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Antibiotic Resistance Patterns and Sequencing of Class I Integron in Uropathogenic *Escherichia coli* in Lebanon

by

Nina Goerge Najjar (El)

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Dr. Sima Tokajian

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Thesis approval Form (Annex III)

Student Name: Nina El Najjar I.D. #: 200603287

Thesis Title : **Antibiotic Resistance Patterns and Sequencing of Class I Integron in Uropathogenic *Escherichia Coli* in Lebanon**

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School : **School of Arts and Sciences**

Approved by: Members of committee

Thesis Advisor: Dr. Sima Tokajian

Member : Dr. Fuad Hashwa

Member : Dr. Roy Khalaf

Date 9.06.09.

(This document will constitute the first page of the Thesis)

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ABSTRACT

Antibiotic Resistance Patterns and
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by Nina George Najjar (El)

Improper monitoring of antibiotic usage has lead to the development of antimicrobial resistance among uropathogenic *Escherichia coli* (UPEC). The rapid dissemination of antibiotic-resistance is mediated by gene transfer mechanisms involving mobile genetic elements including the recently characterized gene cassettes in integrons. Integrons are conserved DNA sequences associated with multi-drug resistance. The aim of this study was to generate data on the prevalence and molecular basis of antimicrobial resistance in UPEC in Lebanon. The phenotypic resistance profiles of 9 currently used antimicrobial agents were determined using the agar diffusion test on a total 100 UPEC isolates. Results obtained revealed that 60% of the isolates were resistant to ampicillin, 55% to trimethoprim/sulfamethoxazole, 53% to streptomycin and only 5% to netilmicin. PCR assay was used to detect the presence of the class 1 integron variable region (VR) containing the gene cassettes. All VR negative isolates showed more susceptibility towards the used antimicrobial agents compared to the VR positive isolates. The VR positive isolates showed highest resistance to aminoglycosides and trimethoprim/sulfamethoxazole (96.7%). VR negative islolates were mainly resistant to ampicillin (48.6%), aminoglycosides (41%), and tetracycline (40%). The VR amplicons were then characterized by direct partial sequencing and restriction digestion with *AluI*, and accordingly,

30% of the isolates were found to be positive for the Class 1 integron VR, with a size ranging from 0.7 to 2.2 Kbp. VR positive isolates carried the genes *dfrA7*, *dfrA17-aadA5*, *dfrA1-aadA1*, *dfrA12*-orf5-*aadA2* and bla_{OXA-30}-*aadA1*. The predominant resistance genes were *dfrA17* and *aadA5* (47% of the isolates) for trimethoprim/sulfamethoxazole and streptomycin, respectively. The presence of a gene cassette was correlated with high resistance to the corresponding antibiotic, with 85.7% of the isolates harboring the *aadA* gene being resistant to streptomycin. Five different restriction patterns were detected; all isolates with the same class 1 integron VR amplicon size had the same restriction pattern. Characterization of class I integrons from UPEC isolates by direct sequencing revealed that those isolates exhibit a wide repertoire of genetic elements to sustain antimicrobial pressure. This study provided basal information for future pursuit and comparison especially with respect to epidemiologic distribution, antimicrobial resistance and evolution of these important pathogens.

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GLOSSARY

API: analytical profile index

CS: Conserved sequence

E. coli: *Escherichia coli*

ExPEC: extraintestinal pathogenic *E. coli*

Kbp: kilo-base pair

ORF: open reading frame

PCR: polymerase chain reaction

TBE: tris borate EDTA

UPEC: uropathogenic *Escherichia coli*

UTI: urinary tract infection

VR: variable region

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I dedicate this work to my mother and my late father.

Chapter 1

INTRODUCTION

Escherichia coli, originally called "*Bacterium coli* commune," was first isolated from the feces of a child in 1885 by the scientist Theodor Escherich and is currently the best-studied bacterium (De Sousa et al., 2006).

Extraintestinal pathogenic *E. coli* (ExPEC) are a diverse group of strains infecting extraintestinal locations, including the urinary tract infected by uropathogenic *E. coli* (UPEC), the bloodstream infected by sepsis-associated pathogenic *E. coli*, and the meninges of the neonates infected by newborn meningitis-associated *E. coli* (NMEC).

The urinary tract is among the most common sites of bacterial infection in women and UPEC is the most common infecting agent at this site (Kusheria et al., 2006).

The first line of treatment of bacterial infections is through the administration of antibiotics. The increased introduction of antimicrobial agents has resulted in selective pressures on bacterial populations leading to the development of antibiotic resistant bacterial strains. Genetic transfer of resistance genes is a significant driver of the evolution of multiple-drug-resistant pathogenic bacteria. This spread of antimicrobial resistance has become an alarming problem complicating established treatments (Ozgumus et al., 2007). Acquisition and transfer of antibiotic resistance and virulence factor genes by the bacteria via horizontal transfer of the resistance plasmids, transposons and integrons occurs through the processes of conjugation, transduction, and transformation (Ozgumus et al., 2007). Integrons are often part of transposons and plasmids and the probability of their presence increases with multidrug resistance among *Enterobacteriaciae* (Leverstein-van Hall et al., 2003; Naas et al., 2001). Integrons are genetic elements that consist of an

integrase gene with adjacent gene cassettes that commonly contain antibiotic resistance genes. Over 60 different cassettes conferring resistance to a variety of antibiotics have been reported (Yu et al., 2003). Several classes of integrons have been established based on the structure of the integrase. The most frequently encountered integrons are those of class 1 commonly found among *Enterobacteriaciae* (Naas et al., 2001).

Several studies conducted in Lebanon (Fadel et al., 2004; Farah et al., 2007; Sawma-Awad et al., 2009), Egypt (Al-Agamy et al., 2006; Al-Agamy & Ashour, 2004), Saudi Arabia (Al-Tawfiq, 2006), Tunisia (Jouini et al., 2007), Turkey (Ozgumus et al., 2007), and Bangladesh (Lina et al., 2007) explored the antibiotic resistance profile of UPEC. However, most of the cited studies used PCR to determine the presence of class 1 integrons (Al-Agamy et al., 2006; Al- Agamy & Ashour, 2004; Jouini et al, 2007; Lina at al., 2007; & Ozgumus et al., 2007), and only some (Al- Agamy & Ashour, 2004; Jouini et al, 2007; & Ozgumus et al., 2007) used sequencing to study the cassette content of integrons. Moreover, none of these studies used restriction fragment length polymorphism (RFLP) to examine if a correlation exists between the restriction pattern and the integron content.

This study represents the first report from Lebanon and the Arab world that uses sequencing and RFLP to characterize the class 1 integrons in UPEC isolates. In addition, this study is of significance to the medical community given that it could help improve the detection and treatment of *E. coli* mediated UTI and would provide essential information on the prevalence and molecular basis of antimicrobial resistance in Lebanon.

Objectives:

- Understanding the molecular basis of integron mediated multidrug resistance in 100 UPEC isolates collected from two Lebanese hospitals in the years 2006-2007.
- Demonstrating the usefulness of PCR in screening for the presence of class 1 integron gene cassettes.
- Using partial direct sequencing to determine the sequence and order of insertion of the gene cassettes in the PCR positive isolates.
- Typing of class 1 integron amplicons with restriction fragment length polymorphism (RFLP) and examining whether or not a relationship exists between integron content and restriction pattern.
- Determining the phenotypic resistance profiles to 11 antibiotics targeting the UPEC isolates by the use of the Kirby-Bauer disc diffusion method.
- Correlating the presence of the integron VR with the determined resistance profiles of the isolates, as well as evaluating the effect of the integrated cassettes on resistance.

Chapter 2

LITERATURE REVIEW

2.1. *Escherichia coli*

Escherichia coli is comprised of diverse species of bacteria naturally found in the intestinal tract of humans and many animal species (Marrs et al., 2005). They are part of the coliforms that include *Klebsiella, Enterobacter, Serratia, and Citrobacter* (Guentzel, 1996). Most *E. coli* strains are harmless commensals of the intestinal tract while others are major pathogens. The pathogenic *E. coli* are divided into those strains causing disease inside the intestinal tract and others capable of infection at extraintestinal sites, including the urinary tract, in which case they are referred to as uropathogens. There are two types of infection, either noscomial or community-acquired (De Sousa, 2006). UPEC is the causative agent in 70%–95% of community acquired UTI and 50% of all cases of nosocomial infections (Kusheria et al., 2005).

2.2. Urinary tract infections

A UTI is defined as the presence of a significant number of pathogenic organisms in the urine. Asymptomatic bacteruria is the presence of the bacteria in the urinary tract without causing an infection. Cystitis is an infection of the lower urinary tract and the bladder. The most important symptoms of cystitis are the urge to urinate, frequency of urination, and cloudy and smelly urine. Pyelonephritis is a life threatening infection affecting the kidneys. The symptoms include back pain, abdominal pains, fever and bloody urine. If pyelonephritis is untreated, it

often leads to renal failure and systemic spread of the pathogen into the blood stream (De Sousa, 2006; Kusheria et al., 2005).

Community-acquired UTI is one of the most common infectious diseases and a frequent cause for outpatient treatment. Nosocomial UTIs accounts for about half of the hospital acquired infections. Generally, UTIs are mediated by gram-negative bacteria, the most common of these being *Escherichia coli* followed by *Klebsiella pneumoniae* (Lina et al., 2007). Other UTI causing bacteria include *Acinetobacter* and *Enterobacter* spp (Márquez el al., 2008). UTIs affect females more often than males (Lina et al., 2007); approximately half of all women will have a UTI by their late 20s and about 25–30% of women with first UTI will have recurrent infections (Marrs et al., 2005). It is estimated that 150 million cases of UTI occur on a global basis per year resulting in more than 6 billion dollars in direct health care expenditure (Kusheria et al., 2006).

2.3. Uropathogenic *Escherichia coli*

Infection begins when the bowel is colonized with a UPEC strain in addition to the commensal flora. This strain can colonize the periurethral area and ascends the urethra to the bladder (Kaper et al., 2004). Type 1 pili are the most commonly expressed virulence factors in UPEC followed by P fimbriae. Additional virulence factors include outer membrane antigens like Dr pili, S pili, flagella, capsule polysaccharide, lipopolysaccharide O and iron transport systems, and secreted toxins such as hemolysin, cytotoxic necrotizing factor 1 and Sat. The host immune responses to these virulence factors include Toll-like receptor mediated immune responses, antimicrobial peptides and neutrophil attack. Adaptive immune responses also play a role in clearing the infection (Kaper et al., 2004).

