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Original Article

Multiple-Antibiotic Resistance Mediated by Plasmids and Integrons in Uropathogenic *Escherichia coli* and *Klebsiella pneumoniae*

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Antibiotic resistance in urinary tract infection (UTI) is a growing public health problem in the world. In this study, a total of 182 uropathogens were isolated from patients with symptoms of urinary tract infection (UTI). *Escherichia coli* (88%) was the most prevalent isolate, while *Klebsiella pneumoniae* was recovered from 12% cases. The male/female ratio was 1:3. About 56% female and 51% male patients belonged to the age group >40 years. The antibiotic resistance rates of the isolates to fifteen different drugs were investigated. *E. coli* and *K. pneumoniae* showed variable pattern of susceptibility. The percentage of resistance to different drugs was higher in *E. coli* isolates compared to that of *K. pneumoniae*. Among the total number of isolates about 87% were resistant to at least three commonly used antibiotics. All the isolates were sensitive to imipenem. Analysis of the plasmid DNA had shown that the plasmid pattern was very diverse in both *E. coli* and *K. pneumoniae.* All the isolates contained multiple numbers of plasmid ranging from 1.0 to >140 MDa. Middle-ranged plasmids (30 to 80 MDa), the transferable resistance plasmids, were found to be present in 86% *E. coli* and 85% *K. pneumoniae* isolates. The strong association observed between plasmid profiles and drug resistance patterns suggest that plasmids other than the common plasmids may have epidemiological significance. The presence of class 1 and class 2 integrons were also investigated. A relatively high occurrence of class 1 integrons, that are associated with lateral transfer of antibacterial resistance genes, was observed in *K. pneumoniae* (88%) than in *E. coli* isolates (54%). Class 2 integrons were not found in any of the *E. coli* and *K. pneumoniae* isolates. These results show the high rate of drug resistance and the presence of high rate of transferable elements in these MDR isolates.

Keywords: Uropathogens, *Escherichia coli, Klebsiella pneumoniae,* Multidrug-resistant (MDR) bacteria, Plasmid profiles, Integrons

Introduction

Urinary tract infection (UTI) is a serious health problem affecting millions of people each year. This is the second most common type of infection in human. Urinary tract infection is a common community-acquired bacterial disease, which frequently affects female than male[1]. Increasing rates of resistance among bacterial uropathogens has caused growing concern in both developed and developing countries[2]. *Escherichia coli*, the most common member of the family Enterobacteriaceae, accounts for 75-90% of all urinary tract infections in both in-patients and out-patients. *Klebsiella pneumoniae* is the second most frequently found organism in UTI patients[2]. Antibiotics are the typical treatment for UTIs. Therefore, multidrug-resistant organisms are frequently found in urinary tract infection (UTI)[3].

The emergence and spread of antibiotic resistance is a cause of increasing concern[2]. Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. It is one of the major causes of failure in the treatment of infectious diseases that results in increased morbidity, mortality, and costs of health care[4]. Understanding the molecular mechanism by which resistance genes are acquired or transmitted may contribute to the creation of new antimicrobial strategies as well as some preventive measure to stop further spreading of resistance determinants among the pathogens[5]. There is a large reservoir of resistant genes, in bacterial genomes and in extra-chromosomal pieces of DNA that encode different mechanisms of drug resistance[6]. Although the mechanisms by which organisms acquire resistance are often well understood, including the selective pressures arising from exposure to antimicrobials, the precise role of drug usage in selection of drug resistance has yet to be fully elucidated. Indiscriminate use of antimicrobial by healthcare providers or by way of self-prescribing and over-the-counter availability are major risk factors for the development of high levels of antimicrobial resistance, which is common in rural Bangladesh and other developing countries. Other factors are overcrowding, poor hygienic practices prevalent in rural people of low socio-economic status, and an increasingly mobile population contributed to facilitate the dissemination of antibiotic resistance determinants among the pathogens[7]. Some other

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factors contributing towards resistance include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients, and the use of antibiotics as livestock food additives for growth promotion[4].

