Antibiotic-Resistant Plasmid Profiles in

Escherichia coli

Isolated from Urine Culture of Patients

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Submitted in partial fulfillment of the requirement for the

Degree of Master of Science in Medical Laboratory Sciences

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TO MY FAMILY

AND

FRIENDS

FOR THEIR SUPPORT AND LOVE

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Finally, my family must receive the greatest credit for their continuous encouragement, support and love.

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Abstract

This is a prospective study that was conducted on multiresistant *E. coli* isolated from urine cultures submitted from inpatients and outpatients at the Jordan University Hospital (JUH) over the period of 2 years. A total of 3583 out of 12021 (29.8 %) and 3140 out of 11328 (27.7 %) urine cultures were found positive for significant bacterial growth during 2000 and 2001, respectively. The total number of *E. coli* isolates out of all uropathogens was 1160 (32.4 %) in 2000, and 1175 (37.4 %) in 2001, respectively. The ratio of distribution of *E. coli* isolates between outpatients and inpatients was 3: 1 and approximately similar in both years. The ratio of female patients to male patients is 4: 1 during the 2- year study.

The majority of *E. coli* isolates were susceptible to nitrofurantoin (80 %, 78%), to cefuroxime (63 %, 67 %) and to norfloxacin (63 %, 59 %) during the period of study.

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Lowest susceptibility rates were found with ampicillin (9 %, 13 %) and cotrimoxazole (22 %, 24 %) and to a lesser extent to tetracycline (25 %, 26 %), augmentin (39 %, 27 %) and gentamicin (51 %, 53 %). A total of 15 out of 53 (28 %) of the examined multiresistant *E. coli* strains were able to transfer most or part of their resistance to the recipient *E. coli* K12 by using eight different conjugation methods. Twenty representative multiresistant *E. coli* strains were tested for Minimal Inhibitory Concentrations (MICs). This has shown that ampicillin and cotrimoxazole have the highest MICs, whereas ciprofloxacin has the lowest MICs, and to a lesser extents gentamicin, nalidixic acid and tetracycline.

Plasmid profiles of 16 multiresistant *E. coli* strains and their 15 transconjugant strains have shown 13 different plasmid profiles, and plasmid sizes ranged from 2.0 kb to 59.4 kb. A common large plasmid of 28.0 kb was found in 10 out of 15 (67 %) of *E. coli* donor strains. Also, 11 out of 15 (73 %) of *E. coli* strains contained more than one plasmid, but no isolate contained more than six plasmids. The majority of *E. coli* isolates (67 %) with a transferable plasmid of 28.0 kb demonstrated common digested patterns and almost similar resistance profiles with *Hind* III restriction enzyme. This common large plasmid may increase in incidence and contribute to the spread of multiple antibiotic resistance among *E.coli* isolates in future. Further investigation with Polymerase Chain Reaction (PCR) may determine the actual number of resistance genes associated with a common R-plasmid in all multiresistant *E. coli* strains.

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1. INTRODUCTION

Urinary tract infection (UTI), which is caused by a wide variety of pathogens with changing antimicrobial susceptibility, is common among females and males of all age groups (Mathai, *et al.*, 2001, Nicolle, 2001, Stamm *et al.*, 2001, Fluit, *et al.*, 2000, Goldstein, 2000, Wagenlehner *et al.*, 2000). It is more common among women and hospitalized patients (Gupta *et al.*, 2001, Fluit, *et al.*, 2000, Chomarat, 2000). Advance in age of patients and the presence of an underlying disease render hospitalized patients susceptible to urinary tract infections. Nosocomial urinary tract infections have increased dramatically in the last two decades, as the pool of at-risk patients increased. Urinary tract infections are responsible for 40 % – 60 % of all hospital-acquired infections representing a significant cause of morbidity and mortality (Langermann *et al.*, 2001, Nicolle, 2001, Stamm *et al.*, 2001, Wagenlehner & Naber 2000, Jones *et al.*, 1999).

Most of the urinary tract infections are caused by Enterobacteriaceae, and *Escherichia coli (E. coli)* is the most common causative microorganism worldwide (Langermann *et al.*, 2001, Mathai *et al.*, 2001, Fluit *et al.*, 2000, Jones *et al.*, 1999, Malkawi *et al.*, 1996, Bettelheim, 1992, Shehabi, 1980). It accounts for 45 % – 65 % of all uropathogens causing a wide spectrum of clinical syndromes ranging from asymptomatic cystitis to pyelonephritis and sepsis (Bukharie *et al.*, 2001, Blazquez *et al.*, 1999). Virulence factors of uropathogenic *E.coli* that contribute to different stages in the pathogenesis of UTI include; adherence factors (P, type 1, Dr adhesions, fimbriae), toxins (lipopolysaccharide, hemolysin), aerobactin, invasion factors, and

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serum resistance (Nowicki *et al*., 2001, Abraham *et al*., 2001, Blazquez *et al*., 1999, Svenson *et al*., 1983). Specifically type 1 fimbriae are responsible for the attachment of *E. Coli* to mucosal cells (Abraham *et al*., 2001, Langermann *et al*., 2001, Blazquez *et al*., 1999, Bettelheim., 1992).

As antimicrobial drugs have been misused and overused worldwide, many microorganisms have fought back by selection of certain strains that are no longer susceptible to one or more of these drugs. (Trilla *et al*., 1996, Murray 1992). Recently, many uropathogens, whether isolated from a community acquired or a hospital-acquired urinary tract infection, have developed high-level of resistance to various antimicrobials in most countries (Gupta *et al*., 2001, Manges *et al*., 2001 *et al*., 2001, Chomarat, 2000, Jones *et al*., 1999, Shears *et al*., 1988).

There has been a serious increase in resistance of uropathogenic *E. coli* strains to amoxycillin/ampicillin, cotrimoxazole and trimethoprim, observed recently in developing and developed countries (Bukharie *et al*., 2001, Manges *et al*., 2001, Abu Shaqra, 2000, Chomarat, 2000, Fluit *et al*., 2000, Malkawi *et al*., 1996, Ahmad & Ahmad 1995). Moreover, high level resistance to ciprofloxacin in *E. coli* is associated with a reduced expression of type 1 and P – fimbriae and that ciprofloxacin-resistant *E. coli* is more frequently nonfimbriated than ciprofloxacin – susceptible *E. coli* (Abraham *et al*., 2001, Langermann *et al*., 2001, Goettsch *et al*., 2000, Blazquez *et al*., 1999).

It is well documented that transferable resistance plasmids contribute extensively to the wide spread resistance to antibiotic as well as to invasiveness among enteric bacteria, particularly *E.coli* (Eberhard, 1989), and *Salmonella typhimurium* (Vahabouglu, *et al*., 1996, Shehabi, 1995). Many antibiotic resistance determinants are

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carried by mobile genetic elements within plasmids called transposons. Furthermore, the strong selective pressure for nosocomial organisms to express antibiotic resistance

may cause such plasmids to spread rapidly among strains and even among different species and to persist for prolonged periods within hospitals (Manges *et al*., 2000, Vahaboglu *et al*., 1996, Shehabi, 1995). Plasmids may also be involved in the spread of virulence factors and may be selected for on this basis (Murray *et al*., 1995). Therefore, knowledge of the antimicrobial resistance patterns of common uropathogens, especially *E.coli* is essential in providing appropriate and cost-effective therapy.

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Aims of the study

The study aims to:

1- determine the antimicrobial resistance patterns of *E. coli* strains isolated from urine specimens of patients at the Jordan University Hospital (JUH) during the period 2000-2001.

2- investigate the resistance-plasmid profiles in multiresistant urinary *E.coli* isolates.

3- determine the prevalence of conjugative plasmids in urinary *E.coli* strains isolated from hospitalized patients at JUH.

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2. REVIEW OF LITERATURE

2.1. Urinary tract infection

Bacterial infections of the urinary tract affect both sexes of all age groups and they vary in clinical presentation from asymptomatic infection to severe systemic disease. The clinical diagnosis of pyelonephritis is frequently overlooked because of the absence of urinary tract symptoms and pyuria (Bukharie *et al*., 2001, Langermann *et al*., 2001, Blazquez *et al*., 1999, Finegold *et al*., 1978). The correlation of bacteriuria with unsuspected active pyelonephritis has been demonstrated at autopsy. The role of the indwelling catheter in the development of bacteriuria that is frequently accompained by gram–negative enteric bacteremia, has also been well documented in numerous studies (Mathai *et al*., 2001, Chomarat, 2000, Murray *et al*., 1995, Bettelheim, 1992). Indwelling urinary catheters increase patient's risk of developing bacteriuria and UTI and the incidence of catheter – associated bacteriuria increases by 5% each day the catheter is in place (Chomarat, 2000, Murray *et al*., 1995). A patient whose periurethral area is colonized with enteric bacteria or who has diarrhea is at increased risk of developing catheter associated bacteriuria. Whereas 70% of patients who develop catheter-associated bacteriuria eliminate the bacteria while the catheter is still in place, 30% develop symptomatic UTI (Murray *et al*., 1995).

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The shorter female urethra is proposed as an explanation for the higher frequency of UTI in women than in men. It has also been shown that sexual intercourse can cause a 10 – fold increase in bacterial numbers in the urine. Women attending a clinic for sexually transmitted diseases were found to have a slightly higher prevalence (4.9 %) of UTI than those of the general population. However, no relation with the number of sexual partners could be found (Bettelheim, 1992).

Microorganisms that commonly cause UTI include;

Escherichia coli, Klebsiella spp., *Enterobacter* spp., *Serratia* species, *Proteus* species, *Providencia* species, *Morganella* species, *Pseudomonas* species, *Salmonella* and *Shigella* species, *Alcaligenes* species, *Acinetobacter* species, *Enterococcus* species; mostly *Enterococcus faecalis*, Staphylococci; both coagulase positive and coagulase negative, Beta – hemolytic streptococci and *Candida albicans.*

E.coli is the most predominant organism isolated from urine specimens and its frequency varied in different studies but it ranged between 45 % and 65 % of all uropathogens (Mathai *et al*., 2001, Langermann *et al*., 2001, Fluit *et al*., 2000, Jones *et al*., 1999, Blazquez *et al*., 1999, Malkawi *et al*., 1996, Farah, 1994, Shehabi, 1980). Other common uropathogens include; *Enterococcus* spp. (12 % – 14%), *Klebsiella* spp. (7 % – 12 %), *Pseudomonas aeruginosa* (6 % – 8 %) and *Proteus mirabilis* (4 % – 7 %) (Mathai *et al*., 2000, Fluit *et al*., 2000, Jones *et al*., 1999, Farah, 1994, Shehabi, 1980).

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2.2. *Escherichia coli* in Urinary Tract Infection

2.2.1. Early reports: genus *Escherichia*.

Theodor Escherich first described this organism, which he isolated from the feces of neonates in 1885 and called it *Bacterium coli*. It was later renamed *Escherichia coli*, and for many years was considered to be a commensal organism of the large intestine (Bettelheim, 1992). The family Enterobacteriaceae was first proposed by Rahn in 1937 for the genus *Enterobacter* and despite some debate about nomenclature among bacteriologists, this family name was maintained (Bettelheim, 1992).

The characteristics of *Escherichia* that distinguish it from other enteric bacteria are that, it is a gram – negative, non spore forming, rod – shaped bacteria usually 0.5 x 1-3mm, occurring singly, in pairs, or in short chains and generally motile by peritrichously arranged flagella. It can grow well on ordinary laboratory media both under the presence or absence of oxygen (facultative anaerobe) and its metabolism can be either respiratory or fermentative, it is oxidase negative and catalase positive bacterium, forms gas from glucose, ferments lactose, produces indole in most cases, gives a positive Methyl-red reaction and a negative Voges–Proskauer reaction, does not utilize citrate, grows in KCN, decomposes urea and it liquefies gelatin (Bettelheim, 1992, Collee *et al*., 1989).

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Most strains of *E.coli* are fimbriated (80%). The fimbriae in most of them are of type 1, haemagglutinating, adhesive for epithelial cells and mannose sensitive. A few strains are encapsulated and many others form an abundant loose slime layer when

grown on sugar – containing medium at 15˚c – 20˚c (Langermann *et al*., 2001, Abraham *et al*., 2001,Collee *et al*., 1989).

2.2.2.Growth characteristics of *E.coli*

Most *E.coli* strains (90 %) ferment lactose rapidly, colonies on MacConkey agar are rose –pink in color, smooth, glossy and translucent (Collee *et al*., 1989). On Cystiene–Lactose–Electrolyte Deficient (CLED) agar, colonies are smooth, circular,

about 1 – 1.5 mm in diameter, and yellow opaque if lactose – fermenting and blue if non–lactose–fermenting. On blood agar, colonies of some strains are surrounded by a zone of haemolysis (Collee *et al*., 1989). *E.coli* is not inhibited by bile salt in MacConkey agar but is inhibited by the citrate in Leifson's Deoxycholate–citrate agar (DCA) and by sodium selenite, sodium tetrathionate, brilliant green, and other substances used in media selective for Salmonellae and Shigellae. Also, it is inhibited by 7% NaCl in salt media used for isolation of staphylococci (Murray *et al*., 1995, Collee *et al*., 1989, Finegold *et al*., 1978).

2.2.3. Biochemical reactions of *E.coli*

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The biochemical reactions listed in table (1) define those activities of *E.coli* and other species of *Escherichia* that can be used for their differentiation and characterization. However, these organisms have a far wider range of activities than those listed in this table.

The Indole, Methyl red, Voges – Proskauer, and Citrate (IMViC) tests were developed in order to distinguish strains of *E.coli* from related species that also produce acid and gas from the fermentation of lactose. Strains were considered *E.coli* if they were positive in the first two tests and negative in the second two. It is also distinguished from other related bacterial species by its ability to form gas from lactose after incubation at 44°c (thermotolerance test or Eijkman tests) (Bettelheim, 1992, Collee *et al*., 1989).

