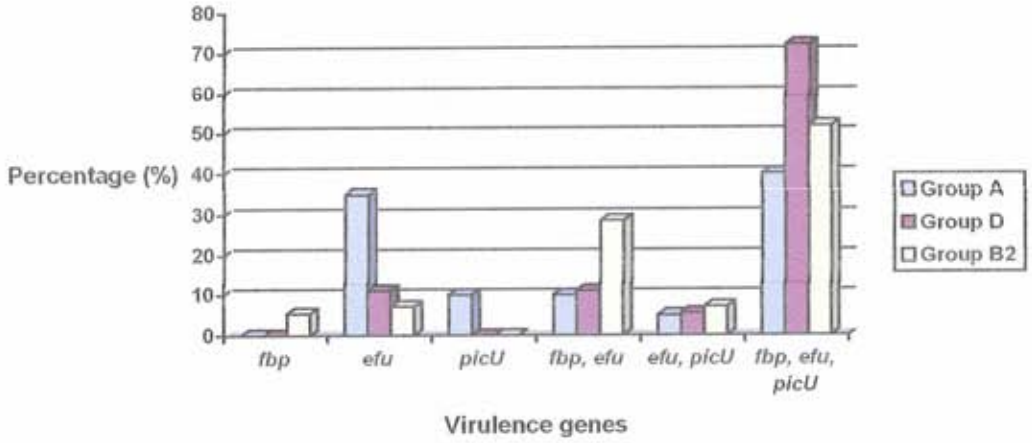

Figure 20b: Relation between the phylogenetic group and virulence genes among netilmicin susceptible isolates.

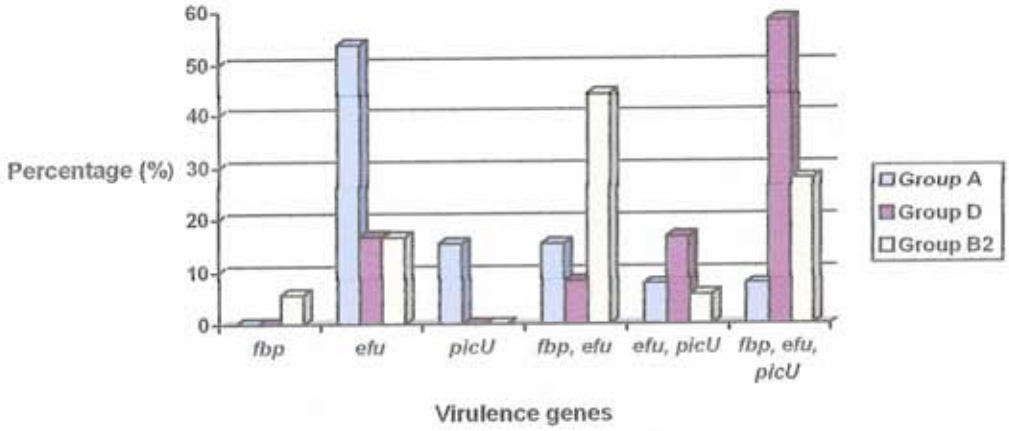

Figure 21a: Relation between the phylogenetic group and virulence genes among tetracycline resistant isolates.

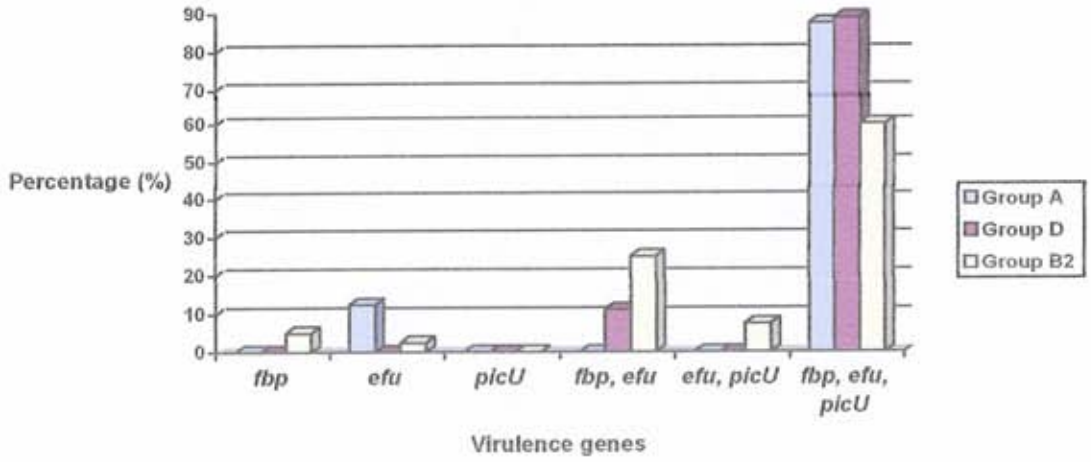

Figure 21b: Relation between the phylogenetic group and virulence among tetracycline susceptible isolates.