Microbes undergo mutation of genes, which can spread from cell to cell by mobile genetic elements such as plasmids, transposons and bacteriophages. Plasmids are extrachromosomal, autonomous DNA that may encode products that aid in virulence, pathogenicity, and the spread of resistance among a wide spectrum of bacteria. They are not indispensable except under certain circumstances. The drug resistance character is most often encoded on plasmids, which can easily be transferred among isolates. Plasmids may effect bacterial virulence and antibiotic resistance and serve as epidemiological markers[8].

Integrons are naturally efficient recombination and expression systems able to capture genes as part of genetic elements known as gene cassettes[9]. Five integrons classes related to antibiotic resistance have been described based on the homology of their integrase genes[9-11]. Class 1 integrons are most commonly found in nosocomial and community environments, followed by class 2 integrons, other integrons classes being scarcely reported to date. Class 1 integrons are associated with lateral transfer of antibacterial resistance genes[9]. Drug resistant genes located on integrons-like structures are being increasingly reported worldwide[12-13].

Therefore, the high isolation rate of drug resistant bacteria especially *E. coli* and *K. pneumoniae* in UTIs implicated a great necessity to study the drug resistance pattern of these organisms and to determine the factors that are responsible for the transfer of this character to other organisms. In this study, the incidence of drug resistance among *E. coli* and *K. pneumoniae* isolated from patient with UTIs was investigated. The frequency of plasmids as well as the relationship among antibiotic resistance, genotypes, and plasmids carriage were also examined. The relationship between antibiotic resistance and the genotypes of these isolates on the basis of *int1* and *int2* genes was also investigated.

Materials and Methods

Bacterial Isolates

A total of 182 isolates of *E. coli* and *K. pneumoniae* were isolated from patients having symptoms of urinary tract infections (UTIs) who submitted their urine samples to Popular Diagnostic Centre, Dhaka during a three month period from September through December 2006. The organisms were identified using standard microbiological technique[14].

Antibiotic susceptibility test

Bacterial susceptibility to antimicrobial agents was determined by the disk diffusion method as recommended by the National Committee for Clinical Laboratory Standards[15] with commercial antimicrobial discs (Oxoid, USA). The antibiotic disks used in this study were ampicillin (10 μg), amoxicillin (10 μg), cotrimoxazole (30 μg), cephalexin (30 μg), cephradine (30 μg), ceftriaxone (30 μg), erythromycin (15 μg), ceftazidime (30 μg), levofloxacin (5 μg), gentamicin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), imipenem (10 μg), and azithromycin (15 μg). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains for susceptibility studies.

Isolation of plasmid DNA

Plasmid extraction procedure was carried out following the methods described by Ish-Horowitz and Burke[16] with a slight modification. The organisms were grown aerobically in 5.0 ml of Luria broth in a test tube at 37°C using a test tube shaker set at 140 rpm. Overnight culture (1.0 ml) was taken in an Eppendorf tube and cells were collected by centrifugation for 7 min at 12,000 rpm. The supernatant was discarded. The pellet was thoroughly suspended in 100 μl of solution I and the solution was kept at room temperature (32°C) for 10 min. Two hundred microlitre of solution II (lysis solution) was added and mixed gently by inverting the tube for a few times. Hundred fifty microlitre of ice-cold solution III (neutralizing solution) was added and mixed vigorously by vortexing for a few sec. The tubes were kept on ice for 5 min. The mixture was then centrifuged at 12,000 rpm for 15 min to pellet the chromosomal DNA. The clear supernatant (approximately 400 μl) was taken to fresh Eppendorf tubes. Two volumes of cold 95% ethanol (800 μl) was added in each tube and vortexed for a few sec to mix well. It was then kept in room temperature for about 1 h for DNA precipitation. The precipitated DNA was collected by centrifugation for 15 min at 12,000 rpm. The supernatant was discarded. The pellet was dried in a drier at 45°C for 45 min. The dried DNA was dissolved in 30 μl Tris-EDTA buffer and kept at 4°C.

