Antibiotic Susceptibility Profile and Detection of Plasmid-Mediated Quinolone Resistant Genes Among Extended Spectrum β-Lactamases (ESBL) Producing Uropathogens in Women

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Abstract

Background/Aim: The most common bacterial diseases in women around the world are urinary tract infections. Aim of this study, was to evaluate the prevalence and current antibiotic resistance rate of uropathogens isolated from the female patients of a tertiary care hospital in Amritsar, Punjab, India.

Methods: Samples were collected from patients showing urinary tract infection (UTI) symptoms and analysed using microscopy, dipstick test and urine culturing followed by identification and characterisation of to identify the uropathogens. Antibiotic susceptibility test and MIC were performed.

Results: The results revealed that *E coli* (35.5 %) was the most prominent uropathogen followed by *Klebsiella spp* (21 %), *Enterobacter spp* (17 %), *Acinetobacter* (11 %), *Enterococcus spp* (6 %), *Pseudomonas spp* (4.5 %), coagulase negative *Staphylococci* (4 %), coagulase-positive *Staphylococci* (0.5 %) and *Corynebacterium aurimucosum* (0.5 %). The antibiotic susceptibility profile study reported eight isolates with multi-drug resistance properties. However, gentamicin, imipenem and meropenem were found to be the most effective antibiotics against the isolated uropathogens. All the extended spectrum β-lactamase (ESBL)-positive isolates possess the quinolone-resistant gene *qnr*B, while *qnr*A was absent.

Conclusion: The current study revealed that for appropriate treatment, it is crucial to be aware of the epidemiological data regarding the disease and to begin any empirical antibiotic treatment.

Key words: Urinary tract infection (UTI); Uropathogens; Antibiotic susceptibility; β-lactamase; Quinolone resistance; Multi-drug resistant.

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Introduction

Urinary tract infections (UTIs) are the most prevalent bacterial infections in women across the globe. These infections may be community-acquired or nosocomial that are acquired from hospital settings such as catheters.[1] UTI is particularly responsible for causing discomfort

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in elderly and immune-compromised patients constituting a risk of septic shock, bacteraemia, respiratory distress syndrome and even death.[2] Patients having UTIs are usually treated by empirical antibiotic treatment.[3] Therefore, to begin with an appropriate treatment, it is crucial to be aware of the epidemiological data regarding the disease.[4] In the present era, where pathogens possess high antibiotic resistance rates, it is even more important to understand epidemiological information before starting the treatment. The main epidemiological factors that influence the type of UTI, causative agents and antimicrobial resistance rates are age group, sex, geographical location and hospital setting.[5]

Although there are many causative agents of UTIs, the members of the family *Enterobacteriaceae* accounts for most of the infections. *E coli* is the most common uropathogen involved in community-acquired UTIs because it belongs to the intestinal microflora of the human intestine and may easily colonise the urinary system. Several investigations on community cases revealed that the most common uropathogens are *E coli, Klebsiella spp, Enterococcus spp and Proteus spp.*[6]

Antibiotic resistance is one of the major growing concern today. The inappropriate use of antibiotics in human medicine and their misuse in the veterinary and agriculture field are the major contributing factors to antimicrobial resistance.[7] Further, the resistance of pathogenic microorganisms to commonly used antibiotics is a serious concern worldwide as it highly affects the treatment of infectious diseases like UTIs.[8, 9]

The World Health Organization published its first list of antibiotic-resistant "priority pathogens" - a database of 12 families of bacteria that represent the most serious threat to human health. Multi-drug resistant bacteria are the most dangerous of all, posing a particular hazard in hospitals, nursing homes and among patients requiring devices such as ventilators and blood catheters. They include *Acinetobacter, Pseudomonas* and several members of *Enterobacteriaceae* family (including *Klebsiella, E coli, Serratia* and *Proteus*). They can cause severe and frequently fatal illnesses such as bacteraemia and pneumonia.[10]

Extended spectrum β-lactamases are a class of genetic alterations that confer resistance by hydrolysing penicillins, 1st, 2nd and 3rd generation cephalosporins and aztreonam. β-lactamase inhibitors can prevent them from growing. Three primary sets of genes encode extended spectrum β-lactamases (ESBLs) ie TEM, SHV and CTX-M and these enzymes are frequently detected in *E coli* and *K pneumoniae*.[11] ESBLs are produced by a variety of bacteria and were initially connected with hospital-acquired infections, but are now increasingly linked with community-acquired illnesses.[12]

Similarly, fluoroquinolones are used to treat UTIs induced by both gram-positive and gram-negative bacteria. The widespread use of these antibiotics has resulted in resistance, particularly among *Enterobacterales*.[13] Due to their potency, broad spectrum of activity, oral absorption and safety profile, cephalosporins and fluoroquinolones are frequently used to treat community-acquired UTIs in non-pregnant women. However, since antibiotic resistance spreads around the world, the efficacy of these antibiotic treatment alternatives may be jeopardised.[12]

Owing to the growing concern of antibiotic resistance UTIs cases in Amritsar (India), aim of this study was to analyse the prevalence of UTI causing bacteria and their antibiotic susceptibility pattern towards different antibiotics. Also, the study was aimed to uncover the presence of ESBL and plasmid-mediated quinolone resistance (PMQR) genes among isolated uropathogens.

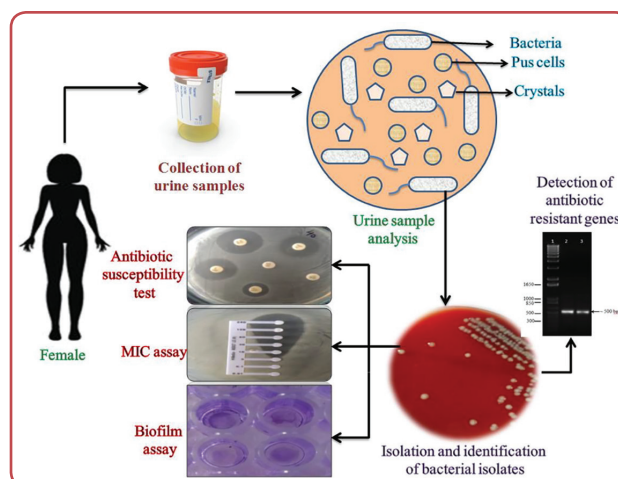## Methods

Flow of the study is presented in Figure 1.

