Isolation and Characterization of Multidrug Resistant Enterobacteriaceae in Urine Sample of Patients Suffering from Urinary Tract Infection with Diabetes and Nephropathy

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ABSTRACT: Multidrug-resistant (MDR) organisms are spreading widely and becoming an issue of utmost importance to deal with. In the current study, ten urine samples from diabetic patients suffering from multiple complications, including urinary tract infection (UTI) and nephropathy were investigated. Antibiogram assays of the bacterial isolates from collected samples demonstrated resistance against most of the antibiotics tested. Further studies were conducted to determine the types of resistant bacteria that caused UTI. Analyzing the 16S rDNA sequence and phylogenetic tree, 3 isolates were identified as *Escherichia coli*, 5 as *Klebsiella pneumoniae* and the rest 2 as *Enterobacter asburiae*. The findings of this research indicate the necessity of urgent attention to find an effective alternative drug for treating infections caused by these resistant isolates.

Key words: UTI, MDR, Enterobacteriaceae, Antibiogram

INTRODUCTION

Every year about 150 million people are affected by urinary tract infections (UTIs) worldwide.[1] UTIs are the most common infection in females, although the male is also affected with lower frequency.[2] Bacteria are the major causative agent of UTIs, and the most commonly found bacteria in the process are *Escherichia coli*, *K. pneumoniae*, and *Enterobacter* spp.[3]

Antimicrobial resistance is an increasing threat posing serious health concerns. Infectious diseases that were previously treatable with antibiotics are often not cured presently with those antibiotics any more. As a result, mortality, morbidity and overall health care cost are rising.[4] Bacteria adapt various genetic and biochemical mechanisms to survive against new antibiotics.[5] Resistant strains of bacteria are continuously evolving by spreading antibiotic resistance genes through mobile genetic elements such as plasmids, transposons and integrons.[6-8] Therefore, it is important to assess routinely whether the bacterial strains develop any resistance against available antibiotics. This would help to address the challenges of treating indigenous diseases caused by Multidrug-Resistant (MDR) bacteria and to discover new treatment options by studying the resistance mechanism.

The UTI patients who remain unresponsive to various antibiotics have been shown to respond effectively against polymyxins and colicins.[9-11] Polymyxins are known to represent the most used antimicrobial options against carbapenem-resistant *K. pneumoniae*. Indeed, polymyxin E (colistin) has been considered as a last resort antimicrobial agent to fight against MDR *K. pneumoniae* infections.[11] However, in many cases especially in diabetic neuropathy and

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nephropathy, this drug could not be prescribed due to its possible adverse renal and neurological effects.[12,13] As a result, more analysis and care should be taken while prescribing these drugs to diabetic patients.[14] A recent report of colistin-resistant *K. pneumoniae* isolate also raise concern for limiting further antimicrobial treatment options in these type of infections.[15]

In the current study, patients suffering from urinary tract infection along with diabetes and showing unresponsiveness to several antibiotic drugs were investigated for the presence of MDR bacteria. The 16S rDNA gene was used for the identification of bacteria for phylogenetic studies.[16] Besides highly conserved sites were used to design primer, and 16S rDNA gene which contains hypervariable regions were used to compare bacteria and their identification.[17-18] Other DNA regions were also used for phylogenic relation prediction.[19-20]

Isolation and characterization of the MDR bacteria revealed the nature of drug resistance in the patient. This study analyzed resistance patterns of MDR bacteria, identified MDR bacteria and established their phylogenetic relationship.

MATERIALS AND METHODS

Collection of urine samples and culture: For sample collection, we choose 10 UTI patients suffering from diabetes and nephropathy and were unresponsive to antibiotics treatment. Clean-catch midstream urine samples were collected using sterile wide-mouth glass containers. Samples were plated on Nutrient agar and MacConkey agar media (Hi media, India) using calibrated wire loops and then incubated aerobic atmosphere at 37°C for 24 hours. A significant infection was considered if urine cultures yield > 10^5 colony-forming units (CFU/ml). The pure culture was preserved for further use. The individual bacterial strain was characterized by visual observation of the colony and also by gram staining and other tests with standard protocol.[21]