Plasmid DNA was separated by horizontal electrophoresis in 1% agarose slab gels in a Tris-borate EDTA (EDTA) buffer at room temperature at 100 volt (50 mA) for 3 h. Briefly, the gel was stained with 0.5 μg/ml of ethidium bromide for 30 min at room temperature. DNA bands were visualized and photograph was taken using an MP4 land camera with UV transilluminator. Plasmids present in strains *E. coli* PDK-9, R1 and V517 were used as molecular weight standard.

Polymerase chain reaction (PCR)

Detection of class 1 and class 2 integrons was performed by polymerase chain reaction (PCR). The primers used for detection of *int1* and *int2* genes by PCR method are presented in Table 1. A single colony of each isolate was suspended in 25 μl of reaction mixer containing 2.5 μl of 10x PCR, 1.5 μl of 50 m*M* $MgCl_2$, 2 μl of 2.5 m*M* dNTP, 1 μl of primer (forward and reverse) together with 1 unit of *Taq* DNA polymerase (5 U/μl). Volume of the reaction mixture was adjusted by adding filtered deionised water. PCR assays were performed in a DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, 480220/220 V. 50 Hz). Each PCR test used the same basic set-up: 94ºC for 12 min followed by 30 cycles of 1 min at 94ºC, 30 sec at $T_{\text{Annealing}}$ (60ºC) and Ti_{Elongate} (2 min) at 72ºC, where $T_{\text{Annealing}}$ is the specific annealing temperature and Ti_{Elongate} is the specific elongation time for each reaction, with a final

extension at 72°C for 10 min. A reagent blank, which contained all components of the reaction mixture with the exception of the bacteria, was included in every PCR procedure. ATCC *E. coli* 25922 strain was used as negative control for all PCR. *E. coli* ur-31, and *E. coli* ur-60 were used as positive controls for *int1*, *int2* gene respectively. Amplification products were subjected to horizontal gel electrophoresis in 1% agarose gel (type II, Sigma, USA) in TBE (Tris-borate EDTA) buffer at room temperature at 100 volt (50 mA) for 1 h. DNA bands were visualized by staining the gel with ethidium bromide (0.5 µg/ml) for 30 min and photographed.

Table 1. *Oligonucleotide primes used in the PCR assay*

Primer	Oligoneucleotide sequence (5' to 3')	Position of amplicon
Int 1-F	GGTCAAGGATCTGGATTTCG	786-766
Int 1-R	ACATGCGTGTAAATCATCGTC	303-324
Int 2-F	CACGGATATGCGACAAAAAGGT	219-240
Int 2-R	GTAGCAAACGAGTGACGAAATG	1,007-986

Results

E. coli and *K. pneumoniae* were isolated from 182 cases, of which 73.6% (n = 134) were from female patients and 26.4% (n = 48) were from male patients. Among the female and male patients, respectively 56.0% (n = 75) and 52.1% (n = 25) cases belonged to the age group >40 years.

The bacterial isolates were tested against different antibiotics to determine the antibacterial susceptibility pattern of the isolates. Fig. 1 and 2 show the antibacterial resistance pattern of the *E. coli* and *K. pneumoniae* isolates. Both of these organisms showed variable pattern of susceptibility. In case of *E. coli*, the resistance towards azithromycin was 98%. Nearly 85% *E. coli* isolates exhibited resistance to ampicillin and amoxicillin, about 74-84% isolates to ciprofloxacin, nalidixic acid and tetracycline, and about 62% isolates to cephalexin, cephradine and levofloxacine. Moreover, 40-50% *E. coli* isolates also showed resistance to gentamicin, ceftazidime, ceftriaxone and erythromycin. All of the *K. pneumoniae* isolates