2.2.4. Antigenic structure of *E.coli*

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There are three kinds of surface antigens demonstrable in aggluti-nation tests and are observed by serotyping of *E.coli*; the O (somatic), K (capsular) and H (flagellar) antigens. Fimbrial antigens also take part in the agglutination reaction of bacilli that are phenotypically in the fimbriate phase, they are widely shared between strains of different serotypes, so that misleading cross – reactions may be obtained unless serotyping tests are done with bacilli from non – fimbriate – phase cultures (e.g. agar – grown cultures) or bacilli defimbriated by heating at 100˚c for 1 hour (Duguid, 1985).

For the antigenic classification of strains of *E.coli,* the primary subdivision is made according to the specific characteristic of the lipopolysaccharide O antigen of the cell wall. A different "O group" of the strains is defined by the presence of each different O antigen, the original classification included 25 O groups. The O groups are subdivided into serotypes according to the K and H antigens present. At least 100 different K antigens and over 50 different H antigens are known. The serotype of a

strain is defined by its full antigenic formula, including O, K, and H antigens. (Murray *et al*., 1995, Bettelheim, 1992, Collee *et al*., 1989).

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Table 1: Main biochemical characteristics of species of the genus *Escherichia* (Bettelheim, 1992)

Character	*E. coli*	*E. blattae*	*E. fergusonii*	*E. hermanii*	*E. vulneris*
Indole	98	0	98	99	0
Methyl red	99	100	100	100	100
Voges-Proskauer test	0	0	0	0	0
Citrate (Simmons)	1	50	17	1	0
Hydrogen sulfide (Triple Sugar Iron)	1	0	0	0	0
Urease	1	0	0	0	0
Phenylalanine deaminase	0	0	0	0	0
Lysine decarboxylase	90	100	95	6	85
Arginine dihydrolase	17	0	5	0	30
Ornithine decarboxylase	65	100	100	100	0
Motility (36°C)	95	0	93	99	100
Gelatin hydrolysis (22°C)	0	0	0	0	0
Growth in KCN	3	0	0	94	15
Malonate utilization	0	100	35	0	85
D-Glucose, acid	100	100	100	100	100
D-Glucose, gas	95	100	95	97	97
Fermentation of:					
Lactose	95	0	0	45	15
Sucrose	50	0	0	45	8
D-Mannitol	98	0	98	100	100
Dulcitol	60	0	60	19	0
Salicin	40	0	65	40	30
Adonitol	5	0	98	0	0
myo-Inositol	1	0	0	0	0
D-Sorbitol	94	0	0	0	1
L-Arabinose	99	100	98	100	100
Raffinose	50	0	0	40	99
L-Rhamnose	80	100	92	97	93
Maltose	95	100	96	100	100
D-Xylose	95	100	96	100	100
Trehalose	98	75	96	100	100
D-Mannose	98	100	100	100	100
Cellobiose	2	0	96	97	100
α-Methyl-D-glucoside	0	0	0	0	25
Erythritol	0	0	0	0	0
Mellibiose	75	0	0	0	100
D-Arabitol	5	0	100	8	0
Glycerol	75	100	20	3	25
Mucate	95	50	0	97	78
Esculin hydrolysis	35	0	46	40	20
Tartrate (Jordan's)	95	50	96	35	2
Acetate utilization	90	0	96	78	30
Lipase (corn oil)	0	0	0	0	0
DNase at 25°C	0	0	0	0	0
Nitrate reduction	100	100	100	100	100
Oxidase (Kovac's)	0	0	0	0	0
o-Nitrophenylgalactoside hydrolysis	95	0	83	98	100
Yellow pigment	0	0	0	98	50

•Each number gives the percentage of positive reactions after 2 days incubation at 36°C.
From Farmer et al. (1985a).

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2.2.5. *E.coli* as a urinary pathogen

According to the serotyping of *E.coli,* certain types were involved in UTI. An investigation of isolates during the course of chronic urinary tract infection showed in some instances that strains were subject to both serological and biochemical variation over time. In addition, it was shown that urine was a good medium for the growth of *E.coli*, and that the urine of pregnant women, who are more prone to UTI was particularly suitable for the growth of *E.coli* (Gupta *et al*., 1999, Malkawi *et al*., 1996).

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The most frequent serogroups of *E. coli* that were responsible for UTI include O1, O2, O4, O6, O7, O9, O11, O18, O39 and O75. The serogroups associated with urinary tract infection corresponded to those that predominate in faeces and this correlation led to the “Prevalence theory” that a limited number of O groups were involved simply because they were dominant in the colon which is the reservoir of the organisms. The alternative, “special pathogenicity theory” postulates that the *E.coli* strains which cause infection, in addition to being prevalent, are specially equipped to colonize the periurethral area and subsequently invade the urinary tract. The possession of one or both of two different structures (K antigens; fimbriae) has been demonstrated with increasing frequency in strains that cause asymptomatic bacteriuria, cystitis and pyelonephritis (70 % – 90 % of strains), in that order, and least often in faecal strains. This correlation has been best documented in childhood disease (Collee *et al*., 1989).

Bettelheim, K. (1992) found that the serogroups O6, O7, and O75 were the most common ones found in UTI. Also, in an investigation of bacteriuria in schoolgirls and women in the USA, it was noted that type O6 was the most frequent, followed by O75, O4, O1, O2, O7, O18, O9, while O11 was the least frequent. Some of these O groups have also been associated with outbreaks of UTI. An example of such outbreaks occurred in 1970 in Czechoslovakia when 39 neonates contracted UTI due to *E.coli* strain O4 (Veleminsky *et al*., 1974). A review of serotypes from Nigeria encountered the serotypes O2, O6, O75, O8, O15, O20, O21, O23 and O73 (Adetosoye, 1981).

2.2.6. Virulence factors in urinary *E.coli*

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Urinary tract infection can be caused by some strains of *E.coli* but not by others due to the presence of a number of extraintestinal virulence factors. Strains producing hemolysin, colicin V, serum sensitivity, and adherence to urinary epithelial cells may particularly be well adapted to causing UTI and could be considered to be particularly "aggressive " (Ranucci *et al*., 1992, Bettelheim, 1992).

Scherberich *et al*. (1977) showed that there may be specific receptors in the urinary tract, particularly the kidney, for certain type of *E.coli.* Also, Eden *et al*. (1977) showed that strains of *E.coli* isolated from cases of acute pyelonephritis adhered to uroepithelial cells. Epithelial cells from the periurethral region of healthy girls had a significantly lower mean number of the pyelonephritic strain of *E.coli* than those from girls prone to repeated UTI. Svenson *et al*. (1983) found that there was a correlation between the ability of strains of *E.coli* to cause UTI and their ability to adhere to uroepithelial cells which led to the observation that pyelonephritogenic *E.coli* agglutinated human A1, Rh^+ erythrocytes in a D – mannose – resistant manner.

By employing human erythrocytes of various blood group phenotypes, it was established that the fimbriae of the pyelonephritogenic *E.coli* were recognized by receptors related to the antigens of the human P blood group system. As a result, these fimbriae were named P fimbriae.

Particles to which this P- antigen – specific glycoside was attached could be used in a specific particle agglutination test to detect *E.coli* carrying P-fimbriae. Therefore, it appears that the P fimbriae are probably the most important virulence factor for pyelonephritogenic *E.coli*. (Blackwell *et al*., 1997, Bettelheim, 1992, Collee *et al*., 1989).

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2.3. Antibiotic – Resistance in *Escherichia coli* and related Gram-negative bacteria

Widespread use of antimicrobials in hospital and the community has been associated with the emergence of multidrug – resistant microorganisms, and that created an enormous challenge for clinicians, infection control personnel, and hospital administrators. It has been estimated that as much as 50% of antimicrobial use is inappropriate. Antimicrobial – resistant pathogens are becoming a prevalent cause of hospital – acquired infections, particularly in the intensive care units (ICUs), and are resulting in prolonged hospital stay, increased antimicrobial use and increased morbidity, mortality, and cost (Waterer and Wunderink, 2001 Shehabi *et al*., 2000, Blondeau and Vaughan, 2000, Flournoy *et al*., 2000, Jarvis, 1996).

2.3.1. Mechanisms of drug resistance

There are four mechanisms by which bacteria can resist the action of drugs as a result of spontaneous or induced mutations (Brooks *et al*., 1998): -

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1- Adoption by the organism of an alternative metabolic pathway in order to bypass the inhibited reaction, e.g., those bacteria resistant to sulfonamides adapt to using preformed folic acid.

2- Production of enzymes that destroy the antibiotic, e.g., penic-illinases (β–lactamases) which inactivate almost all types of penicillins and cephalosporins, or production of enzymes by some bacteria that inactivate aminoglycoside drugs by acetylation, adenylation, or phosphorylation.

3- Change in permeability or decreased uptake of the drug by the cell or some special parts of the cell like that which occurs for tetracycline's and polymyxins.

4- Altered target structure of the drug, so that it is no longer susceptible to the drug. Examples are macrolides and chloramphenicol where the affinity of the specific ribosomes for them decreases due to change in the 50S rRNA to which they bind.

Extended spectrum β–lactamases

Extended spectrum β–lactamases (ESBLs) are constantly evolving group of enzymes capable of inactivating a wide variety of β–lactam agents including expanded

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spectrum penicillins and cephalosporins. The enzymes prefer the oxyiminocephalosporins (ceftazidime, ceftriaxone, cefotaxime) and the monobactam aztreonam as substrates (Urban and Rahal, 1997, Jacoby, 1996). The genes for ESBLs are usually carried on plasmids and these often encode resistance traits for other classes

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of antibacterial agents as well (Jacoby, 1996). More important, once *Klebsiella* strains have mobilized these genes on plasmids, they can be disseminated rapidly to other members of Enterobacteriaceae including *E.coli,* and species of *Enterobacter, Citrobacter, Serratia* and *Proteus* (Urban and Rahal, 1997).

Expanded – spectrum cephalosporins (cefotaxime, ceftazidime) have been specifically designed to resist degradation by the older broad – spectrum β – lactamases such as TEM– 1, TEM– 2, and SHV– 1. With the use of these antibiotics in vivo, extended spectrum β– lactamases have been selected, these ESBLs most often are mutants of these older enzymes and carry a limited number of amino acid substitution (Silva *et al*., 2000, Jacoby *et al*., 1996). There is also a small but growing family of plasmid – mediated ESBLs that are not related to TEM or SHV β-lactamases, such as CTX– M and Toho, that preferentially hydrolyze cefotaxime and that belong to Ambler class A. In addition, there has been a World – wide emergence of novel β– lactamases, mainly among members of the family Enterobacteriaceae, that hydrolyze expanded – spectrum β- lactams. Most of these ESBLs are plasmid mediated and include the PER– 1, PER– 2, VEB– 1, CblA, and CepA enzymes (Girlich *et al*., 2001, Silva *et al*., 2000, Schiappa *et al*., 1996, Blazquez *et al*., 1993, Bingen *et al*., 1993, Sirot *et al*., 1991).

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Recently, Stapleton *et al*. (1999) found that carbapenem – resistance in *E.coli* can arise from high level expression of plasmid – determined CMY – 4 β- lactamase combined with an outer membrane protein (OMP) deficiency. Furthermore, in the

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presence of an OMP deficiency, the level of expression of a plasmid – mediated class C β – lactamase is an important factor in determining whether *E.coli* isolates are fully resistant to carbapenems or not. It has been observed that clinical bacterial strains that produce extended – spectrum β-lactamases are becoming more frequent in hospitals, and this type of resistance may be very difficult to detect by most methods used for testing antimicrobial susceptibility (Murray *et al*., 1995).

2.3.2. Mechanism of action of antibiotics

Owing to their specific inhibitory action on cell metabolic reactions, antibiotics have proved to be of great value in fundamental molecular biological research. The inhibitory action on a particular biosynthetic reaction by an antibiotic takes place by specific, covalent or non-covalent binding and results in the inactivation of a substrate, an enzyme or a factor. This inhibition leads to the cessation of growth or indeed to the death of the affected cell. To achieve this, often only very low concentrations of the antibiotics are required, it only requires a few molecules per cell for the most active antibiotics to inhibit the most susceptible microorganisms. Knowledge of the mechanism of antibiotic action often provides an explanation of the specificity of the action, and consequently the tolerance or toxicity of an antibiotic (Brooks *et al*., 1998).

2.4. *E.coli* plasmids and Transposons

2.4.1. *E.coli* plasmids

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Plasmids are nonessential, autonomous replicating units that are generally stable and maintained within the host cell. All known plasmids are circular molecules of DNA. They can vary in size from a few to several hundred kilobases (Kb), and the number of copies per cell can vary from one to hundreds per genome (Salyers *et al*., 1997, Bettelheim, 1992).

Plasmids are dispensable, that is, they are not required for survival of the cell in which they reside (Salyers *et al*., 1997). In many cases, however, they are essential under certain environmental conditions, such as in the presence of an antibiotic (Salyers *et al*., 1997, Gardner *et al*., 1991). The autonomous replication of the plasmids occurs in such a way that their copy number is maintained (Salyers *et al*., 1997). Plasmid replication is under the genetic control of the plasmid itself in the form of a negative feedback loop. Most natural plasmids are rarely lost from the cells (Bettelheim, 1992), whereas certain other plasmids can be spontaneously lost from or readily acquired by a host strain. Consequently, epidemiologically related isolates can exhibit common or different plasmid profiles (Salyers *et al*., 1997, Gardner *et al*., 1991). Since many plasmids carry antibiotic resistant determinants contained within mobile genetic elements (transposons) that can be readily acquired or deleted, the DNA composition of plasmid can change rapidly (Salyers *et al*., 1997). Furthermore, the strong selective pressure of antibiotics on nosocomial organisms to express antibiotic resistance may cause such plasmids to spread rapidly among strains and even among different species

and to persist for prolonged periods within an institution. Plasmids may also be involved in the spread of virulence factors and may be selected for on that basis. For

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example, among enterotoxigenic and enteroinvasive *E.coli* isolates, the pathogenic toxins, adhesion and invasion are often plasmid associated. Many clinical isolates lack plasmids and are therefore non-typeable; others carry only one or two plasmids, a situation that provides relatively poor discriminatory power for epidemiological studies (Murray, 1995).

