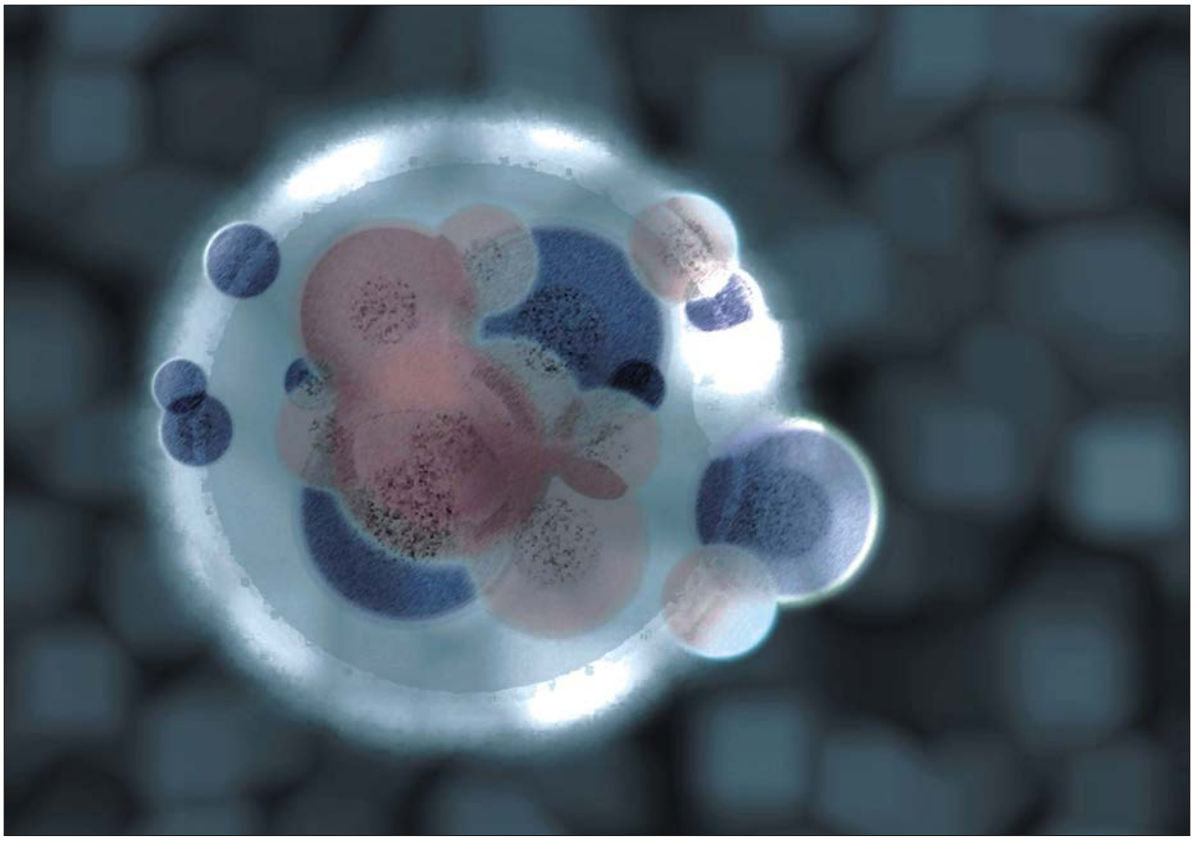

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Plasmid Profiles of Multidrug Resistant Local Uropathogenic *Escherichia coli*, *Klebsiella* spp., *Proteus* spp. and *Pseudomonas* spp. Isolates

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Abstract: Fifty-two multidrug resistant local uropathogenic *Escherichia coli, Klebsiella* spp., *Proteus* spp. and *Pseudomonas* spp. isolates were tested for susceptibility to 10 antimicrobial agents. Tetracycline (100.0%) and ampicillin (94.2%) showed the highest rates of resistance and ciprofloxacin (50.0%) demonstrated the lowest. The majority of the multiple drug resistant (MDR) isolates showing concurrent resistance to all 10 agents (n = 17) was a component of 32.7%. A total of 23 antimicrobial resistance patterns was observed. The predominant phenotype (32.7%) amongst the isolates included resistance to ciprofloxacin, cefuroxime, ceftriaxone, ampicillin, gentamicin, nalidixic acid, nitrofurantoin, co-trimoxazole, streptomycin and tetracycline. A 38.5% (n = 10) of the isolates were carrying plasmids of sizes estimated above 2.1 kb. After curing, most of the mutant strains lost their plasmids as was corroborated by their improved susceptibilities. Given that uropathogens are demonstrating increasing antimicrobial resistance, epidemiological investigation using the molecular characterization tools is highly necessary.

