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URINARY TRACT INFECTIONS ASSOCIATED WITH MULTIDRUG RESISTANT ENTERIC BACILLI: CHARACTERIZATION AND GENETICAL STUDIES

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ABSTRACT

Urinary tract infections (UTIs) are among the most commonly prevalent infections in clinical practice. *Escherichia coli* is the causative agent in about 85% of community-acquired UTIs, followed by Klebsiella that accounts for 6 to 17% of such infections. Present study is based on the isolation-identification and antibiotic resistance pattern of about 60 indigenous bacterial isolates from UTI patients. Prevalence rates were consistent with those from major recent studies reported in the literature, i.e. 73% isolates were identified as *E. coli,* 16% as *K. pneumoniae* and 11% as Proteus sp. Bases of identification included morpho-cultural and biochemical characteristics. To assess the breadth of multidrug resistance among these isolates, culture medium incorporation method was employed using ampicillin, fosfomycin, chloramphenicol, tetracycline, and three aminoglycosides (kanamycin, gentamicin, and streptomycin). Of these isolates, 30% offered multidrug resistance to three or more agents. Among multidrug resistant isolates, 100% were resistant to ampicillin, 47% to streptomycin, 41% to chloramphenicol, gentamicin and tetracycline, 35% offered resistance to kanamycin while only 6% showed resistance to fosfomycin. After curing treatment with acridine orange, some of the isolates lost their resistance, thereby indicating the extrachromosomal location of the resistance determinants. Plasmid DNA (bearing multidrug resistant genes) was isolated from the uncured cells, and was stably transformed into the competent cured recipient cells.

INTRODUCTION

Urinary tract infections (UTI) are among the most commonly observed infections in clinical practice, and more than 25% of all women experience some form of UTI at least once during their lifetime. It also contributes the most common nosocomial infection in many hospitals, and accounts for approximately 35% of all hospital acquired infections. Majority of UTIs are not life threatening and do not cause any irreversible damage. However, when the kidneys are involved, there is a risk of irrepairable tissue damage with an increased risk of bacteremia (Hvidberg *et al*., 2000).

The Enterobacteriaceae, were the most frequent pathogens detected, causing 84.3% of the UTIs (Gales *et al*., 2000). *Escherichia coli* causes about 85% of community-acquired UTIs, 50% of nosocomial UTIs, and more than 80% of cases of uncomplicated pyelonephritis (Bergeron, 1995). A vacuolating cytotoxin expressed by uropathogenic *E. coli*, elicits defined damage to kidney epithelium (Guyer *et al*., 2002). The medically equally important Klebsiella account for 6 to 17% of all nosocomial UTIs and show an even higher incidence in specific groups of patients at risk (Bennett *et al*., 1995). *Proteus mirabilis* is a common cause of UTI in individuals with long-term urinary catheters in place or individuals with complicated urinary tracts. *P. mirabilis*,

despite its antibiotic sensitivity, can be difficult to clear by antibiotic treatment. It has been hypothesized that bacteria within a stone matrix are protected from antibiotic treatment (Li *et al*., 2002).

Multiple antimicrobial resistances among gram-negative organisms have been a long term and well-recognized problem with urinary tract infections. Resistance has been observed in multiple genera including Escherichia, Enterobacter, Klebsiella, Proteus, Salmonella, Serratia, and Pseudomonas (Cohen, 1992). Fosfomycin is routinely and effectively used for the treatment of uncomplicated lower urinary tract infections. The clinical cure rate is estimated to be as high as 99 percent (Patel *et al*., 1997). The mechanisms whereby bacteria circumvent drug action are many and varied, ranging from intrinsic impermeability to acquired resistance involving plasmids, transposons, and mutations (Gutmann, 1985). Transformation is the technique commonly used for gene transfer in recombinant DNA studies. Competence can be induced in species that are not naturally transformable (example, *E. coli*) by chemical treatments such as ice-cold $CaCl_2$ treatment followed by a brief heat shock. The discovery of techniques for transformation of *E. coli* together with *in vitro* DNA manipulations was of crucial importance in establishing genetic engineering methods associated with the development of modern biotechnology (Mandel and Higa, 1970).