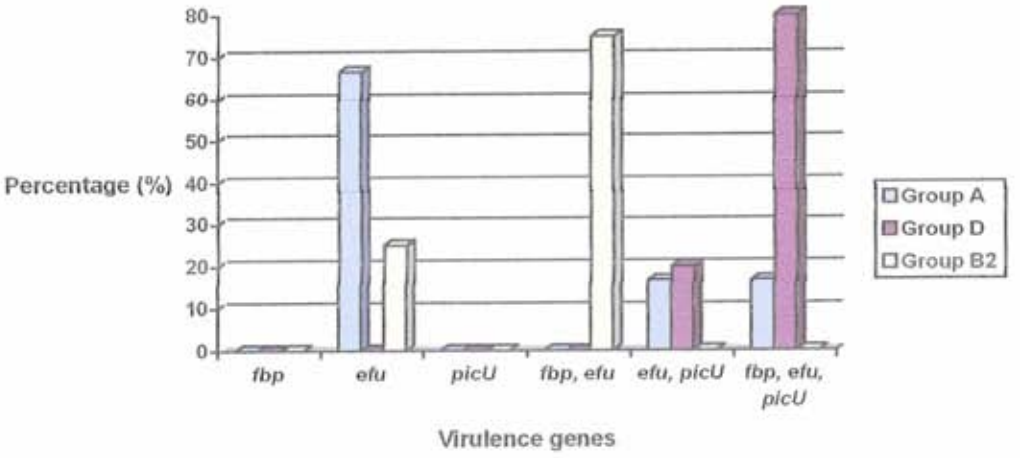

Figure 22a: Relation between phylogenetic group and virulence genes among gentamicin resistant isolates.

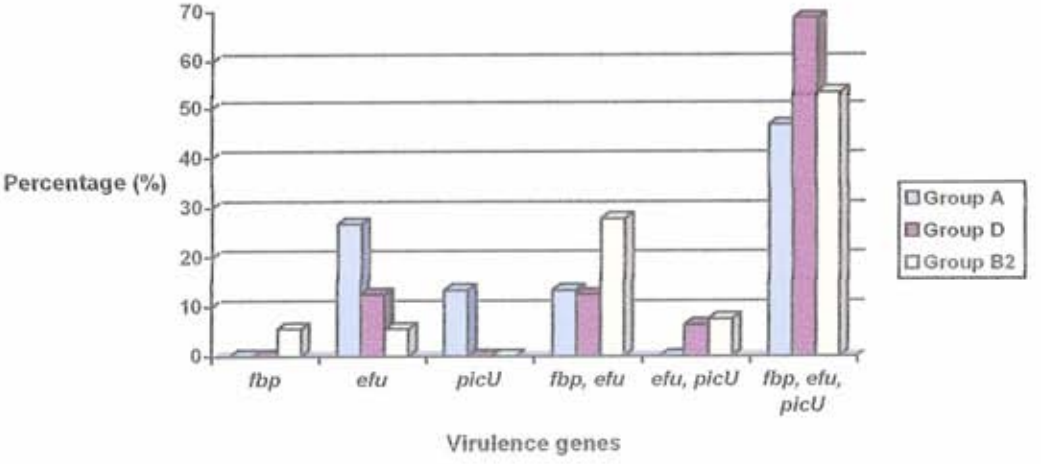

Figure 22b: Relation between the phylogenetic group and virulence genes among gentamicin susceptible isolates.