Antibiotic susceptibility testing: Antimicrobial susceptibility testing was done on Mueller-Hinton agar plate (Oxoid, England) using Kirby Bauer disk diffusion method.[22] Commercially available discs of amikacin, ampicillin, azithromycin, carbenicillin, cefepime, cefixime, cefotaxime, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, colistin, cotrimoxazole, gentamicin, imipenem, lomefloxacin, mecillinam, meropenem, netilmicin, nitrofurantoin, norfloxacin, piperacillin+tazobactam, polymyxin B (300 U), and tobramycin were used. The resistance and susceptibility of antibiotics were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Identification of bacteria using 16S rDNA sequencing: A partial sequence of 16S rDNA was amplified for each isolate, using Polymerase Chain Reaction (PCR). To do so, a colony was picked from the solid plate culture of bacteria and was suspended into nanopure water. The cell suspension was boiled for 5 minutes at 100°C. The boiled cell suspension was centrifuged at 5,000 rpm for 5 minutes. The supernatant was taken and was used as a template for PCR. The primer pair (27 F-AGA GTT TGA TCM TGG CTC AG and 1492 R- CGG TTA CCT TGT TAC GAC TT) was chosen to amplify three hypervariable regions (V1-V3)[23] of 16S rDNA. PCR amplification was done for 30 cycles and the condition was 1 minute of denaturation at 94°C, 45 second at 57°C for primer annealing, and 2 minutes at 72°C for primer extension, followed by a final step at 72°C for 10 minutes and cooling to 4°C. Products were analyzed by electrophoresis in 1.5% (w/v) ethidium bromide containing agarose gels. The PCR product was purified and quantified and diluted to the concentration required for DNA sequencing. PCR DNA of all 10 isolates tested was sequenced using cycle sequencing followed by capillary electrophoresis, in the Center for Advanced Research in Sciences (CARS), University of Dhaka. The quality of the sequences was assessed and sequences were stored in fasta format. All sequences were analyzed using the web interface of the blast program.[24] The sequences were searched against the 16S ribosomal DNA database of bacteria and archaea using the Megablast algorithm. Then phylogenetic trees for each isolate were constructed using the Maximum likelihood method, taking the top 10

sequences returned by the blast result using MEGA version 5.[25] Analyzing the distant trees, the most closely related sequences were identified for each isolate, and the alignment result was recorded. According to the maximum match the species name was assigned.

RESULTSANDDISCUSSION

Colony characteristics and structural morphology of bacterial isolates from urine samples. When the bacterial isolates were grown on nutrient agar plates at 37°C temperature under aerobic condition, they produced characteristic colony features. The features of their colony and structural morphology are shown in Table 1. All of them were Gram (-) ve and either short rod or rod in shape. The similarities in characteristics were found among isolates-1, 6, 7, 8 and 9; isolates 2, 3 and 10; and isolates 4 and 5 (Table 1).

Table1. Colony characteristics and structural morphology of bacterial isolates.

	Isolates									
Characteristic	1	2	3	4	5	6	7	8	9	10
Colony morphology	grayish white, circular, mucoid	white, circular, Smooth, opaque	white, circular, Smooth, opaque	opaque, grey, smooth, mucoid	grey, smooth, convex, mucoid	grayish white, circular, mucoid	grayish white, circular, mucoid	grayish white, circular, mucoid	grayish white, circular, mucoid	grayish white, circular, opaque
Gram staining	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve
Shape	straight rod	short rod	Short rod	bacilli	bacilli	Straight rod	straight rod	straight rod	straight rod	short rod
Motility	Non motile	motile	motile	Non motile	Non motile	Non motile	Non motile	Non motile	Non motile	motile

Table 2. Antibiogram of isolates obtained from urine samples of UTI patients suffering from diabetes and nephropathy.