Key words: Uropathogen, antimicrobial susceptibility, multidrug resistance, plasmids, curing

INTRODUCTION

Bacteria are by far the most frequent cause of urinary tract infections and aerobic Gram negative bacilli predominate (Clarridge *et al*., 1987; Ryan 1996; Schaberg *et al*., 1991). Urinary Tract Infections (UTI) are common infectious diseases that can be associated with substantial morbidity and significant expenditures worldwide. Its is estimated that about 40% of women had a UTI at some point in their lives (Clarridge *et al*., 1987). The incidence of UTI increases with age and the annual cost to health care service is staggering reaching $2 billon in the United state (Forman *et al*., 2000). The pathogens causing UTIs are almost always predictable, with *E. coli*, the principal etiological agent among both outpatients and in patients (Zhanel *et al*., 2000). Their common presence is a major reason for antibiotic prescriptions. They have shown dramatic increase in resistance to the β lactams (Stratchounski *et al*., 1999), with specific reference to the more recent cephalosporins (Jacoby, 1994), to aminoglycosides (Hardy *et al*., 1980) and even to the newer fluoroquinolones (Hooper, 2001). As bacterial resistance continues to evolve, some pathogens that were once considered routine to treat are developing resistance to almost all antibacterial agents (Lister, 2000). Multidrug Resistance (MDR) in bacteria is generally attributed to the acquisition of multiple transposons and plasmids bearing genetic determinants for different mechanisms of resistance (Gold and Moellering, 1996; Alekshun and Levy, 1997).

In this study, we report the MDR phenotypes and plasmid profiles of these uropathogens isolated from ciprofloxacin-administered outpatients.

MATERIALS AND METHODS

Bacterial strains: All 52 clinical strains used in the study were isolated from patients with urinary tract infection that were treated with ciprofloxacin. The identification and characterization of isolates were by standard method (Holt, 1984).

Susceptibility testing: Antimicrobial susceptibility testing of the bacterial isolates was done using the disc diffusion (Kirby-Bauer) method as described by the National Committee for Clinical Laboratory Standards (NCCLS, 1997). The antimicrobial agents used were: ciprofloxacin, Cf (5 μg), cefuroxime, Cxm (30 μg), ceftriaxone, Cro (30 μg), ampicillin, Amp (25 μg), gentamicin, Gen (10 μg), nalidixic acid, Nal (30 μg),

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nitrofurantoin, Nit (200 μg), co-trimoxazole, Cot (25 μg), streptomycin, Str (25 μg) and tetracycline, Tet (25 μg). Resistance was evaluated according to the reference zone diameter interpretative standards of NCCLS (1999).

Plasmid isolation: Extraction of plasmid was done using the alkaline lysis method of Birnboim and Doly (1979). Loopfuls of 24 h old culture of the isolates were each transferred into 300 μL of TES buffer containing glucose (25 mM Tris.Cl, pH 8.0, 10mM EDTA, pH 8.0 and 50 mM Glucose) and mixed vigorously by vortexing. After which, 600 μL of 0.2 N NaOH and 1% SDS was added to the solution. The suspension was mixed gently by inverting the tubes rapidly 5 times and the tubes were stored on ice for 5 min. To the suspension, 450 μL of ice cold 3 M sodium acetate, glacial acetic acid and distilled water was added. This was then vortexed for 10 sec and the tubes stored on ice for 3-5 min. After this, the tubes were centrifuged at 12,000×g for 5 min at 4°C and the supernatant was transferred to a fresh tube. An equal volume of phenol-chloroform was added and mixed again by vortexing. After centrifuging, the supernatant was transferred to a fresh tube. The DNA was precipitated with 2 volumes of ethanol and kept on ice for 1 h. It was then mixed by vortexing and allowed to stand for 2 min at room temperature. The mixture was centrifuged. After which, the supernatant was discarded and the pellet was dried in air. The pellet was washed with 1 mL of ethanol at 4°C, centrifuged and allowed to air-dry for 5 min. The nucleic acids were redissolved in 50 μL of TE (pH 8.0, 10 mM Tris.Cl, 1 mM EDTA, distilled water).