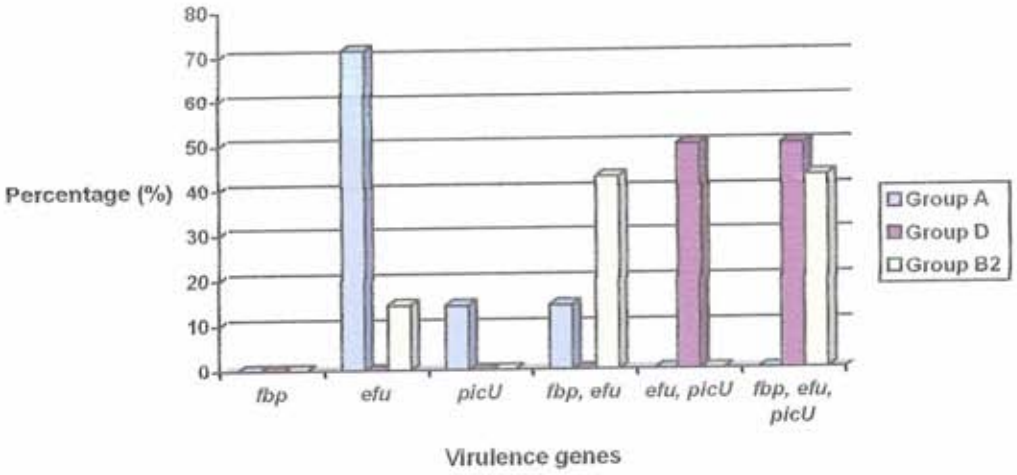

Figure 23a: Relation between the phylogenetic group and virulence genes among chlroramphenicol resistant isolates.

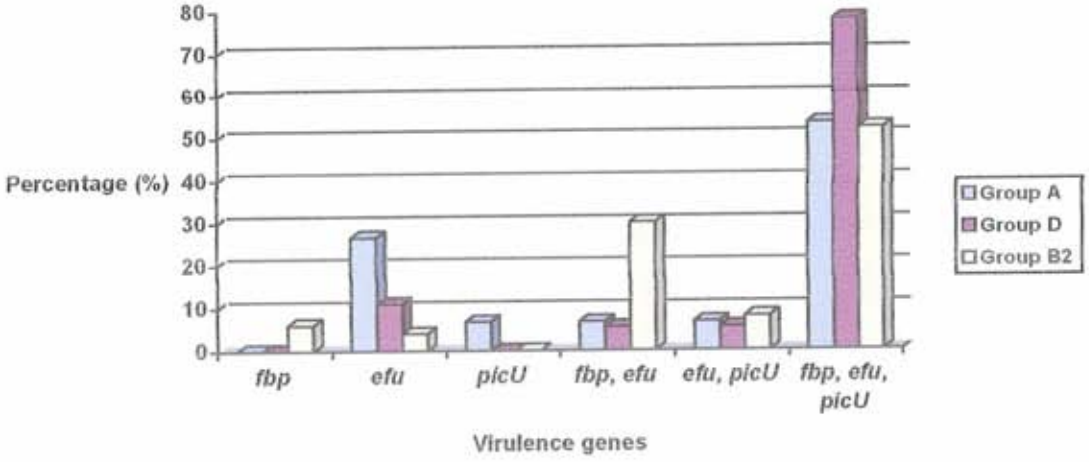

Figure 23b: Relation between the phylogenetic group and virulence genes among chloramphenicol susceptible isolates.

Chapter 5

DISCUSSION

The subject of UPEC is receiving increasing awareness given the high degree of morbidity and mortality. *Escherichia coli* isolates, collected in 2006, were obtained from two different health care institutes (Notre Dame de Secours and St Georges) located in different regions (Jbeil and Achrafieh). Besides the fact that isolates were taken from two hospitals at different locations, patients from other cities and rural areas also tend to visit them, as they are considered as major health care centers. Accordingly, the isolates used in our study are most likely to be representatives of UPEC population in Lebanon.

A total of 100 UPEC isolates were analysed for their phylogenetic groups, prevalence of three associated loci (*fbp*, *efa*, and *picU*), and antimicrobial susceptibility profiles. Phylogenetic grouping involves the classification of *E. coli* by triplex PCR into four main groups, designated A, B1, B2, and D (Clermont et al., 2000; Zhang et al., 2002). The assignment of *E. coli* clones to either one of these groups is the core of phylogenetic studies of the species (Duriez et al., 2001). Extremely extraintestinal virulent strains belong to group B2 and D, whereas commensal strains with lesser virulence belong to group A and B1 (Duriez et al., 2001; Maynard et al., 2004). In this study, and among the 100 UPEC isolates undertaken, B2 group represented the majority of the tested isolates (58%, n=58), followed and with equal proportions by groups A and D (21%, n=21 for each). A high prevalence of group B2 and approximately similar proportions for both groups A and D were also observed by Johnson et al. (2005a),

where 55% of *E. coli* isolated from women with acute uncomplicated cystitis belonged to group B2, 20% to group A, 19% to group D, and 6% to group B1. However, in contrast to Johnson et al. (2005a) none of the studied isolates belonged to group B1. Thus, our results further confirmed that extraintestinal pathogenic isolates mainly belonged to the virulent phylogenetic group B2.