	Name of the antibiotics																						
Isolates	Amikacin	Ampicillin	Azithromycin	Carbenicillin	Cefepime	Cefixime	Cefotaxime	Ceftazidime	Ceftriaxone	Cefuroxime	Ciprofloxacin	Colistin	Cotrimoxazole	Gentamicin	Imipenem	Lomefloxacin	Mecillinam	Meropenem	Netilmicin	Nitrofurantoin	Norfloxacin	Piperacillin+ Tazobactam	Polymyxin B	Tobramycin
1	S	R	S	R	R	R	R	R	R	R	R	S	R	R	S	R	R	S	R	R	R	R	S	R
2	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	S	R	R	R	R	R	S	R
3	R	R	R	R	R	R	R	R	R	R	R	S	I	S	R	R	S	R	R	R	R	**I**	S	R
4	R	R	R	R	R	R	R	I	R	R	R	S	R	R	S	R	R	S	S	I	R	R	S	R
5	S	R	I	R	R	R	R	R	R	R	R	S	R	R	S	R	I	S	R	R	R	R	S	R
6	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	S	R
7	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	S	R
8	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
9	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	R	R	R	R	S	R
10	R	R	R	R	R	R	R	R	R	R	R	S	R	R	I	R	R	I	R	R	R	R	S	R

S= Susceptible, R= Resistant, I = Intermediate.

Bacterial isolates showed resistance against most antibiotics tested. In the antibiotic susceptibility test, we used 24 different antibiotics. Our result revealed that all of the tested bacteria showed resistance against most of the antibiotics. Only 2 antibiotics (colistin and polymyxin B) were found sensitive to all bacterial isolates except isolate 8, which showed resistance against all the antibiotics tested. Amikacin, imipenem, mecillinam, meropenem were found relatively sensitive (not so strong effect as colistin) to some isolates. Among the isolates, isolate-1 was found sensitive against 6 antibiotics (amikacin, azithromycin, colistin, imipenem, meropenem and polymyxin B), isolate-2 against 4 antibiotics (colistin, imipenem, Mecillinam, and polymyxin B), isolate-3 against 4 antibiotics (colistin, gentamycin imipenem, and polymyxin B) isolate-4 against 5 antibiotics (colistin, imipenem, meropenem, netilmicin, and polymyxin B), isolate 9 against 3 antibiotics (amikacin, colistin and polymyxin B), and isolates 6, 7 and 10 against only 2 antibiotics (colistin and polymyxin B) (Table 1 and Figure 1). These results revealed that the isolates were MDR in nature and very few antibiotics became sensitive to them. This result indicates difficulties to treat the patients suffering from UTI and other complicy like diabetes and nephropathy.

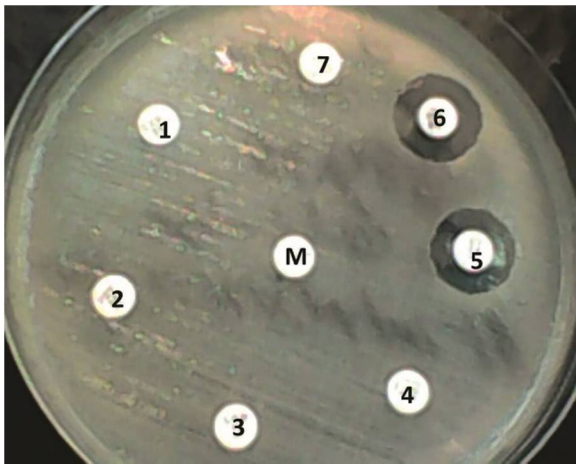

Figure 1. A representative antibiogram figure of the isolate-6. 1- Ampicillin; 2- Cefepime; 3- Ceftriaxone; 4- Imipenem; 5- Colistin; 6- Polymyxin B; 7- Norfloxacin; M-mock (without antibiotics). This isolates showed sensitivity against only Colistin and Polymyxin B.

PCR and sequencing of 16S rDNA gene. For the amplification of 16S rDNA, colony PCR was performed. The gel electrophoresis showed an appropriate band of 514bp (Figure 2). After the purification of the PCR amplified 16S rDNA gene, the sequence for all isolates was obtained through cycle sequencing and capillary electrophoresis. The quality of the sequences was satisfactory. The sequences were then analyzed to identify the isolates.