MATERIALS AND METHODS

Reagents

MacConkey's agar no. 3 (BioM Laboratories, USA) was used for the screening of cultures and for antibiotic resistance. It is a selective-differential medium for the growth and identification of Gram-negative bacilli. Triple sugar iron (TSI) agar (BioM Laboratories, USA), Simmons citrate agar (Difco Laboratories, USA), peptone tryptone broth (for indole test), Clarck's medium, and peptone nitrate broth were used for identification. Brain heart infusion broth (Oxoid, UK) was used for curing experiments. Acridine orange (Merck, Germany) was used as curing agent. Luria Basal agar was used for the bacterial transformation studies. Agar (2%) of Difco Laboratories (USA) was added in order to solidify the medium. Seven antibiotics i.e, ampicillin (Amp), chloramphenicol (Cm), fosfomycin (Fos), gentamicin (Gm), kanamycin (Km), streptomycin (Sm), and tetracycline (Tc) of Sigma (USA), were used in this study. Stock solutions (10mg/mL) were prepared, sterilized by Millipore 0.45μm filters (Millipore Filter Corp., Bedford, USA) and kept frozen in small vials.

Bacterial strains

A total of 56 clinical isolates (Gram-negative bacilli causing urinary tract infections) collected from different diagnostic laboratories of Karachi (mainly Quality Laboratory, Karachi, Pakistan) were evaluated in this study.

Purification and maintenance of clinical isolates

Clinical isolates were purified on MacConkey's agar plates. Before maintenance, their purity was checked by Gram's staining and identification was done by performing characteristic biochemical test i.e. TSI (Triple sugar iron), and IMViC (Indole, Methyl red, Voges-Proskauer, Citrate) (Cheesbrough, 1989). Cultures were then maintained in nutrient broth containing 4% glycerol and kept in freezer at $-4^{o}C$ until use.

Determination of antibiotic resistance profiles

Isolates were subjected to antibiotic resistance screening by replica plate method. For this purpose, cultures were streaked on MacConkey's agar plates to obtain isolated colonies. A pure

isolated colony was then plated on to a master plate, incubated overnight at 37°C and next day replicated on to MacConkey's agar plates containing different concentrations of antibiotics. Controls were also run. The highest concentration of an antibiotic showing growth was taken as the resistance level of the strain for that particular antibiotic.

Plasmid curing

In order to determine the location (plasmid-borne or chromosomal) of the drug resistance marker(s), the curing (elimination) experiments were performed using acridine orange (Salisbury *et al*., 1972). For this purpose, 0.2 mL of overnight culture was added in 5 mL BHI broth and incubated (at 37°C) in shaking incubator for 4 hours. The log phase 200µL culture quantity was then added in 5mL BHI broth tubes containing different concentrations of acridine orange. Positive and negative controls were also run. Positive control contained only cells and no acridine orange while negative control contained only acridine orange and no cells. All the tubes were incubated (in dark) at 37°C for overnight. Next day tubes containing the highest concentration of acridine orange in which growth was present were selected and loopful was streaked on MacConkey's agar plates and incubated as earlier. Colonies from the plates were then checked for the loss of antibiotic resistance by replica plate method.

Plasmid isolation and transformation

Isolation of plasmid DNA was performed by alkaline lysis method (Sambrook *et al*., 1989). 5 mL of overnight culture was transferred to eppendorff tubes and centrifuged for 5 min. at 10,000r.p.m. to pellet the cells. The supernatant was discarded and cell pellet was resuspended in 100µL of solution A (50mM glucose, 10mM EDTA, 25mM Tris) and incubated at 37°C for 10 minutes. After thorough vortexing, 200µL fresh solution B (0.2M NaOH, 1% SDS) was added, rapidly invert mixed and left for another 10 min. at room temperature. To this suspension, 150µL ice cold solution C {5M potassium acetate (60 mL), glacial acetic acid (11.5mL), water (28.5mL)} was added, invert mixed and left on ice for 10 min, centrifuged for 5 min. at 10,000 r.p.m. Supernatant was transferred to new eppendorff tube, and 650µL chilled isopropanol was added, mixed by vortexing and left for 10 minutes. Centrifuged it for 10 min. at 10,000 r.p.m. to precipitate the DNA. The supernatant was poured off and 1 mL of cold 70% ethyl alcohol was added, kept in ice bath for 10 min. and centrifuged for 10 min., supernatant was poured off and ethanol was removed by aspiration. The pellet was dried, resuspended in 50 µL TE buffer {10mM Tris, 1mM EDTA (pH 8)} and kept in freezer.