Figure 1: Flow of the study

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Study area and population

The present study was carried out in the Amritsar city of Punjab, India. The urine samples were collected from Guru Nanak Dev Hospital, Amritsar. The study was carried out from April to September 2017. A total of 200 female patients with signs and symptoms of UTI who visited the outpatient department (OPD) of the hospital were selected for this study. The age of patients was between 21 to 60 years. The non-inclusion criteria for this study is the age of patients (< 21 years), patients who were on antibiotics, patients with a history of any implants and patients with a history of hospital admission a week before an OPD visit to rule out nosocomial infections. A questionnaire was prepared to have questions related to the signs and symptoms of the UTI, previous history of the UTI and medication or antibiotics taken. The study was approved by the institutional ethical committee of Guru Nanak Dev University, Amritsar, Punjab (India) (Ref No: -659/HG; Date: 29-Mar-2016). The work was done according to the guidelines provided by Indian Council of Medical Research (ICMR). The purpose of the study was clearly stated and a written consent was taken from each patient involved in the study before sample collection.

Sample collection

The clean-catch midstream of freshly voided urine was collected in a sterile screw-capped container (50 mL). The instructions were given to the patients on how to collect the sample in the container. The collected samples were labelled and transported to the laboratory within 2 h of collection for analysis.

Urinalysis

Urine microscopy. In the preliminary urine analysis, 10 mL of well-mixed urine was taken in a centrifuge tube and centrifuged at 3000 rpm. The supernatant was discarded and the pellet/deposits left in the tube were smeared on the glass slide to observe under the light microscope for any cells, crystals and casts present in the urine.

Urine dip-stick test. The preliminary examination of the urine samples was done using 10 test strips coated with chemicals (*Orinasys, ARKRAY Healthcare Pvt Ltd*, Santacruz East, Mumbai, India). To perform the test, the manufacturer's methodology was followed and the results were also read accordingly. The chemical-coated strips were dipped in the un-centrifuged urine samples and taken out immediately on a blotting paper. The results were noted by observing the colour change on the patches.

Urine culture. The urine culture was done with the help of a calibrated loop. One µL of un-centrifuged, well-mixed urine sample was inoculated with the help of inoculating loop on the surface of MacConkey and blood agar plates (*HiMedia Laboratories*, Mumbai, India). For counting the bacterial colonies, a measured amount of urine (1 µL) was inoculated onto the nutrient agar plates with the help of calibrated inoculating loop. The colony-forming unit count was more or equal to 10^4 CFU/mL for a single potential pathogen interpreted as positive bacteriuria.

Identification of bacterial isolates

The isolated bacteria were identified using standard microbiological methods as stated in Bergey's Manual of Systematic Bacteriology.[14] Differential mediums were used such as mannitol salt agar and eosin methylene blue agar to identify bacteria. Different biochemical tests were performed for bacterial identification including IMViC, sugar fermentation (glucose, lactose, sucrose, mannitol), motility, oxidation/fermentation, oxidase, catalase, urease, coagulase and triple sugar iron. Gram staining was performed to differentiate between Gram-positive and Gram-negative bacteria. Few isolates were further confirmed by 16s rRNA gene sequence analysis. For molecular characterisation, each bacterium was grown on nutrient agar plates and DNA was isolated by following the protocol described by Kaur et al.[15] The 16s rRNA gene sequence of the bacterial samples was amplified using primers as described by Lane.[16] The primer sequences were: 27F (5'-CAGGCCTAACACATGCAAGTC-3') and 1492R (5'- GGGCGGWGTGTACAAGGC-3'). Polymerase chain reaction (PCR) was used to amplify the 16s rRNA gene in a 20 µL reaction mixture as described by Kaur et al.[15] The PCR was performed in a thermocycler (*Agilent Technologies*, Santa Clara, CA, USA). The PCR-amplified product was purified using a Gel Extraction Kit (*IBI Scientific*, Dubuque, IA, USA) following the instructions of the manufacturer. The purified PCR products were sequenced using the same primers provided by DNA sequencing services of 1st BASE, *Axil Scientific Pte Ltd*, Singapore. The sequences obtained were used for a gene similarity search against the National Center for Biotechnology Information (NCBI) database using the BLAST algorithm. The 16s rRNA gene sequences of the isolates were

submitted to NCBI GenBank using *BankIt* (www.ncbi.nlm.nih.gov/Banklt/).

Antibiotic susceptibility pattern of the isolates

Antibiotic susceptibility test. The antibiotic susceptibility pattern of the bacterial isolates was evaluated by the disc diffusion method as given by Bauer and Kirby with few modifications.[17] The isolated bacteria were initially grown in Luria Bertani broth and the cell density was set equivalent to 0.5 McFarland standards by diluting the grown culture with fresh medium. The agar plates were prepared with Mueller-Hinton agar medium and the diluted bacterial culture (0.1 mL) was spread on each agar plate. A sterile forceps were used to place the antibiotic discs over the agar surface. The plates were incubated at 37 ºC in a BOD incubator for 24-48 h. A total of 24 antibiotics belonging to different groups based on their mode of action were procured from *HiMedia Laboratories* (Mumbai, India) and were tested. The antibiotic susceptibility of the isolates was determined by measuring the zone of inhibition around the discs and the results were interpreted according to the guidelines of CLSI.[18] *E coli* ATCC 25922 was used as the control strain.

Multiple antibiotic resistance (MAR) index. The MAR index was calculated by observing the antibiotic susceptibility pattern of the isolates. It was calculated by the method given by Krumperman.[19] Total number of antibiotics to which an isolate was resistant was divided by total number of antibiotics tested against it.