Moreover, the phylogenetic distributions of *E. coli* isolates varied according to the API 20E numerical profile. In the present study, 25 different numerical profiles were obtained. The majority of the isolates (30%, n=30) had the numerical profile 19. The differences between the observed profiles were mainly with the following tests: β-Galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, oxidation/fermentation of saccharose, melibiose, and sorbitol. Isolates with the numerical profile 19 belonged to either group A, D, or B2. However, those that were positive for all the above mentioned tests were exclusively from group B2. These observations could indicate that group B2 isolates had metabolic variabilities enabling them to adapt to different environments.

Among *E. coli* isolates collected from Notre Dame de Secours hospital, 69.1% (n=47) were from female patients versus 30.9% (n=21) from males. Most of the isolates collected from males and females belonged to group B2, with B2 isolates being more prevalent in males (71.4%, n=15) compared to females (46.8%, n=22). Groups A and D isolates were mainly observed in isolates collected from females (27.7% and 25.5%, respectively) compared to males (14.3% for both groups). A possible cause for the high prevalence of UTIs among women could be linked to hygienic aspects or to anatomical differences. The proximity of the anus to the urethral opening and the

shortened urethra could possibly make women more prone to develop a bladder infection. Accordingly, this increases the possibility of group A, D or B2 *E. coli* to move from the gut and colonize and possibly infect the urinary tract environment. In contrast, establishing a urinary tract infection in males is more difficult due to the long urethra and thus this could explain the prevalence of group B2 as these isolates are highly virulent having the mechanisms needed to establish uropathogenesis.

According to Zhang et al. (2002), strains of groups B2 and D carry virulence determinants that are absent in groups B1, and A. In this study, three virulence loci were studied by triplex PCR: the *fbp*, *efa*, and *PicU*. All 100 isolates possessed at least one virulence determinant and 52% (n=52) of them had all the three studied virulence genes with different combinations. Isolates with *fbp*, *efa*, and *picU* virulence genes represented 38.1% of group A, 71.5% of group D, and 50% of group B2. The *fbp* locus involved in iron acquisition (Parham et al., 2005) was present in 77% (n=77) of the isolates. According to Parham et al. (2005), the *fbp* locus had low prevalence among non-B2 groups ECOR strains (3.5%) and a higher occurrence among the B2 strains (66.7%). Our results were in harmony with that of Parham et al. (2005), where *fbp* gene was mainly associated with B2 isolates (86.2%), but was also highly detected in group D (81%), while to a lesser extent in group A (47.6%). Several investigations emphasized the importance of iron acquisition for full virulence (Brown et al., 2001; Carniel, 2001). The presence of iron acquisition system offers a kind of adaptation for *E. coli* to the iron-limiting urinary tract environment by scavenging this vital nutrient (Johnson et al., 2004; Parham et al., 2005; Lloyd et al., 2007).

The *picU* gene, which belongs to the serine protease autotransporter of the *Enterobacteriaceae* (SPATE) family, is in turn widely involved in virulence (Parham et al., 2005). The PicU protein is a multifunctional enzyme that can digest mucin like its enteric counterpart (Pic) and breaches the protective layer of mucin that lines the urinary tract (Parham et al., 2005). Given the structural homology between PicU and Hbp, which is involved in recruiting iron through the degradation of hemoglobin, the possibility that PicU has also a similar function has yet to be investigated (Parham et al., 2005). *picU* may also enhance the ability of *E. coli* to invade the bloodstream (Parham et al., 2005), and according to Parham et al. (2005) 7% of the non-B2 ECOR strains and 53.3% of group B2 possessed this locus. This study revealed that the gene was present in 52.4% (n=11) of group A isolates, in 81% (n=17) of group D, and in 56.9% (n=33) of group B2.