Type 1 pili are involved in the first stages of infection. Type 1 fimbriated *E. coli* attach to epithelial cells via the adhesin subunit FimH located at the fimbrial tip. Attachment triggers apoptosis and exfoliation; following exfoliation, neutrophils infiltrate into the bladder and urothelium to help clear out the infection (Mulvey et al., 2000; De Sousa et al., 2006). Invasion of the bladder epithelium is accompanied with formation on the bladder surface of pod-like bulges containing bacteria encased in a polysaccharide-rich matrix. These bulges could act as a reservoir for recurrent infection in response to environmental triggers. In strains that cause cystitis, the infection is confined to the bladder. In pyelonephritis strains, the expression of type 1 pili is turned off and UPEC is released from bladder and ascends through the ureters to the kidneys, where the organism can attach by P pili to the kidney epithelium. At this stage, hemolysin could damage the renal epithelium and, together with other bacterial products including LPS, an acute inflammatory response recruits PMNs to the site. Secretion of Sat, a vacuolating cytotoxin, damages the glomeruli and the surrounding epithelium. In some cases, the barrier provided by the one-cell-thick proximal tubules can be breached and bacteria can enter the bloodstream, leading to bacteremia (Kaper et al., 2004), (Figure 1).

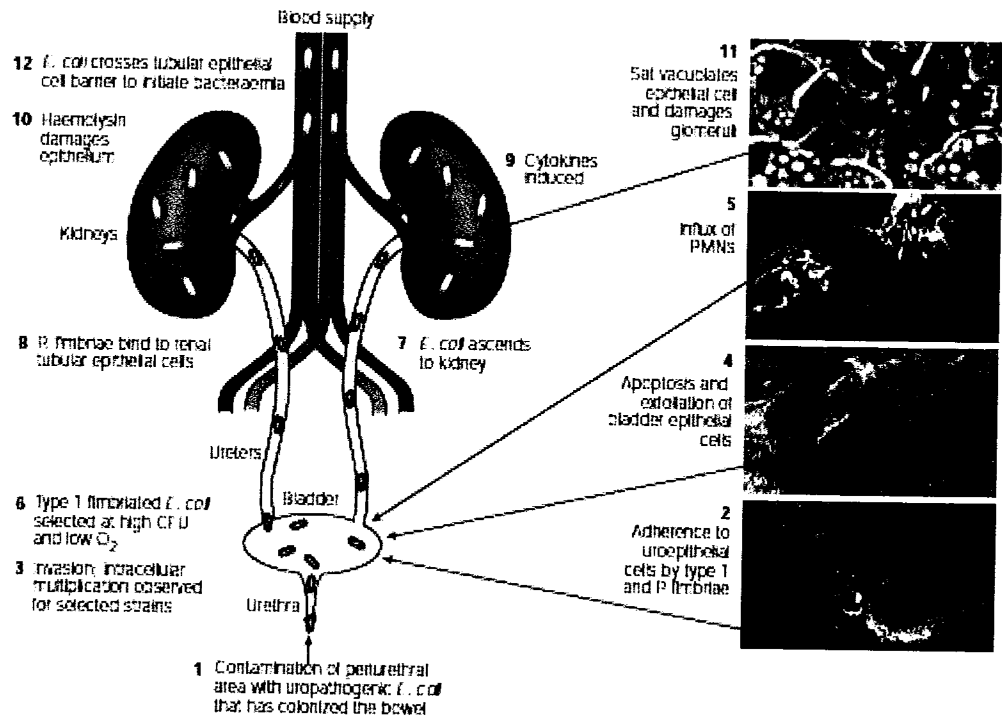

Figure 1. Schematic of UPEC pathogenic pathway (Kaper et al., 2004).

2.4. Antimicrobial therapy

An antibiotic can be either bacteriostatic or bactericidal depending on its concentration. A bacteriostatic agent prevents the bacterial cell from growing, and a bactericidal agent kills bacteria (Yoneyama & Katsumata, 2006).

According to their mode of action, antibiotics are divided into inhibitors of cell wall synthesis (including β-lactams and fosfomycin), inhibitors of protein synthesis (including aminoglycosides, tetracycline, and chloramphenicol), inhibitors of nucleic acid synthesis (quinolones) and antimetabolites like inhibitors of folic acid synthesis (sulfonamides and trimethoprim) (Yoneyama & Katsumata, 2006).

The treatment of UTI is based on the frequency of pathogens, the antimicrobial resistance rates and the illness severity. Oral regimens are commonly prescribed to treat acute cystis, and the ones usually prescribed are trimethoprim-sulfamethoxazole, nitrofuarantoin, β-lactams, quinilones, and fosfomycin (Gupta, 2003). Intravenous cephalosporins and aminoglycosides are prescribed for patients with acute pyelonephritis (Ronald & Alfa, 1996).

2.5. Antibiotic resistance

The widespread use of antibiotics imposes strong selection pressure for the development of antibiotic resistance. Occurrence of antibiotic resistance is inevitable, that is, it is only a question of time. As the frequency of antibiotic use increases, the speed of resistance development also increases, thereby reducing the effectiveness of the antibiotic (Yoneyama & Katsumata, 2006).

Antibiotic resistance can be either intrinsic or acquired. In general, resistance is acquired by mutational change or by the acquisition of resistance-encoding genetic material. The three major mechanisms of antibiotic resistance are (i) prevention of accumulation of antibiotics either by decreasing uptake or increasing influx as has been observed for tetracycline and quinilone resistance, (ii) inactivation of antibiotics either by hydrolysis or by modification as is the case for chloramphenicol and β-lactams resistance, and (iii) alteration of the target either qualitatively which reduces the affinity for antibiotics or quantitatively by overproduction of the target. This mechanism has been observed for β-lactam, quinilone and trimethoprim-sulfonamide resistance (Yoneyama & Katsumata, 2006) (Figure 2).

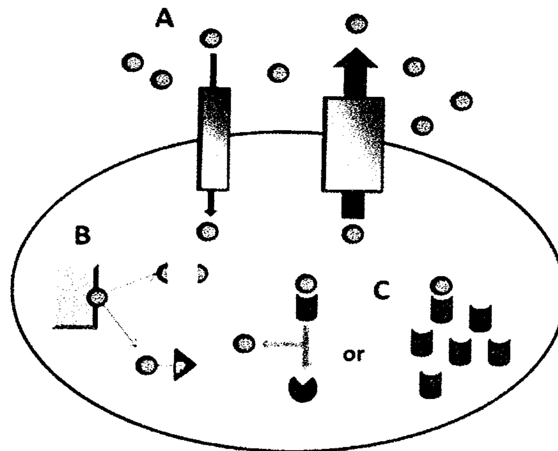

Figure 2. Principle mechanisms of antibiotic resistance. **A.** Prevention of accumulation of antibiotics by decreasing uptake and increasing efflux. **B.** Inactivation of antibiotics by hydrolysis or modification. **C.** Alteration or overproduction of targets (Yoneyama & Katsumata, 2006).

2.6. Integrons and antibiotic resistance

Much often, microorganisms acquire genetic material from other bacteria. The chief mechanisms of transfer between bacteria are conjugation, transformation, and transduction. Resistance factors are usually located on fragments of DNA referred to as transposons, which allow the resistance gene to move easily form one plasmid to another. Transposons are often associated with a more complex DNA fragment called an integron. However, integrons are not always located on transposons but can exist independently on several groups of broad host range plasmids (Croft et al., 2007). First identified by Stokes and Hall in 1989, integrons have become recognized as a primary means by which bacteria acquire antimicrobial resistance (Roe & Pillai, 2003).

Integrons possess two conserved segments, the 5' CS and the 3'CS, separated by a variable region which includes integrated antibiotic resistance genes. The 5' CS contains the *int* gene, a gene cassette insertion site, *attI* and, on the opposite strand, a common promoter region, *P1-P2*, directed toward the site of integration. Most inserted cassettes lack their own promoter and are expressed from the common promoter region. Integrons are classified according to the integrase sequence. To date, 5 different integron classes have been found, the most common of which in *Enterobacteriaceae* are the class 1 integrons, followed by class 2 integrons (Lévesque et al., 1995; Moura et al., 2007). All five classes are physically linked to mobile DNA elements, such as insertion sequences, transposons and conjugative plasmids. Integron classes 1, 2, and 3 are 'historical' classes that are involved in the multiple-antibiotic-resistance phenotype. Classes 4 and 5 have been characterized through their involvement in the development of trimethoprim resistance in *Vibrio cholerae.* Class 1 integrons are found extensively in clinical isolates and most of the known antibiotic resistance gene cassettes belong to this class. To date, more than 80 different gene cassettes have been described from class 1 integrons, as opposed to only 6 associated with class 2 integrons (Mazel, 2006). The 3' conserved segment of class 1 integrons contains the *qac*ED*1* and *sulI* genes and an open reading frame, orf5. The *qac*ED*1* and *sulI* genes determine resistance to ethidium bromide and quaternary ammonium compounds and to sulfonamide, respectively. At the downstream end of each inserted cassette is a short imperfect inverted repeat element, the *attC* also referred to as the 59-base element, which functions as a recognition site for the site-specific integrase. Each of the inserted genes has its own version of this element. Circular gene cassettes are inserted individually via a single site specific recombination event (Lévesque et al., 1995; Moura et al., 2007). The general structure of a class 1 integron is shown in Figure 3.

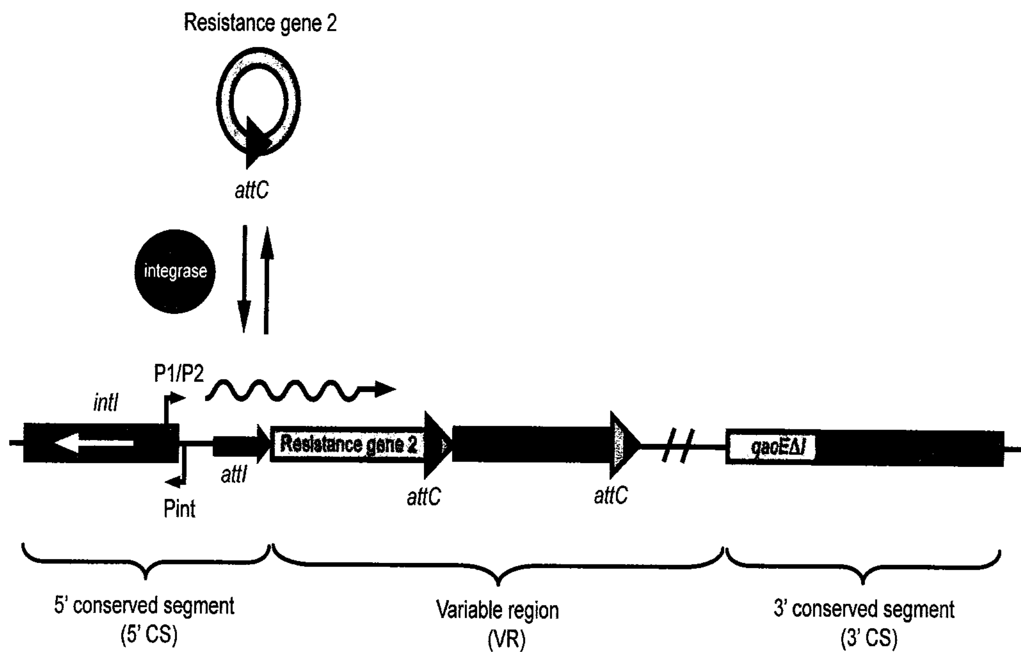

Figure 3. General structure of class 1 integrons. The arrows show the direction of transcription (Farah, 2007).