The importance of plasmids has become increasingly recognized during the last two decades (Salyers *et al*., 1997, Gardner *et al*., 1991). They are known to have major practical significance in two areas: -

1- The spread of multiple antibiotic resistance in pathogenic bacteria.

2- The instability of industrially important microorganisms. i.e., in *streptococcus lactis* and related bacteria used in cheese processing, multiple plasmids have been identified and shown to carry gene coding for enzymes important in the fermentation processes involved in making cheese.

2.4.1.1. Types of bacterial plasmids: -

Three major types of bacterial plasmids have been extensively studied (Gardner *et al*., 1991): -

1- **F–plasmids,** the F factors responsible for DNA transfer during conjugation. It codes for at least nine proteins and contains two origins of replication (*ori* V) and

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(*ori* S) but it is not known whether both origins function in replication (Bettelheim, 1992).

2- **R–plasmids**, DNA molecules carrying genes for resistance to various antibacterial drugs.

3- **Col–plasmids**, plasmids that code for proteins called colicins, which kill sensitive *E.coli* cells.

2.4.1.2. Transfer of plasmids by conjugation

Conjugation was discovered in 1946 by J. Lederberg and E.L. Tatum who are the 1958 Nobel Prize corecipients. During conjugation, DNA is transferred from a donor cell to a recipient cell through a specialized intercellular connection, or conjugation tube, that forms between them (Fig.1). The donor and recipient cells are sometimes referred to as male and female cells, respectively. The transfer of genetic information is thus a one-way transfer during conjugation. Cells that have the capacity to serve as donors during conjugation are differentiated by the presence of specialized cell-surface appendages, rope-like fibers, called F pili (fig. 1). The synthesis of these F pili is controlled by nine genes that are carried by a small circular molecule of DNA "minichromosome" with about 94.500 base-pairs long called F factor (fertility factor, also called sex factor or F plasmid).

There are three states of an *E.coli* cell with respect to the F factor (Fig. 2): -

1) **F^- cell**, containing no F factor.

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2) **F^+ cell**, containing an autonomously replicating F factor.

3) **Hfr cell** (high-frequency recombinant), containing an F factor integrated into the *E. coli* chromosome.

Cells carrying an F factor (donor cells) form a conjugation tube and initiate DNA transfer after making contact with a cell not carrying an F factor, called F^- cell (recipient cell). Conjugation occurs when an Hfr or an F^+ cells contacts an F^- cell and forms a conjugation tube. When F^+ mates with F^-, only the F factor is transferred, whereas in Hfr mating, the Hfr chromosome is nicked within the integrated F factor, and a sequential transfer of chromosomal gene follows. (Fig.3). The integration of the F factor is believed to be mediated by short DNA sequences called IS elements (insertion sequences), each of which is about 800 – 1400 base pairs in length (Gardner *et al.*, 1991).

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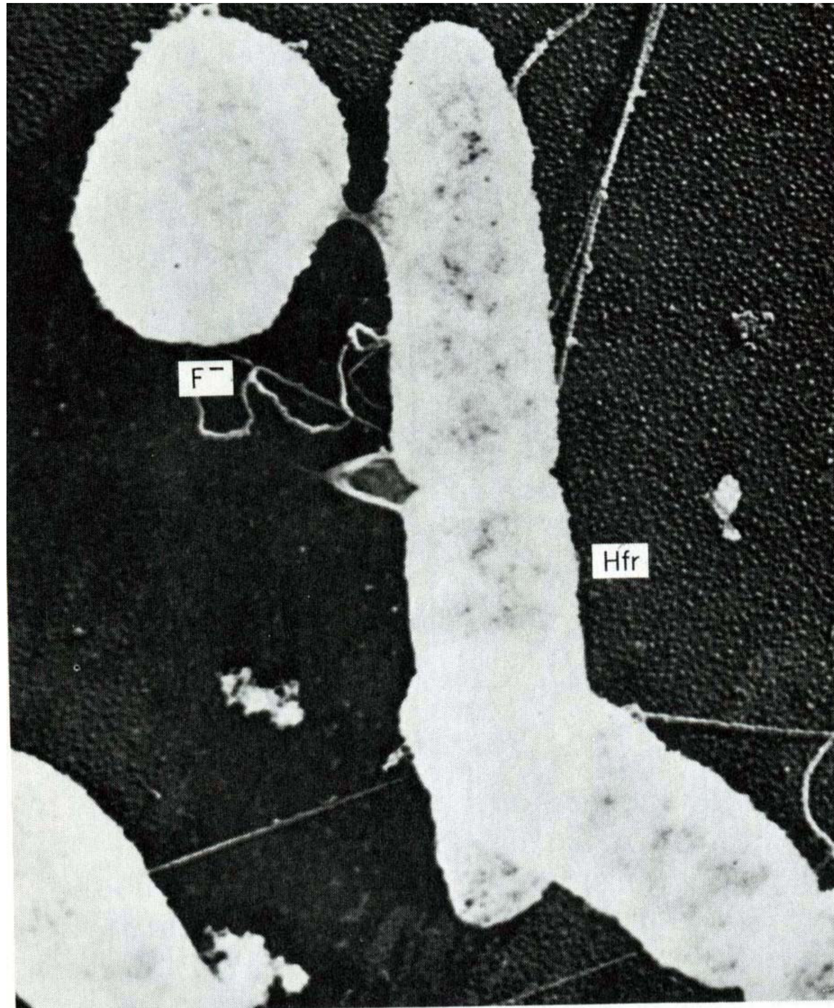

Figure 1: An electron micrograph of conjugating *E.coli* cells. The elongated donor (Hfr) cell (on the right) is undergoing cell division. It is joined to the short, plump recipient (F^-) cell (top left) by a conjugation tube, through which the Hfr chromosome passes. The rope-like fibers on the Hfr cell are F pili (Gardner *et al*., 1991).

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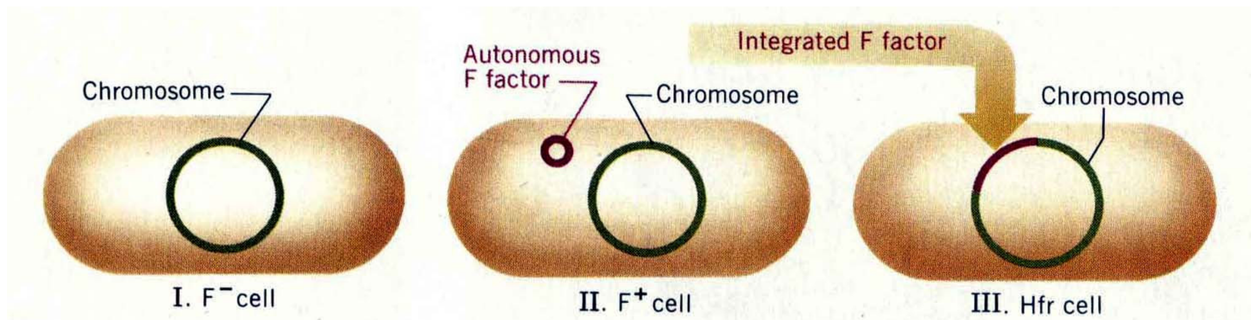

Figure 2: The three states of an *E.coli* cells with respect to the F factor: (i) F^- cell (ii) F^+ cell (iii) Hfr cell (Gardner *et al*., 1991).

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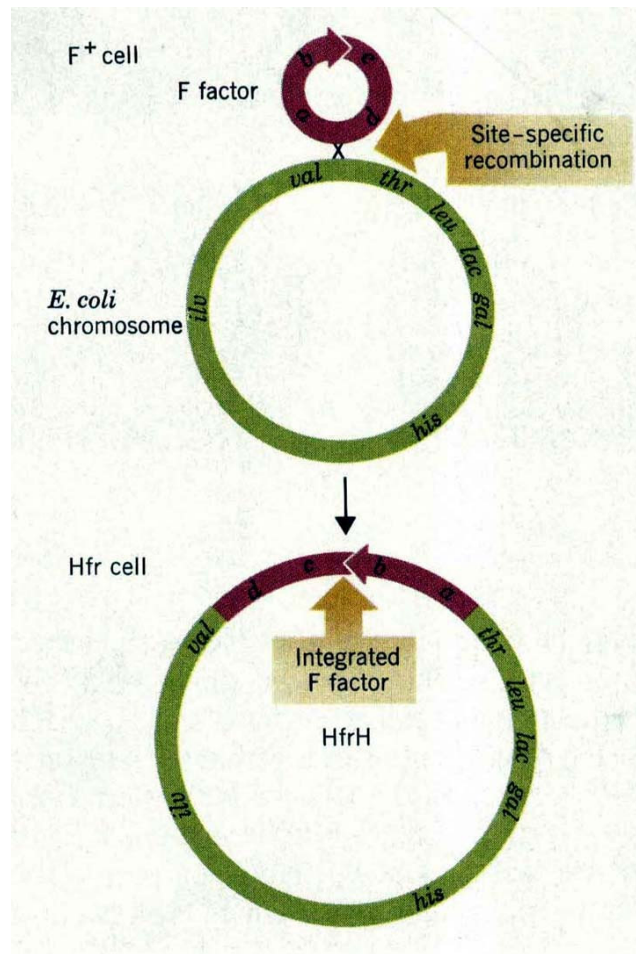

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Figure 3: Conversion of an F^+ cell to an Hfr cell by the integration of the autonomous F factor into the host chromosome through sit – specific recombination (IS elements) (Gardner *et al*., 1991).

2.4.1.3. Mechanism of DNA transfer during conjugation (Fig. 4):

1- After cell contact, a conjugation tube forms between the two cells.

2- An endonuclease cleaves one strand of DNA at the unique sit (the "origin" of transfer) on the F factor (either integrated in the case of an Hfr or autonomous in the case of an F^+ cell).

3- The 5′ end of the cleaved strand is then displaced, as in normal rolling circle replication, except that during conjugation, the 5′ end moves through the conjugation tube into the recipient cell.

4- Transfer occurs concurrently with, possibly driven by, rolling circle replication, with the intact circular strand serving as a template for the synthesis of complementary strand in the donor cell and the transferred linear strand being replicated discontinuously (by the synthesis of short 5′ → 3′ Okazaki fragments that are then joined by DNA ligase in an over all 3′ → 5′ reaction) in the recipient cell immediately after transfer.

5- In F^+ by F^- mating, both exconjugants will be F^+, since both will have a complete copy of the F factor. In the Hfr by F^- mating, the donor cell will remain an Hfr and

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the recipient cell will usually remain F^-, since a portion of the integrated F factor is the last segment of DNA to be transferred.

6- After conjugation is completed, the conjugation tube and chromosome usually break spontaneously in Hfr by F^- mating before the entire chromosome is transferred. In the rare cases where the entire Hfr chromosome is transferred, the recipient cell becomes an Hfr after conjugation (Bettelheim, 1992, Gardner *et al*., 1991).

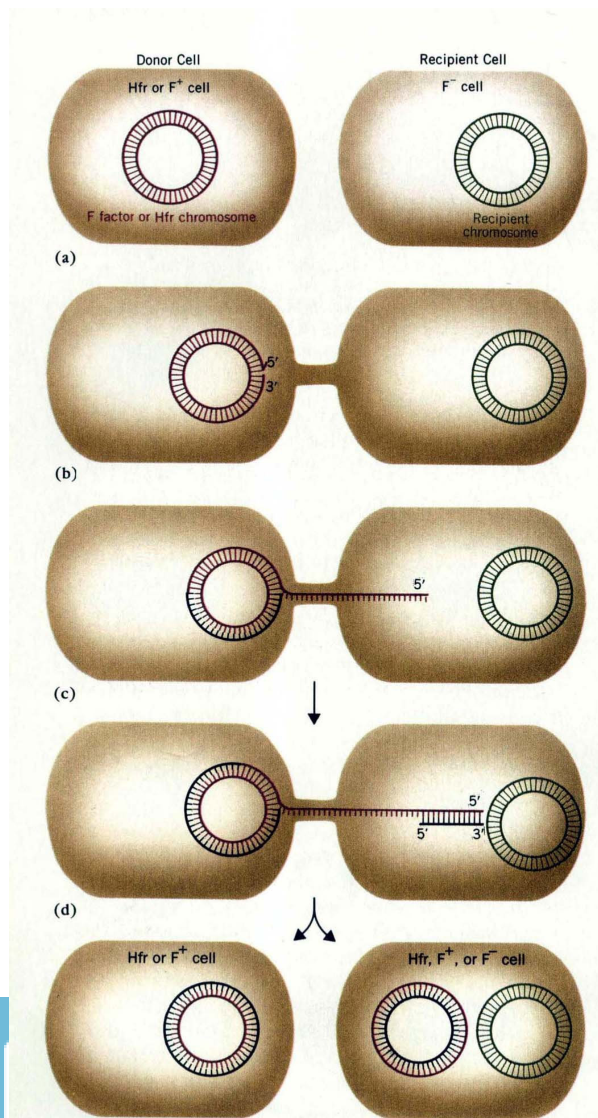

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Figure 4: Mechanism of DNA transfer during conjugation. Donor cell represents if it is either a Hfr cell or F^+ cell (Gardner *et al*., 1991).