Curing of plasmid: The method of Tomoeda *et al.* (1968) was used. Overnight cultures in complete broth were diluted to 100-fold and 0.5 mL volumes were added to 30 mL nutrient broth. These were incubated overnight and 10% SDS stock solution was added to give the required final concentration of 1% w/v. The cultures were incubated at 37°C with gentle shaking for up to 72 h. Then, 100 μL of the cultures were diluted and plated on MacConkey agar plates. Discreet single colonies were marked and subcultured onto antibiotic minimal plates. The colonies that failed to grow on the minimal plates were selected and screened for plasmids as previously described (Birnboim and Doly, 1979). Those colonies that failed to grow on the minimal plates and were found to have lost their plasmids were selected as cured strains.

Electrophoresis of plasmid DNA: Agarose gel electrophoresis was performed using 0.8% horizontal slab gels in TBE buffer. Gels were stained with ethidium bromide (2 μL) and ran at 61 volts for about 1 h. DNA molecular weight marker VI (Boehringer Mannheim GmbH, Germany) was used.

RESULTS

The antimicrobial susceptibility results for all 52 isolates analyzed are summarized in Table 1. For the 10 agents tested, tetracycline (100.0%) and ampicillin (94.2%) showed the highest rates of resistance and ciprofloxacin (50.0%) demonstrated the lowest.

Table 2 showed that majority of multidrug resistant isolates were resistant to 10 antimicrobials and these accounted for 32.7% of total isolates. All isolates were resistant to 3 or more antimicrobials and defined as multidrug resistant, MDR. The list of 23 MDR phenotypes identified is shown in Table 3, with majority showing concurrent resistance to the 10 agents (n = 17; 32.7%).

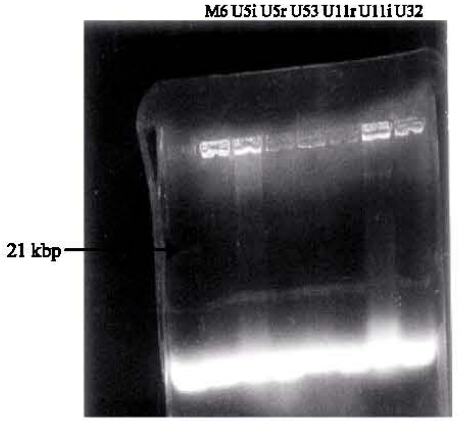

Fig. 1: Plasmid profilie of isolates. Lane 1: MWMVI with sized 154-2176 bp. Lane 2: *E. Coli.* Lanes 4-7: *Klebsiella* spp. Lane 8: *Pseudomonas* spp.

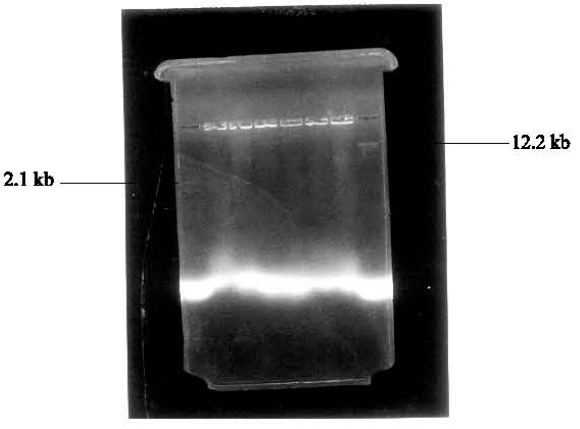

Fig. 2: Plasmid profile of isolates. Lane 1: MWM VI. Lane 8: MWM X. Lanes 2-4: *E. coli. Proteus* spp. Lanes 6-7: *Klebsiella* spp.