The *efu* gene product was mainly associated with sugar metabolism and was found to be involved in the degradation of fucosylated structures present on the surface of uroepithelial cells (Parham et al., 2005). Parham et al. (2005) indicated that although the *efu* locus showed a wide distribution among non-B2 strains (29.8%), it was present at a higher frequency in the B2 group (80%) than in the non-B2 group. Our results agreed with that of Parham et al. (2005) with the *efu* locus being widely distributed in non-B2 isolates, with 90.5% (n=19) of group A isolates and all members of group D having the *efu* gene. However, the prevalence of this gene was approximately similar in both non-B2 (95.2%, n=40) and B2 groups (94.8%, n=55). A possible explanation for the observed discrepancies could be linked to differences in the isolate sources. Isolates used in this study were isolated from urine samples, whereas Parham surveyed the phylogenetic distribution of these genes among the ECOR collection, which varies in terms of source (animals or humans) and geographical

location (Parham et al., 2005). Moreover, several studies have stressed on the importance of these three genes in UTIs and on their distribution among cystitis or pyelonephritis isolates. Rasko et al. (2001) showed that the *fbp* locus was more prevalent in cystitis than among pyelonephritis strains. Parham et al. (2005) on the other hand, showed that statistically significant differences were not observed among the distribution of *fbp*, *efu*, and *picU* loci among strains associated with cystitis or pyelonephritis. In contrast, Heimer et al. (2004) found that the *picU* had a wider distribution among isolates causing pyelonephritis than those associated with cystitis.

From a therapeutical point of view, a major issue facing the health care sector is the increase in antimicrobial resistance (Kiffer et al., 2007). National and international examination programmes are needed to monitor the level of antimicrobial resistance (Kahlmeter, 2003). During the past decades, the rate of resistance has increased significantly, notably in some geographical locations (Moreno et al., 2006) with *E. coli* becoming increasingly resistant to commonly prescribed antibiotics (Karlowsky et al., 2001). Although, many factors contribute to the increase in resistance, the selective pressures regarding the inappropriate use of antibiotics remain the principal cause (Kiffer et al., 2007). Therefore, antimicrobial resistance patterns of uropathogenic isolates should be determined so as to prescribe the appropriate treatment. In this study, eight antibiotics were tested against UPEC by the disk diffusion method: trimethoprim/ sulfamethoxazole (SXT), ciprofloxacin (CIP), tetracycline (TE), chloramphenicol (C), gentamicin (CN), netilmicin (NET), ampicillin (AMP), and nitrofurantoin (F). Among 100 isolates tested in this study using the above mentioned antimicrobial agents, 21% were susceptible to all the genes. UPEC resistance to SXT (38%, n=38) exceeded the

threshold (<10-20%) recommended by the Infectious Diseases Society of America (IDSA) (Warren et al., 1999). Resistance to trimethoprim/sulfamethoxazole mandates the use of second line agents such as fluoroquinolones (Warren et al., 1999). The fluoroquinolones developed in the 1980s have an improved activity against *Enterobacteriaceae* and are widely used to treat both community and hospital acquired infections (Robert et al., 2001). Ciprofloxacin (fluoroquinolone) is the principal drug used to treat UTIs, especially in Lebanon (personal communication). Resistance to ciprofloxacin is of great concern, and *E. coli* resistance to fluoroquinolones is increasing (Kiffer et al., 2007). In Brazil, the rate of ciprofloxacin resistance increased from 9% in 2000 till 14% in 2003 (Kiffer et al., 2007), while it was much higher (29%) according to the results of this study. High resistance levels were also observed for ampicillin (68%), and tetracycline (43%). Accordingly, these results preclude the use of trimethoprim/sulfamethoxazole, ciprofloxacin, ampicillin, and tetracycline in the empirical treatment of UPEC. On the other hand, the resistance rates to chloramphenicol, gentamicin, nitrofurantoin, and netilmicin were 16% (n=16), 15% (n=15), 8% (n=8), and 3%, respectively. Nitrofurantoin still exhibited low resistance rates in many countries despite its use for more than fifty years (Hooton et al., 2004). The high resistance rate associated with most of the tested antibiotics could be explained by the high and uncontrollable consumption of these drugs in the treatment of UTIs or any other infection. Although the present data seem to encourage the perscription of chloramphenicol, gentamicin, netilmicin or nitrofurantoin, further clinical studies assessing the clinical efficacy and safety profiles of these drugs are warranted.