Several reports in different areas of the world have detected a strong association between antibiotic resistance and the presence of class 1 integrons in both nosocomial and community acquired strains of uropathogenic *Escherichia coli.* These reports include studies in Slovenia (Rijavec et al., 2006); The United States (Solberg et al., 2006) Uruguay (Marquez et al., 2008); Korea (Yu et al., 2003), Turkey (Ozgumus et al., 2007), and many other countries.

2.7. Molecular characterization of integrons

2.7.1. Restriction Fragment Length Polymorphism (RFLP)

Restriction endonucleases are components of the restriction modification systems that occur ubiquitously among bacteria. Their main function is to defend their host against foreign DNA. This is achieved by cleaving incoming DNA that is recognized as foreign at defined sites within the recognition sequence. The host DNA is resistant to cleavage as the DNA is methylated at these sites. There are three different types of restriction enzymes. Type II restriction enzymes are used in molecular studies since they cleave in an ATP independent manner (Pingoud et al., 2005). These restriction enzymes recognize specific sequences, usually 4-6 nucleotides in length. They cut both strands at these sites producing unique fragments. The obtained fragments are extremely useful since they allow detailed mapping of the DNA in question (Pingoud et al., 2005; Yang et al., 1976).
Characterization of class 1 integrons in *Escherichia coli* by restriction fragment length polymorphism has been used in many reports to determine if the PCR amplicons for the variable region having the same size and restriction patterns carry the same resistance cassettes (Essen-Zandbergen et al., 2007; Heir et al., 2004; Machado et al., 2005).

2.7.2. DNA sequencing

The first bacterial genome to be published was that of *Haemophilus influenza* in 1995. Since then sequencing has been used to characterize bacterial genes and to identify important mutations. The genome of *Escherichia coli* was published by Blattner et al in 1997 (Hall, 2007).
The Sanger sequencing method devised in 1977 relies on synthesizing DNA on a single stranded template while randomly incorporating chain terminators. This

generates a range of different fragment sizes that correspond to the positions of the terminators. The older methods would require four reactions per template, one for each base. The reactions are then run on a gel to identify the size of each fragment. Improvements were later made with the use of different colored fluorescent dyes to label terminators, so that all of the terminators can be incorporated in a single reaction. The gels were replaced by capillaries, which simplified the separation step and increased the length of reads (Hall, 2007). Many reports have used partial direct sequencing to evaluate the cassette contents of integrons (Essen-Zandbergen et al., 2007; Heir et al., 2004; Machado et al., 2005; Solberg et al., 2006; Ozgumus et al, 2007). In all of these studies, the antibiotic resistance profiles determined by the disc diffusion method, the broth microdilution method (National committee for Clinical Laboratory Standards, NCCLS), or the E-test (AB BIODISK, Solna, Sweden) were highly associated with the integron content. Hence, most of specimens carrying specific antibiotic resistance cassettes were resistant to the correspondent antibiotics, providing proof that integrons play a very important role in the dissemination of resistance.

Chapter 3

MATERIALS & METHODS

3.1. Clinical isolates and storage conditions

A total of 100 *Escherichia coli* isolates recovered from patients diagnosed with urinary tract infections were screened in this study. 50 Isolates were kindly donated by Dr. George Abdelnour from Notre dame de Secours and designated EM and 50 by Dr. Ziad Daoud form St. George Hospital and designated as ER. The isolates were confirmed to be *E. coli* by the API 20E strips (BioMérieux, Marcy-L'Etoile, France) (Sawma-Aouad, 2007) and were cryobanked at -20 and -80 °C. The *E. coli* strain Ec1484R containing the class I integron was kindly donated by Dr. Veronique Dubois of the Faculté des Sciences Pharmaceutique, Université Victor Segalen, Bordeux, France, and was used as a positive control (Dubois et al., 2003). The *E. coli* strain HB101 and RNase-free water were included as negative controls.

3.2. API Identification System

The total 100 *E. coli* strains were confirmed to belong to the species *Escherichia coli* by the API 20E identification system for *Enterobacteriaceae* (Biomérieux, France).

3.3. Plasmid extraction

Isolates were grown overnight on Tryptone Soy Agar (Mast Group Ltd., Merseyside, U.K.) media at 37°C. Plasmid DNA was extracted using the Quicklyse Miniprep kit (Qiagen, Germany) following the manufacturer's instructions.

3.4. Detection and sizing of class 1 integron variable regions by PCR

The forward primer 5'-GGC ATC CAA GCA GCA AG-3', which anneals at positions 1206 to 1190 of the *intI1* gene, and the reverse primer 5'-AAG CAG ACT TGA CCT GA-3', which anneals at positions 1342 to 1326 (Lévesque et al., 1995), were used to detect the presence of gene cassettes and determine the size of the variable region.

Amplification reactions were carried out on a conventional thermal cycler with 2 µl of DNA (70ng/ µl), 200 µM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.4 µM of primers, and 0.4 µl of Gold Taq polymerase. Water was added to bring the final volume to 20 µl. The PCR cycle consisted of denaturation at 95 ^{0}C for 12 min, followed by 30 cycles of denaturation for 30 seconds at 94 ^{0}C, primer annealing for 30 s at 55 ^{0}C, extension for 1 min at 72 ^{0}C, and a final extension at 72 ^{0}C for 10 min. The reaction products were detected by gel electrophoresis of 10 µl of the reaction mixture on 1.5% agarose gel in 1x TBE containing 4 µg ml^{-1} (Zhao et al, 2001).

3.5. PCR- restriction fragment length polymorphism

Typing of class 1 integrons of similar gel sizes was performed by restriction fragment length polymorphism (RFLP) analysis. PCR products corresponding to the amplification of the 5' CS-3' CS region of class 1 integrons were digested with 1 µl of the enzyme *AluI* (Fermentas), a type 2 restriction endonuclease of the microorganism *Arthrobacter luteus* (Machado et al., 2005, Yang et al., 1976). The fragments obtained were separated in a 2.5% agarose gel and visualized under UV light after staining with ethidium bromide.

3.6. DNA sequencing analysis

Purified PCR products were subjected to direct sequencing using the BigDye Terminator Kit (ABI biosystems) and 2 pmol of the 5'CS and 3'CS primers in separate reactions. Sequence homology and identity comparison was performed with the NCBI BLAST sequence search (Chang et al., 2007).

3.7. Determination of antibiotic resistance profiles by the standard disc diffusion method

Antimicrobial resistance was determined for the following 11 antibiotics using the standard disc diffusion method (Clinical and Laboratory Standards Institute) and according to the manufacturer's instructions: ampicillin, chloramphenicol, ciprofloxacin, gentamicin, netilmicin, ofloxacin, oxacillin, spectinomycin, streptomycin, tetracycline, and trimethoprim/sulfamethoxazole. Resistance or susceptibility profiles were established according to the NCCLS.

Chapter 4

RESULTS

4.1. Antibiotic resistance profiles:

4.1.1. Drug Resistance

All uropathogenic *Escherichia coli* isolates undertaken in this study were screened for their resistance to 9 different antibiotics. The antimicrobial agents used were chosen to cover different classes of antibiotics and based on resistance genes associated with integrons (Annex II). The resistance and/or susceptibility profiles for each isolate are shown in Annex 1. 60% of the isolates were resistant to ampicillin, 55% to trimethoprim/sulfamethoxazole, 53% to streptomycin and only 5% to netilmicin (Figure 4).

Patterns of resistance established based on the mode of action revealed that 60% of the isolates were resistant to β-lactams (ampicillin), 59% were resistant to aminoglycosides and only 11% were resistant to phenicols (Table 1).

4.1.2. Multiple drug resistance

Most of the isolates were resistant to either 4 (17%), 5 (15%) or 3 (13%) of the antibiotics tested in this study. Only 1 isolate (1%) showed resistance to all tested antibiotics while 20% were susceptible to all tested drugs (Table 2).

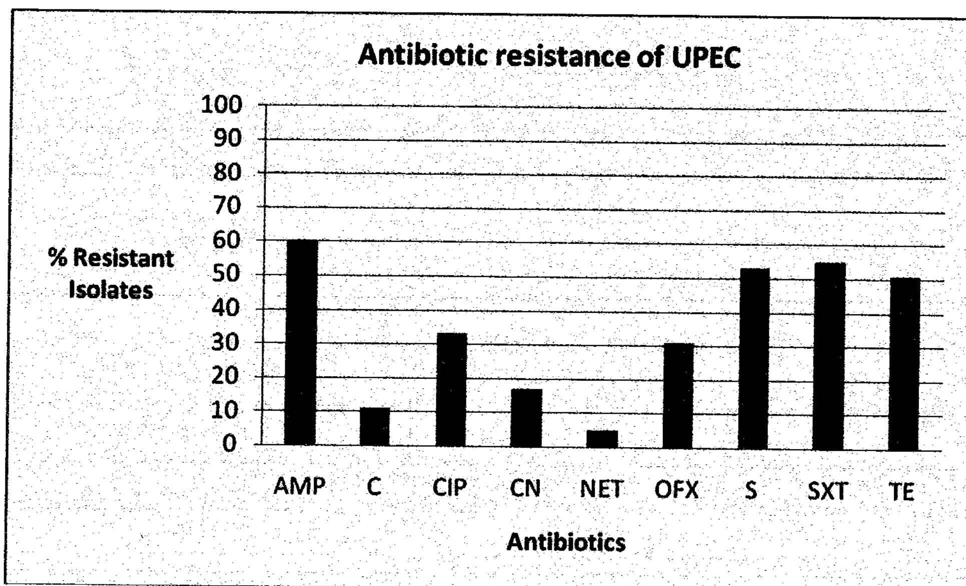

Figure 4. Percentage of resistance within UPEC against the tested antimicrobial agents. (AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; CN, gentamycin; NET, netilmicin; OFX, ofloxacin; S, streptomycin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline).

Table 1. Resistance profiles of UPEC based on the mode of action of tested antibiotics.

Antibiotic modes of action	Antibiotic categories	Number of isolates (%)
Inhibition of cell wall synthesis	penicillins (AMP)	60
Inhibition of protein synthesis	aminoglycosides (CN, NET, S)	59
	tetracyclines (TE)	51
	phenicols (C)	11
Inhibition of nucleic acid synthesis	fluroquinilones (CIP, OFX)	35
Anti-metabolites	trimethoprim/sulphamethoxazole (SXT)	55

Refer to Figure 4 for abbreviations of antibiotics.

Table 2. Percent occurrence of multiple resistance among UPEC.

Number of antibiotics that isolates were resistant to	Total number of isolates (%)
0	20
1	10
2	10
3	13
4	17
5	15
6	5
7	7
8	2
9	1

4.2. Variable region PCR

4.2.1. Occurrence of the variable region

Plasmids recovered from the UPEC were further analyzed by the amplification of the variable region (VR) to determine its presence and size (Figure 5). Results obtained showed that 30% of the isolates were positive having an amplicon size in the range of 0.7- 2 Kbp (Table 5).