2.4.2. Transposons

Conjugative transposons are DNA segments, ranging in size from 18 to over 150 kbp, which are normally integrated into the bacterial genome (Salyers *et al*., 1997, Gardner *et al*., 1991). Many genes are carried on them such as *tet*M*, tet*Q*, erm*F*,* and *erm*G. To transfer conjugative transposons, they first excise themselves to form a nonreplicating circular intermediate. The circular intermediate is then transferred by conjugation to a recipient, where it integrates into the recipient genome. Integration is mediated by an integrase carried on the conjugative transposon. Conjugative transposons differ from conventional transposons in that they have a circular intermediate, transfer by conjugation, and do not create target site duplication when they integrate (Salyers *et al*., 1997).

Transposons were first discovered in gram-positive cocci and *Bacteroides* species (Salyers *et al*., 1997), but now they are being found in a variety of bacterial

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genera including the enteric bacteria and it is likely that they are as widespread and diverse as self-transmissible plasmids (Salyers *et al*., 1997). The relatively recent discovery of conjugative transposons (Salyers *et al*., 1997), at a time when conjugal transfer had become synonymous with plasmid transfer, raises the question of whether there are still other types of conjugal gene transfer elements that have yet to be identified. Plasmids and conjugative transposons are proving to be very interactive gene transfer elements. It has been known that self-transmissible plasmids, such as the IncP plasmid can mobilize other plasmids residing in the same donor strain, either by providing the mating apparatus through which the other plasmid moves (*trans* mobilization) or by forming a cointegrate with the other plasmid (*cis* mobilization)

(Salyers *et al*., 1997). Recent studies have shown that plasmid mobilization can occur even if the mobilizing plasmid and the plasmid being mobilized are in two different bacterial cells (Salyers *et al*., 1997).

2.5. Detection and isolation of *Escherichia coli* plasmids

2.5.1. Rapid procedures: -

There are different techniques used for the isolation plasmids, all of which have the same principle (Lewin, 1997, Lodish *et al*., 1995, Akada, 1994, Takahashi and Nagano, 1984, Kado and Liu, 1981, Birnboim and Doly, 1979). This involves:

1) Growing bacteria in enrichment broth (like L-broth) overnight at 30ºc – 37ºc to get as much a high copy number of plasmids as possible .

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2) Lysing the cells by using lysing solution to allow the cell contents to be released.

3) Precipitation of proteins, high molecular weights RNA and chromosomal DNA.

4) Extraction and precipitation of plasmids.

5) Determining the quality of isolated plasmids by agarose gel electrophoresis.

6) Determining purity by digestion with restriction endonucleases. The three most commonly used methods are the following:

1- **Birnboim and Doly Method (1979**). This method is suitable for a rapid alkaline extraction procedure for screening recombinant plasmid DNA. The bacterial cells is treated with lysozyme to weaken the cell wall and then lysed completely with alkaline

sodium dodecyl sulfate (SDS) and NaOH. Chromosomal DNA, still in very high molecular weight form, is selectively denatured and when the lysate is neutralized by acetic sodium acetate (pH 4.8), the mass of chromosomal DNA renatures and aggregates to form an insoluble network. Simultaneously, the high concentration of sodium acetate causes precipitation of protein-SDS complexes and of high molecular weight RNA. In this way, most of the three major contaminating macromolecules are

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co-precipitated and may be removed by a single centrifugation in a bench-top centrifuge. Plasmid DNA, and residual low molecular weight RNA, are recovered from the supernatant by ethanol precipitation. Plasmid DNA may be analyzed by gel electrophoresis either intact in the covalently closed circular (CCC) form or after digestion with a restriction enzyme.

2- **Kado and Liu Method (1981**). This method is directed for detection and isolation of large (up to 350 megadaltons) and small (2.6 megadaltons) plasmids. The method utilizes the molecular characteristics of covalently closed circular deoxyribonucleic acid (CCC DNA) that is released from cells under conditions that denature chromosomal DNA by using alkaline sodium dodecyl sulfate (pH 12.6) at elevated temperatures. Proteins and cell debris are removed by extraction with phenol-chloroform. Under these conditions, chromosomal DNA concentrations are reduced

or eliminated. The purified extract is used directly for electrophoretic analysis. This procedure also permits the selective isolation of plasmid DNA that can be used directly in nick translation, restriction endonuclease analysis, transformation, and DNA cloning experiments.

3- **Takahashi and Nagano Method (1984).** They have developed a rapid isolation method of plasmid DNA for application in epidemiological studies. By this method,

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plasmid DNA ranging in molecular weight between 2.0 and $122X10^6$ could be detected. This procedures involves the following steps:-

1) Lysis of cells by lysostaphin.

2) Neutralization of solution by using sodium acetic acid (pH 5.5) which renatures the mass of chromosomal DNA and aggregates it to an insoluble network.

3) Addition of an equal volume of chloroform to remove proteins and cell debris.

4) Precipitation of plasmid DNA by ethanol.

The plasmid DNA obtained could be directly used for restriction endonuclease analysis without further purification.

2.5.2. Dot blot technique: -

It is an efficient technique commonly used to detect a bacterial colony carrying a particular DNA clone (plasmid of interest) by pressing a piece of absorbent paper on Petri dish with colonies of bacteria containing recombinant plasmids to get a replica. This replica is treated with alkali to disrupt the cells and denature DNA to get SS DNA, and then hybridized to a highly radioactive DNA probe specific to the plasmid

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of interest. These bacterial colonies that have bound the probe are identified by autoradiography (Lewin, 1997).

2.5.3. In Situ hybridization: -

It is used to detect the location of specific genes on chromosomes. Denaturation of the bacterial DNA with alkaline solution to get SS DNA is done first, then a radiolabeled probe specific to the gene of interest is added and detected by an X-ray film (autoradiograph) (Lodish *et al*., 1995).

2.5.4. Polymerase chain reaction (PCR): -

It is the best-studied and most widely used target amplification technique. This method uses repeated cycles of oligo-nucleotide-directed DNA synthesis to perform in vitro replication of target nucleic sequences. The oligonucleotide whose sequence is determined by the target nucleic acid is synthesized to be complementary to its annealing sites with the two different strands forward and reverse strand. Each cycle of PCR consists of three steps: (i) denaturation step, in which

the target DNA is incubated at high temperature so that the target strands are melted apart and thus made accessible to hybridization by specific oligonucleotide primers. (ii) an annealing step, in which the reaction mixture is cooled to allow the primers to anneal to their complementary target sequences, and (iii) an extension reaction, usually done at an intermediate temperature, in which the primers are extended on the DNA template by a DNA polymerase. These three incubation steps are linked in what is referred to as a

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thermal cycler. A typical PCR protocol comprises 30 to 50 thermal cycles. Each time a cycle is completed there is a theoretical doubling of the target sequence. Thus, repeating the thermal cycle results in a geometric accumulation of amplified target sequences (Lewin, 1997, Lodish *et al*., 1995).

Study protocol

This is a prospective study that consists of

1- Collection of 160 multiresistant *E.coli* isolates from urine cultures of inpatients and outpatients admitted to JUH during the period 2000-2001.

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2- Performance of conjugation tests to detect the presence of transferable resistant-plasmid by eight different culture conditions.

3- Plasmid screening of multi-resistant *E.coli* isolates by using two methods for extraction of plasmid DNA.

4- Determining the minimal inhibitory concentrations (MICs) of representative multiresistant *E.coli* isolates to commonly used antibiotics (ampicillin, ciprofloxacin, cotrimoxazole, gentamicin, nalidixic acid, and tetracycline).

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3. Materials and methods

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3.1.1. Materials and Reagents

- MacConkey agar – Oxoid, Unipath Ltd., Hampshire, England.
- Brain Heart infusion broth – Oxoid, Unipath Ltd., Hampshire, England.
- L B-broth – Difco, USA.
- Mueller-Hinton agar – Oxoid, Unipath Ltd., Hampshire, England.
- Mueller-Hinton broth – Oxoid, Unipath Ltd., Hampshire, England.
- Kligler`s iron agar – Oxoid, Unipath Ltd., Hampshire, England.
- SIM medium – Oxoid, Unipath Ltd., Hampshire, England.
- Urea agar – Oxoid, Unipath Ltd., Hampshire, England.
- Simmon citrate agar – Oxoid, Unipath Ltd., Hampshire, England.
- Absolute Ethanol – Labscan Ltd., Dublin, Ireland.
- Chloroform – Sigma, USA.
- Glycerol – Merk, Germany.
- Phenol – Sigma, USA.
- Glacial acetic acid – Merk, Germany.
- Sodium dodecyl sulfate (SDS), Promega, USA.
- Sodium citrate, Gibco – BRL, Scotland.
- Sodium hydroxide, Riedl – de Haen AG, Germany.
- Di-Sodium EDTA, Biochemical, BDH, England.
- Sodium acetate, Gibco – BRL, Scotland.
- D. Glucose an hydrous, Promega, USA.
- Sucrose, Promega, USA.
- Tris – base, Promega, USA.

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- Tris – HCl, Sigma, USA.
- LE agarose, Promega, USA.
- Boric acid, Riedl – de Haen, Germany.
- Ethidium bromide, Sigma, USA.
- Bromophenol blue, Sigma, USA.
- Lysozyme powder, Promega, USA.
- Nuclease – free water, Promega, USA.
- Antibiotics powder, Dar Al-Dawa, Jordan.
- Antibiotic disks, Mast groups Ltd., UK.
- Sterile (autoclaved) distilled water.
- Lambda DNA/*Eco*R I +*Hind* III Markers, Promega, USA.
- Lambda DNA, Promega, USA.
- *Hind* III Restriction enzyme, Promega, USA.
- Blue/orange 6X Loading Dye, Promega, USA.
- DNA Purification System A 7500, Promega, USA.

The system consist of the following components:

1- Resuspension solution (50 mM Tris (pH 7.5), 10 mM EDTA, 100 ug/ml RNase A).

2- Cell lysis solution (0.2 M NaOH and 1% SDS).

3- Wizard Minipreps DNA purification Resin.

4- Neutralization solution (1.32 M potassium acetate).

5- Column wash solution (80 mM potassium acetate, 8.3 mM Tris –HCl (pH 7.5), 40 uM EDTA and 55% ethanol).

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6- Wizard Minipreps Column.

- Cryotubes (1.8 ml) – AB gene House, Epsom, UK.
- Eppendorf tubes (1.5 ml), AB gene House, Epsom, UK.
- A calibrated 0.001 ml loop of approximately 10^7 CFU/ml.
- *E.coli* standard strains

1- *E.coli* strain V517 (Marcina *et al*., 1978), containing eight reference plasmids was used for plasmid isolation. Its plasmids are not coded for antibiotic resistance.

2- *E.coli* K12 strain (1485-1 F^- Nal ^{R}) was used as a recipient in conjugation tests (Shehabi, 1984).

3- *E.coli* strain ATCC 25922 was included for quality control in antibiotic susceptibility of both diffusion and MIC tests.

3.1.2. Equipment's

- Water bath with Shaker, Clifton, Germany.
- Laminar Flow Cabinet.
- Electrophoresis system: Onchor probe tech I – Gaithersburg, USA.
- UV Camera, Monitor and Printer, UVP, USA.

3.2. Methods

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3.2.1. Urine culture

The first phase of this study included collection of culture results of all urine specimens received by the clinical bacteriology laboratory/JUH during the period from Jan., 2000 through Dec., 2001 for culture and susceptibility testing. Specimens were cultured on MacConkey and blood agar plates and incubated for 24 hours at 37°c. Bacterial species were identified using standard biochemical tests. Urine cultures were generally recorded positive if their bacterial growth showed the presence of $\geq 10^5$ colony forming unit (CFU)/ml unless clinical data indicated UTI where cultures with lower counts were also included. Positive cultures were submitted for antibiotic susceptibility using disk diffusion method. Susceptibility results were expressed as susceptible or resistant (NCCLS, 1997). Urine culture results were classified according to the following characteristics:

1- Pattern of organisms isolated.

2- Classification of *E.coli* isolates in relation to the patient's sex, and whether infection is community or hospital acquired.

3- Antimicrobial resistance patterns of *E.coli* isolates.

3.2.2. Multiresistant *E.coli* isolates

The second phase of the study included collection of 160 multiple antibiotics – resistant *E.coli* strains (resistant to 2 or more antibiotics) which were randomly obtained from positive urine cultures of inpatients and outpatients of all age groups. The

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E.coli isolates were confirmed by biochemical standard tests; including Kligler`s iron agar, citrate utilization, indole and urease production (Brooks *et al*., 1998, Collee *et al*., 1989).

Each *E.coli* isolate was subcultured on MacConkey agar plate and incubated for 18 - 24 hours at 37˚c. Eight to ten colonies of each pure culture of *E.coli* were taken and inoculated in one ml of brain heart infusion broth containing 40 % glycerol and then stored at – 20˚c for further investigation.

3.2.3. Bacterial conjugation

53 out of 160 *E.coli* isolates were included in the conjugation experiments. Three out of 53 were highly resistant to nalidixic acid as judged by the MIC test, 50 were sensitive to nalidixic acid and were used in conjugation experiments. Recipient *E.coli* strain (K12) was resistant to nalidixic acid only. All isolates and control strains were tested for antibiotic susceptibility prior to use.

3.2.3.1. Sample preparation:

All *E.coli* strains were inoculated in Brain heart infusion broth and incubated at 37˚c for 2 – 3 hours before they were subcultured on MacConkey agar and incubated at 37˚c for 18-24 hours before used.