Minimum inhibitory concentration (MIC) assay. MIC of the selected bacterial strains was determined by using commercial *HiComb MIC^TM* strips that contain antibiotic concentrations gradient-wise (*HiMedia Laboratories* Mumbai, India). Briefly, it is based upon the diffusion of the antibiotic into the medium so that MIC values could be estimated directly using a single culture plate. Mueller Hinton agar plates were prepared and the *HiComb MIC^TM* strips were placed on them after inoculating them with desired bacterial culture (cell density equivalent to 0.5 McFarland standards). The MIC was recorded at the point of intersection of the clear zone with the point on the scale of the MIC test strip. In the present study, the sixteen antibiotics selected for the MIC test were: polymyxin B, ciprofloxacin, amoxicillin/clavulanic acid, gentamicin, levofloxacin, imipenem, tobramycin, nitrofurantoin, norfloxacin, na-

lidixic acid, piperacillin, aztreonam, meropenem, amikacin, cefepime and cefpirome were assessed using MIC strips (*Himedia*). *E coli* ATCC 25922 was used as the control strain.

Phenotypic detection of ESBL producers

Double-disc synergy test (DDST). The ESBL production of the isolated Gram-negative bacteria was tested by the modified double disc synergy test (MDDST) given by Kaur et al.[20] In this test, a disc of amoxicillin/clavulanic acid (20/10 µg) along with four cephalosporins; ceftriaxone, ceftazidime cefpodoxime (third generation cephalosporin) and cefepime (fourth generation cephalosporin) were used. A lawn culture of the isolate was made on a Mueller-Hinton agar plate as recommended by CLSI.[18] The amoxicillin/clavulanic acid disc (20/10 µg) was placed in the centre of the plate and the other discs of 3GC and 4GC were placed at 15 mm and 20 mm distance from centre to centre to that of the amoxicillin/clavulanic acid disc. Any distortion or increase in the inhibition zone towards the amoxicillin/clavulanic acid disc was considered positive for ESBL production. *Klebsiella pneumoniae* ATCC 700603 was used as a positive control strain.

Combination disc test (CDT). The test was performed according to the guidelines of CLSI in which the discs containing cephalosporin alone and with the clavulanic acid were used.[18] In this study, a ceftazidime (30 µg) disc alone and in combination with clavulanic acid (30 µg/10 µg) was used. The inhibition zone around the two discs was compared and an increase in the zone diameter ≥ 5 mm of the cephalosporin disc with clavulanic acid was interpreted as positive.

Detection of antibiotic resistance genes in MAR uropathogens. The Gram-negative isolates which were resistant to more than seven antibiotics were further explored for the presence of antibiotic-resistant genes. The plasmid-mediated β-lactamase genes (bla_{SHV}, bla_{CTX-M}, bla_{TEM}, bla_{AmpC}) and quinolone-resistant genes (qnrA and qnrB) were studied among two isolates of *E coli* RBRJ005 and RBRJ013 (Accession No: MN294475, MN294482), two isolates of *K pneumoniae* RBRJ019, RBRJ024 (Accession No: MN294488, MN294493), one isolate of *Acinetobacter baumannii* RBRJ027 (Accession No: MN294496) and one isolate of *Enterobacter cloacae* RBRJ017 (Accession No: MN294486) which were resistant to more than seven antibiotics.

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Plasmid DNA was isolated from these bacterial isolates using High-Speed Plasmid Mini Kit Cat. No IB47101 (*IBI, Scientific*, Dubuque, IA, USA). A single colony of each bacterium was grown in the Luria-Bertani broth for plasmid DNA isolation and the extracted DNA was used as a template for amplification. The multiplex PCR (*Agilent Technologies*, Santa Clara, CA, USA) was used to identify these plasmid-mediated ESBL and quinolone-resistant genes. PCR was performed in a thermocycler (*Agilent Technologies*, USA). The final reaction mixture was 20 µL containing various components.

Biofilm assay. This assay was performed according to the method given by O'Toole with some modifications.[21] The cultures of the selected bacteria were grown overnight in the Luria broth and diluted in a ratio of 1:100 using a fresh medium. From the diluted medium, 100 µL was added to a sterile 96-well microtiter plate. The microtiter plate was covered and incubated for 24 and 48 h at 37 ºC in a BOD incubator. For the quantitative purpose, the assay was performed in the triplicate wells for each culture. After incubation of 24 and 48 h, the bacterial culture was dumped by flipping the microtiter plate and gently shaking to remove the liquid medium from the wells. The wells were washed twice with phosphate-buffered saline (1 x), thus removing the media components and planktonic cells if attached to the wells. About 125 µL 0.1 % crystal violet was used to stain the wells and after 10-15 min the stain was removed by flipping the plate and gently shaking. The autoclaved distilled water was used to wash the microtiter plate until all the excess stain was removed. After that, the plate was shaken and blotted vigorously on a stack of tissue paper and was then dried at room temperature overnight. Thirty three percent glacial acetic acid was added into each well to solubilise the dye for quantification purposes. The microtiter plate was incubated for 10-15 min at room temperature and the optical density (OD) was measured at 590 nm using a microtiter plate reader (*BioTek, Model Synergy HT*). Thirty three percent acetic acid was taken as blank. The biofilm mass and bacterial adhesion were expressed as OD590 nm values. Bacteria were classified according to the scheme of Stepanović et al on the basis of the cut-off OD (ODc) value as non-biofilm producer (OD < ODc), weak biofilm producer (ODc < OD ≤ 2 × ODc), moderate biofilm producer (2 × ODc < OD ≤ 4 × ODc), strong biofilm producer (OD > 4 × ODc).[22]

Results

The age distribution of the patients was between 21-60 years with mean age of 36.5 ± 11.87. The results of the present study revealed that patients of age group 21-30 and 31-40 years showed maximum symptoms of burning micturition, dysuria and pyuria (Table 1).

Table 1: *Patients reporting UTI symptoms*

Age (Years)	No of patients showing these symptoms (n = 200)				
	Burning micturition	Frequency/ Urgency	Dysuria	Haematuria	Pyuria
21-30	49	17	59	5	29
31-40	57	32	63	27	60
41-50	37	21	33	9	39
51-60	13	19	11	2	22

Patients gave multiple responses; UTI: urinary tract infection;

The maximum cases of pyuria were in the age group 31-40 years. In this study, the pyuria was confirmed by both the dipstick test and microscopic examination. The presence of pus cells in the sample was shown in Figure 2, as well as the presence of crystals in the urine samples.