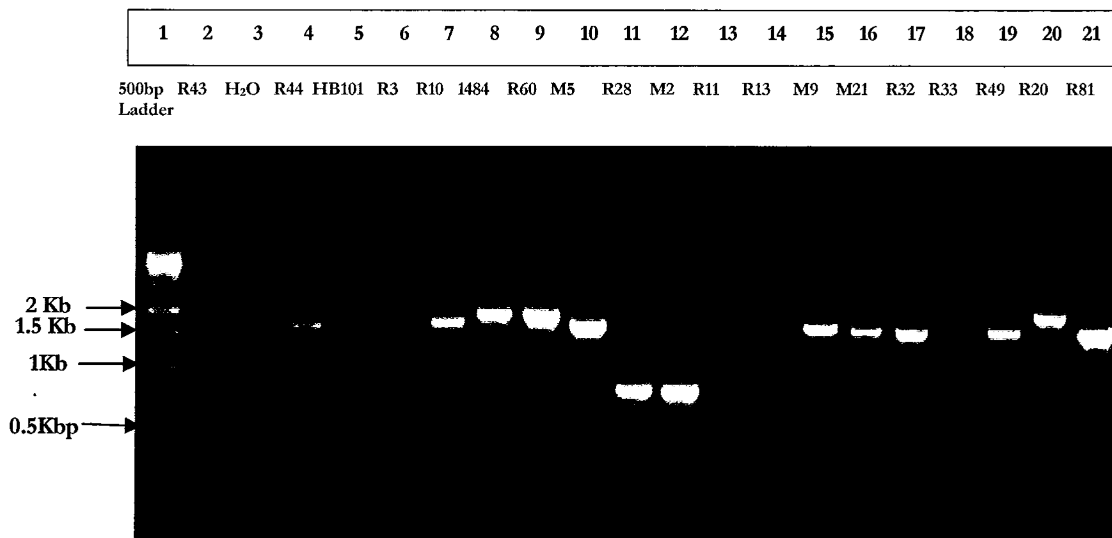

Figure 5. Gel electrophoresis showing the VR PCR products. Lane 1: DNA ladder; lane 3: H_2O; lane 5: the negative control (*E. coli* strain HB101); lane 8: positive control (*E coli* strain 1484); lanes 2, 4, 7, 8, 9, 10, 11, 12, 15, 16, 17, 19, 20 and 21: amplified variable regions of various sizes; lanes 6, 13, 14 and 18: isolates containing no integrons.

4.2.2. Variable region and antibiotic resistance

VR positive isolates were found to be mainly resistant to trimethoprim/sulfamethoxazole (96.7%), ampicillin (86.7%), and streptomycin (83.3%). However, the lowest percentage of resistance among those isolates was for chloramphenicol (16.7%) and netilmicin (10%). None of the VR positive isolates had a complete susceptibility profile. On the other hand, all VR negative isolates showed more susceptibility towards the used antimicrobial agents compared to the VR positive isolates, with 25% being susceptible to all tested drugs, 48.6% resistant to ampicillin, 40% to streptomycin and tetracycline, 8.6% to chloramphenicol, and 2.9% to netilmicin. (Figure 6).

The percentage of VR positive vs. VR negative isolates resistant to at least one antibiotic within each of the studied classes of antimicrobial agents was also as-

sessed (Table 3). The VR positive isolates showed highest resistance to aminoglycosides and trimethoprim/sulfamethoxazole (96.7%), followed by tetracycline (76.6%). VR negative islolates were mainly resistant to ampicillin (48.6%), aminoglycosides (41%), tetracycline (40%), and trimethoprim/sulfamethoxazole (37%). All tested isolates whether VR positive or negative were highly sensitive to the studied phenicols.

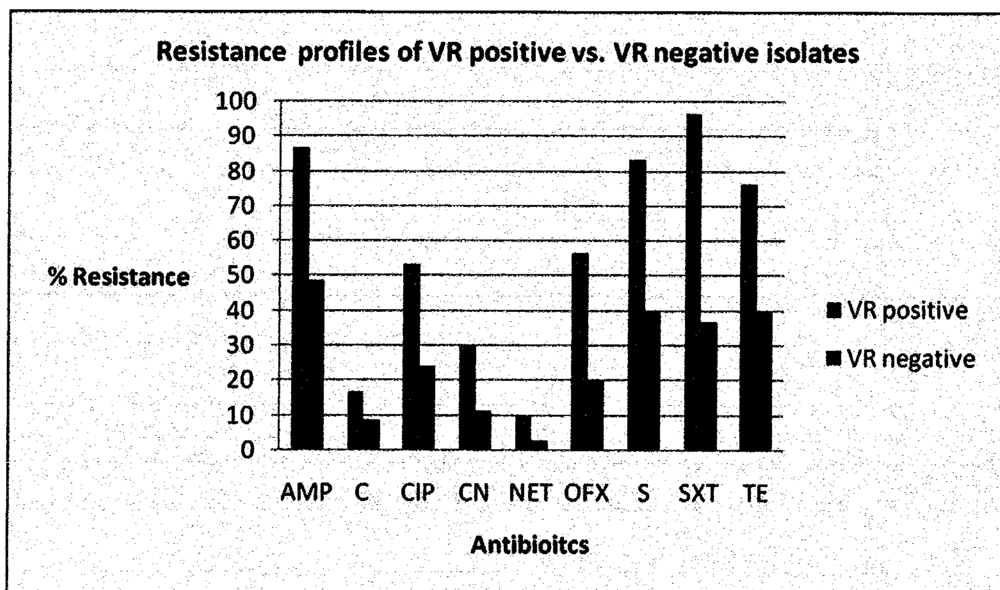

Figure 6. Percentage of resistance within VR positive vs. VR negative isolates to each of the 9 tested antibiotics.

Table 3. Correlation between resistance profiles and occurrence of VR in UPEC.

		VR positive		VR negative	
Antibiotic modes of action	Antibiotic categories	# of isolates (n=30)	%	# of isolates (n=70)	%
Inhibition of cell wall synthesis	penicillins (AMP)	26	86.7	34	48.6
Inhibition of protein synthesis	aminoglycosides (CN, NET, S)	29	96.7	29	41.4
	tetracyclines (TE)	23	76.6	28	40
	Phenicols (C)	5	16.7	6	8.6
Inhibition of nucleic acid synthesis	fluroquinilones (CIP, OFX)	17	56.7	18	25.7
Anti-metabolites	trimethoprim/sulphamethoxazole (SXT)	29	96.7	26	37.1

4.2.3. Variable region and multiple drug resistance

All VR positive isolates were resistant to at least 2 of the antibiotics used in this study, with most being resistant to 4 (33.3%), 5 (26.7%), or 7 (20%) antibiotics. Only 3.3% of the samples were resistant to 8 antibiotics. It is noteworthy that within the VR negative UPEC one isolate was resistant towards all tested drugs. The remaining were mainly resistant to three (17.1%), two (12.2%), or one (14.2%) tested agent, with 28.6% showing complete susceptibility (Table 4).

Table 4. Multiple antibiotic resistance of VR positive vs. VR negative samples

	VR positive		VR negative	
Number of antibiotics	**# of isolates (n=30)**	**%**	**# of isolates (n=70)**	**%**
0	0	0	20	28.6
1	0	0	10	14.2
2	1	3.3	9	12.9
3	1	3.3	12	17.1
4	10	33.3	7	10
5	8	26.7	7	10
6	3	10	2	2.8
7	6	20	1	1.4
8	1	3.3	1	1.4
9	0	0	1	1.4

4.3. Direct partial sequencing of VR positive amplicons

4.3.1. Cassette content of integrons

The Variable region amplicons were sequenced to determine their cassette contents and sequences obtained were analyzed using the NCBI database. The tested integrons had one of 4 different cassette arrays: *dfrA17-aadA5* (46.7%); *dfrA1-aada1* (33.3%); *dfrA12-orf5-aadA12* (10%); *dfra7* (6.7%); and *bla*$_{OXA\text{-}30}$*-aadA1* (3.3%). The amplicon sizes and the integron contents of all positive samples are shown in Table 5. Enzymes encoded for by genes located in those integrons and their functions are shown in Annex II.

Table 5. Amplicon sizes, integron cassette content, antibiotic resistance profiles and restriction patterns of VR positive isolates. Antibiotics in bold represent resistance genes located within the tested integrons.

Sample Name	Amplicon size (Kb)	Integron content	Antibiotic resistance	# of antibiotics showing resistance	Retriction pattern
ER5	1.8	*dfrA12, orf5, aadA2*	AMP, CIP, CN, OFX, **SXT,** TE	6	3
ER9	1.65	*dfrA17, aadA5*	AMP, CIP, CN, NET, OFX, **SXT**, TE	7	1
ER10	1.65	*dfrA17, aadA5*	C, CIP, CN, NET, OFX, **S**, **SXT**, TE	8	1
ER15	1.6	*dfrA1, aadA1*	CIP, OFX, **S**, **SXT**, TE	5	2
ER20	2	*bla*$_{OXA-30}$,*aadA1*	OFX, **S**	2	5
ER28	0.7	*dfrA7*	AMP, CIP, OFX, **SXT**, TE	5	4
ER32	1.6	*dfrA1, aadA1*	AMP, **S**, **SXT**	3	2
ER41	1.65	*dfrA17, aadA5*	AMP, CIP, NET, OFX, **S**, **SXT**, TE	7	1
ER43	1.6	*dfrA1, aadA1*	AMP, **S**, **SXT**, TE	4	2
ER44	1.6	*dfrA1, aadA1*	AMP, **S**, **SXT**, TE	4	2
ER45	1.65	*dfrA17, aadA5*	AMP, C, CIP, CN, OFX, **SXT**, TE	7	1
ER46	1.6	*dfrA1, aadA1*	AMP, **S**, **SXT**, TE	4	2
ER49	1.6	*dfrA1, aadA1*	AMP, CIP, OFX, **S**, **SXT**, TE	6	2
ER50	1.65	*dfrA17, aadA5*	AMP, C, **S**, **SXT**, TE	5	1
ER51	1.65	*dfrA17, aadA5*	AMP, CIP, CN, OFX, S, SXT, TE	7	1

Sample Name	Amplicon size (Kb)	Integron content	Antibiotic resistance	# of antibiotics showing resistance	Retriction pattern
ER60	1.8	*DfrA12, orf5, aadA2*	AMP, CIP, OFX, S, SXT	5	3
ER67	1.65	*dfrA17, aadA5*	AMP, CIP, CN, OFX, S, SXT, TE	7	1
ER69	1.65	*dfrA17, aadA5*	AMP, CIP, CN, OFX, S, SXT, TE	7	1
ER73	1.8	*dfrA12, orf5, aadA2*	AMP, C, CN, S, SXT	5	3
ER78	1.65	*dfrA17, aadA5*	AMP, CIP, CN, OFX, SXT	5	1
ER81	1.6	*dfrA1, aadA1*	AMP, CIP, OFX, **S**, **SXT**, TE	6	2
ER84	1.6	*dfrA1, aadA1*	AMP, **S**, **SXT**, TE	4	2
ER93	1.65	*dfrA17, aadA5*	AMP, CIP, OFX, **S**, **SXT**	5	1
EM2	0.7	*dfrA7*	AMP, C, **S**, **SXT**, TE	5	4
EM5	1.65	*dfrA17, aadA5*	CIP, OFX, **S**, **SXT**	4	1
EM9	1.65	*dfrA17, aadA5*	AMP, **S**, **SXT**, TE	4	1
EM10	1.6	*dfrA1, aadA1*	AMP, **S**, **SXT**, TE	4	2
EM17	1.65	*dfrA17, aadA5*	AMP, **S**, **SXT**, TE	4	1
EM21	1.6	*dfrA1, aadA1*	AMP, **S**, **SXT**, TE	4	2
EM26	1.65	*dfrA17, aadA5*	AMP, **S**, **SXT**, TE	4	1

4.3.2. Integron encoded resistance

All the VR positive isolates were resistant to trimethoprim/sulfamethoxazole except for ER20, being the only isolate not harboring the *dfrA* gene cassette.