Table 1: Antimicrobial susceptibility results for isolates

Antimicrobial agent	No. of isolates	% isolates (No.) classified as[a]: Sensitive	Intermediate	Resistant
Ciprofloxacin, Cf	52	26.9 (14)	23.1 (12)	50.0 (26)
Cefuroxime, Cxm	52	0	9.6 (5)	90.4 (47)
Ceftriaxone, Cro	52	0	7.7 (4)	92.3 (48)
Ampicillin, Amp	52	3.9 (2)	1.9 (1)	94.2 (49)
Gentamicin, Gen	52	5.8 (3)	9.6 (5)	84.6 (44)
Nalidixic acid, Nal	52	1.9 (1)	21.2 (11)	76.9 (40)
Nitrofurantoin, Nit	52	9.6 (5)	9.6 (5)	80.8 (42)
Co-trimoxazole, Cot	52	5.8 (3)	9.6 (5)	84.6 (44)
Streptomycin, Str	52	1.9 (1)	5.8 (3)	92.3 (48)
Tetracycline, Tet	52	0	0	100.0 (52)

[a] according to NCCLS (M100-S9) criteria

Table 2: Resistance to one or more antimicrobials among isolates tested against 10 agents

	No. of agents to which isolates were resistant							
	3	4	5	6	7	8	9	10
No. of resistant isolates	2	0	2	1	5	12	13	17
% resistant isolates	3.9	0	3.9	1.9	9.6	23.1	25.0	32.7

All 52 isolates were resistant to 3 or more antimicrobials and defined as MDR

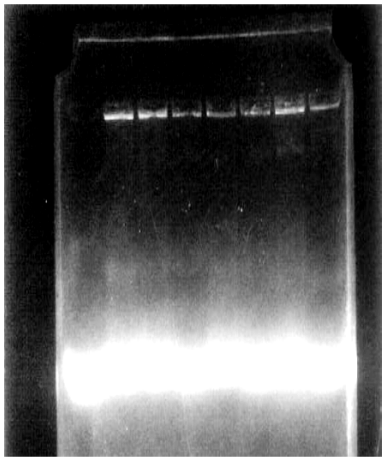

Fig. 3: Plasmid profile of plasmid-cured mutants. Lane 1:MWM VI. Lanes 2-4, 7, 8: *E. coli*. Lanes 5, 6: *Klebsiella* spp.

Ciprofloxacin was used to select resistant mutants. Figure 1 and 2 showed the plasmid profiles of 50.0% (26 out of 52) of the ciprofloxacin resistant MDR isolates screened. 38.5% (10 out of 26) of these isolates carried plasmids of various sizes, estimated mostly at above 2.1 kb and were plasmid-cured as shown in Fig. 3. Most of these mutants lost their plasmids as was corroborated by their improved susceptibilities to the antimicrobial agents used (data not shown).

DISCUSSION

Ciprofloxacin has maintained a high level of activity against UTI isolates compared with other commonly used agents such as ampicillin, co-trimoxazole and amoxycillin (Gupta *et al*., 1999). This study demonstrated that the prevalence of tetracycline (100.0%) and ampicillin (94.2%) resistance among urinary tract isolates recently collected from outpatients was high and ciprofloxacin (50.0%) demonstrated the lowest (Table 1). The majority of MDR isolates was shown to be resistant to all 10 antimicrobials (n = 17; 32.7%). Resistance to 6 agents showed the lowest rate (1.9%) while resistance to 9 agents was a component of 25.0% (n = 13). Of the 23 MDR phenotypes identified, concurrent resistance to ciprofloxacin, cefuroxime, ceftriaxone, ampicillin, gentamicin, nalidixic acid, nitrofurantoin, co-trimoxazole, streptomycin and tetracycline accounted for 32.7% of the total isolates. The current data also demonstrated that a ciprofloxacin resistance phenotype without concurrent resistance to other classes of antimicrobials is uncommon (Table 3). These data in addition to other reports highlight the continued deterioration of tetracycline, ampicillin, streptomycin and ceftriaxone activities against urinary tract pathogens, especially against isolates with concurrent resistance to other antibiotics (Zhanel *et al*., 2000; Sahm *et al*., 2001).