Prevalence of uropathogens

The 16S rRNA PCR amplification gave approximately 1450 bp amplicons of different bacterial isolates. The alignment of partial 16S rRNA sequences against the NCBI database suggested that they belong to different bacterial species (Table 2). In the present study, the most frequently identified uropathogen using urine culture method was *E coli* (35.5 %) followed by *Klebsiella spp* (21 %), *Enterobacter spp* (17 %), *Acinetobacter spp* (11 %), *Enterococcus spp* (6 %), *Pseudomonas spp* (4.5 %) coagulase-negative *Staphylococci* (4 %), coagulase-positive *Staphylococci* (0.5 %) and *Corynebacterium aurimucosum* (0.5 %) (Table 2).

Antibiotic susceptibility pattern of uropathogens

All the isolated uropathogens were susceptible to gentamicin while, two isolates were resistant to amikacin and tobramycin (Table 3). Among the penicillins group, the only effective antibiotic was carbenicillin as only three isolates were resistant to it. In the cephalosporins group, most uropathogens were found to be resistant to third and fourth-generation antibiotics. About 8.5 % were resistant to ceftazidime, while 7.5 % were resistant to cefepime and 9.5 % to cefpirome. An

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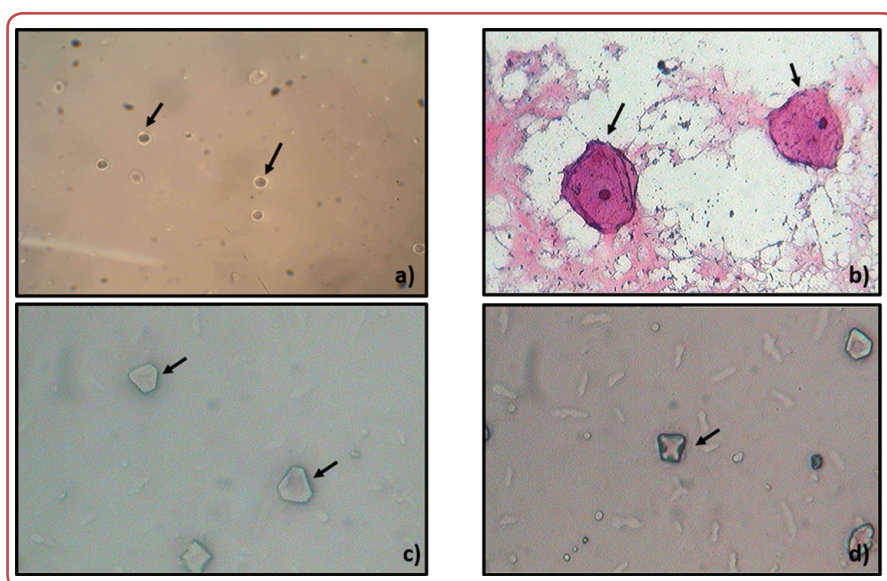Kaur et al. Scr Med 2023 Dec;54(4):315-28.

Figure 2: a) Pus cells, b) epithelial cells, c) cystine crystals and d) calcium oxalate crystals under light microscope (40x)

antibiotic belonging to group monobactams aztreonam proves to be ineffective against 11.5 % of the isolated urinary microbes. All the isolates were susceptible to carbapenems ie imipenem and meropenem, hence, can be used to treat recurrent UTIs. About 13.5 % isolates were resistant to nalidixic acid and ciprofloxacin was found to be ineffective against 7.5 % of the isolates. Polymyxin B was found to be ineffective against 3 % of the isolates. Trimethoprim/sulfamethoxazole was found to be ineffective against 12.5% of the uropathogens.

Table 2: a) Pus cells, b) epithelial cells, c) cystine crystals and d) calcium oxalate crystals under light microscope (40x)

Isolated bacteria	Number (n = 200)	Percentage
Escherichia coli	71	35.5
Klebsiella spp	42	21.0
Enterobacter spp	34	17.0
Pseudomonas spp	9	4.5
Enterococcus faecalis	12	6.0
Coagulase positive *Staphylococci*	1	0.5
Coagulase negative *Staphylococci*	8	4.0
Acinetobacter spp	22	11.0
Corynebacterium aurimucosum	1	0.5

Table 3: Antibiotic susceptibility of uropathogens towards different antibiotics

Mode of action of antibiotics	Antibiotic groups	Antibiotics	Antibiotic concentration (µg)	No of resistant isolates
Inhibitor of protein synthesis	Aminoglycosides	Amikacin	30	2 (1.0 %)
		Tobramycin	10	2 (1.0 %)
		Gentamicin	10	0 (0.0 %)
Inhibitor of cell wall synthesis	Beta-lactam antibiotics (Penicillins)	Amoxicillin/ clavulanic acid	30	19 (9.5 %)
		Ampicillin	10	26 (13 %)
		Carbenicillin	100	3 (1.5 %)
		Piperacillin	100	17 (8.5 %)
	Beta-lactam antibiotics (Cephalosporins)	Cefadroxil	30	9 (4.5 %)
		Cefuroxime	30	4 (2.0 %)
		Ceftriaxone	30	2 (1.0 %)
		Ceftazidime	30	17 (8.5 %)
		Cefepime	30	15 (7.5 %)
		Cefpirome	30	19 (9.5 %)
	Monobactams	Aztreonam	30	23 (11.5 %)
	Carbapenems	Imipenem	10	0 (0.0 %)
		Meropenem	10	0 (0.0 %)

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	Quinolones	Nalidixic acid	30	27 (13.5 %)
Inhibitor of nucleic acids	Fluroquinolones	Ciprofloxacin	5	4 (2.0 %)
		Levofloxacin	5	7 (3.5 %)
		Norfloxacin	10	15 (7.5 %)
	Furans	Nitrofurantoin	300	2 (1.0 %)
Inhibitor of membrane function	Polymyxins	Polymyxin B	300 U	6 3.0 %
Inhibitor of metabolic processes	Sulphonamides	Trimethoprim/ sulfamethoxazole	25	25 (12.5 %)

Table 4: Multiple antibiotic resistance (MAR) and biofilm formation potential of the bacterial isolates