On the other hand, 24 (85.7%) isolates out of 28 harboring the *aadA* gene were resistant to streptomycin. Isolate ER20 was sensitive to ampicillin although it carried the bla_{OXA-30} cassette which confers resistance to β-lactams while EM2 didn't have the *dfrA* gene within the integron and yet was resistant to streptomycin (Table 5). Resistance to other antibiotics was not accounted for by Integrons. Isolates with the most common arrays namely *dfrA17-aadA5* and *dfrA1-aadA1* were resistant to 6 and 4 antibiotics respectively. Isolates with the *dfrA17-aadA5* cassette array and compared to the ones with *dfrA1-aadA1* showed resistance to ciprofloxacin, netilmicin, and chloramphenicol (Figure 7).

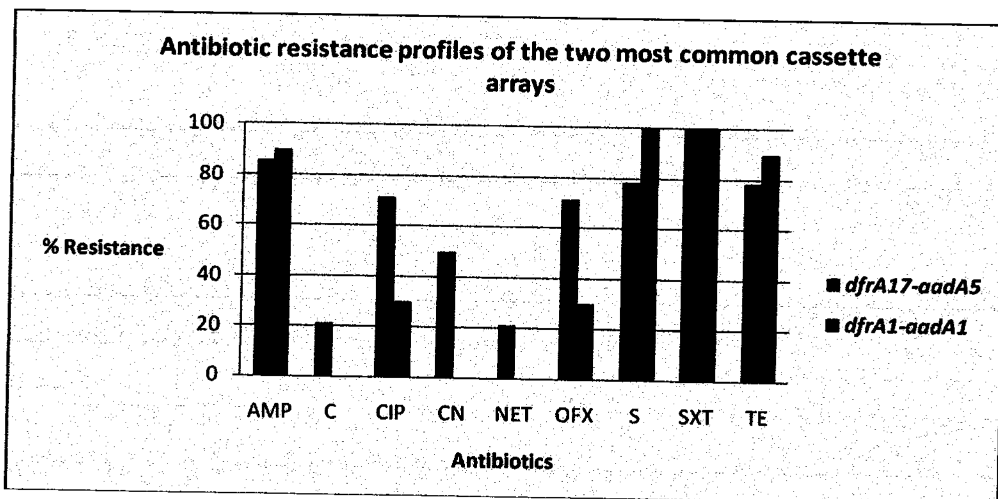

Figure 7. Antibiotic resistance profiles for isolates with the *dfrA17-aadA5* vs. isolates with the *dfrA1-aadA1* cassette array.

4.4. Restriction fragment length polymorphism (RFLP)

The VR positive amplicons were digested with the enzyme *Alu1* to determine if the amplicons of the same size harbored the same integrons. Results revealed that amplicons of the same size carried the same cassette array and had the same restriction pattern (Table 5). Figure 8 shows all 5 restriction patterns for different representative samples.

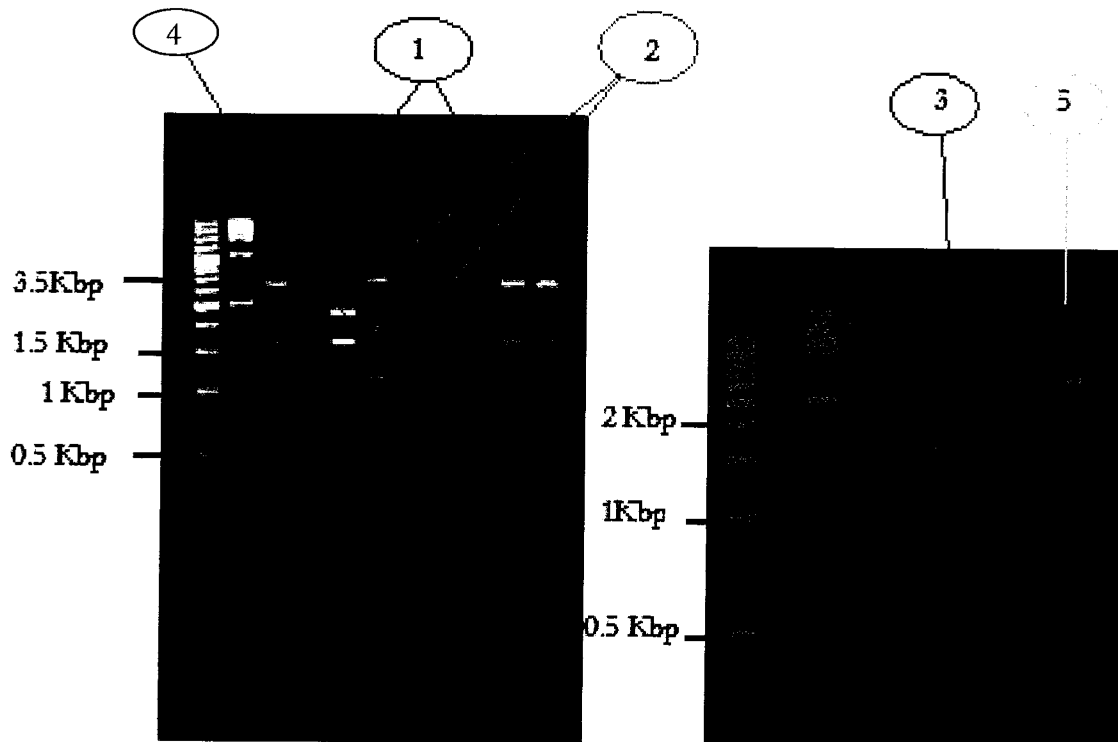

Figure 8. Different restriction patterns for VR positive amplicons. Refer to table 5.

Chapter 5

DISCUSSION

The probability of integron presence was found to increase with multidrug resistance among *Enterobacteriacea* and has been found on the rise in several geographic locations (Yu el al., 2003). In this study, the molecular basis of integron mediated antibiotic resistance in 100 uropathogenic *Escherichia coli* (UPEC) isolates was examined.

The highest levels of resistance among isolates were detected for ampicillin (60%), trimethoprim/sulfamethoxazole (55%), streptomycin (53%), and tetracycline (51%). These antimicrobial agents have been therapeutically present for a long time, thus accounting for their increased resistance (Yoneyama & Katsumata, 2006). Results obtained in Lebanon by Farah (2007), were in agreement with the ones obtained in this study for gentamicin, ofloxacin, and trimethoprim/sulfamethoxazole, while differences ranging between 7-15% were detected with ampicillin, tetracycline, and netilmicin. In both studies, the highest resistance levels were detected for ampicillin. Sawma-Awad et al. (2009) also reported a high level of resistance to ampicillin (68%), and lower resistances to tetracycline (43%), and trimethoprim/sulfamethoxazole (38%); similar results to ours were obtained for ciprofloxacin (29%), chloramphenicol (16%), gentamicin (15%), and netilmicin (3%). Moreover, Borg et al. (2006) and Daoud & Hakime (2003) obtained comparable results to ours for ampicillin, gentamicin, and ciprofloxacin, with resistance levels of about 55%, 18% and 30%, respectively. However, other studies were conducted in Lebanon and differences were observed with chloramphenicol, gentamicin, tetracycline, trimethoprim/sulfamethoxazole, and ciprofloxacin (Araj & Zaatari, 2007; Matar et al., 2005; Moubareck et al., 2005). These studies, and compared to our results, showed higher resistance levels to the above mentioned antibiotics except for Matar et al. (2005) who reported

only 33% resistance to aminoglycosides. This heterogeneity might be attributed to the types of *E. coli* (general vs. UPEC) and the assay employed in assessing the resistance profiles.

Furthermore, and in contrast to our results, *E. coli* isolates in Kuwait and Saudi Arabia exhibited low resistance profile to gentamicin (4%) and trimethoprim/sulfamethoxazole (35%) (Al Sweih et al., 2005; Al-Tawfiq, 2006).

In Jordan, UPEC isolated between 2000-2001 showed high resistance levels to ampicillin (89%), tetracycline (74%), and trimethoprim/sulfamethoxazole (77%) (Shehabi et al., 2004). Similarly, high resistance levels were detected in Taiwan for ampicillin, chloramphenicol, streptomycin, and trimethoprim/sulfamethoxazole (95%, 74%, 94%, and 86% respectively) (Chang et al., 2007).

Similar results were obtained in a study in Turkey only with gentamicin (13.6%). All other tested drugs including ampicillin, chloramphenicol, trimethoprim/sulfamethoxazole, tetracycline, and ciprofloxacin showed lower resistance, while a higher percentage was reported for netilmicin. Moreover, 38% of the isolates showed complete sensitivity to all tested antibiotics and 13.5% were resistant to only one antibiotic as opposed to 20% and 10% according to the results of this study (Ozgumus et al., 2007; Ozyurt et al., 2008). Differences or similarities in the antibiotic resistance profiles can be attributed to the antibiotic treatment regimens adopted or to the geographical locations.

Within the 100 UPEC isolates screened in this study, 30% were positive for the plasmid located class 1 integron variable region. Farah, (2007) detected that 36% of UPEC isolates in Lebanon carried a class 1 integeron. Chang et al. (2000) found that 52% of *E. coli* isolates collected in 1993-1994 from clinical specimens in Taiwan harbored a class 1 integron VR as opposed to 74% in specimens col-

lected in 2002 (Chang et al., 2007).On the other hand, Martinez-Freijo et al. (1998) detected class 1 integron VR in 43% of gram negative isolates from different European hospitals, while it was 47% in California (Solberg et al., 2006).

The class 1 integron VR size obtained in previous studies ranged from as small as 0.5 Kbp to as large as 3.5 Kbp (Chang et al., 2000; Chang et al., 2007; Martinez-Freijo et al., 1998; Solberg el al., 2006; Yu et al., 2003). The size range in this study was between 0.7 Kbp and 2.2 Kbp. Chang et al. (2000) and Martinez-Freijo et al. (1998) detected a larger range of VR size, reflecting the difference in the number and types of integrated cassettes within different *E. coli* pathotypes within different geographical locations. Farah (2007), detected integron sizes in the range of 0.75 -2.5 Kbp in Lebanon, which is in agreement with the results of this study.