Multiple drug resistant isolates (resistance to at least three drugs) was another important aspect and was observed in 42% of the isolates. From a therapeutic point of view, multiple resistances are particularly worrisome as they decrease the range of antimicrobial agents that could be possibly used. However, despite the increase resistance of *E. coli* to antimicrobial agents, several data suggest that they are less virulent than their susceptible counterparts, belonging mainly to non-B2 groups, with their success being derived predominantly from their antibiotic resistance rather than from their pathogenecity potential (Johnson et al., 2002; Vila et al., 2002; Johnson et al., 2004). This study supported this tendency with multiple drug resistant isolates exhibiting lower prevalence of virulence factors and belonging mainly to non-B2 groups compared to susceptible isolates. Resistance to at least three drugs was observed in 61.9% of group A, 52.5% of group D, and 31% of group B2. Phylogenetic differences were detected, with susceptible isolates being mainly belonging to group B2. Moreover, susceptible isolates exhibited a higher prevalence of *fbp*, *efa*, and *picU* (90.5%, n=19; 100%, n=21; and 76.2%, n=16, respectively) compared to multiple drug resistant isolates (64.3%, n=27; 95.2%, n=40; 43%, n=18, respectively). If virulence determinants were compared according to phylogenetic groups, 7.7% (n=1) of group A multiple drug resistant isolates (n=13) possessed the three virulent loci, 72.7% (n=8) in group D and 22.2% (n=4) in group B2. However, 53.3% of B2 isolates susceptible to all tested drugs carried the three virulence determinants, while all susceptible isolates from both groups A and D were positive for the three tested genes. Therefore, the multiple drug resistant isolates exhibited lower prevalence of virulence factors except for *efa* locus, which was also prevalent in multiple drug resistant isolates. It is noteworthy that the prevalence of virulence genes among susceptible isolates was still high. A possible explanation of how

resistant isolates that are avirulent or less virulent compared to susceptible ones can cause diseases in healthy individuals could be through having unrecognized virulence genes that compensated for the lack of known genes (Johnson et al., 2005b). If the isolates are really avirulent, enhanced host susceptibility could present another alternative explanation.

Recently, it has been shown that *E. coli* resistant to fluoroquinolones were associated with a categorical shift away from group B2 and were less virulent than susceptible strains according to clinical behavior and/or virulence determinants profiles (Johnson et al., 2002; Vila et al., 2002; Johnson et al., 2003; Johnson et al., 2004; Johnson et al., 2005b; Moreno et al., 2006). In the present study, ciprofloxacin resistance was associated with a shift away from B2 towards group A (41.4%, n=12), while, the majority of the isolates susceptible to ciprofloxacin belonged to group B2 (67.6%, n=48). Ciprofloxacin resistant isolates exhibited a lower prevalence of virulence factors compared to susceptible ones except for the *efa* locus, where 96.6% (n=28) of resistant isolates possessed it compared to 94.4% (n=67) in susceptible ones. Among ciprofloxacin resistant isolates, 58.6% (n=17) and 37.9% (n=11) had the *fbp* and *picU* loci, respectively, compared to 84.5% (n=60) and 70.4% (n=50) in susceptible ones. According to Moreno et al. (2006), fluoroquinolones resistant group B2 strains were associated with low inferred virulence potential. In harmony with Moreno's finding, among resistant group B2 isolates, 20% (n=2) had the three virulence genes versus 56.2% (n=27) in those that were susceptible. Moreno et al. (2006) also reported that with resistant strains, the magnitude of the shift away from group B2 depends on the specific antimicrobial resistance pattern being greater for fluoroquinolones than for trimethoprim/sulfamethoxazole.

Additionally, no net difference in the virulence potential between trimethoprim/sulfamethoxazole resistant and susceptible strains was detected by Johnson et al. (2005b). Our results were in agreement with Johnson et al. (2005a) findings, with group B2 representing the majority of both resistant and susceptible isolates. A decrease in virulence potential upon resistance was clearly seen with both *picU* and *fbp* genes. The *efa* locus was present in 97.4% (n=37) of resistant isolates compared to 93.3% (n=56) in susceptible ones. Group B2 was also the dominant type within the susceptible and resistant isolates to ampicillin. Similar findings were also reported by Johnson et al. (2005a), with *E. coli* isolates collected from women with uncomplicated acute cystitis. No net change in the virulence potential among resistant or susceptible strains was observed by Horcajada et al. (2003) and Johnson et al. (2005b). In our study, the prevalence of *fbp*, *picU* and *efa* genes was higher in ampicillin susceptible isolates than in the resistant ones. On the other hand, with nitrofurantoin, B2 was common within both the susceptible and resistant isolates, with the *picU* being mainly detected in those isolates showing resistance (87.5%). In contrast to other drugs, resistance was associated with isolates from groups A and B2 carrying all the three tested virulence genes. However, with netilmicin, all B2 isolates were susceptible, while the resistant isolates were mainly from group D and to a lesser extent group A. Both *efa* and *picU* genes were more prevalent among resistant isolates. Additionally, all the netilmicin resistant group D isolates had the three virulence determinants compared to 72.2% in their susceptible counterparts. Although, a role of *efa* locus in pathogenesis is unlikely due to its wide distribution among all isolates whether resistant or susceptible and due to the similar prevalence among cystitis and pyelonephritis, it is one of the top fifteen genes upregulated during growth in urine (Parham et al., 2005).