Bacterial isolates	MAR index calculation			Biofilm formation	
	Total number of antibiotics tested (y)	N of antibiotics to which the isolate was resistant (x)	MAR index (x/y)	24 h	48 h
E coli RBRJ005	24	7	0.29	+	++
E coli RBRJ013	24	8	0.33	++	+++
K pneumoniae RBRJ019	24	10	0.41	+	++
K pneumoniae RBRJ024	24	8	0.33	++	+++
Enterobacter cloacae RBRJ017	24	8	0.33	+	++
Acinetobacter baumannii RBRJ027	24	12	0.50	++	+++
Enterococcus faecalis RBRJ015	25	9	0.36	++	+++
Staphylococcus aureus RBRJ010	25	8	0.32	0	+

(0) non-biofilm producer; (+) weak biofilm producer; (++) moderate biofilm producer; (+++) strong biofilm producer;

Antibiotic susceptibility profile of multi-drug resistant uropathogens

Eight bacterial isolates were resistant to > 7 antibiotics and were considered multi-antibiotic resistant based on their antibiotic susceptibility profile. The bacterial isolates, namely *E coli* RBRJ005 and RBRJ013, *K pneumoniae* RBRJ019, RBRJ024, *Acinetobacter baumannii* RBRJ027, *Enterobacter cloacae* RBRJ017, *Enterococcus faecalis* RBRJ015 and *Staphylococcus aureus* RBRJ010 were resistant to > 7 antibiotics (Table 4).

Figure 3 shows the antibiotic resistance pattern, while Figure 4 shows the MIC values of the antibiotics against multi-drug resistant strains. The two *E coli* strains ie RBRJ005 and RBRJ013 were found to be resistant to antibiotics commonly used to treat UTIs. The antibiotic profile of the strain RBRJ005 showed that it was resistant to trimethoprim/sulfamethoxazole, ceftazidime, aztreonam, ampicillin, piperacillin, amoxicillin/ clavulanic acid, nalidixic acid and cefepime, while the strain RBRJ013 was found to be resistant to antibiotics: trimethoprim/sulfamethoxazole, carbenicillin, ceftazidime, aztreonam, ampicillin, nalidixic acid, cefpirome and cefepime. The MIC assay revealed that both the strains were resistant to amoxicillin/clavulanic acid, nalidixic acid, piperacillin and aztreonam.

Similarly, the two strains of *K pneumoniae* RBRJ019 and RBRJ024 were found to be resistant to various antibiotics used to treat UTIs. The MIC assay revealed that the strain RBRJ019 was resistant to ciprofloxacin, amoxicillin/clavulanic acid and gentamicin, while the strain RBRJ024 was resistant to ciprofloxacin, amoxicillin/clavulanic acid, gentamicin, piperacillin, aztreonam and norfloxacin at the specified concentrations given by CLSI. The strain RBRJ017 of *Enterobacter cloacae* was also observed for antibiotic resistance pattern and found that it was resistant to cefuroxime, levofloxacin, cefadroxil, ampicillin, nitrofurantoin, norfloxacin, amoxicillin/clavulanic acid and polymyxin-B at the concentrations specified by CLSI. The other Gram negative bacteria *Acinetobacter baumannii* RBRJ027 of family *Moraxellaceae* was also explored for its antibiotic susceptibility profile and was found to be resistant to many antibiotics, namely amoxicillin/clavulanic acid, nitrofurantoin, piperacillin, aztreonam and cefepime.