It is noteworthy that a great diversity within antibiotic resistance genes carried on the integrons has been previously reported in different environments, with a change in the gene cassette content being the outcome of high antibiotic selective pressures (Partridge et al 2001; Yu et al., 2003). Our results however, revealed a low diversity and hence stability of class 1 integrons, which was also in agreement with other studies (Machado et al., 2005; Mazel et al., 2000).

Direct sequencing of the VR PCR amplicons revealed 5 different cassette arrays. *dfrA17-aadA5* (46.7%), *dfrA1-aadA1* (33.3%), *dfrA7* (6.7%), *dfrA12-orf5-aadA2* (10%) and $bla_{OXA\text{-}30}$-*aadA1* (3.3%). These cassette arrays had amplicon sizes of 1.65 Kbp, 1.6 Kbp, 0.7Kbp, 1.8 Kbp and 2.2 Kbp, respectively. Amplicons of the same size harbored the same cassette content. The results of this study generally corroborate the high prevalence of the *aadA* and *dfrA* gene cassettes observed in UPEC isolates in both Asia and Europe, namely being *aadA5* and *dfrA17* cassettes (Chang et al., 1997; Grape et al., 2000; Mathai et al., 2004; Yu et al., 2003). Chang et al. (2007) and Du al. (2005), contradictory to this study, detected that *dfrA12-orf5-aadA2* was the most prevalent cassette array, followed by *aadA5*-

dfrA17. In all previous studies, the *aadA5* cassette was detected always with the *dfrA17* cassette, indicating that *dfrA17* and *aadA5* were introduced to class 1 integrons almost simultaneously (chang et al., 2003; Ozgumus et al., 2007; Solberg et al., 2006; Yu et al., 2003).

Chang et al. (2007), using the same primers employed in this study for VR amplification and sequencing detected, and similar to our results, the 0.7 Kbp *dfrA7* cassette and the 1.6 kbp amplicon with *dfrA1-aadA1* genes. This might indicate that the corresponding integrons were identical in both studies. On the other hand, and in contrast to our results, a 1.6 Kbp amplicon contained the *dfrA17-aadA5* array and a 1.9 Kbp amplicon contained the *dfrA12-orf5-aadA2* genes. This can be attributed to the fact that the VR amplicons were only partially sequenced in this study. Hence, the obtained sequence revealed only a part of the entire integron, and even though the same cassette array was detected in both studies, the integrons implicated were not identical. Moreover, Sanchez et al. (2000) detected the *aadA5-dfrA17* cassette array in a 1.7 Kbp amplicon, Solberg et al. (2006) detected the same cassette array in a 1.8 Kbp amplicon, while Chang et al. (2000) and Solberg et al. (2006) detected the $bla_{OXA\text{-}30}$-*aadA1* array in a 2.2 Kbp amplicon, which is in accordance with our results.

The occurrence of the same combinations of cassette genes in class 1 integrons in different areas of the world suggests that international travel and the import and export of animals might be contributing to the dissemination of class 1 integrons or their host strains to different areas in the world. Chang et al. (2000) and Martinez-Freijo et al. (1998) proposed that gene cassettes become stably integrated over long periods, and that the transfer of an entire integron via plasmids or transposons is much more frequent than single-gene mobilization or integration within the integron (Martinez-Freijo et al., 1998). This further facilitates the rapid spread of integrons between different strains and geographical locations (Martinez-Freijo et al., 1998).

A correlation between the VR gene cassettes and drug resistance was detected in this study, where 85.7% of isolates harboring the *aadA* gene were resistant to streptomycin as opposed to 38.8% of isolates not harboring the gene within the integron. All isolates carrying the *dfrA* gene were resistant to trimethoprim/sulfamethoxazole. The *dfr* genes were found to be located directly next to the 5' CS, closest to the promoter, thereby ensuring high level of gene expression and conditional resistance (Grape et al., 2003). A strong association between the presence of gene cassettes and resistance to specific antibiotics was also confirmed by earlier studies (Chang et al., 2000; Rao et al., 2008; Roe et al., 2003). However, in some isolates containing gene cassettes, the corresponding antibiotic resistance phenotype was not always present. The isolate harboring the bla_{OXA-30} gene was sensitive to ampicillin and not all isolates carrying an *aadA* cassette were resistant to streptomycin, which can be attributed to the inefficient expression of the inserted gene cassettes by the integron promoter. A similar observation was reported by Roe et al. (2006) & Zhao et al. (2001) where a low resistance profile to streptomycin in isolates carrying the *aadA* gene was detected.
On another note, VR positive isolates showed an overall higher level of resistance to all used antimicrobial agents compared to the VR negative isolates. Some of the genes involved could be located outside the sequenced region. High resistance to all or most used antibiotics has been confirmed in previous studies and was attributed to the association of integrons with conjugative plasmids carrying additional resistance genes (Chang et al., 2007; Machado et al., 2005; Yu et al; 2003). These broad-spectrum plasmids are able to capture integrons which can acquire determinants encoding resistance to frequently used antibiotics (Machado et al., 2005). Furthermore, VR Positive isolates were resistant to at least 2 antibiotics, with most being resistant to 4, and none showing complete susceptibility. Isolates that were resistant to 4 or more antibiotics but were negative for the inte-

gron class 1 VR possibly had the genes on an integron other than class 1, or had the genes dispersed in the genome rather than clustered in an integron.

Typing of class1 integron VR was performed in this study by RFLP analysis and different VR patterns were designed by numbers. Restriction of the same sized amplicons with the *Alu1* enzyme generated identical restriction patterns and had the same cassette content. In contrast, Essen-Zandbergen (2007) & Machado et al. (2005) obtained different restriction patterns with amplicons of similar sizes. Machado et al. (2005) also used *Alu1* to type class 1 VR integrons, and RFLP analysis generated the same restriction patterns for the *dfrA1-aadA1* and *dfrA12-orf5-aadA2* cassette arrays as the ones obtained in our study. This further confirmed that the same integrons were implicated in both studies and gave further support on the dissemination of integron mediated resistance between different bacterial strains and geographical locations.

Finally, we recommend having a follow up study which involves screening of a greater number of isolates to get more comprehensive results covering the UPEC population. Moreover, internal primers should be designed to complete the sequencing of the VR and hence ascertain the relation between integrons and antibiotic resistance in Lebanon.

Chapter 6

CONCLUSION

- This study assessed the antibiotic resistance profiles of a 100 clinical UPEC isolates in Lebanon and revealed that the majority were resistant to ampicillin (60%), trimethoprim/sulfamethoxazole (55%), streptomycin (53%), and tetracycline (51%). Only 5% of the isolates were resistant to netilmicin hence encouraging the use of this antibiotic in the treatment of UPEC.
- 30% of the isolates were positive for the class 1 integron variable region PCR, which revealed amplicons in the range of 0.7 Kbp to 2.2 Kbp.
- The study was the first in Lebanon to employ sequencing for the characterization of the cassette content of class 1 integrons.
- Five different cassette arrays were revealed: *dfrA17 -aadA5* (46.7%), *dfrA1-aadA1* (33.3%), *dfrA7* (6.7%), *dfrA12-orf5-aadA2* (10%) and $bla_{OXA\text{-}30}$-*aadA1* (3.3%).
- Amplicons of equal size had the same cassette content. The cassette arrays obtained in this study were reported in different regions of the world.
- A correlation was detected between the presence of gene cassettes and resistance to the corresponding antibiotics.
- The VR positive isolates showed an overall higher level of resistance to all used antimicrobial agents than the VR negative isolates.
- RFLP analysis generated identical restriction patterns for amplicons of equal size, further supporting the fact that these isolates harbor the same integron.

Chapter 7

BIBLIOGRAPHY

Al-Agamy, M., & Ashour, M. S. E. (2004). Phenotypic and genotypic characterization of antimicrobial resistance in Egyptian *Escherichia coli* isolates. *Clinical Microbiology and Infection, 10*, 486-512.

Al-Agamy, M., M.S.E. Ashour, & Wiegand, I. (2006). First description of CTX-M genes from Egyptian *Escherichia coli* strains. *International Journal of antimicrobial Agents, 27*, 545-48.

Al- Tawfiq, J. A. (2006). Increasing antibiotic resistance among isolates of *Escherichia coli* recovered from inpatients and outpatients in a Saudi Arabian hospital. *Infection Control and Hospital Epidemiology, 27*, 748-53.

Al Sweih, N., Jamal, W., & Rotimi, V. O. Spectrum and antibiotic resistance of uropathogens isolated from hospital and community patients with urinary tract infections in two large hospitals in Kuwait. (2005). *Medical principles at practice, 14*, 401-07.

Araj, G. F., & Zaatari, G. S. (2007). Antimicrobial susceptibility patterns of bacterial isolates. Beirut: AUB Medical Center.

Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., et al. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science, 277*, 1453-74.

Borg, M. A., Scicluna, E., De Kraker, M., De Sande-Bruinsma, N. V., Tiemersma, E., Gur, D., et al. (2006). Antibiotic resistance in the southeastern Mediterranean–Preliminary results from the ARMED project. *Eurosurveillance, 11*, 1-5.

Chang, C. Y., Chang, L. L., Chang, Y. H., & Lee, T. M. (2000). Characterization of drug resistance gene cassettes associated wit class 1 integrons in clinical isolates of *Escherichia coli* from Taiwan, ROC. *Antimicrobial Resistance, 49*, 1097-1102.

Chang, L. L., Chang, J. C., Chang, C. Y., Chang, S. F., & Wu, W. J. (1997).Genetic localization of the type I trimethoprim resistance gene and its dissemination in urinary tract isolates in Taiwan. *Kaohsiung Journal of Medical Sciences, 13*, 525–33.

Chang , L. L., Chang T. M., & Chang C. Y. (2007). Variable gene cassette patterns of class 1 integron-associated drug-resistant *Escherichia coli* in Taiwan. *Kaohsiung Journal of Medical Sciences, 23*, 273-280.

Croft, A. C., D'Antoni, A. V., & Terzulli, S. L. (2007). Update on the antibacterial resistance crisis. *Medical Science Monitoring, 13*, 103-18.

Daoud, Z., & Hakime, N. (2003). Prevalence and susceptibility patterns of extended-spectrum beta-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* in a general university hospital in Beirut, Lebanon. *Sociedad Española de Quimioterapia, 16*, 233-38.

De Sousa, C. P. (2006). *Escherichia coli* as a specialized bacterial pathogen. *Revista de Biologia e Ciencis da Terra, 6,* 341-52.

Du, X.., Shen, Z., Wu, B., Xia, S., & Shen, J. Characterization of class 1 integrons-mediated antibiotic resistance among calf pathogenic *Escherichia coli. FEMS Microbiology Letters, 245*, 295-98.

Dubois, V., Arpin, C., Quentin, C., Texier-Maugein, J., Poirel, L., & Nordmann, P. (2003). Decreased susceptibility to cefepime in a clinical strain of *Escherichia coli* related to plasmid- and integron-encoded OXA-30 beta lactamase. *Antimicrobial Agents and Chemotherapy, 47,* 2380-81.