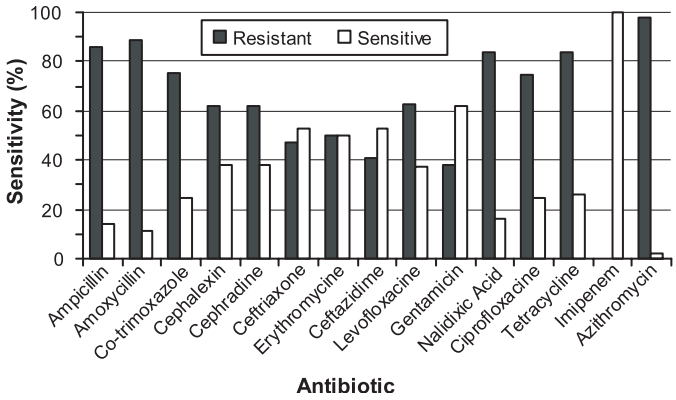

Figure 1. *Antibiotic resistance/sensitivity pattern of the Escherichia coli isolates (n = 160) recovered from UTI patients.*

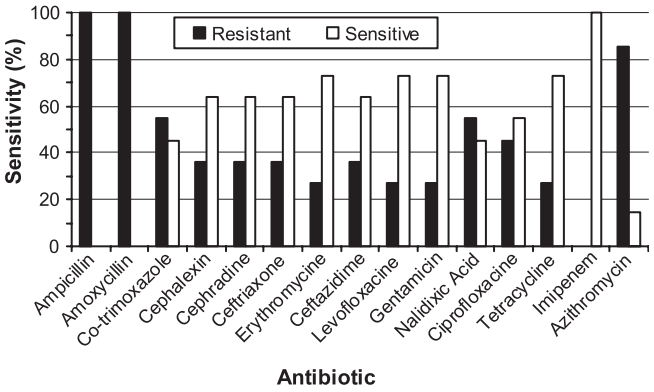

Figure 2. *Antibiotic resistance/sensitivity pattern of the Klebsiella pneumoniae isolates (n = 22) recovered from UTI patients.*

were found to be resistant to ampicillin and amoxicillin, and resistance to azithromycin was observed with 86% isolates. About 45-54% *K. pneumoniae* isolates also showed resistance toward ciprofloxacin, nalidixic acid and cotrimoxazole, about 27% to erythromycin, tetracycline, gentamicin and levofloxacine, and about 36% to cephalexin, cephradine, ceftriaxone and ceftazidime. None of the *E. coli* or *K. pneumoniae* isolates was found resistant to imipenem.

All the multidrug-resistant (MDR) isolates were examined for the presence of plasmids. Analysis of plasmid DNA by agarose gel electrophoresis revealed that all the isolates contained multiple numbers of plasmid ranging from 1 to more than 140 MDa, forming a unique banding pattern (Figure 3 and 4). Middle ranged plasmid (30 to 80 MDa) was found to be present in 86% *E. coli* and 85% *K. pneumoniae* isolates.

PCR was performed for all the MDR isolates. In all the isolates the PCR products were successfully amplified to the expected 1,900 bp for *int1* gene. Class 1 integron was present in 54% *E. coli* and 88% *K. pneumoniae* isolates. None of the isolates contain any *int2* gene.

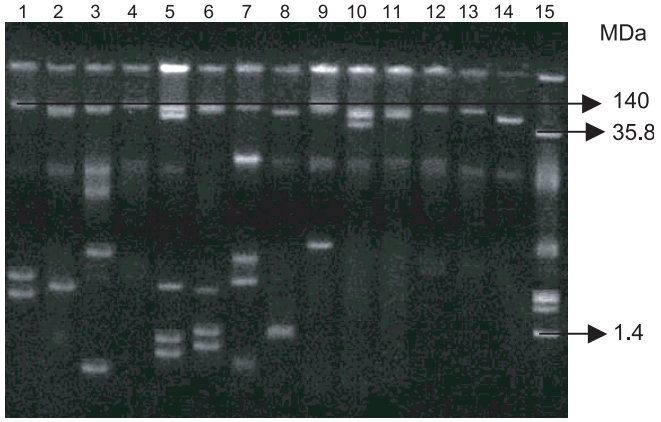

Figure 3. *Plasmid profile of Escherichia coli isolates. Lane 1: E. coli PDK-9 (marker); lanes 2 to 13: E. coli clinical isolates; lane 14: E. coli R1 (marker); lane 15: E. coli V-517 (marker).*