The two Gram positive bacteria *Enterococcus faecalis* RBRJ015 and *Staphylococcus aureus* RBRJ010 were investigated for their susceptibility towards different antibiotics including methicillin. The strain RBRJ015 was resistant to ceftazidime, aztreonam, amikacin, nalidixic acid,
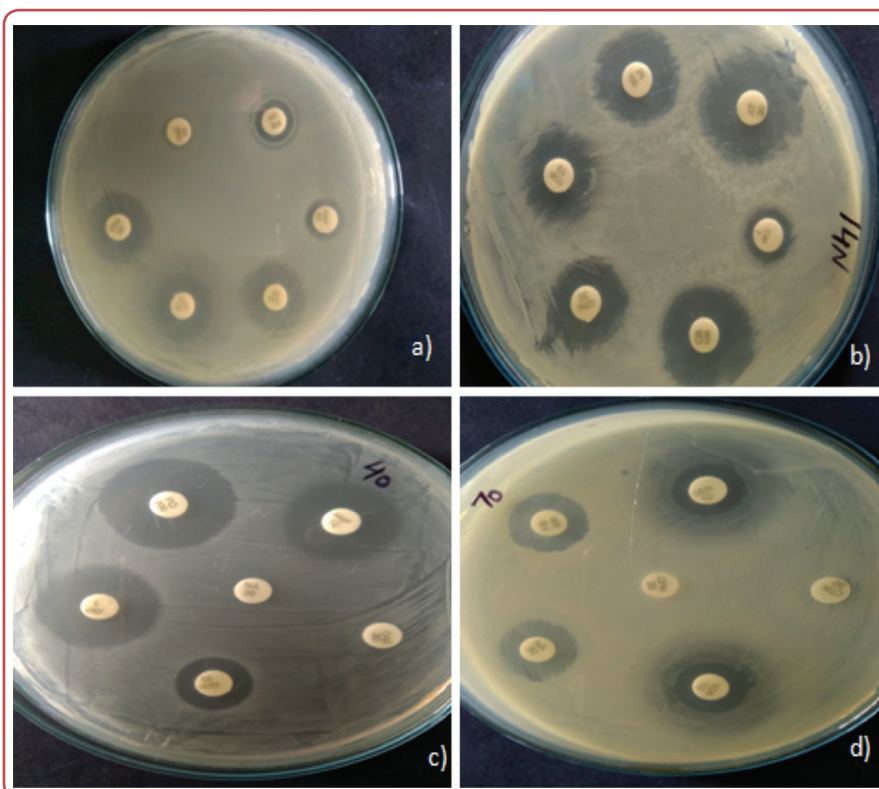
Figure 3: Antibiotic susceptibility of a) Enterobacter cloacae b) Escherichia coli c) Enterococcus faecalis d) Acinetobacter baumannii towards different antibiotics
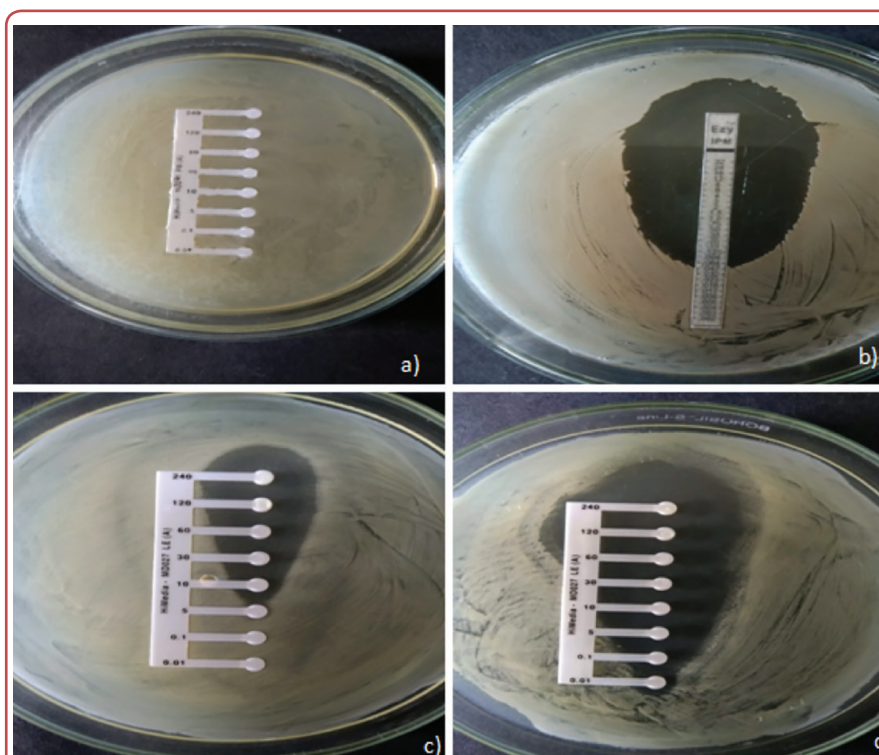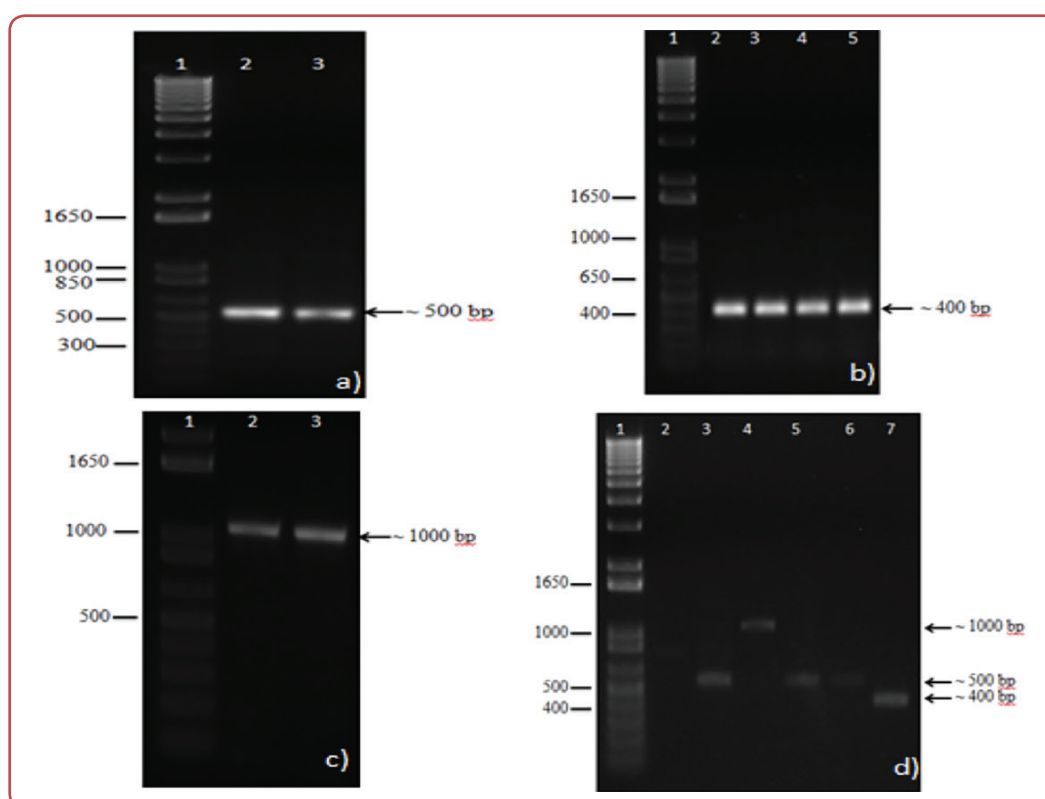

Figure 4: Minimum inhibitory concentration (MIC) of the antibiotics towards different bacterial isolates; a) Enterococcus faecalis b) E coli c) Enterobacter cloacae d) Acinetobacter baumannii

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Table 5: Antibiotic resistant genes present in bacterial isolates

Type of gene	Bacterial isolates					
	E coli RBRJ005	E coli RBRJ013	K pneumoniae RBRJ019	K pneumoniae RBRJ024	Enterobacter cloacae RBRJ019	Acinetobacter baumannii RBRJ027
bla_{SHV}	+	+	+	+	+	+
bla_{TEM}	+	+	+	+	+	+
bla_{CTX-M}	-	+	+	+	+	+
bla_{AmpC}	+	+	+	+	+	+
qnr_A	-	-	-	-	-	-
qnr_B	+	+	+	+	+	+

Gene: '+' present; '-'absent;

Figure 5: Gel electrophoresis of the PCR products

a) Gel electrophoresis of the PCR products of bla_{CTX-M} gene; lane 1: DNA Ladder (1 Kb), lane 2-3: 500 bp band of PCR product in K pneumoniae RBRJ019 and Acinetobacter baumannii RBRJ027;

b) Gel electrophoresis of the PCR products of qnr_B gene; lane 1: DNA Ladder (1 Kb), lane 2-5: 400 bp band of PCR product in E coli RBRJ013, K pneumoniae RBRJ019, K pneumoniae, RBRJ024, Enterobacter cloacae RBRJ017;

c) Gel electrophoresis of the PCR products of bla_{SHV} and bla_{TEM} gene in E coli RBRJ005; lane 1: DNA Ladder (1 Kb), lane 2-3: 1000 bp band of PCR product;

d) Gel electrophoresis of the PCR products of bla_{TEM} (lane 4) in K pneumoniae RBRJ024 (1000 bp); bla_{AmpC} (lane 3, 5, 6) in E coli RBRJ013, K pneumoniae RBRJ019 and Enterobacter cloacae RBRJ017 (500 bp); qnr_B (lane 7) in Acinetobacter baumannii RBRJ027 (400 bp); lane 1: DNA Ladder (1 Kb); lane 2: negative result.