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3.2.3.2. Bacterial conjugation procedures

Eight procedures of conjugation were performed to detect the best results for transfer R – plasmids from multiresistant *E.coli* strains (donors) to *E.coli* K12 (recipient).

1- A Donor to recipient ratio of 1: 2, overnight incubation at 37˚c without shaking (Shehabi, 1984).

2- A Donor to recipient ratio of 1: 2, overnight incubation at 37˚c with shaking (Mamun *et al*., 1993).

3- A Donor to recipient ratio of 1: 2, overnight incubation at 30˚c without shaking (Mamun *et al*., 1993).

4- A Donor to recipient ratio of 1: 2 with overnight shaking at 30˚c.

5- A Donor to recipient ratio of 1: 10, overnight incubation at 37˚c without shaking.

6- A Donor to recipient ratio of 1: 10 with overnight shaking at 37˚c.

7- A Donor to recipient ratio of 1: 10, overnight incubation at 30˚c without shaking (Silva *et al*., 2000).

8- A Donor to recipient ratio of 1: 10 with overnight shaking at 30˚c.

The steps that were followed up in all studies were:

1- Few colonies of each *E.coli* donor strain and *E.coli* K12 were inoculated separately in brain – heart infusion broth and incubated overnight at 37˚c.

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2- Each of the mixtures was diluted 100 – fold with fresh broth and incubated separately in a sterile screw cap glass tube for four hours with shaking and airiation.

3- Mixing of Donor and recipient cells with each other's in a proportion of 1: 2 and 1: 10 (donor to recipient) and incubating the mixture overnight at 30˚c and 37˚c with (150 rev/ min.) and without shaking.

4- A volume of 1 - 2 ul of mixtures was spread on MacConkey agar plates containing a mixture of nalidixic acid (50 mg/L) and ampicillin (25 mg/L), and incubated at 37˚c for 18 hours.

5- Growth of *E.coli* strains on selective MacConkey agar plates indicates a successful R-plasmid transfer process. The *E.coli* transconjugants were investigated for transfer of one or more antibiotic resistance factors by antibiotic susceptibility tests using the disk diffusion method.

3.2.3.3. Antibiotic susceptibility tests

a- The disk diffusion method using Mueller – Hinton agar (Kirby – Bauer, 1966) has been used for confirmation of resistance factor transfer in transconjugants.

b- Minimal Inhibitory Concentrations (MICs) were determined by the agar dilution method. Serial two fold dilutions of antibiotics were made in Mueller – Hinton agar. The bacterial suspensions used were 4 – 5 hours

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Mueller – Hinton broth cultures. A calibrated 0.001 ml loop of approximately 10^7 CFU/ml was spotted onto the surface of agar plates.

MICs and inhibition zones for each antibiotic were interpreted according to guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 1997).

The antimicrobial agents used were ampicillin (A), ciprofloxacin (Cip), cotrimoxazole (Ts), gentamicin (GM), nalidixic acid (NA), and tetracycline (T).

- *Esherichia coli* ATCC 25922 was used as a standard control strain

3.2.4. Rapid screening of plasmid DNA

Three methods for the extraction of plasmid DNA were used:

a) Alkaline lysis method of Birnboim and Doly (1979).

b) DNA purification system, A7500 (Promega, USA).

c) Quick-check method to test the size of *E.coli* plasmids (Akada, 1994).

3.2.4.1. Alkaline lysis method of Birnboim and Doly

The following reagents were prepared:

a- **reagent I: lysozyme solution**. 2 mg /ml Lysozyme, 50 mM Glucose, 10 mM EDTA, 25mM Tris–Hcl (pH 8.0). Prepared fresh daily from crystalline lysozyme and stock solutions of the other components. Stored at 0˚c.

b- **reagent II: alkaline SDS solution**. 0.2 N NaOH, 1 % Sodium dodecyl sulfate (SDS). Stored at room temperature and stable for about 1 week.

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c- **reagent III: high salt solution**. 3M sodium acetate (pH 4.8). Prepared by dissolving 3 moles of sodium acetate in a minimal volume of water, adjusted to a pH of 4.8 with glacial acetic acid and then volume was adjusted to 1 L before being stored at room temperature.

Procedure

- After sample preparation of each frozen *E.coli* isolate and *E.coli* strain V517 as a control, selected colonies were grown in 2.5 ml of LB-broth containing 100 ug /ml ampicillin for each isolate but without ampicillin for control *E.coli* strain.
- After 18 hours of incubation, heavy growth was obtained and 0.5ml of culture was transferred to a 1.5 ml Eppendorf tube for plasmid extraction, and the rest was stored at – 20˚c after the addition of 40% glycerol.

All steps were carried out at room temperature unless otherwise indicated.

- The tubes were then centrifuged at 10000 rpm for 15 seconds. The supernatant was carefully aspirated with a fine-tip pipette and the cell pellets were thoroughly suspended in 100 ul of solution I.
- After 30 minutes of incubation at 0˚c; 200 ul of solution II was added and the tube was gently vortexed. The suspension became almost clear and slightly viscous. The tubes were maintained for 5 minutes at 0˚c.
- 150 ul of solution III was added. The contents of the tube were gently mixed by inversion for a few seconds where a clot of DNA formed. The tubes were maintained at 0˚c for 60 minutes to allow most of the protein, high molecular weight RNA and

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chromosomal DNA to precipitate. Centrifugation for 5 minutes yielded an almost clear supernatant.

- 0.4ml of the supernatant was aspirated and transferred to a second centrifuge tube. One ml of cold ethanol was added to each tube and kept at – 20˚c for 30

minutes. The precipitate was collected by centrifugation at 10,000 rpm for 2 minutes and the supernatant was aspirated.

- The pellet was dissolved in 100 ul of 0.1M sodium acetate / 0.05M Tris – HCl (pH 8.0) and reprecipitated with 2 volumes of cold ethanol. After 10 minutes at – 20˚c, the precipitate was again collected by centrifugation as before.
- The pellet was dissolved in 40 ul distilled water and then 10ul of 5X sample buffer was added (5X sample buffer contained 25% Sucrose, 5mM Sodium acetate, 0.05% Bromophenol blue, 0.1% SDS).
- 10 to 20 ul of the dissolved pellet was applied to an agarose gel for electrophoretic analysis.

3.2.4.2. Extraction of plasmid DNA using DNA Purification System Procedure

- Sample preparation was performed using frozen *E.coli* isolates and *E.coli* V517 as control. Selected colonies were grown in 3ml of LB-broth. Two methods were used in sample preparation:

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a) A 3ml of LB-broth containing 100 ug / ml ampicillin was used for all *E.coli* samples except control (*E.coli* V517).

b) A 3 ml of L-broth without ampicillin was used for all *E.coli* samples and control (*E.coli* V517).

- After Incubation at 37˚c for 18 hours heavy growth was obtained.
- Following centrifugation of bacterial broth cultures for 1-2 minutes at 10,000 rpm in a microcentrifuge, the supernatants were poured off and the tubes kept upside – down on a paper towel to remove excess media. The cell pellets were completely resuspended in 200 ul solution, and the cells were transferred to a 1.5 ml Eppendorf tube.
- A 200 ul cell lysis solution was added and mixed by inverting the tube 4 times. The cell suspension became clear immediately.
- A 200ul of neutralization solution was added and mixed by inverting the tube 4 times. The lysate was centrifuged at 10,000 rpm in a microcentrifuge for 5 minutes, and the cleared lysate was carefully removed to a new tube.
- For each sample, one minicolumn of 3ml disposable syringe was used, and 1 ml of the resuspended resin was pipetted into the barrel (after warming the resin to 37˚c for 10 minutes, and cooling to 30˚c).
- All of the cleared lysate of each sample was transferred into the barrel of the minicolumn /syringe assembly containing the resin. The syringe plunger was inserted carefully and the slurry was pushed gently into the minicolumn. The syringe from the minicolumn was detached and the plunger from the syringe barrel was removed. The syringe barrel was reattached to the minicolumn.

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- A 2ml of column wash solution was pipetted into the barrel of the minicolumn/syringe assembly, and the plunger was inserted into the syringe and the column wash solution gently pushed through the minicolumn.
- The syringe was removed and the solution was transferred from minicolumn to a 1.5ml Eppendorf tube. The tube was centrifuged at 10,000 rpm for 2 minutes to dry the resin. Finally the minicolumn was transferred to a new 1.5 ml Eppendorf tube

and 50ul of pre-heated at 75°c nuclease – free water was added to the minicolumn. After 1 min, it was centrifuged at 10,000 rpm for 20 seconds to elute the plasmid DNA, and stored at – 20°c.

3.2.4.3. Quick – Check method to test the size of *E.coli* plasmid

Procedure: -

- Overnight *E.coli* liquid culture was grown in LB broth with or without addition of antibiotics. 100 ul of culture broth and 50ul of phenol / chloroform was added in a ratio of 1:1 in an Eppendorf tube.
- 10 ul of loading dye was added (0.25 % Bromophenol blue and 40 % Glycerol) and the mixture was vortexed for 10 seconds.
- Centrifugation at 10000 rpm was performed for 3 minutes. The supernatant was aspirated and transferred to a new Eppendorf tube and stored at 4°c.

3.2.4.4. Gel electrophoresis

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A 10x TBE buffer stock solution was prepared by dissolving 55 gram Boric acid, 108 gram Tris base, and 7.4 gram EDTA in 0.5 litter of distilled water followed by adjusting to pH 8 in a final volume of 1 litter. For usage; one part of stock solution was diluted with 9 parts of distilled water. A 0.7 gram of agarose was dissolved in 100 ml of 1 x TBE buffer by boiling. After the agarose completely dissolved, it was cooled to 50˚c and 2.5 ml of 1 % ethidium bromide was added. The same concentration of ethidium bromide was added to the running buffer.

sample size: 20ul sample + 4 ul 6 x loading dye. Control *E. coli* strain was treated as the *E. coli* isolates.

marker size: 2ul (λ DNA *Eco*R I + *Hind* III) + 2 ul 6 x loading dye + 8 ul of distilled water.

Agarose gel electrophoresis was run for 2 hours at 95 volts using horizontal electrophoresis apparatus.

3.2.4.5. Restriction enzyme digestion

Plasmid DNA and lambda DNA marker were restricted by *Hind* III restriction enzyme.

- **Restriction conditions:** for each 25 ul sample, 2.5 ul of restriction enzyme buffer, 0.25 ul of Bovine serum albumin (as activator) and 0.4ul of *Hind* III enzyme were added and incubated overnight at 37˚c.
- Lambda DNA marker treated as a sample with the exception that 10 ul instead of 25ul samples was loaded in the gel electrophoresis.

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3.2.5. Statistical Analysis

All data were entered in a computer database and analyzed using the SPSS statistical program to compare variables, using two tailed paired- samples T-test with significant cut – off value of 0.05 (P- value < 0.05 significant, while P- value > 0.05 non-significant).

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4. Results

4.1. Urine culture results

The results of urine cultures as recorded at the clinical bacteriology laboratory of JUH during the two year study period are shown in Table 2 and 3. A total of 3583 out of 12021 (29.8 %) and 3140 out of 11328 (27.7 %) urine cultures were found positive for significant bacterial growth during the years 2000 and 2001 respectively.

The ratio of *E. coli* isolates between outpatients and inpatients was 3: 1 and approximately similar in both years, as shown in Table 4. The ratio of male patients to female patients was 1: 4 in the 2- year study (Table 5).

4.2. Antimicrobial Susceptibility results

The antimicrobial susceptibility patterns of 1160 *E. coli* isolates in the years 2000 and 1175 isolates in 2001 are presented in Table 6. Rates of susceptibility of *E. coli* isolates for the 2 years were 80 % and 78 % to nitrofurantoin, 63 % and 67 % to cefuroxime, 63 % and 59 % to norfloxacin, 51 % and 53 % to gentamicin, 39 % and 27 % to augmentin, 25 % and 26 % to tetracycline, 22 % and 24 % to cotrimoxazole and 9 % and 13 % to ampicillin. These overall results indicated that there were no significant differences ($P > 0.05$) in antimicrobial susceptibility of *E. coli* isolates in both years to the antibiotics tested.

The antimicrobial susceptibility patterns of *E. coli* isolates from inpatients and outpatients are presented for comparison in Tables 7 and 8. The prevalence of resistant

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isolates of *E. coli* to gentamicin, norfloxacin and nitrofurantoin among inpatients is significantly more ($P < 0.05$) than among outpatients during the 2-year study period.

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Table 2: Distribution of microorganisms isolated from urine cultures at JUH, 2000.

	No. (%)
Total No. of urine cultures	12021 (100%)
Total No. of positive cultures	3583 (29.8)
a. Gram-negative bacterial isolates	No. of isolates (%)
	1160 (32.4)
Klebsiella spp.	118 (3.3)
Enterobacter spp.	114 (3.2)
Pseudomonas spp.	107 (3.0)
Proteus spp.	72 (2.0)
Morganella spp.	23 (0.6)
Acinetobacter spp.	21 (0.6)
Providencia spp.	19 (0.5)
Serratia spp.	12 (0.3)
Citrobacter spp.	11 (0.3)
Salmonella & *Shigella* spp.	5 (0.1)
Total	1662 (46.3)
b. Gram-positive bacterial isolates	
staphylococci	274 (7.7)
taphylococci	38 (1.1)
	184 (5.1)
s	
Strept.	41 (1.1)
pt.	37 (1.0)
pt.	10 (0.3)
	28 (0.8)
	22 (0.6)
Total	634 (17.7)
	132 (3.7)
	1155 (32.3)
Grand Total	3583 (100%)

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Table 3: Distribution of microorganisms isolated from urine cultures at JUH, 2001.