Transformation (with plasmid DNA isolated from resistant strains) was carried out according to the $CaCl_2$ protocol (Davis *et al*., 1986). Cured cultures were used as competent recipient. To make the cured cultures competent, cells were grown in 1 mL LB broth for 2-4 hrs in shaking incubator at 37°C; 0.1 mL of this was added to 8 mL fresh LB broth and incubated for 2-4 hrs in shaking incubator at 37°C. Broth culture was chilled in ice bath, and centrifuged for 5 min. at 3000 r.p.m. The pellet was suspended in 4 mL ice-cold 50mM $CaCl_2$ and incubated for 10 min. on ice. After spinning of cells (at 3000 r.p.m.) for 5 min, they were resuspended in 2 mL of ice-cold 50 mM $CaCl_2$ and incubated for 5 min. on ice. After another spinning of cells (at 3000 r.p.m.) for 5 min., 125µL fresh LB broth was added in pellet. In a sterile vial, 5 µL of plasmid DNA was mixed with 50 µL of competent cells and vial was placed on ice for 3 min. The tube was transferred from ice to 42°C water bath for 3 min. 500 µL of the fresh LB medium was added and the cells were incubated at 37°C for 2 hours. After incubation, 100 µL was spread on antibiotic containing MacConkey's agar plates. As a negative control, 100 µL of competent cells was spread on antibiotic containing plates. All the plates were incubated overnight at 37°C.

RESULTS AND DISCUSSION

The purpose of the present study was to describe the susceptibility profiles with specific attention to the prevalence and genetic studies of multidrug resistant isolates from urinary tract infection (pertaining to the isolation-characterization of clinical indigenous gram-ve bacilli). Identification of the causative organism and its susceptibility to antimicrobials is important, so that proper drug is chosen to treat the patient in early stages of UTI (Khan and Shah, 2000). About 60 isolates from different pathological laboratories and hospitals of Karachi (Pakistan) were procured. Percentage of different Gram-negative isolates in urinary tract infections is depicted in Fig. 1.

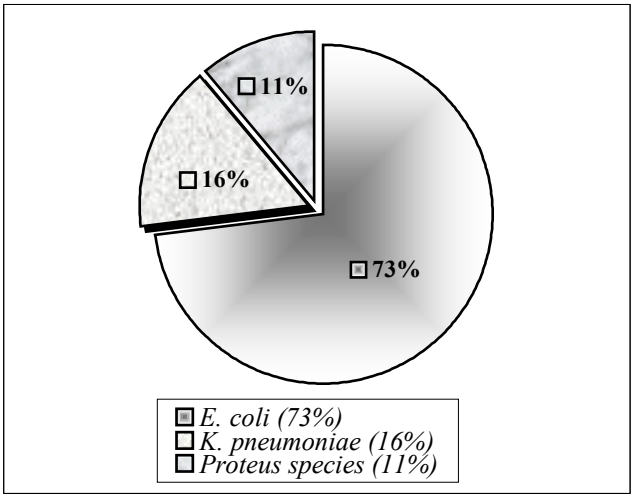

Fig. 1: Frequency of different gram-negative enteric bacilli causing urinary tract infections.

The frequency of gram-negative enteric bacilli causing urinary tract infections was 41/56 *Escherichia coli* (73%), 9/56 *Klebsiella pneumoniae* (16%), and 6/56 Proteus species (11%). Organisms responsible for UTI include *Escherichia coli*, *Proteus mirabilis, Klebsiella pneumoniae*, *Staphylococcus* and *Pseudomonas* (Ali, 2000). Nosocomial infections are a problem for the successful therapeutic treatments (Lyon and Skurray, 1987). About 50% of all nosocomial infections caused by Enterobacteriaceae pertain to urinary tract (Zaman *et al*., 1999). Previous studies have also demonstrated that *Escherichia coli* is the most frequent etiological agent causing community- and hospital acquired UTIs (Brosnema *et al*., 1993, Weber *et al*., 1997).

All the isolates were screened for drug resistance profile. Table-1 and Fig.2 indicate the resistance level (at different concentrations) against commonly used antibiotics in urinary tract infection. All the isolates offered high degree of resistance against the commonly used antibiotics (via one or other resistance mechanism). These resistant organisms can then pass on their resistance genes to their offspring by replication or to related bacteria through conjugation. Mechanisms of resistance such as plasmid mediated and/or reduced outer membrane permeability could be involved in the resistance to β-lactams. The production of extended spectrum β-lactamase (ESBL) among *E. coli* and *K. pneumoniae* also contributed significantly to the resistance of these isolates (Jenks *et al*., 1995). Resistance to aminoglycosides and chloramphenicol in gram-negative bacilli is often mediated by β-lactamases which are unaffected

by exposure of the bacterium to the potential drugs (Shafran, 1990). The resistance (pattern) to ampicillin, streptomycin, tetracycline, chloramphenicol and gentamicin has been the most common according to the present findings.