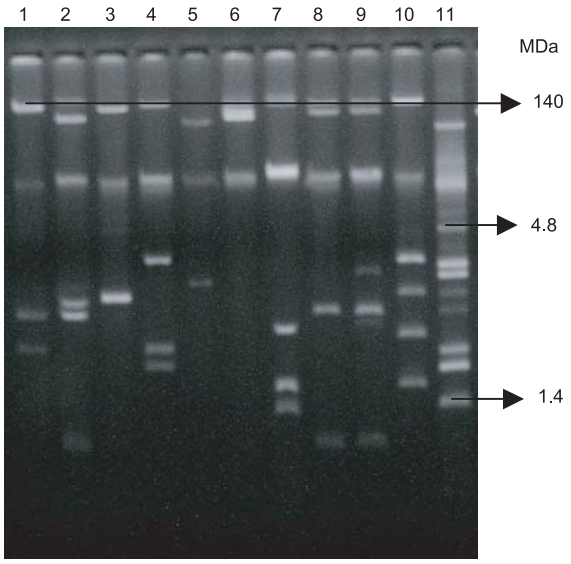

Figure 4. *Plasmid profile of Klebsiella pneumoniae isolates. Lane 1: Escherichia coli PDK-9 (marker); lanes 2 to 10: K. pneumoniae clinical isolates; lane 11: E. coli V-517 (marker).*

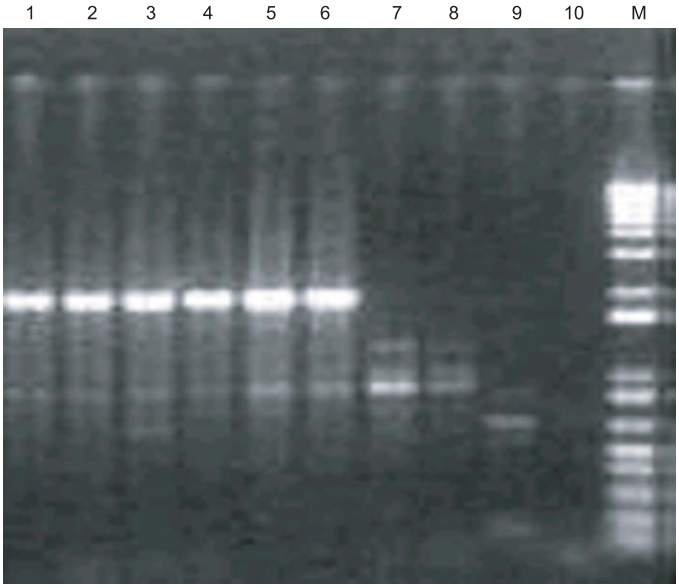

Figure 5. *Class 1 integron (int1; 1,900 bp) PCR of Escherichia coli and Klebsiella pneumoniae isolates. Lanes 1-3: int1-positive E. coli isolates; lanes 4-6: int1-positive K. pneumoniae isolates; lanes 7-9: int1-negative E. coli isolates; lane 10: int1-negative K. pneumoniae isolates; M: 1 kb DNA ladder*

Discussion

UTI ranks as one of the most important causes of morbidity even today in the developing countries like Bangladesh[17]. This may be attributed to lack of proper research, abuse of chemotherapeutic agents and most importantly ignorance of people and little or no preventive measure. There have been several studies on bacterial cause of UTI in Bangladesh[18-19]. This study had been designed to investigate the drug resistance pattern and the frequency of plasmids as well as the relationship between antibiotic resistance, genotypes, and plasmids carriage of the MDR *E. coli* and *K. pneumoniae* isolates in UTI patients.