	No. (%)
Total No. of urine cultures	11328 (100%)
Total No. of positive cultures	3140 (27.7)
a. Gram-negative bacterial isolates	No. of isolates (%)
E.coli	1175 (37.4)
Klebsiella spp.	134 (4.3)
Enterobacter spp.	100 (3.2)
Pseudomonas spp.	96 (3.1)
Proteus spp.	56 (1.8)
Acinetobacter spp.	28 (0.9)
Morganella spp.	12 (0.4)
Providencia spp.	10 (0.3)
Serratia spp.	3 (0.1)
Citrobacter spp.	2 (0.1)
Total	1616 (51.5)
b. Gram-positive bacterial isolates	
Coagulase-negative staphylococci	154 (4.9)
Coagulase-positive staphylococci	38 (1.2)
Enterococcus spp.	122 (3.9)
Streptococcus groups	
Non – hemolytic Strept.	36 (1.1)
β – hemolytic Strept.	26 (0.8)
α – hemolytic Strept.	10 (0.3)
Micrococcus spp.	18 (0.6)
Diphtheroid bacilli	12 (0.4)
Total	416 (13.2)
c. *Candida* spp.	120 (3.8)
d. Mix growth	988 (31.5)
Grand Total	3140 (100%)

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Table 4: Distribution of *E.coli* isolated from urine cultures among in – and outpatients at JUH (2000/2001).

Year	Inpatients No. (%)	Outpatients No. (%)	Total
2000	317 (27.3)	843 (72.7)	1160 (100%)
2001	355 (30.2)	820 (69.8)	1175 (100%)

Table 5: Distribution of *E.coli* isolated from urine cultures according to sex at JUH (2000/2001).

Year	Male No. (%)	Female No. (%)	Total
2000	281 (24.2)	879 (75.8)	1160 (100%)
2001	290 (24.7)	885 (75.3)	1175 (100%)

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Table 6: Percent antimicrobial susceptibility of *E.coli isolates* from urine cultures during 2000 and 2001 at JUH.

Antimicrobial agent	percent susceptible	
	2000	2001
Ampicillin	9	13*
Cotrimoxazole	22	24
Tetracycline	25	26
Augmentin	39	27
Gentamicin	51	53
Nalidixic acid	58	47
Norfloxacin	63	59
Cefuroxime	63	67
Nitrofurantoin	80	78

* Except the last two months (Nov. & Dec. / 2001)

P- value = 0.407; not significant for the 2-year study.

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Table 7: Distribution of percent antimicrobial susceptibility of *E.coli* strains isolated from in – and outpatients at JUH (2000)

Antimicrobial agent	percent susceptible	
	inpatient	outpatient
Ampicillin	9	9
Cotrimoxazole	21	23
Augmentin*	21	46
Tetracycline	28	23
Nalidixic acid*	31	68
Gentamicin*	32	58
Norfloxacin*	36	73
Nitrofurantoin*	47	92
Cefuroxime	56	65

* P- value = 0.002; significant between in-and outpatients (2000).

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Table 8: Distribution of percent antimicrobial susceptibility of *E. coli* strains isolated from in – and outpatients at JUH (2001).

Antimicrobial agent	percent susceptible	
	inpatient	outpatient
Ampicillin #	11	14
Augmentin	21	29
Tetracycline	26	26
Cotrimoxazole	27	23
Gentamicin*	35	61
Cefuroxime*	38	78
Nalidixic acid	43	49
Norfloxacin*	46	64
Nitrofurantoin*	58	87

Except the last two months (Nov. & Dec.)

* P- value = 0.006; significant between in-and outpatients (2001).

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4.3. Bacterial conjugation

In conjugation experiments, a total of 15 out of 53 (28 %) of the examined multiresistant *E. coli* strains were able to transfer most or part of their resistance to the recipient *E. coli* K12 (Table 9).

Each one of the eight procedures that were used for bacterial conjugation gave almost the same results. The most common transferable resistance was demonstrated in 7 out 8 of *E. coli* isolates with 4–drug resistance patterns (AGMTTs). This 4–drug resistance was transferred as a triple resistance (ATTs) and (AGMTs) in both high and low frequency, respectively. In addition, each one of the following *E. coli* strains were capable of transferring one or more drug resistance in high or low frequency; one strain (ACXMGMNANORTTs), two strains (ACXMGMTTs), one strain (ACXMGMTs), four strains (AGMTTs), one strain (ACXMGMT), one strain (ACXMTTs) and one strain (ACXMGMNiTTs).

4.4. MICs of 20 multiresistant *E. coli* strains

A total of 20 multiresistant *E.coli* strains were tested for MICs. Table 9 shows that ampicillin and cotrimoxazole have the highest MICs whereas ciprofloxacin has the lowest MIC for *E.coli* strains, and to a lesser extent, gentamicin, nalidixic acid and tetracycline. Three isolates were resistant to ampicillin, ciprofloxacin, cotrimoxazole,

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gentamicin, nalidixic acid and tetracycline, 13 were resistant to ampicillin, cotrimoxazole, gentamicin and tetracycline, two were resistant to ampicillin, cotrimoxazole and gentamicin, one was resistant to ampicillin, cotrimoxazole and tetracycline and one was resistant to ampicillin, gentamicin and tetracycline. The MICs of *E. coli* isolates are presented in Table 9.

Table 9: Minimal Inhibitory Concentrations (MICs) of multiresistant *E. coli* strains

E.coli strains (No.strains)	Resistance pattern of *E.coli* isolates	Mean MICs (mg / l) of *E.coli* strains					
		A	Cip	Ts	GM	NA	T
(3)	**ACXMGMNANORTTs**	≥ 256	≥ 64	≥256	≥ 64	≥ 128	≥ 64
	ACXMGMNANORTTs	≥ 256	32	256	64	≥ 128	32
	ACXMGMNANORTTs	≥ 256	≥ 64	≥ 256	32	≥ 128	≥ 64
(2)	**ACXMGMNiTTs**	64	4	64	32	16	32
	ACXMGMNiTTs	≥ 256	4	≥ 256	64	8	64
(2)	**ACXMGMTTs**	≥ 256	2	≥ 256	16	8	32
	ACXMGMTTs	≥ 256	2	≥ 256	32	16	32
(1)	**ACXMGMTs**	≥ 256	2	≥ 256	32	8	4
(8)	**AGMTTs**	≥ 256	4	≥ 256	64	8	32
	AGMTTs	≥ 256	1	≥ 256	32	16	32
	AGMTTs	≥ 256	4	≥ 256	32	16	≥ 64
	AGMTTs	≥ 256	2	≥ 256	32	8	32
	AGMTTs	≥ 256	2	≥ 256	16	16	64
	AGMTTs	≥ 256	4	≥ 256	64	8	32
	AGMTTs	128	≤ 1	256	32	16	32
	AGMTTs	64	2	64	16	8	32
(1)	**ACXMGMT**	32	4	≤ 8	16	≤ 4	64
(1)	**ACXMTTs**	≥ 256	≤ 1	≥ 256	64	16	32
(1)	**ATTs**	≥ 256	4	≥ 256	4	16	64
(1)	**AGMTs**	128	≤ 1	64	32	8	4
(20)	Total						

A: Ampicillin, **CXM**: Cefuroxime, **GM**: Gentamicin, **T**: Tetracycline, **Ts**: Cotrimoxazole, **NA**: Nalidixic acid, **NOR**: Norfloxacin, **Ni**: Nitrofurantoin, **Cip**: Ciprofloxacin.

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4.5. Resistant phenotypes

The resistant phenotypes of multiresistant *E. coli* isolates are presented in Table 10. Resistance phenotype of 4–drug resistance (AGMTTs) accounted for the majority of isolates (26 / 53; 49 %), followed by 3–drug resistance (9.4 %) of both ATTs and AGMT. Other resistance phenotypes were less common (≤ 7.5 %) among *E. coli* isolates.

4.5.1. Plasmid profiles

Transferable resistance and plasmid profiles of 16 multiresistant *E. coli* strains, and 15 *E. coli* transconjugants strains are shown in Table 10. The results showed 13 different plasmid profiles, and plasmid sizes ranged from 2.0 kb to 59.4 kb. A common large plasmid (28.0 kb) was found in 10 out of 15 (67 %) *E. coli* donor strains. Also, 11 out of 15 (73 %) of *E. coli* strains contained more than one plasmid, but no isolate contained more than 6 plasmids. The three used plasmid extraction methods gave almost similar results. Agarose gel electrophoresis of plasmid profiles are shown in Figures 5, 6 and 7.

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4.5.2. Restriction enzyme digestion

All plasmids presented in gel electrophoresis were treated with *Hind* III restriction enzyme. The results demonstrated common patterns in plasmid of the same

molecular size (28.0 kb) with mostly similar antimicrobial resistance pattern, whereas different digest patterns were observed in plasmids with different resistance patterns. Figure 8 and 9 shows *Hind* III restriction pattern of plasmids in *E. coli* strain and their transconjugants.

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Table 10: Resistance phenotypes, transferable resistance and plasmid profiles of 53 multiresistant Escherichia coli strains isolated from urine specimens at JUH – 2001.

No. Of strains Donor / Recipient [a]	Resistance phenotype	Transferable resistance (No. of strains)	Transfer frequency [b]	No. of plasmid(s) Donor / Recipient	Plasmid profile (Kb) Donor / Recipient
1 / 1	ACXMGMNANORTTs	AGMNORTTs	High	4 / 1	12.2, 7.4, 6.6, 3.0 / 7.4
2 / 1	ACXMGMNiTTs	ACXMNiTs	Low	1 / 1	28.0 / 28.0
	ACXMGMNiTTs [c]	Nil	—	6 / —	59.4, 48.2, 40.0, 28.0, 6.4, 2.2 / —
4 / 2	ACXMGMTTs	ACXMGMTs(1)	High	1 / 1	29.2 / 29.2
	ACXMGMTTs	A (1)	High	5 / 2	48.0, 28.0, 3.94, 3.2, 2.12 / 48.0, 28.0
1 / 0	AGMNiTTs	Nil	—		
1 / 1	ACXMGMTs	AGM	High	6 / 2	47.4, 28.0, 10.4, 6.0, 3.03, 2.0 / 47.4, 28.0
26 / 7	AGMTTs	ATTs(2)	High	4 / 3	45.4, 42.4, 26.6, 2.8 / 45.4, 42.4, 26.6.
				1 / 1	28.0 / 28.0
	AGMTTs	AGMTs(1)	Low	3 / 3	28.0, 6.6, 4.4 / 28.0, 6.6, 4.4
	AGMTTs	GM(2)	Low	4 / 2	49.8, 29.2, 4.0, 3.2 / 49.8, 29.2.
			High	5 / 1	28.0, 11.2, 6.2, 3.0, 2.2 / 28.0
	AGMTTs	A(2)	High	2 / 1	28.0, 7.0 / 28.0.
				2 / 1	28.0, 18.0 / 18.0
1 / 1	ACXMGMT	A	High	2 / 2	29.2, 25.4 / 29.2, 25.4
1 / 1	ACXMTTs	ACXM	High	1 / 1	28.0 / 28.0
5 / 1	ATTs	ATTs	Low	1 / 1	28.0 / 28.0
5 / 0	AGMT	Nil	—	—	—
4 / 0	AGMTs	Nil	—	—	—
1 / 0	ATs	Nil	—	—	—
1 / 0	ACXM	Nil	—	—	—
53 / 15					

a. *Esherichia coli* k12, F^{-} Nal^{r}.
b. Low indicates $\leq 10^2$ recipient / donor cells, High indicates $\geq 10^4$ recipient / donor cells.
c. Multiresistant *E. coli* strain with nontransferable R-plasmid.
A: Ampicillin, **CXM**: Cefuroxime, **GM**: Gentamicin, **T**: Tetracycline, **Ts**: Cotrimoxazole, **NOR**: Norfloxacin, **Ni**: Nitrofurantoin.

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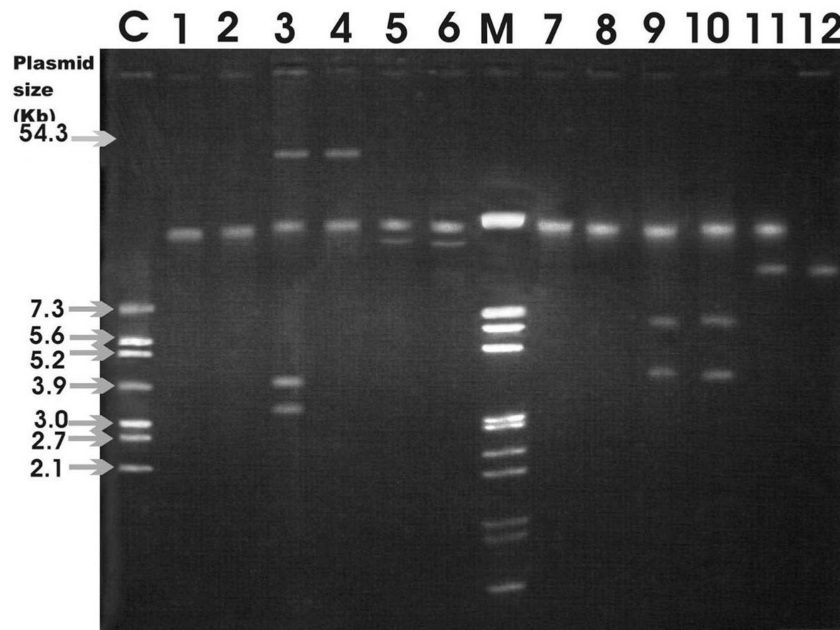

Figure 5: Agarose gel electrophoresis of plasmid DNA from representative of different *E.coli* strains. C: Positive control, *E.coli* V517 containing eight reference plasmids. Lanes 1 – 12: *E.coli* strains & its transconjugants. M: Lambda DNA/*Eco*R I + *Hind* III Marker.