Table-1
Antibiotic resistance offered by urinary tract isolates at different concentrations

Antibiotics	Number of resistant isolates at different concentration (μg/mL)						
	5	**10**	**25**	**50**	**100**	**250**	**500**
Ampicillin	55	53	51	48	44	41	38
Chloramphenicol	-	-	55	43	30	21	15
Fosfomycin	-	-	14	8	6	4	3
Gentamicin	-	-	35	29	28	23	12
Kanamycin	-	-	40	34	30	15	9
Streptomycin	-	45	46	43	35	21	17
Tetracycline	-	52	51	47	38	22	16

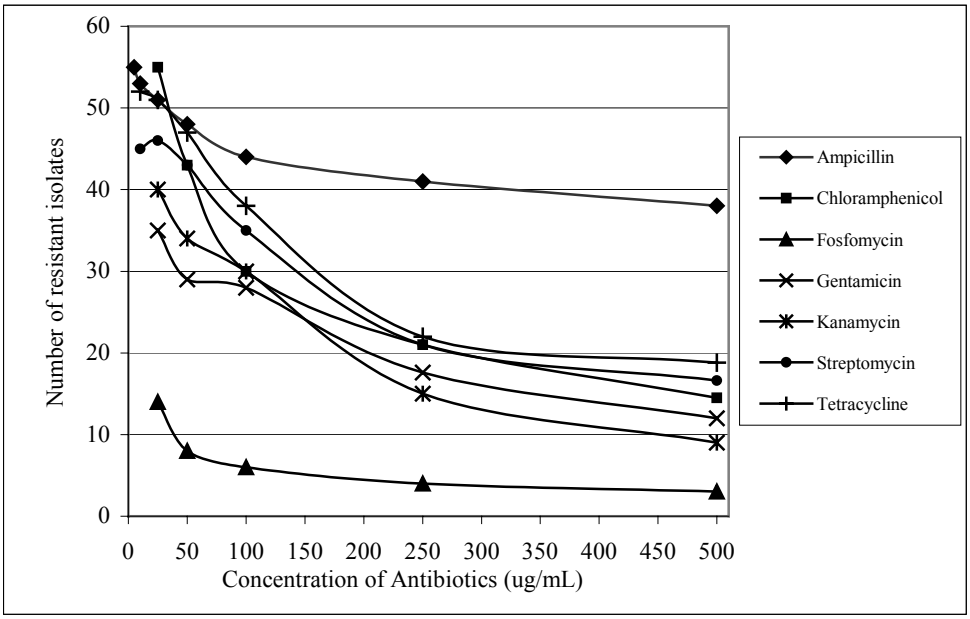

Fig. 2: Antibiotic resistance offered by urinary tract isolates at different concentrations.

Earlier it has been reported by Sahm *et al.* (2001) that ampicillin has no more effect on any of the isolates of UTI. Our results also revealed that 68% isolates could resist upto 500μg/mL of ampicillin, which may be due to the frequent-haphazard use of ampicillin. Resistance against

streptomycin, tetracycline and chloramphenicol was also significant as 30%, 29% and 27% isolates resisted upto 500µg/mL of these drugs respectively. Resistance to gentamicin (21%) and kanamycin (16%) at 500µg/mL was less significant. Most of the isolates were found sensitive to fosfomycin even at low concentrations whereby only 5% could resist 500µg/mL.

Fosfomycin, a broad-spectrum, oral antibiotic, is approved for single dose therapy and rated as an established therapy for uncomplicated lower urinary tract infections (Arav-Boger *et al*., 1994). One of the reasons for the lowest rate of fosfomycin resistance may involve its inhibitory effect on MurA enzyme (member of series of Mur enzymes), which catalyzes the cytoplasmic steps of biosynthesis of peptidoglycan precursors (E1 Zoeiby *et al*., 2003). Resistance mechanisms common for the tetracyclines, the fosfomycins, and the aminoglycosides may include metabolic changes in the target microorganisms that allow the decreased entry of a drug molecule or changes that provide the microorganism with a modified process for increasing the rate of drug removal from the cell. For example, tetracycline uptake in the Enterobacteriaceae is a biphasic process. In an initial energy-independent rapid phase, the tetracycline molecule binds to cell surface layers and passes by diffusion through the outer layers of the cell. In a second, energy-dependent phase, the drug molecule will cross the cytoplasmic membrane—probably by means of a proton-motive force, although the precise transport system has not been identified. But there also exists an inducible resistance-encoded plasmid that carries the genetic information necessary to create an environment that is unfavorable for the biphasic drug accumulation process (William and Wyandt, 2001).