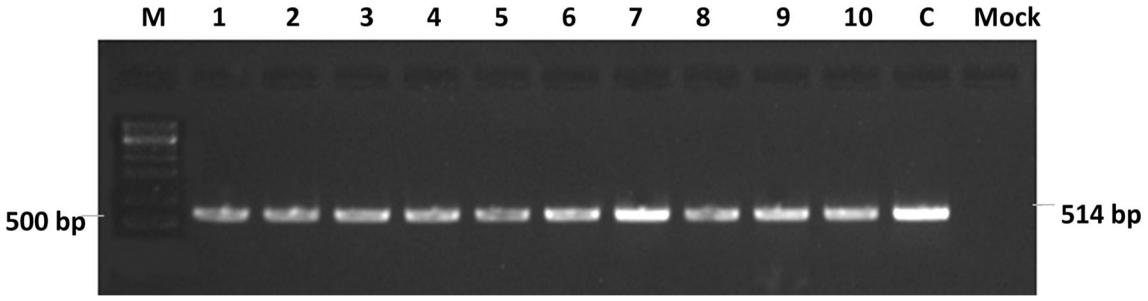

Figure 2. Gel electrophoresis. A purified PCR product of partial 16S rDNA (514 bp) was visualized in gel electrophoresis. M- was for 100 bp DNA ladder, while 1 through 10 were for purified PCR product of the isolates, C-positive control and Mock-PCR without any DNA template. No band in this mock lane indicates absence of DNA contamination in the reagents.

Phylogenetic and 16S rDNA sequence analyses revealed the isolates belonging to Enterobacteriaceae family. Using the blast program, the partial 16S rDNA gene sequences were compared against the nucleotide database, and the isolates were identified based on the sequence similarity (Table 3). The phylogenetic tree was constructed by the maximum likelihood method

using MEGA version 5. Distance tree analysis demonstrated that the isolates 1,6,7,8 and 9 were closely related to *K. pneumonia* (Table 3 and Figure 3) showing 98% - 99% similarity to the reference sequence in the database. Isolate-2, 3 and 10, were closely related to *E. coli* showing 98%, 96% and 97% similarity to the reference sequence, respectively. Isolates 4 and 5 were found to be closely related to *E. asburiae* showing 97% similarity to the reference sequence. For all the isolates close match was found based on the shortest distance in the phylogenetic tree, and maximum identity, the genus and species were suggested according to the maximum match (Table 3).

Table 3. Blast analysis of partial 16S rDNA sequence searched in nucleotide database.

Isolate ID	Query cover	E value	Identity	Species to which the max match belongs to
1	100%	0.00	99%	*Klebsiella pneumoniae*
2	100%	0.00	98%	*Escherichia coli*
3	100%	0.00	96%	*Escherichia coli*
4	100%	0.00	97%	*Enterobacter asburiae*
5	100%	0.00	97%	*Enterobacter asburiae*
6	100%	0.00	99%	*Klebsiella pneumoniae*
7	100%	0.00	98%	*Klebsiella pneumoniae*
8	100%	0.00	98%	*Klebsiella pneumoniae*
9	100%	0.00	99%	*Klebsiella pneumoniae*
10	100%	0.00	97%	*Escherichia coli*