polymyxin-B, cefpirome and cefepime, while the strain RBRJ010 was resistant to trimethoprim/sulfamethoxazole, aztreonam, ampicillin, piperacillin, amoxicillin/clavulanic acid, nalidixic acid, polymyxin-B and cefepime antibiotics. The strain RBRJ010 was resistant to polymyxin-B, amoxicillin/clavulanic acid, nalidixic acid, piperacillin, aztreonam and cefepime, while the antibiotics polymyxin-B, nalidixic acid, aztreonam, amikacin and cefepime were proved ineffective against the strain RBRJ015. The bacterium *Staphylococcus aureus* RBRJ010 was susceptible to antibiotic methicillin giving inhibition zone of 18 mm, while the bacterium *Enterococcus faecalis* RBRJ015 was resistant to it.

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Detection of antibiotic resistant genes

All the multi-antibiotic resistant Gram-negative bacterial strains ie RBRJ005, RBRJ013, RBRJ019, RBRJ024, RBRJ017 and RBRJ027 were ESBL producers.

The presence of the β-lactamase genes *viz bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{ampC}$ and quinolone-resistant genes *viz qnr*A and *qnr*B among bacterial isolates is given in Table 5. *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{ampC}$ were present in all the members of the family *Enterobacteriaceae*, while *bla*$_{CTX-M}$ was present in RBRJ013, RBRJ019, RBRJ024 strains. The strain RBRJ027 was found to be positive for *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{ampC}$. The quinolone genes *viz qnr*A was absent in all the MAR isolates, while *qnr*B was present in all the isolates. The bands in the agarose gel showed the presence of various genes in extra-chromosomal DNA of the bacterial isolates (Figure 5).

Biofilm assay

The isolates, *E coli* RBRJ013, *K pneumoniae* RBRJ024, *Acinetobacter baumannii* RBRJ027 and *Enterococcus faecalis* RBRJ015 were moderate biofilm producers within 24 h and strong biofilm producers within 48 h (Table 4). On the other hand, the strain RBRJ010 of *Staphylococcus aureus* was unable to form biofilm within 24 h. The study results showed that most isolates possess strong adhesion potential to form biofilms.

Discussion

In the preliminary analysis of the urine samples, the results of the microscopic study and dip sticks observations were recorded. The UTI symptoms like burning micturition, dysuria, frequency/urgency, pyuria and haematuria were recorded from the questionnaires filled by the patients and by direct and microscopic observations of the samples. Only those samples were selected for the study which showed these symptoms and gave positive culture results. The females were selected for this study as they are more prone to UTI's. The main reasons for UTI to be more common in females is due to their shorter urethra as compared to males, reduction in normal microflora ie *Lactobacilli*, less acidic pH of the vagina, poor hygiene and sanitation conditions.[23, 24]

The patients of all age groups showed the condition of dysuria with other symptoms like urine urgency, frequency, painful micturition, nocturia and bladder discomfort. These all symptoms and conditions among adult females were also reported by Wrenn (1990).[25] The main cause of dysuria is stones in the urinary tract or inflammation of the bladder (cystitis), inflammation of the kidney (pyelonephritis) and inflammation of the urethra (urethritis).[26] According to Kurowski, in adult women, the pain felt due to the passage of urine over the inflamed vaginal labia indicates external dysuria which may be due to vaginal infection or inflammation, while the pain felt inside the body is due to internal dysuria which may be due to bacterial cystitis or urethritis.[27]

The urinary urgency and frequency were more observed in the age group 31-40 years followed by 41-50 years. Urinary urgency was mainly caused by trigonal or posterior urethral irritation which may be due to the presence of stones, inflammation or tumours. The discharge from the urethra was mainly linked with the condition of urethritis.[28] The history of the frequency of normal urination is somewhat difficult to obtain as it is different for every individual depending upon their bladder capacity and fluid intake. The urgency to urinate may arise with or without urination and is highest in incontinence. The urge to urinate may become constant in lower urinary tract inflammation by eliminating only a few millilitres of urine during each voiding.[25, 29]

The increased number of pus cells or leukocytes (WBCs) (≥ 10 WBC/high power field) in microscopic examination or the positive leukocyte esterase test of the urine samples indicates pyuria and evidence for the inflammation of the genitourinary tract.[30]

The formation of kidney stones is due to the accumulation of dissolved minerals on the kidney's inner lining. These minerals in the urine lead to the formation of crystals. Most of the stones are composed of calcium followed by uric acid, struvite and cystine. These are mainly present in infected urine so also called infection stones. Inside the kidneys, the urine backs up in the tubes when the urinary tract is blocked by these stones. The bacteria that may trap in the urine due to blockage cause UTI; also the excessive pressure on the kidneys results in swelling (hydronephrosis) and kidney damage.[31]

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The alignment of partial 16S rRNA sequences against the NCBI database suggested that bacterial isolates belong to different bacterial species. The results of the present study are in accordance with the other researchers who reported *E coli* as the prime aetiological agent in causing UTIs among females.[32-37] The presence of bacteria in the urine indicates UTI or bacteriuria. The presence of at least 10^4 bacteria/mL in a freshly voided midstream urine sample indicates significant bacteriuria. Bacteria invade the urinary tract by ascending or descending invasion causing UTIs. The ascending pathway is the more common mode where the normal faecal microbiota gets access to the urinary tract by colonising the urethra. The bacterium mainly involved in UTIs is the bowel microbiota ie *E coli* in most cases causing ascending infection.[38, 39]