Essen-Zandbergen, A. V., Smith, H., Veldman, K., & Mevius, D. (2007). Occurance and characteristics of class 1, 2, and 3 integrons in *Escherichia coli, Salmonella,* and *Campylobacter* spp. in the Netherlands. *Journal of Antimicrobial Chemotherapy, 59,* 746-750.

Fadel, R., Dakdouki, G., Kanafani, A., Araj, G., & Kanj, S. (2004). Clinical and microbiological profile of urinary tract infection at a tertiary-care center in Lebanon. *Infection Control and Hospital Epidemiology, 25,* 82-5.

Farah, M. (2007). Antibiotic resistance patterns & characterization of class 1 integron in uropathogenic *Escherichia coli* in Lebanon. Unpublished master's thesis, Lebanese American University, Blat, Lebanon.

Grape, M., Farra, A., Kronvall, G., & Sundstrom, L. (2005). Integrons and gene cassettes in clinical isolates of co-trimoxazole-resistant Gram-negative bacteria. *Clinical Microbiology of Infections, 11*, 185–92.

Grape, M., Sundstrom, & Kronvall, G. (2003). New dfr2 gene as a single-gene cassette in a class 1 integron from a trimethoprim-resistant *Escherichia coli* isolate. *Microbial Drug Resistance, 9*, 317-22.

Guentzel, M. N. (1996). *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter,* and *Proteus.* In S. Baron (Ed.), Medical microbiology (4th ed., p. 262). Galveston, TX: University of Texas Medical Branch.

Gupta, K. (2003). Addressing antibiotic resistance. *Disease Monitoring, 49*, 99-110.

Hall, N. (2007). Advanced sequencing technologies and their wider impact in microbiology. *Journal of Experimental Biology, 209*, 1518-25.

Heir, E., Lindstedt, B. A., Leegaard, T. M., Gjernes, E, & Kapperud, G. (2004). Prevalence and characterization of integrons in blood culture *Enterobacteriaceae* and gastrointestinal *Escherichia coli* in Norway and reporting of a novel class 1 integron-located lincosamide resistance gene. *Annals of Clinical Microbiology and Antimicrobials, 3*, 1-9.

Jouini, A., Vinué, L., Ben Slama, K., Sáenz, Y., Klibi, N., Hammami, S., et al. (2007). Characterization of CTX-M and CHV extended-spectrum β- lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia, *Journal of Antimicrobial Chemotherapy, 60*, 1137-41.

Kaper, J. B., Nataro, J. P., Harry, L. T., & Mobley, H. L. T. (2004). Pathogenic enteric *Escherichia coli. Nature Reviews Microbiology, 2,* 123-40.

Kusheria, R., Dasgupta, P., Sacks, S. H., Khan, M. S., & Sheerin, N. S. (2005). Urinary tract infections: New insights into a common problem. *Postgraduate Medical Journal, 81,* 83-6.

Ladhani, S., & Grandsen, W. (2003). Increasing antibiotic resistance among urinary tract isolates. *Archives of Disease in Childhood, 88,* 444-5.

Leverstein-Van Hall, M. A., Paauw, A., Box, A. T. A., Blok, H. E. M., Verhoef, J., & Fluit, A. C. (2002). Presence of integron-associated resistance in the community is widespread and contributes to multidrug resistance in the hospital. *Journal of Clinical Microbiology, 40,* 3038-40.

Lévesque, C., Piché, L., Larose, C., & Roy, P. H. (1995). PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrobial Agents and Chemotherapy, 39,* 185-91.

Lina, T. T., Rahman, S. R., & Gomes, D. J. (2007). Multiple antibiotic-resistance mediated plasmids and integrons in Uropathogenic *Escherichia coli* and *Klebsiella pneumonia. Journal of Microbiology, 24,* 19-23.

Machado, E., Canton, R., Baquero, F., Galan, J., Rollan, A., Peixe, L., et al. (2005). Integron content of extended-spectrum-β-lactamase-producing *Escherichia coli* strains over 12 years in a single hospital in Madrid, Spain. *Antimicrobial Agents and Chemotherapy, 49,* 1823-29.

Martinez-Freijo, P., Fluit, A. C., Schmitz, F. J., Grek, V. S. C., Verhoef, J., & Jones, M. E. (1998). Class 1 integrons in Gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotc compounds. *Journal of Antimicrobial Chemotherapy, 42,* 689-96.

Márquez, C., Labatte, M., Raymondo, C., Fernández, J., Gestal, A. M., Holley, M., et al. (2008). Urinary tract infections in South American populations: Dynamic spread of class 1 integrons and multidrug resistance by homologous and site specific recombination. *Journal of Clinical Microbiology, 46,* 3417-25.

Marrs, C. F., Zhang, L., & Foxman, B. (2005). *Escherichia coli* mediated urinary tract infections: Are there different Uropathogenic *E. coli* (UPEC) pathotypes? *FEMS Microbiology Letters, 252,* 183-90.

Matar, G. M., Al Khodor, S., El Zaatari, M., & Uwaydah, M. (2005). Prevalence of the genes encoding extended-spectrum β- lactamases, in *Escherichia coli* resistant to β-lactam and non-β-lactam antibiotics. *Annals of Tropical Medicine and Parasitology, 99,* 413-17.

Mathai, E., Grape, M., & Kronvall, G. (2004). Integrons and multidrug resistance among *Escherichia coli* causing community-acquired urinary tract infection in southern India. *APMIS, 112,* 159–64.

Mazel, D., Dychinco, B., Webb, V. A., & Davies, J. (2000). Antibiotic resistance in the ECOR collection: Integrons and identification of a novel *aad* gene. *Antimicrobial Agents and Chemotherapy, 44,* 1568–74.

Mazel, D. (2006). Integrons: Agents of bacterial evolution. *Nature Reviews, 4*, 608-20.

Moubarek., C., Daoud, Z., Hakimé, N. I., Hamzé, M., Mangeney, N., Matta, H., et al. (2005). Countryside spread of community and hospital acquired extended spectrum β-lactamase (CTX-M15) producing *Enterobacteriaceae* in Lebanon. *Journal of Clinical Microbiology, 43*, 3309-13.

Moura, A., Henriques, I., Ribeiro, R., & Correia, A. (2007). Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *Journal of Antimicrobial Chemotherapy, 60*, 1243-50.

Mulvey, M. A., Schilling, J. D., Martinez, J. J., & Hultgren, S. G. (2000). Bad bugs and beleaguered bladders: Interplay between uropathogenic *Escherichia coli* and innate host defenses. *PNAS, 97*, 8829-35

Naas, T., Mikami, Y., Imai, T., Poirel, L., & Nordmann, P. (2001). Characterization of In53, a class 1 plasmid and composite Transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. *American Society for Microbiology,183*, 235-49.

Ozgumus, O. B., Celik-Cevim, C., Alpay-Karaoglu, S., Sandalli, C., & Sevim, A. (2007). Molecular characterization of antibiotic resistant *Escherichia coli* strains isolated from tap and spring waters in coastal region in Turkey. *The Microbiological Society of Korea, 45*, 379-87.

Ozyurt, M., Haznedaroqlu, T., Sahiner, F., Oncul, O., Ceylan, S., Ardic, N., et al. (2008). Antimicrobial resistance profiles of community-acquired uropathogenic *Escherichia coli* isolates during 2004-2006 in a training hospital in Istanbul. *Microbiology bulletin, 42*, 231-43.

Partridge, S., Collis, R. C., & Hall, R. M. (2002). Class 1 integrons containing a new cassette, *aadA10*, with Tn*1404* from R151. *Antimicrobial Agents and Chemotherapy, 46,* 2400–08.

Pingoud, A., Fuxreiter, M., & Wende W. (2005). Type II restriction endonucleases: Structure and mechanism. *Cellular and Molecular Life Sciences, 62,* 685-707.

Rao, S., Maddox, C. W., Hoien-Dalen, P., Lanka, S., & Weigel, R. M. (2008). Diagnostic accuracy of class 1 integron PCR method in detection of antibiotic resistance in *Salmonella* isolates from swine production systems. *Journal of Clinical Microbiology, 46*, 916-20.

Rijavec, M., Erjavec, M. E., Avgustin, J. A., Reissbrodt, R., Fruth, A., Krizan-Hergouth, V., et al. (2006). High prevalence of multidrug resistance and random distribution of mobile genetic elements among Uropathogenic *Escherichia coli* (UPEC) of the four major phylogenetic groups. *Current Microbiology, 53*, 158-62.

Roe, M. T., & Pillai, S. D. (2003). Monitoring and identifying antibiotic resistance mechanisms in bacteria. *Poultry Science, 82,* 622-26.

Ronald, A. R., & Alfa, M. J. (1996). Microbiology of the genitourinary system. In S. Baron (Eds.), Medical microbiology (4th ed., p.526). Galveston, TX: University of Texas Medical branch.

Sanchez, S., McCrackin Stevenson, M. A., Hudson, C. R., Maier, M., Buffington, T., Dam, Q., et al. (2002). Characterization of multidrug-resistant *Escherichia coli* isolates associated with nosocomial infection in dogs. *Journal of Clinical Microbiology, 40*, 3586-95.

Sawma-Aouad, G. (2007). Antimicrobial resistance in relation to virulence determinants and phylogenetic background among uropathogenic *Escherichia coli* in Lebanon. Unpublished master's thesis, Lebanese American University, Blat, Lebanon.

Sawma-Awad, G., Tokajian, S., & Hashwa, F. (2009). Antimicrobial resistance in relation to virulence determinants and phylogenetic background among uropathogenic *Escherichia coli* in Lebanon. *Journal of Chemotherapy, 21*, 118-123.

Shehabi, A. A., Mahafzah, A. M., & Al- Khalili, k. Z. (2004). Antimicrobial resistance and plasmid profiles of urinary *Escherichia coli* isolates from Jordanian patients. *East Mediterranean Health Journal, 10*, 322-8.

Solberg, O. D., Ajiboye, R. M., & Riley, L. W. (2006). Origin of class 1 and 2 integrons and gene cassettes in a population-based sample of Uropathogenic *Escherichia coli. Journal of Clinical Microbiology, 44*, 1347-51.

Yang, R. C. A., De Vourde, A., & Fiers W. (1976). Specific cleavage and physical mapping of Simian-Virus-40 DNA by the restriction endonuclease of *Arhtrobacter luteus*. *European Journal of Biochemistry, 63*, 119-38.

Yoneyama, H., & Katsumata, R. (2006). Antibiotic resistance in bacteria and its future for novel antibiotic development. *Bioscience, Biotechnology and Biochemistry, 70,* 1060-75.

Yu, H. S., Lee, J. C., Kang, H. Y., Ro, D. W., Chung, J. Y., Jeong, Y. S., et al. (2003). Changes in gene cassettes of class 1 integrons among *Escherichia coli* isolates from urine specimens collected in Korea during the last two decades. *Journal of Clinical Microbiology, 41,* 5429-33.

Zhao, S., White, D. G., Ge, B., Ayers, S., Friedman, S., English, L., et al. (2001). Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. *Applied and Environmental Microbiology, 67*, 1558-64.