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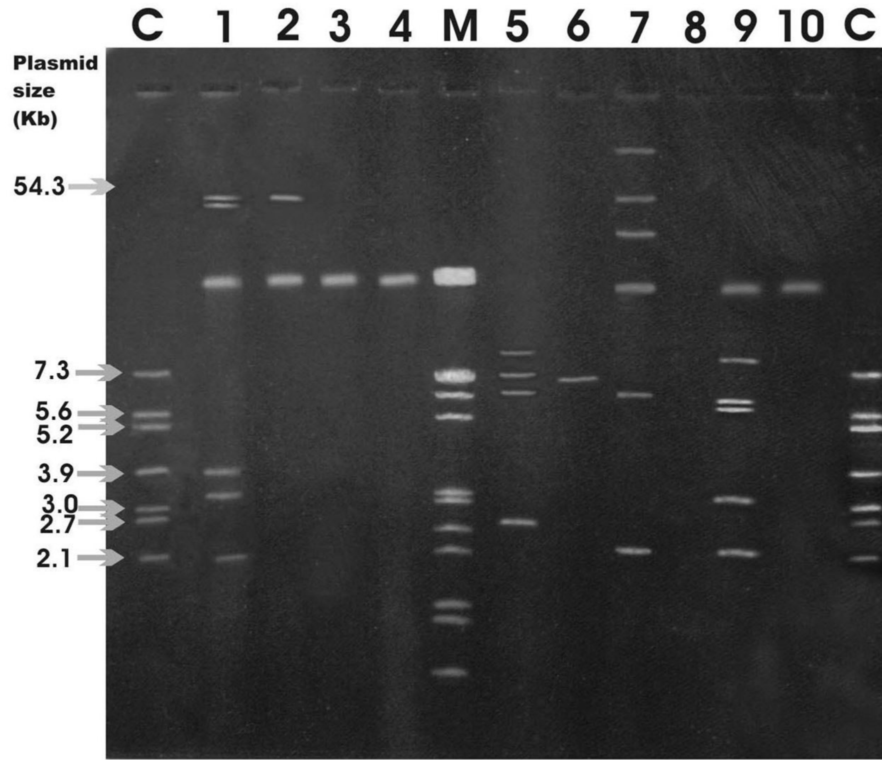

Figure 6: Agarose gel electrophoresis of plasmid DNA from representative of different *E.coli* strains. C: Positive control, *E.coli* V517 containing eight reference plasmids. Lanes 1 – 10: *E.coli* strains with its transconjugants. M: Lambda DNA/*Eco*R I + *Hind* III Marker. Lane 7: not transferable *E.coli* strains. Lane 8: Negative control, *E.coli* K12.

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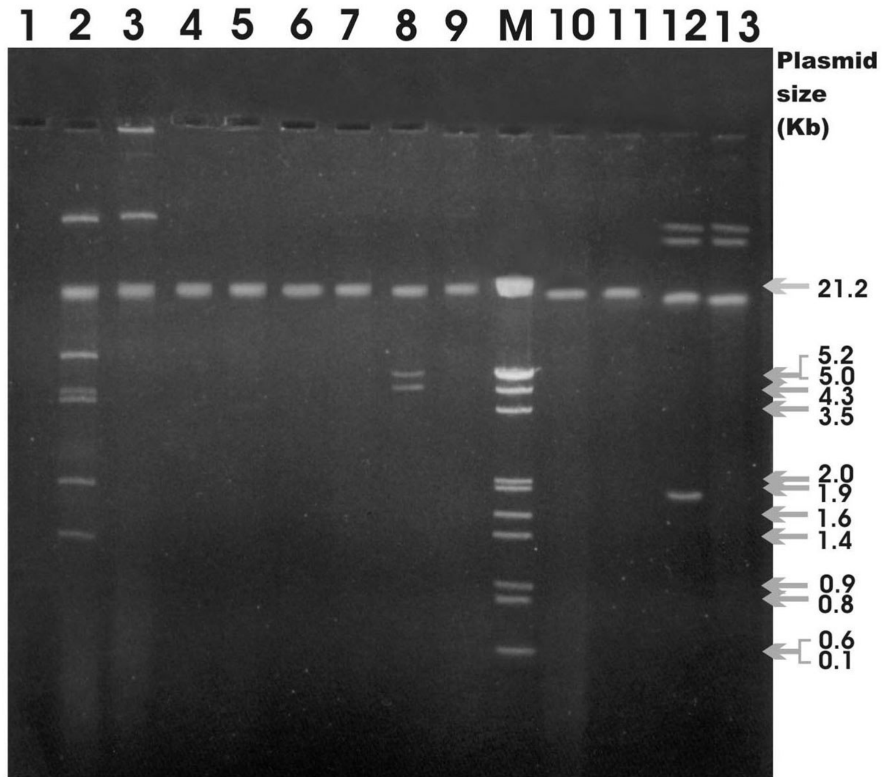

Figure 7: Agarose gel electrophoresis of plasmid DNA from representative of different *E.coli* strains. Lane 1: Negative control, *E.coli* K12. Lanes 2 – 13: *E.coli* strains & its transconjugants. M: Lambda DNA/ *Eco*R I + *Hind* III Marker.

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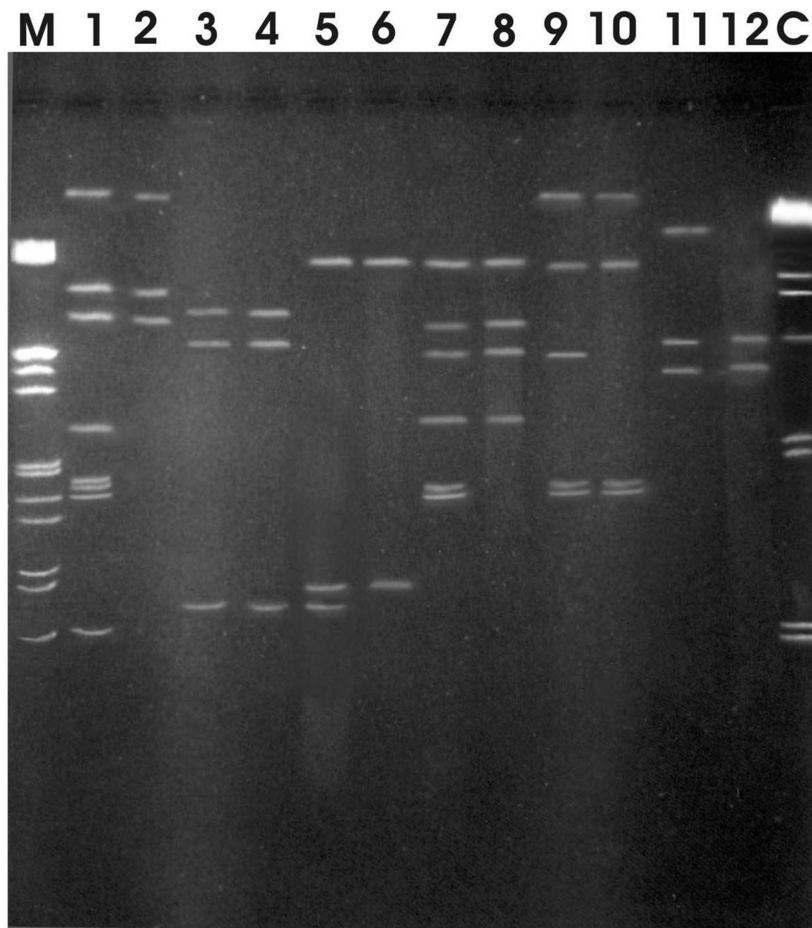

Figure 8: *Hind* III restriction pattern of plasmids from *E.coli* strains before and after transconjugants. M: Lambda DNA/ *Eco*R I + *Hind* III Marker. 1 – 12: *E.coli* strains & its transconjugants. C: Control, Lambda DNA.

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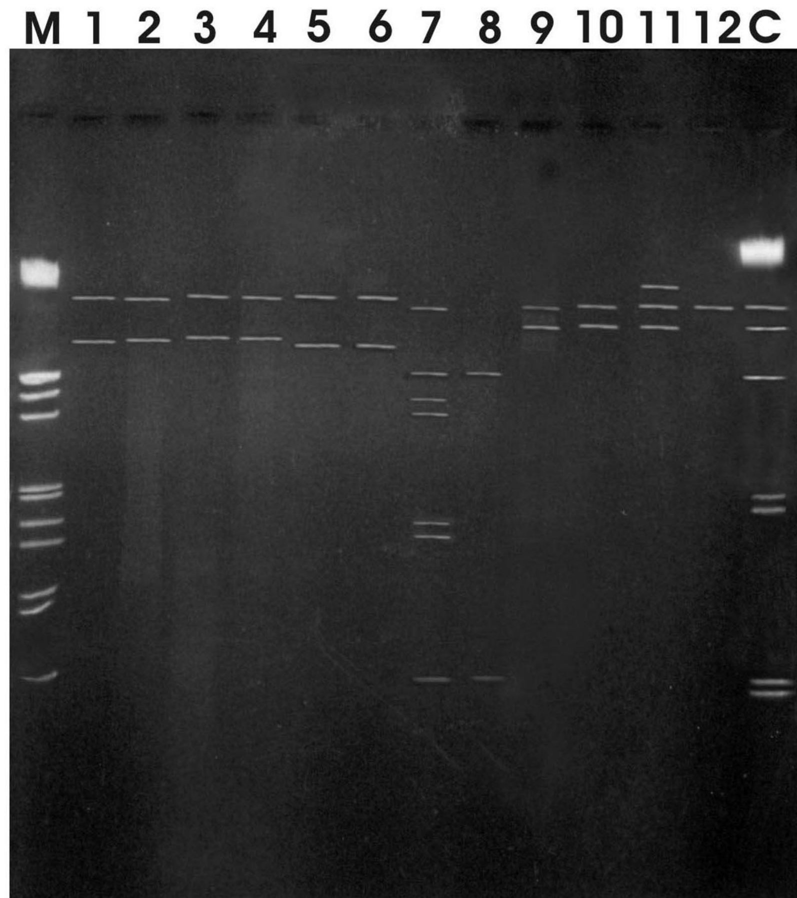

Figure 9: *Hind* III restriction pattern of plasmids from *E.coli* strains before and after transconjugants. M: Lambda DNA/ *Eco*R I + *Hind* III Marker. 1 – 12: *E.coli* strains & its transconjugants. C: Control, Lambda DNA.

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5. Discussion

5.1. Prevalence of multi-resistance urinary *E.coli*

Bacterial infection of the urinary tract is a common health problem worldwide, especially among women of all age groups (Mathai *et al*., 2001, Chomarat, 2000, Jones *et al*., 1999). Nearly, 80 % of UTIs occurring in nonhospitalized patients in the absence of obstruction are caused by *E. coli* that originate from the fecal flora of the colon (Stamm & Norrby 2001, Shehabi *et al*., 2000). Nosocomial urinary tract infections are caused by a wide spectrum of microorganisms, including *E. coli* and many gram – negative enteric bacteria. Worldwide UTIs in hospitalized patients are commonly

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caused by bacterial strains resistant to multiple antimicrobial drugs which make these infections more difficult to treat (Stamm & Norrby 2001, Mathai *et al.*, 2001, Shehabi *et al.*, 2000, Chomarat, 2000, Jones *et al.*, 1999).

The first study to document the spectrum of uropathogens and their susceptibility to antimicrobial drugs in Jordan, was reported by Shehabi, (1980). This study showed that 42 % of all positive urine cultures of inpatients and outpatients admitted to JUH in 1978 were due to *E. coli.* The antimicrobial resistance rates of *E. coli* isolates were very high to ampicillin (88 %), cotrimoxazole (78 %), and tetracycline (83 %), whereas resistance rates to nalidixic acid, nitrofurantoin, and gentamicin were low and ranged between 13 % and 16 %. A second study carried out in 1995 in northern Jordan reported a prevalence of *E. coli* of 52.7 % in urine cultures of patients at Princes Basma Hospital, Irbid, with 93 % of the isolates being resistant to ampicillin, 72 % to tetracycline, and only 1 % to gentamicin (Malkawi *et al.*, 1996). A third study covered patients with community acquired urinary tract infections in Al-Zarqa Governorate, Jordan, indicated that *E. coli* accounted for 82 % of isolates from urine cultures (Abu Shaqra, 2000). This study also found high rates of resistance among *E. coli* isolates to ampicillin (95 %), tetracycline (86 %), cotrimoxazole (48 %), and amoxycillin/ clavulanic acid (45 %). A fourth recent study from Jordan (Abu-Elteen *et al.*, 2000), found that *E. coli* was the most frequently isolated organism (75.4 %) from patients with community acquired urinary tract infections over a period of 5 years and resistance rates to ampicillin and cephaloridine were observed in 90.8 % and 87.5 % of these

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isolates, respectively. These previous four studies and the current study which cover various major regions of Jordan over a period of 23 years (1978 – 2001) demonstrated that uropathogenic *E. coli* were highly resistant (70 % – 95 %) to ampicillin, cephaloridine, cotrimoxazole and tetracycline. Also, moderate to high rates of resistance (30 % – 50 %) have been observed to other commonly used antibiotics like gentamicin, cefuroxime and norfloxacin among uropathogenic *E. coli* isolates as demonstrated in the present study (Table 11)

Table 11: Comparison between antibiotic-resistance of uropathogenic *E.coli* from patients admitted to JUH in 1978 (total isolates no. 479) and 2001 (total isolates no. 1175).

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Antibiotic	1978	2001
Ampicillin*	88	87
Co-trimoxazol*	78	76
Gentamicin	13	53
Nalidixic acid	16	47
Nitrofurantoin*	16	22
Tetracycline*	83	74

* P- value = 0.710; not significant for the 2-year study.