It has been argued that there is a direct relation between the antibiotic used and the frequency and kinds of antibiotic-resistant strains in human beings (Kupersztoch, 1981). The resistance to antimicrobial agents can readily be transferred among bacteria by transmissible elements/plasmids (Neu, 1994).

All antimicrobial agents may eventually encounter resistant organisms. Analysis of bacterial collections from the pre-antibiotic era indicates that although plasmids were present in some of the strains but did not harbour antibiotic resistance genes (Hughes and Datta, 1983). It seems, the development of antibiotic resistance among bacteria occurred after the introduction of antibiotics into clinical use. Epidemiological studies have suggested that antibiotic resistance genes emerge in microbial populations within 5 years of the therapeutic introduction of an antibiotic (Chakrabarty *et al*., 1990). Further, the antibiotic resistance genes (found in human and animal isolates) could have originated in the industrial microbes that are used for the production of antibiotics (Webb and Davis, 1993).

The mechanisms of homologous recombination, site-specific recombination and transposition play a key role in the evolution of new combinations of multiresistant plasmids, followed by their spread through classical *in vivo* mechanisms (Sohail and Dyke, 1995). In order to confirm the location of the antibiotic resistance determinants, curing experiments were conducted using acridine orange. Although "curing" provides only the preliminary evidence that genetic traits are of extrachromosonal nature, but loss of growth on antibiotic containing plates shows that the multidrug resistant genes may be plasmid-borne. Effects of curing on the drug resistance determinants of the isolates are presented in Table-2. Four representative isolates were selected for this purpose. Gentamicin, kanamycin and streptomycin resistance markers were cured in one isolate while chloramphenicol and kanamycin resistance was lost in another isolate. However, ampicillin and tetracycline resistance determinants were not lost in any of the selected isolates. This may be due to the fact that resistance determinants in these isolates are often transposable

which exist in both plasmid and chromosomal locations (flip-flop mechanism) (DeFlaun and Levy, 1989). Transposons with individual resistance for kanamycin, tetracycline, chloramphenicol and trimethoprim have also been reported (Cohen, 1976). It is however, important to note that not all antibiotic resistance genes are plasmid mediated (Shoemaker, 1992). It may be noted that copies of the plasmid lying closer to the membranes are completely eliminated by chemical agents while those lying closer to the nucleus may escape the curing effect, thereby; one may observe partial curing (Jenks *et al*., 1995).

Table-2
Effect of acridine orange mediated plasmid curing on the drug resistance pattern of urinary tract isolates

Isolates	Isolate No.	Pre-Curing (at 500µg/mL)	Post-Curing at 500µg/mL)	Markers Cured
K. pneumoniae NN-10	NN-10	ACKST	ACT	KS
K. pneumoniae NN-23	NN-23	AFGKST	AFT	GKS
E. coli NN-44	NN-44	ACGKST	ACT	GKS
K. pneumoniae NN-54	NN-54	ACKT	AT	CK

Transformation is the oldest studied mechanism to introduce new genetic information into microorganisms. Four representative isolates (NN-10, NN-23, NN-44 and NN-54) were selected for transformation experiments. These isolates were processed for plasmid DNA isolation by alkaline lysis method. Plasmid DNA of these (uncured) isolates was transformed into cured competent recipient cells.

The transformants were scored on the plates containing the 500µg/mL of antibiotics and stability check was followed. This experiment then confirms that the drug resistant genes are located on plasmids. Results of the transformation experiments are given in Table 3. The resistant-stable transformants were detected, indicating the transfer of plasmid genes carrying the multidrug resistance marker(s). It may be concluded, that urinary tract isolates being exposed to antibiotics are developing high level of antibiotic resistance, which involves the intrageneric transfer of the corresponding genes (Lennon and Decicco, 1991).

Table-3
Transformation efficiency

Plasmid (Uncured)	Source (Donor)/recipient (cured)	Transformants/mL (Total drug resistant recombinants)	Transformation frequency
pNN-10	*K. pneumoniae* NN-10	140	140×10^{-7}
pNN-23	*K. pneumoniae* NN-23	170	170×10^{-7}
pNN-44	*E. coli* NN-44	230	230×10^{-7}
pNN-54	*K. pneumoniae* NN-54	110	110×10^{-7}

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