Annex 1. Antibiotic resistance profile of UPEC. (AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; CN, gentamycin; NET, netilmicin; OFX, ofloxacin; OX, oxacillin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline). Blue: VR positive; Yellow: Resistant; Green: intermediate resistance. T refers to the total number of antibiotics a sample is resistant to.

Sample Name	AMP	C	CIP	CN	NET	OFX	S	SXT	TE	T
ER1	2.2	2	2.5	1.8	1.9	2	1.6	2.1	1.9	1
ER2	2	2.1	2.4	1.6	1.8	2	1.7	2	2	1
ER3	1.8	2.1	0	2	1.9	3	[illegible]	2.4	2	2
ER4	2.2	2.2	2.7	1.9	2.1	3	[illegible]	2.5	2.1	1
ER5	0	2.5	0	0	1.5	0	[illegible]	0	0	7
ER6	2	2.5	3	2	2	2.5	1.5	2.5	2	1
ER7	0	2.2	2.6	1.8	1.8	2.5	[illegible]	2.5	2.1	2
ER8	2.1	1.9	2.2	1.8	2	2.2	1.5	1.9	2	1
ER9	[illegible]	2.2	0	0	[illegible]	[illegible]	[illegible]	0	[illegible]	8
ER10	0	[illegible]	0	0	[illegible]	[illegible]	[illegible]	0	[illegible]	9
ER11	[illegible]	2	2.6	1.6	1.9	3	1.6	2	[illegible]	2
ER12	0	2	2.4	1.5	1.9	3.2	[illegible]	2.1	2.5	3
ER13	0	2	3	2.1	2	2.6	1.5	2.2	[illegible]	3
ER14	2	2	2.5	1.5	2.1	2.3	1.5	2.1	2.2	1
ER15	2	2	0	2	2.3	[illegible]	[illegible]	[illegible]	[illegible]	6
ER16	0	2.5	3	0	1.8	3	[illegible]	1.8	[illegible]	5
ER17	0	[illegible]	[illegible]	2	2.5	2	[illegible]	[illegible]	[illegible]	4
ER18	1.8	2.1	2.2	1.8	2	1.7	[illegible]	[illegible]	[illegible]	2
ER19	1.9	2.5	3.5	2	2.3	3.1	1.5	2.7	2.3	1
ER20	1.8	2.1	2.2	1.8	2	[illegible]	[illegible]	[illegible]	2	3
ER21	0	0	3	1.8	2	3.1	[illegible]	[illegible]	[illegible]	6
ER22	0	0	3	2	2.2	2.5	[illegible]	[illegible]	[illegible]	6
ER23	0	2.5	0	2	2	0	[illegible]	2.5	2	4
ER24	0	2.2	0	0	1.2	0	[illegible]	1.8	[illegible]	8
ER25	1.9	2.5	3.1	1.8	2	3.1	[illegible]	2.5	2.2	1
ER26	0	2.5	3.2	1.7	2.1	3	[illegible]	0	2.1	4
ER27	2	2.1	3.6	2	2	3	[illegible]	2.5	2.2	1

ER28	[illegible]	2.1	[illegible]	1.6	2.2	[illegible]		[illegible]	[illegible]	6
ER29	[illegible]		2.5	2	2		2	2	[illegible]	3
ER30		1.8	2.6	1.6	1.8	2.6	[illegible]	2.4	2.2	2
ER31	[illegible]	[illegible]	3.4	[illegible]	2.4	3.5	[illegible]	[illegible]	[illegible]	7
ER32	[illegible]	2.6	3.2	1.8	1.9	2.9	[illegible]	[illegible]	2.2	4
ER33	[illegible]	2.3	[illegible]	1.9	1.7	[illegible]	1.5	2.4	2.2	4
ER34	1.9	2.2	3	1.6	2	3		2.2	1.9	1
ER35	[illegible]	2.4	3	1.7	2.5	2.5	[illegible]	[illegible]	2.1	4
ER36	[illegible]	2.8	[illegible]	2	2.1	[illegible]	[illegible]	[illegible]	2.3	6
ER37			2.6	1.9	2	1.7	[illegible]	[illegible]	1.9	3
ER38	[illegible]	2		[illegible]	1.8	[illegible]	1.5	[illegible]	1.9	5
ER39	[illegible]	2.3	3	2	2.3	3	[illegible]	[illegible]	[illegible]	5
ER40	[illegible]		2.9	1.8	2.1	1.6	1.9	2.5	2.1	2
ER41	[illegible]	1.9	[illegible]	1.7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	8
ER42	[illegible]	2.3	3	2.9	2.2	3.4	[illegible]	[illegible]	[illegible]	5
ER43	[illegible]	2.4	3.5	2.1	2.1	2.9	[illegible]	[illegible]	[illegible]	5
ER44	[illegible]	2	3.4	1.9	2	2.1	[illegible]	[illegible]	[illegible]	5
ER45	[illegible]	[illegible]	[illegible]	[illegible]	1.6	[illegible]	1.5	[illegible]	[illegible]	8
ER46	[illegible]	2	3	1.8	1.9	1.9	[illegible]	[illegible]	[illegible]	5
ER47		2.2	2.8	1.8	2.1	2	1.5	2.5	2	1
ER48	[illegible]	2	2.5	1.5	1.7	2.5		2.4	[illegible]	3
ER49	[illegible]		[illegible]	2		[illegible]	[illegible]	[illegible]	[illegible]	7
ER50	[illegible]	[illegible]	2.4	1.6	2.1	2.1	[illegible]	[illegible]	[illegible]	6
ER51	[illegible]	2	[illegible]	[illegible]	1.7	[illegible]	[illegible]	[illegible]	[illegible]	8
ER52	2	2.5	3	1.9	2.1	3		2.6	2.2	1
ER57	[illegible]	2.6	[illegible]	1.8	2.1	[illegible]	[illegible]	[illegible]	2.2	6
ER58	1.8	2	[illegible]	2	2.3	[illegible]	[illegible]	2	2.3	4
ER59	1.8	2.1	3	1.8	2.3	2.8		2.9	2	1
ER60	[illegible]	2.2	[illegible]	1.9	1.8	[illegible]	[illegible]	[illegible]	2	6
ER62				1.7	1.8	2		[illegible]		2
ER63	1.9	2	3.5	1.5	1.7	2.8	[illegible]	[illegible]	[illegible]	4
ER66	1.8	2.1	2.4		1.6	2.5	[illegible]	[illegible]		3
ER67	[illegible]	1.8	[illegible]	[illegible]	1.5	[illegible]	[illegible]	[illegible]	[illegible]	8

ER68	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	9
ER69	[illegible]		[illegible]	[illegible]	1.9	[illegible]	[illegible]	[illegible]	[illegible]	8
ER71	2	2	2.5	1.9	1.9	2.6	1.5	2.8	2.1	1
ER72	2	2	3.5	1.8	2.3	2		2.7	2.1	1
ER73	[illegible]	[illegible]	2.6	[illegible]	1.8	2.2	[illegible]	[illegible]		6
ER74	[illegible]	2.5	3	1.6	2.1	2.8	[illegible]	1.7	[illegible]	4
ER75	1.8			1.7	1.9	2		[illegible]	[illegible]	3
ER76		2	3		2.1	2	1.5	[illegible]	[illegible]	3
ER77	[illegible]	[illegible]	[illegible]	2	2	[illegible]		[illegible]	[illegible]	7
ER78	[illegible]	1.8	[illegible]	[illegible]	2	[illegible]		[illegible]		6
ER79	[illegible]	2.3	[illegible]	1.6	2	[illegible]	1.5	2.8	2.1	4
ER80	1.9	2.3	3.5	1.7	2.2	2.4		2.6	2.1	1
ER81	[illegible]	2.4	[illegible]	1.9	2.4	[illegible]	[illegible]	[illegible]	[illegible]	7
ER83	1.9	2.1	[illegible]	2	2	3	1.5	2.8	2.2	2
ER84	[illegible]	2.1	2.5	1.6	1.9	2.5	[illegible]	[illegible]	[illegible]	5
ER85	[illegible]	2.8	3	[illegible]	2.1	2.4	[illegible]	[illegible]	[illegible]	6
ER86		2.2	[illegible]	1.9	2	[illegible]	1.5	2	2	3
ER87	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	10
ER88	[illegible]	2	3	2	2	2.1	[illegible]	[illegible]	[illegible]	5
ER89		2.2	2.3	1.5	2	2		2.2	[illegible]	2
ER92	[illegible]	2.1	[illegible]	2	2.5	1.7	[illegible]	[illegible]	[illegible]	6
ER93	[illegible]		[illegible]		1.5	[illegible]	[illegible]	[illegible]		6
EM1	[illegible]	2.4		1.5	1.8	2	[illegible]	[illegible]	[illegible]	5
EM2	[illegible]	[illegible]	2.6	1.8	2.1	2.2	[illegible]	[illegible]	[illegible]	6
EM3	[illegible]	2.2	[illegible]	[illegible]		[illegible]		2.5	2	5
EM4	[illegible]	2.4	3.6	1.7	2.2	2.6	[illegible]		[illegible]	4
EM5		2	[illegible]		1.8	[illegible]	[illegible]	[illegible]	2	5
EM6	[illegible]	2.2	3	1.9	1.8	2.7	1.5	2.6	1.9	2
EM7	2.4	2.5	2.7	1.7	2.2	2.3		2.5	2	1
EM9	[illegible]	2.2	3.2	1.7	1.8	3	[illegible]	[illegible]	[illegible]	5
EM10	[illegible]	2.1	2.6	2.1	2.5	2.2	[illegible]	[illegible]	[illegible]	5
EM11		2.4	2.5	2.1	2	2.5	1.5	2.4	2	1
EM15	1.9	1.8	[illegible]	2.2	2.2	[illegible]	[illegible]	[illegible]	[illegible]	6

EM13	0		1.4	1.5	2.1		1.5	2	0	4
EM16	2.2	2.2	3	1.7	2.2	2.5		2.5	2.1	1
EM17	0	2.1	3.5	1.7	2	3	0	0	0	5
EM19		2.1	2.3	2.1	1.7	2	1.7	2.4	2	1
EM21	0	2.5	3.5	1.9	2	3	0	0	0	5
EM22		2.4	2.5	1.5	1.6	2.6	0	0	0	4
EM26	0	1.8	2.5	1.9	2.5	2.8	0	0	0	5

Annex II. Integron gene cassettes, their products and functions (Solberg et al., 2006).

Gene	Product	Function
aadA1	aminoglycoside adenyl transferase	resistance to streptomycin & spectinomycin
dfrA1	dihydrofolate reductase	trimethoprim resistance
aadA5	aminoglycoside adenyl transferase	resistance to streptomycin & spectinomycin
dfrA17	dihydrofolate reductase	trimethoprim resistance
dfrA7	dihydrofolate reductase	trimethoprim resistance
aadA2	aminoglycoside adenyl transferase	resistance to streptomycin & spectinomycin
dfrA12	dihydrofolate reductase	trimethoprim resistance
bla_{OXA-30}	oxacillinase	confers resistance to beta-lactam
orf5	unknown	unknown