Accordingly, we suggest that virulence and antimicrobial resistance are not always mutually exclusive in *E. coli* clinical isolates. The relationship between virulence and antimicrobial differs according to the particular resistance phenotype. The observed phylogenetic differences between susceptible and resistant isolates for each of the eight tested drugs seemed to favour the notion that resistant and susceptible isolates appear to derive from different bacterial population. Therefore, the resistant subgroups might represent distinct populations rather than emerging from susceptible isolates by the acquisition of resistance capabilities and potential. The observed phylogenetic differences could be responsible for the observed variations in the virulence profiles, leaving probably no evidence for a direct relationship between resistance and virulence, such as that which may occur when virulence factors are lost upon a mutation that leads to resistance. Moreover, Johnson et al. (2005b) didn't find detectable virulence alterations concomitant with transition to resistance, which further opposes the direct linkage between virulence and resistance. A possible alternative explanation for this yet unexplained relationships between virulence, antimicrobial resistance, and phylogenetic groups, especially with the negative association between resistance and virulence factors for the majority of the tested drugs could be that resistance happens more easily in non-B2 isolates or those having fewer virulence potential. However, due to the much higher number of commensals in the intestine, the probability that they would emerge as resistant upon drug use is much greater than among the few pathogens that might be present. Less pathogenic non group B2 isolates would therefore become resistant and cause disease, particularly in susceptible hosts. Although the source of resistant isolates could be the intestinal microbiota of patients receiving a drug therapy (Johnson et al, 2005b), there is no consensus as to the source

of the urovirulent resistant clones that inhabit the colon and may be more dependable with an animal or an environmental source. Moreover, the urovirulent resistant isolates are possibly commensals, but their association with diseases depends on their opportunistic nature.

Finally, the difference at the molecular level between human UPEC clinical isolates versus intestinal isolates and animal isolates should be assessed in the future to decipher the molecular basis of pathogenesis and to validate the hypothesis that some human *E.coli* are derived from animal sources by foodborne transmission.

CONCLUSION

- Phylogenetic grouping revealed that the majority of the tested isolates belonged to group B2 (58%), followed and with equal proportions by groups D and A (21%, for each). None of the tested isolates belonged to group B1. These results further emphasized that extraintestinal pathogenic isolates mainly belonged to the virulent group B2.

- The phylogenetic distribution of *E. coli* isolates varied according to the API 20E numerical profile, where isolates reacting positively with the test substrates being mainly from group B2.

- *E. coli* isolates were mainly isolated from females (69.1%). The majority of the isolates whether from males (30.9%) or females belonged to group B2.

- All isolates had at least one virulence gene with 52% of them possessing all the three tested virulence determinants.

- The *fbp* locus, involved in iron acquisition, was present in 77% of the isolates, with the majority of those isolates mainly belonging to group B2.

- The *PicU* gene, involved in mucin digestion, was more prevalent among group D (81%) isolates.

- The *efa* locus, involved in the degradation of fucosylated structures lining the urinary tract, was dominant among all isolates with 90.5% of group A, 94.8% of group B2, and all isolates within group D possessing this locus.

- The high resistance potential detected using trimethoprim/sulfamethoxazole, ciprofloxacin, ampicillin, and tetracycline preclude the use of these antibiotics for the empirical treatment of UTIs.

- Although the present data seem to encourage the use of chloramphenicol, gentamicin, netilmicin, and nitrofurantoin, further clinical efficacy and safety profiles are warranted.

- Multiple drug resistant *E. coli* isolates accounted for 42% of the isolates and were more prevalent among non-B2 isolates, while susceptible isolates mainly belonged to the virulent group B2. In multiple drug resistant isolates, a number of virulence determinants were detected.

- The majority of the isolates resistant to trimethoprim/sulfamethoxazole were from group B2 (52.7%) and 45% of them were positive for the three tested virulence determinant genes.

- Ciprofloxacin resistance mainly occurred in *E. coli* group A (41.4% resistant) isolates.

- Virulence and antimicrobial resistance are not always mutually exclusive in *E. coli* clinical isolates. The relationship between virulence and antimicrobial resistance varies according to the particular resistance phenotype.