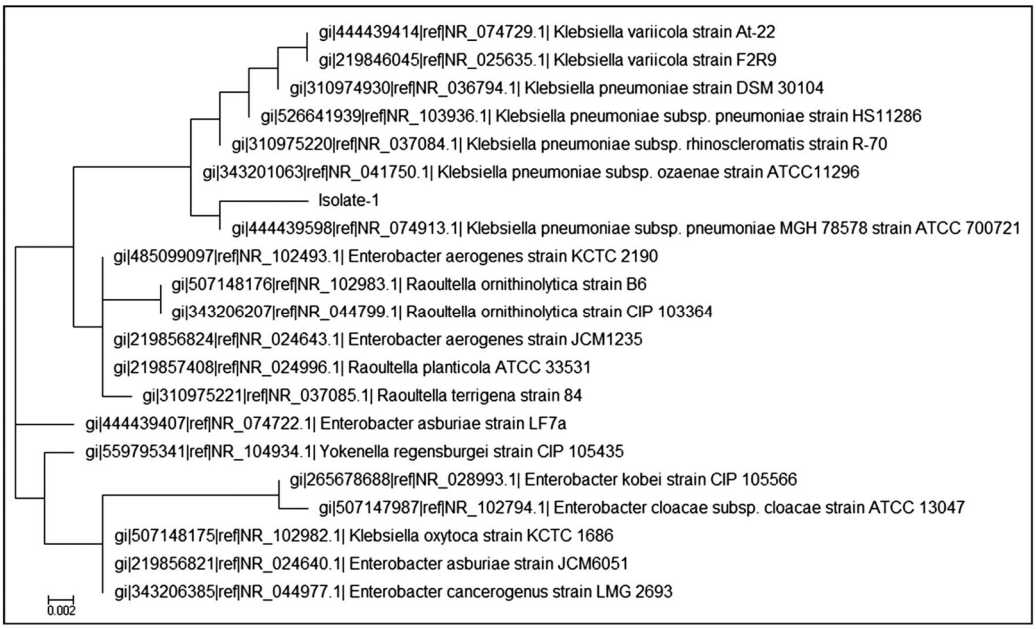

Figure 3. A representative figure of phylogenetic treeanalysis.Isolate-1 was found closely related to *Klebsiella pneumonia*.

Our results demonstrated that all the MDR strains were resistant to antibiotics like ampicillin, carbenicillin, cefepime, cefixime, cefotaxime, ceftazidime, ceftriaxone, cefuroxime, and ciprofloxacin, cotrimoxazole, lomefloxacin, nitrofurantoin, piperacillin+tazobactam, and tobramycin rendering them obsolete for present-day application. Amikacin, azothomycin, imipenem,

mecillinam, meropenem remain capable of killing some of the MDR strains. This indicates that resistance to these antibiotics is very much present, but not widespread. Colistin and polymyxin B have shown to be effective against all the bacterial isolates except isolate 8 which is *K. pneumonia*e. However, these antibiotics have been shown to cause neurotoxicity and nephrotoxicity.[10,12-15] We, therefore, need to develop effective drugs against these kinds of MDR pathogens for better clinical management of the diseases.

Using the 16S rDNA gene to identify the isolates, the ~500 bp sequence was searched in the database, and phylogenetic analyses were conducted. The search result was returned with sequences with a significant match, the E-value for the maximum match was zero for each isolate. Sequences were found 96% to 99% identical to at least one sequence in the database. There is always a debate about the exact percentage of identity that a sequence should possess to conclude that it belongs to a particular species. However, more than ~97% identity is sufficient to decide the species of a bacterium.[26] The partial 16S rDNA sequence for both the isolates lies very close to so many diverse genus and species that make it hard to reach any conclusion about the species level identity of the isolates. However, in this study species-level identity was suggested based on maximum similarity.

According to the maximum similarity to sequences deposited in the database, isolate-1 and 6-8 were *K. pneumoniae*, isolate-2, 3 and 10 were *E. coli*, isolate-4 and 5 were *E. asburiae*. All of them belong to a large family of bacteria, Enterobacteriaceae. So, it can be concluded that MDR bacteria of the Enterobacteriaceae family were responsible for causing multiple antibiotic-resistant urinary tract infections (UTI) along with diabetes, nephropathy and other complications in patients.

In this study, all the strains of bacteria were successfully identified and their phylogenetic relationships were established. From their antibiogram, the resistance profile was also revealed. As these organisms are abundantly associated with human disease, antimicrobial agents have to be available to control them; otherwise, serious epidemics can occur. The resistance genes occur naturally in the bacteria of diverse taxonomical variations. Bacteria are capable of exchanging those genes between the same as well as different species. Moreover, mutations may help to produce more variations capable of defending a wide spectrum of antibiotics. Because of these adaptive mechanisms, bacteria acquire a fighting tendency against any newer antibiotic possible.[27] Targeted synthetic or antimicrobial agents can be developed specifically to the mechanism of resistance only if the molecular mystery in the organisms is known. This study directs future research on the genome, proteome and metabolome of this Enterobacteriaceae to predict potential therapeutic targets conserved in all three pathogens. Therapeutic targets could be identified through analysis of metabolic pathways, comparison of pathogen and host protein sequences, identification of essential proteins and analysis of protein-protein interactions.

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