The symptomatic infections are linked with the virulence of the causing organism which competes with the innate defence system of the host and the inflammation or injury is due to the host's immune response not because of bacterium.[40] The bacteria colonise the urethral opening often called microbiota is routinely present in the urine in both men and women. But, the bacteria present in the urine in the urethra are often flushed out during micturition. In women, the shorter distance to the bladder makes it easy for the uropathogens to access and colonise the bladder easily before being removed by urination. Also, the closer proximity of the urethral opening, vaginal cavity and rectum make it easier for the bacterial colonisers to get easy access to the bladder.[6, 34] Sexual activity may also directly transfers bacteria from the vaginal cavity to the urethra or indirectly through oral sex. Mostly uncomplicated UTIs are associated with sexual activities and are more common among the females of the age group 18-29 years.[6, 41]

Resistance to commonly used antibiotics used against various infections is now a serious global problem. The current study revealed the antibiotic resistance among isolated uropathogens against commonly prescribed antibiotics in UTI's. Despite the large availability of antibiotics, UTIs are still the most common among females.[42] Antibiotic course during UTI affects the normal vaginal and gastrointestinal flora to great extents.[43] Antimicrobial resistance among uropathogens varies from one region to another depending upon many factors. The most common one is prescription of antibiotics by physicians without any culture sensitivity testing and their haphazard use by laypeople leads to increasing resistance among bacteria. Also, the improper dose, duration and leaving the antibiotic course in between make the uropathogens more resistant.[44] Besides this, the use of antibiotics in fish farms and the animal farming sector makes animals and poultry resistant thus, transferring resistant strains to humans.[45] Another main reason for antibiotic resistance among uropathogens is mainly due to horizontal antibiotic gene transfer.[46] It also leads from one bacterium to other and through this process, bacteria become resistant to more than one antibiotic at once. The bacterial plasmid DNA possesses multidrug resistance genes which they transfer to other enterobacterial species.[47, 48]

The treatment of UTIs is increasingly getting complicated because bacteria develop resistance to various antibiotics. The increasing antibiotic resistance among bacteria often leads to treatment failures which have serious effects on critically ill patients.[49] The resistant bacteria, particularly *E coli*, *Klebsiella spp*, *Pseudomonas spp*, *Enterobacter spp*, *Staphylococcus spp* and *Enterococcus spp* are more commonly emerging in community-acquired as well as in nosocomial infections.[50] The susceptible bacterial population may acquire resistance to antimicrobial agents through mutation and selection or through genetic information from other bacteria that encodes resistance involving different mechanisms such as conjugation, transformation and transduction.[46] Eight bacterial isolates were resistant to > 7 antibiotics and were considered multi-antibiotic resistant based on their antibiotic susceptibility profile. The bacterial isolates, namely *E coli*, *K pneumoniae*, *Acinetobacter baumannii*, *Enterobacter cloacae*, *Enterococcus faecalis* and *Staphylococcus aureus* were resistant to > 7 antibiotics. The results are similar with the studies published from other regions of the country.[51-54]

All the multi-antibiotic resistant Gram-negative bacterial strains ie RBRJ005, RBRJ013, RBRJ019, RBRJ024, RBRJ017 and RBRJ027 were ESBL producers. The frequency of ESBL-producing members of the family *Enterobacteriaceae* isolated from urine samples varies in different regions of the country and was studied by many authors.[37, 55]

UTIs are the most common bacterial infections in women and *E coli* is the primary pathogenic agent in these infections. There were many reports across the country on antibiotic resistance

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and ESBL-producing Gram-negative bacilli isolated from urine samples. Gajamer et al investigated the major ESBL-producing uropathogens in female patients of Sikkim and Darjeeling.[37] They found that the $bla_{CTX-M-I5}$ group was more predominant in the isolates than all other ESBL genes. In a similar study, Ojdana et al observed the prevalence of bla_{CTX-M}, bla_{SHV} and bla_{TEM} genes in *K pneumoniae*, *E coli* and *Proteus mirabilis* strains.[56] They revealed that thirty-six of the tested strains exhibited bla_{CTX-M} genes, twelve strains harboured bla_{SHV} genes and twenty-five strains showed the presence of bla_{TEM} gene respectively. Gajamer et al studied the occurrence of Extended Spectrum β lactamase genes coexisting with carbapenemase, AmpC and aminoglycoside resistance gene in uropathogens and reported the high prevalence of carbapenemase resistance among ESBL positive isolates.[57]

Similarly, the prevalence of quinolone resistance genes in uropathogenic *E coli* was observed by Malekzadegan et al and revealed that 33.1 % of the isolates were positive for *qnr*S gene and 12.4 % of the isolates were positive for *qnr*B genes respectively, while, none were found to be positive for *qnr*A gene.[58] The present study also reported similar findings. Tayebi et al investigated the plasmid-mediated quinolone resistance genes in ESBL-producing *E coli* isolated from UTIs and found that the widespread presence of plasmid-mediated quinolone resistance genes in ESBL-positive isolates is increasing at an alarming rate.[59] Presented study findings were also concordant with these as all the Gram-negative *Bacilli* possess *qnr*B gene. The co-dissemination of these genes among bacterial isolates is a major threat to public health.

There were many reports on uropathogenic *E coli* (UPEC) which is the primary causal agent of UTIs, forming biofilms on different sources. In a recent study, Eberly et al reported that *E coli* forms biofilms on catheters as well as on and within urinary bladder epithelial cells.[60] Biofilms mainly protects these isolates from antibacterial agents, environmental conditions and the host's immune system. In another study, Zheng et al characterised the biofilm formation by *Enterococcus faecalis* isolates derived from UTIs in China.[61] Alves et al, Taya et al and Karigoudar et al have seen a significant association between the antibiotic resistance pattern and biofilm formation among clinical isolates from UTIs.[62-64]

Conclusion

The current study revealed that the incidence of UTIs is more prominent in the age groups 21-30 and 31-40 years. The menacing state of drug resistance among Gram-negative bacilli in this geographical region is revealed. The effective group of antibiotics against these isolates are aminoglycosides (gentamicin) and carbapenems (imipenem and meropenem). All the Gram-negative multidrug-resistant isolates were ESBL producers which also possess *qnr*B gene. Furthermore, it is extremely crucial to design a strict antibiotics prescription policy and judicious use of antibiotics should be encouraged.

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Conflict of interest

None.

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