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ANNEX I

API 20E Numerical Profiles among the uropathogenic *Escherichia coli*

Table 1: API 20E numerical profiles

	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number
ONPG	+	+	+	+	-	-	+	+	+	+
ADH	-	-	-	+	-	-	-	-	-	-
LDC	-	-	-	-	+	+	+	+	+	+
ODC	-	+	+	+	+	+	-	-	-	-
CIT	-	-	-	-	-	-	-	-	-	-
H2S	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-
IND	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-
GEL	-	-	-	-	-	-	-	-	-	-
GLU	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+
INO	+	+	+	-	-	-	-	-	-	-
SOR	-	-	-	+	+	+	-	+	+	+
RHA	+	+	+	+	+	+	+	-	+	-
SAC	+	-	+	+	-	+	+	-	-	-
MEL	-	-	+	+	-	-	+	-	-	+
AMY	-	-	-	-	-	-	-	-	-	-
ARA	+	+	+	+	+	+	+	+	+	+
OX	-	-	-	-	-	-	-	-	-	-
	NM1	NM2	NM3	NM4	NM5	NM6	NM7	NM8	NM9	NM10

	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number	Profile number
ONPG	+	+	+	+	+	+	+	+	+	+
ADH	-	-	-	-	-	-		-	-	+
LDC	+	+	+	+	+	+	+	+	+	+
ODC	-	-	-	-	+	+	+	+	+	-
CIT	-	-	-	-	-	-	-	-	-	-
H2S	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-
IND	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-
GEL	-	-	-	-	-	-	-	-	-	-
GLU	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+
INO	-	-	-	-	-	-	-	-	-	-
SOR	+	+	+	+	-	-	+	+	+	+
RHA	+	+	+	+	+	+	+	+	+	-
SAC	-	+	+	+	+	+	-	-	+	-

MEL	+	-	+	+	-	+	-	+	+	-
AMY	-	-	-	+	-	-	-	-	-	-
ARA	+	+	+	+	+	+	+	+	+	+
OX	-	-	-	-	-	-	-	-	-	-
	NM11	NM12	NM13	NM14	NM15	NM16	NM17	NM18	NM19	NM20

	Profile number	Profile number	Profile number	Profile number	Profile number
ONPG	+	+	+	+	+
ADH	+	+	+	+	+
LDC	+	+	+	+	+
ODC	-	-	+	+	+
CIT	-	-	-	-	-
H2S	-	-	-	-	-
URE	-	-	-	-	-
TDA	-	-	-	-	-
IND	+	+	+	+	+
VP	-	-	-	-	-
GEL	-	-	-	-	-
GLU	+	+	+	+	+
MAN	+	+	+	+	+
INO	-	-	-	-	-
SOR	+	+	+	+	+
RHA	+	+	+	+	+
SAC	-	+	-	-	+
MEL	+	+	-	+	+
AMY	-	-	-	-	-
ARA	+	+	+	+	+
OX	-	-	-	-	-
	NM21	NM22	NM23	NM24	NM25

Table 2: Virulence determinants in relation to antimicrobial resistance phenotype for each of the tested drugs among *E. coli* isolates.

	Resistant			Susceptible		
	fbp	*efu*	*picU*	*fbp*	*efu*	*picU*
SXT	61% n=23	97.4% n=37	52.6% n=20	86.7% n=52	93.3% n=56	65% n=39
CIP	58.6% n=17	96.6% n=28	37.9% n=11	84.5% n=60	94.4% n=67	70.4% n=50
F	75% n=6	87.5% n=7	87.5% n=7	78% n=71	95.6% n=87	59.3% n=54
AMP	76.4% n=52	94.1% n=64	58.8% n=40	82.1% n=23	96.4% n=27	67.9% n=19
CN	53.3% n=8	100% n=15	46.7% n=7	81.2% n=69	94.1% n=80	63.5% n=54
NET	66.7% n=2	100% n=3	66.7% n=2	77.7% n=73	94.7% n=89	61.7% n=58
TE	58.1% n=25	93% n=40	44.2% n=19	91.2% n=52	96.5% n=55	73.7% n=42
C	50% n=8	93.8% n=15	37.5% n=6	81.9% n=68	95.2% n=79	66.3% n=55

Ampicillin (AMP), Chloramphenicol (C), Ciprofloxacin (CIP), Gentamicin (CN), Netilmicin (NET), Nitrofurantoin (F), Tetracycline (TE), Trimethoprim/sulfamethoxazole (SXT).