Knowledge of antimicrobial susceptibility patterns of uropathogens in the community and hospitals is important in determining empiric antimicrobial treatment of UTIs.

Numerous studies from the developed world have demonstrated an increasing antibiotic resistance among uropathogens causing both community- and nosocomially acquired UTIs. Even in these countries, increasing resistance to ampicillin (40 % – 50 %), cephalosporines (20 % – 30 %), and cotrimoxazole (20 % – 30 %) have been observed in uropathogenic *E. coli* (Gupta *et al.*, 2001, Fluit *et al.*, 2001, Mathai *et al.*, 2001, Goldstein, 2000, Jones *et al.*, 1999).

Most studies from North America and Europe reported an increasing fluoroquinolone resistance among uropathogenic *E. coli* and other enterobacteria as well as extended-spectrum β-lactamase-mediated resistance in gram-negative bacteria and

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vancomycin-resistance in enterococci (Stamm *et al*., 2001). However, most of these studies have observed that resistance to ciprofloxacin and norfloxacin among uropathogenic *E. coli* has been within 10 % (Mathai *et al*., 2000, Jones *et al*., 1999), whereas the overall norfloxacin-resistance among *E. coli* isolates in this study was higher (40 %) and mostly found in hospitalized patients. Also, a recent study carried out in 1997 at the JUH found that there was a slight to moderate increases in the incidence of resistance of *E. coli* and *Enterobacter* spp. to extended-spectrum β-lactam drugs over a period of 3 years, but a significant increase in the resistance of *Klebsiella pneumoniae* to ceftazidime and aztreonam (Shehabi *et al*., 2000). In addition, a comparison of antibiotic-resistance patterns of uropathogenic *E. coli* isolates in 1978 and 2001 at JUH has revealed that gentamicin resistance increased from 13 % to 53 % and nalidixic acid from 16 % to 47 %, whereas resistance rates to ampicillin, cotrimoxazole, nitrofurantoin and tetracycline were much similar during this long period (Shehabi, 1980).

A recent study from Saudi Arabia found that the prevalence of resistance among uropathogenic *E. coli* isolates from a university Hospital was 54 % to ampicillin, 50 % to tetracycline, 39 % to trimethoprim-sulfamethoxazole, and resistance rates for amoxicillin-clavulanate, nitrofurantoin, nalidixic acid, and norfloxacin were between 9 % and 15 % (Bukharie *et al*., 2001). A second study from Saudi Arabia has reported a very high proportion of strains of *E. coli* (86 %) and *Klebsiella* spp. (94.6 %) that were found to be resistant to ampicillin (Ahmad and Ahmad, 1995). In Lebanon, a study

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from the American University Medical Center found that ampicillin resistance rate was 65 % among *E. coli* strains isolated from various clinical specimens (Araj *et al*., 1994). The overall results of these studies are similar in many aspects to our study, and indicated that antimicrobial resistance patterns in these Arab countries are clearly higher than in the developed countries.

It has been reported that organisms causing nosocomial infections often change as a function of medical practice and due to the extensive use of antibiotics (Arlet *et al*., 1994, New, 1993). In recent years, the number of multiresistant bacteria causing nosocomial infections have been increasing, and an important percentage of these strains were associated with frequent use of invasive devices and intensive care in both the developing and developed countries (Eltahawy *et al*., 2001, Shehabi *et al*., 2000, Elhag *et al*., 1999, Mc Gowan, 1996). More than 20 % of all patients admitted to European intensive care units developed a nosocomial infection (Spencer, 1996). Hospital – acquired urinary tract infections have a great impact on clinical medicine. Urinary tract infections (UTIs) are responsible for 40 % – 60 % of all nosocomial infections, particularly among elderly patients where co-morbidity renders hospitalized patients susceptible to urinary infection (Sotto *et al*., 2001,Wagenlehner and Naber, 2000).

A Jordanian study has reported that 30 % (155/519) of all patients consecutively admitted in 1993 to the adult intensive care unite at the JUH had microbial infections as documented with positive cultures. Gram-negative bacteria were involved in 49 %, gram-positive in 31 %, mixed bacterial species in 11 % and *Candida*

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spp. in 9 %. UTIs that are caused by *E. coli* accounted for 26 % of all gram-negative isolates (Shehabi *et al*., 1996).

5.2. Conjugation study and plasmid profiles

Conjugation study has shown that 28 % (15 out of 53) of *E. coli* isolates with multiple antibiotic resistance were able to transfer their resistance. Of these, partial resistance was transferred, particularly in association with a large size plasmid of molecular size 28.0 kb. The frequency of conjugative resistant plasmids was found to be high in association with seven, six and three drug-resistant *E. coli* strains. All *E. coli* strains with transferable plasmids were resistant to ampicillin, cotrimoxazole, gentamicin, and tetracycline. Transferable resistance plasmids were not detected among *E. coli* strains to ciprofloxacin or nalidixic acid. Therefore, this type of resistance may be chromosomal or is associated with non-transferable plasmids. These results are similar to other studies, which reported that complete or partial resistance in enteric bacteria can be transferred by conjugative plasmids (Malkawi *et al*., 1996, Shehabi, 1995, Shears *et al*., 1988, Shehabi *et al*., 1986).

A study from Bangladesh has reported that 52 % of coliform bacterial strains from feces with multiple antibiotic resistance have transferred completely or partially their resistance, most frequently by single plasmids (Mamun *et al*., 1993). Malkawi *et al*. (1996) found that a large size plasmid with a molecular size of 27.0 kb was present in all multiresistant *E. coli* strains isolated from urinary tract infections. Also, a study reported by Shehabi (1995) has shown that multiresistant *Salmonella typhimurium*

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strains were able to transfer most or part of their resistance by conjugation to *E. coli* K12. There was a common large plasmid (55 Mdal) in 10 out of 12 *Salmonella* strains isolated from patients at the JUH (1 Megadalton = 1515.7 bp). Similar findings were obtained by Shears *et al*. (1988) at children's emergency Hospital in Khartoum, Sudan. Their study found 45 different plasmid profiles with sizes that ranged from 160 Mdal to 2.8 Mdal, and a 62 Mdal plasmid was present, either alone or in combination with plasmids of other sizes in 62% of strains with different resistance patterns. All of these studies reported that there were no consistent relationships between plasmid profile and antibiotic resistance pattern among examined clinical bacterial isolates (Malkawi *et al*., 1996, Shehabi, 1995, Mamun *et al*., 1993, Shears *et al*., 1988). In this study plasmid analysis of the *E. coli* isolates showed the presence of a wide range of plasmid sizes, but there were also no consistent relationships between plasmid profile and antimicrobial resistance patterns, and in one case a multiresistant strain with six–drugs–resistant markers (ACXMGMNiTTs) was non – transferable.

There are many methods currently used for plasmid DNA extraction. Birnboim and Doly method is the most commonly used in literature. Therefore, it has been used in association with two other methods in order to ensure the isolation of all available plasmids in *E. coli* strains by agarose gel electrophoresis. The amount of plasmid DNA extracted by Birnboim and Doly was about 1.0 ug/ 1ml culture of covalently closed circular (CCC) DNA, while by using the commercial DNA purification system the yield was greater than 2.0 ug/ 1 ml with 80 % supercoiled

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plasmid, and that gave a good results when the plasmid was digested by restriction enzymes. There were also no differences between using ampicillin in LB–broth to

increase the selection of plasmids in *E.coli* strains or not during sample preparation for DNA extraction method. The third DNA extraction, Quick-check method is applied to test the size of *E.coli* plasmids. It is a simple method and does not require specific equipments or reagents, there is no need for large sample size, and it is not time – consuming like the other two methods (it takes only 5 minutes for each sample or 15 minutes for 12 samples). The disadvantages of this method are the high impurity content, and that not all plasmid DNA appear clearly similar to the large intensive bands like 28.0 kb, while small and faint bands can not appear due to masking by chromosomal DNA.

This study could not confirm the presence of epidemic R-plasmid(s) in *E. coli* strains, since the common large plasmid of 28.0 kb was found both in *E. coli* isolates from hospitalized patients and non hospitalized patients with nearly similar rates (47% versus 53%).

Further investigation with Polymerase Chain Reaction (PCR) may determine whether the resistance genes found on a common plasmid are the same in all multiresistant uropathogenic *E. coli* strains or not.

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6. Summary and conclusion

- A total of 3583 (29.8 %) and 3140 (27.7 %) urine culture specimens were positive for significant bacterial growth in the clinical bacteriology laboratory at JUH in Amman, during the two-year study 2000 and 2001.

- *E.coli* accounted for 32.4 % and 37.7 % of the isolates recovered from urine cultures at the JUH during 2000 and 2001, respectively.

- The *E.coli* isolates were highly resistant to ampicillin (87 %, 91 %), followed by cotrimoxazole (76 %, 78%), tetracycline (74 %, 75 %), augmentin (61 %, 73 %). Less rates of resistance were observed in gentamicin (49 %, 47 %), nalidixic acid (42 %, 53 %), norfloxacin (37 %, 41 %), cefuroxime (33 %, 37 %) and nitrofurantoin (20 %, 22 %).

- Transferable plasmids have been detected in 15 out of 53 (28%) strains of *E.coli*. The ratio of transferable plasmids among inpatients to outpatients was close (47 % Vs 53 %).

- The results revealed 13 different plasmid profiles, and plasmid sizes ranged from 2.0 kb to 59.4 kb. A common large plasmid (28.0 kb) was found in 10 out of 15 (67 %) *E. coli* donor strains.

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- This study suggests that this common plasmid may increase the incidence and spread of multiple antibiotic resistance among *E.coli* isolates in future.
- Further investigation using Polymerase Chain Reaction (PCR) may reveal the actual number of resistance genes associated with the common large plasmid.
- Knowledge of antimicrobial resistance patterns among uropathogens in Jordan can improve the selection of effective empirical treatment with antimicrobial drugs.
- This study suggests that nitrofurantoin, cefuroxime, norfloxacin and nalidixic acid may be used in empirical urinary tract infections.

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أنواع البلازميدات المقاومة للمضادات الحيوية في بكتيريا الإيشريشياكولي المعزولة من زراعة البول للمرضى

إعداد

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الملخص

تم دراسة نتائج زراعة عينات البول بين المرضى المقيمين والمراجعين لمستشفى الجامعة الأردنية على مدى عامي 2000 و2001 م . وأظهرت الدراسة أن 3583 عينة من أصل 12012 عينة (29.8%) عام 2000 و 3140 عينة من اصل 11328 عينة (27.7%) عام 2001 كانت ايجابيه للانتان الجرثومي. وكانت إيشريشياكولي هي المسبب للانتان البولي في 1160 عينة (32.4%) خلال عام 2000 م و 1175(37.4%) خلال عام 2001 م. و كانت نسبة عينات البول الإيجابية بين المرضى من خارج المستشفى إلى المرضى المقيمين فيها 1:3 و هذه النسبة كانت متشابهة في كلا العامين ، كما كانت نسبة الاصابه بانتان المجاري البولية بين الإناث إلى الذكور 1:4. وتبين أن حساسية سلالات إيشريشياكولي المعزولة للمضادات الحيوية في العامين كالتالي: نايتروفيورانتوين 80% و 78%وسيفروكزيم 63% و 67% ونورفلوكساسين 63% و 59% ، بينما كانت هذه السلالات أقل حساسية للامبسلين 9% و 13% و الكوتريموكسازول 22% و 24% .

كما كانت نسبة التحسس للتتراسايكلين 25% و 26% و الاوغمنتين 39% و 27%و الجنتامايسيين 51% و 53%.

و تبين أن 15 عزلة من اصل 53 عزلة (28%) من سلالات إيشريشياكولي ذات مقاومة عالية للمضادات الحيوية ولها القدرة على نقل هذه المقاومة بشكل جزئي أو كلي للبكتيريا المستقبلة (إيشريشياكولي

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نوع K12). وتبين أن التركيز المثبط الأدنى من المضاد الحيوي في 20 سلالة من إيشريشياكولي ذات المقاومة العالية كان حسب التسلسل التالي من التركيز الأدنى للأعلى: السيبروفلوكساسين و الجنتامايسيين و الناليديكسيك أسد و التتراسايكلين والامبسلين و الكوتريموكسازول. وأظهرت الدراسة أن هناك 13 مجموعة مختلفة من البلازميدات بأحجام تتراوح من 2 إلى 59.4 كيلوبيز توجد في 16 سلالة من إيشريشياكولي ذات المقاومة العالية ولا توجد أي سلالة تحتوي على أكثر من 6 بلازميدات إضافة إلى أن 15 سلالة انتقل منها بلازميد أو أكثر بالاقتران. كما وجد بلازميد كبير ومشترك بحجم 28 كيلوبيز في 10 سلالات من أصل 15 (67%) في إيشريشياكولي. وبينت الدراسة أن معظم سلالات إيشريشياكولي(15/10 ، 67%) التي لها القدرة على نقل البلازميد الكبير المشترك (28 كيلوبيز) تتشابه في أنواع المقاومة للمضادات الحيوية عند إخضاعها لعملية التقطيع باستخدام الأنظيم القاطع Hind III . وتفترض الدراسة أن هذا البلازميد الكبير المشترك ربما يكون له دور في زيادة انتشار المقاومة للمضادات الحيوية في سلالات إيشريشياكولي التي تسبب الانتانات في المستقبل . وباستخدام تفاعل البلمرة المتسلسل يمكن أن يحدد التشابه الحقيقي لهذا البلازميد المقاوم والمشترك بين سلالات إيشريشياكولي المقاومة للمضادات الحيوية بدرجة عالية .

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