Highly resistant clinical isolates, however, have been shown to have multiple mutations, in some cases associated with intensive fluoroquinolone (ciprofloxacin) selective pressure and the likely presence of natural endogenous reservoirs in which organisms with intermediate levels of resistance may persist (Hooper, 1998). This study also showed that the prevalence of plasmids was high (n = 10; 38.5%) among the 26 ciprofloxacin resistant isolates screened for plasmids. In most of the isolates screened, the sizes of the

Table 3: Antimicrobial resistance phenotypes of 52 MDR uropathogenic isolates

Resistance pattern	No. of isolates	% MDR isolates
Cro, Nit, Tet	1	1.9
Cxm, Nit, Tet	1	1.9
Amp, Nit, Cot, Str, Tet	1	1.9
Cxm, Cro, Amp, Nit, Tet	1	1.9
Amp, Gen, Nal, Cot, Str, Tet	1	1.9
Cxm, Cro, Amp, Gen, Cot, Str, Tet	2	3.9
Cxm, Cro, Amp, Gen, Nal, Str, Tet	1	1.9
Cro, Amp, Gen, Nit, Cot, Str, Tet	1	1.9
Cxm, Cro, Amp, Nal, Cot, Str, Tet	1	1.9
Cxm, Cro, Amp, Nal, Nit, Cot, Str, Tet	1	1.9
Cxm, Cro, Amp, Gen, Nit, Cot, Str, Tet	3	5.8
Cf, Cxm, Cro, Amp, Gen, Cot, Str, Tet	1	1.9
Cxm, Cro, Amp, Gen, Nal, Nit, Str, Tet	4	7.7
Cf, Cro, Gen, Nal, Nit, Cot, Str, Tet	1	1.9
Cxm, Cro, Amp, Gen, Nal, Cot, Str, Tet	1	1.9
Cf, Cxm, Cro, Amp, Nal, Cot, Str, Tet	1	1.9
Cxm, Cro, Amp, Gen, Nal, Nit, Cot, Str, Tet	6	11.5
Cf, Cxm, Cro, Amp, Gen, Nal, Cot, Str, Tet	2	3.9
Cf, Cxm, Amp, Gen, Nal, Nit, Cot, Str, Tet	1	1.9
Cf, Cxm, Cro, Amp, Gen, Nal, Nit, Cot, Tet	1	1.9
Cf, Cxm, Cro, Amp, Nal, Nit, Cot, Str, Tet	1	1.9
Cf, Cxm, Cro, Amp, Gen, Nit, Cot, Str, Tet	1	1.9
Cf, Cxm, Cro, Amp, Gen, Nal, Nit, Cot, Str, Tet	17	32.7

plasmids were estimated at above 2.1 kb, the weight of the largest fragment of molecular weight marker VI. This agrees with the findings of Ogunledun *et al.* (2000) where they reported of bacterial isolates carrying low molecular weight plasmids ranging in size from 2.3 to 30 kb which might not be related to bacterial resistance to multiple antibiotics. In order to relate the presence of plasmids to the susceptibility of the mutant strains, curing of the residential plasmids was performed. The plasmid profiles of the mutant strains showed that most of them have lost their plasmids, thus elaborating comparatively improved susceptibility to most antimicrobials used (data not shown). This has led to the suggestion that there may be the presence of multiple plasmids in the mutants or a plasmid carrying multiple resistance determinants (Martinez-Martinez *et al.,* 1996). If drug resistance in a bacterium isolated from community-acquired infection is found to be largely plasmid-mediated, it may confer epidemic status on the incriminated strains because the resistant gene could easily be transferred to susceptible bacterial strains (Ogunledun *et al.*, 2000).

In conclusion, physicians should be aware of current antimicrobial susceptibility patterns of UTI pathogens in their local communities, as antimicrobial susceptibilities change over time and vary geographically (Sahm *et al.,* 2001). We have also demonstrated the plasmid analysis of the isolates. This dimension of epidemiological investigation will enable us compare data on our local uropathogenic isolates with those from other parts of the world.

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