A total of 182 isolates (160 *E. coli* and 22 *K. pneumoniae*) associated with UTI were analyzed. These isolates were isolated and identified according to the standard microbiological method. *E. coli* was found in 87.9% cases, while *K. pneumoniae* in 12.1% cases. The greater dominance of *E. coli* and considerable isolation rates of other organisms from UTI patients are supported by many investigators[20-22]. To determine if there was any correlation of UTI with sex and age, the patient histories were analyzed and it was found that about 74% patient were women. It was previously found that UTI is always a very common phenomenon among the women[23-24]. Women are mosttly at risk of developing a UTI and half of all women develop a UTI during their lifetimes[1]. Men and women of elderly group were found to be very much prone to UTI. In this study, about 56% female and 51% male patients were above 40 years of age. It has been previously reported by several investigators[25-26] that after middle age the incidence of UTI increases in men progressively owing to the development of prosthetic enlargement and consequent instrumentation. In the present study, a much greater prevalence of this infection was observed in male and female at this stage of life.

All the isolates were tested for antibacterial susceptibility. *E. coli* and *K. pneumoniae* showed variable pattern of susceptibility. The percentage of resistance to different drugs was higher in *E. coli* compared to *K. pneumoniae*. But all (100%) were sensitive to imipenem. According to Franklin *et al*.[27], imipenem is most active agent against Gram-negative isolates, which correlates well with this study. In the present study, all the isolates were collected from UTI patients who had been exposed to great antibiotic pressure. Increased resistance might be due to the extensive use of these drugs. Infection caused by the MDR organisms has currently been treated with carbapenems such as imipenem and meropenem. In the present study, among the total number of isolates about 87% (n = 157) were resistant to at least three commonly used antibiotics. However, resistance to two different antibiotics or to one antibiotic occurred at very low frequencies. In fact, this result might reflect to the idea that the multiple antibiotic resistances among the isolates could be conferred by the plasmid and might be attributed from other organisms by any other gene transfer method.

Analysis of plasmid profiles is useful tool with which to document the appearance of plasmid associated with important phenotypic characteristics. Most importantly the drug resistance character is most often encoded on plasmids, which can easily be transferred among isolates. Analysis of the plasmid DNA of *E. coli* and *K. pneumoniae* isolates had shown that all the isolates tested contained multiple numbers of plasmids ranging from 1.0 to more than 140 MDa. The plasmid pattern was very diverse in both *E. coli* and *K. pneumoniae.* Middle ranged plasmid (30 to 80 MDa) was found to be present in 86% *E. coli* and 85% *K. pneumoniae* isolates. Plasmid-mediated multiple antibiotic resistance in different bacterial species are well recognized today.

It appears from a previous study that the transferable resistance plasmid is the middle order plasmid having a molecular weight ranging between 44 and 76 MDa[28]. The present study showed that about 87% isolates were resistant to multiple antibiotics, of them 86% *E. coli* and 85% *K. pneumoniae* isolates harboured this middle order-ranged plasmid. The strong associations observed between plasmid profiles and drug resistance patterns suggested that plasmids other than the common plasmids may have epidemiological significance.

The drug resistant gene located on integron-like structures is being increasingly reported worldwide[12-13]. In this study the presence of class 1 and class 2 integrons were also investigated. Class 1 integron was found to be present in both *E. coli* and *K. pneumoniae.* A relatively high occurrence of class 1 integrons was observed in *K. pneumoniae* than in *E. coli* isolates (88% and 54% respectively). Class 2 integron was not found in any of the *E. coli* and *K. pneumoniae* isolates. Previous studies had shown the association of the drug resistant genes with plasmids from bacteria responsible for nosocomial outbreaks, which were associated with class 1 integrons[29]. Class 1 integrons are frequently reported in clinical isolates of the family Enterobacteriaceae. As in other recent studies, resistance to quinolones was more common among integrons-containing strains[30].

The widespread dissemination of MDR organisms would severely limit the therapeutic options of physicians facing these organisms, because the carbapenems are the only drugs uniformly active against these organisms. It is important that these newer antimicrobial agents be used sparingly and with discretion. Furthermore, continuous monitoring of the antibiotic susceptibility of carbapenems is also necessary to check the effectiveness of this drug.

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