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Molecular identification of multi drug resistant bacteria from urinary tract infected urine samples

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ABSTRACT

Urinary tract infections (UTIs) are of great concern in both developing and developed countries all over the world. Even though the infections are more common in women and children, they are at a considerable rate in men and of all ages. The uropathogens causing the infections are spread through various routes. The treatment generally recommended by the physicians is antibiotic usage. But, most of the uropathogens have evolved antibiotic resistance mechanisms. This makes the present situation hectic in control and prevention of UTIs.

The present study aims to illustrate the multidrug resistance patterns among isolated bacterial strains from infected urine samples in Odisha state, India. Four bacterial strains were isolated and identified as *Proteus* sp. SK3, *Pseudomonas* sp. ADMK77, *Proteus* sp. BLKB2 and *Enterobacter hormaechei* strain CW-3 by 16S rRNA gene sequencing. Phylogenetc analysis indicated the strains belong to three various genera namely, *Proteus*, *Pseudomonas* and *Enterobacter*. The evolutionary timeline of the bacteria was studied by constructing phylogenetic trees by Neighborhood Joining method. The presence of ESBL gene and biofilm forming capability were studied for the four strains. Antibiotic susceptibility patterns of the isolates were studied toward the commonly recommended antibiotics. Both the *Proteus* strains were found commonly susceptible to aminoglycoside and sulphonamide groups. *Pseudomonas* strain was found to be susceptible to cephems, aminoglycosides and fluoroquinolones. *Enterobacter* sp was found to be resistant to almost all antibiotic groups and susceptible to only sulphonamides group. The antibiotic susceptibility patterns of the bacteria help in choosing the empirical antibiotic treatment for UTI.

1. Introduction

Urinary tract infections (UTIs) are significant infections comprising 1/4th of all the infections [1]. The number of hospital admissions due to UTI exceeds 1 million per year [2]. The cost of treatment for a single UTI case exceeds $750 and the annual cost lies beyond $3 billion [3]. It is reported as the fourth most common infection in USA and third common in India [4]. Infants and children are more prone to these bacterial infections [5]. The epidemiological factors include gender, age and genetic susceptibility aspects. Females are more susceptible to the infections compared to males [3]. However, during the first year after birth, males are at higher risk [5]. UTIs may be complicated or uncomplicated depending on the site of presence of uropathogens in urinary tract [6]. UTI may be considered uncomplicated if the pathogens are localized in urinary bladder; and considered complicated if the pathogens reach ureters and kidney. Complicated UTIs if left untreated may result in blocking of urinary tract and kidney failure [7].

Uropathogens include both gram positive and gram negative bacteria [8]. However, the commonly reported uropathogens are gram negative such as *Escherichia coli*, *Klebsiella pneumonia*, *K. oxyntoca*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Serratia marcescens*, *Vibrio cholera*, *Salmonella paratyphi*, *Providencia stuartii*. Enterobacteriaceae was found to be the most prevalent constituting more than 80% of all the published UTI cases [9]. Among Enterobacterioceae family members, *E. coli* was reported to be the most common uropathogen [10]. Gram positive uropathogenic bacteria include *Staphylococcus saprophyticus* and *Enterococcus facecalis* [11].

Detection of bacteria causing infections at early stages has been the major focus in health care and medical sectors [12,13]. Delay in detection at the initial stages would result in deadly effects [14].

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Hence, different methodologies have been introduced for the detection of uropathogens from various biological samples [15]. The discovery of new antibiotics is being neutralized by emerging antibiotic resistance mechanisms by the bacteria [16]. Therefore, the treatment and prevention of various UTIs is becoming hectic day by day.

The aim of the present work is (i) to isolate and identify bacteria from UTI infected urine samples (ii) to evaluate the presence of extended spectrum beta lactamase gene, (iii) biofilm forming capability and (iv) antibiotic resistance patterns of isolated bacteria.

2. Materials and methods

2.1. Collection of urine samples

Urine samples were collected from Department of Urology, SUM hospital located at Bhubaneswar in Odisha, India. The samplings were performed for three months: October, November and December. Clean catch mid stream urine samples were collected from patients, both men and women, suspected with positive urinary tract infections. The age of patients was in the range of 15 years–73 years (Table 1). The samples were collected in sterilized 2 ml vials and immediately transported to laboratory. The samples were used for analysis immediately or stored at 4 °C for further analysis.

2.2. Isolation of bacterial species

Isolation of bacterial species was performed by spread plate technique. The urine samples were diluted 10^5 fold with sterile water and 100 μl of the diluted sample was spread onto nutrient agar containing plates. UTI positive specimens were identified and morphologically dissimilar bacterial colonies were sub cultured on MacConkey agar plates [17]. The plates were incubated at 35 °C for 24 h, and those plates which had no growth at the end of 24 h were incubated further for another 24 h [18].

2.3. Biochemical investigation

Bacterial isolates were characterized by biochemical performances such as staining, catalase production, oxidase production, methyl red, Voges-Proskauer reaction, indole test, starch hydrolysis, citrate test, mannitol test and nitrate test [19,20]. Cell structures were observed microscopically using phase contrast microscope, Olympus trinocular research microscope model BX43 and images were captured using Q-capture Pro 7 software.

Table 1
Patient demographics involved in this study.

SL no.	Group	Number
1.	*Age*	
	a. <20 years	02
	b. 20–50 years	12
	c. >50 years	08
2.	*Sex*	
	a. Male	14
	b. Female	08
3.	*Marital status*	
	a. Married	18
	b. Unmarried	04
4.	*Hospital visit*	
	a. First visit	18
	b. Recurring	04
5.	*UTI*	
	a. Positive	10
	b. Negative	12
	Total	22

2.4. Growth on HiCrome UTI agar medium

The test bacterial isolates were grown on chromogenic agar medium separately. Medium was prepared by dissolving 56.8 g/L HiCrome UTI agar medium (Himedia, India). The bacteria were inoculated by streak plate method and were incubated at 37 °C for 24 h. Pink color colonies after 24 h of incubation were suspected to be *Escherichia*. Blue colonies indicated *Enterobacter*, *Enterococci*, *Klebsiella* or *Seratia* sp. [21]. *Pseudomonas* produced dark green colonies whereas *Proteus* sp. produced brown color colonies [22,23].

2.5. DNA isolation and PCR amplification

DNA extraction was done by phenol-chloroform method using 24 h old bacteria cultures grown in Luria broth [24,25]. The extracted DNA samples were preserved at −20 °C. 16S ribosomal RNA genes were amplified using PCR with a set of universal primers 8 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-GGTTACCTTGTTACGACTT-3′) using the mentioned PCR conditions: 3 min of pre-heating at 94 °C that is followed by 35 cycles of denaturation at 94 °C for 1 min, 45 s of annealing at 55 °C and 1 min primer extension at 72 °C and a final post elongation step for 10 min at 72 °C. Post PCR amplification, 10 μl of the generated amplified sequences were loaded on 1% agarose gel stained with ethidium bromide to perform electrophoresis for 1 h at 70 V, and DNA bands were detected using UV transilluminator.

2.6. Sequencing of 16S rRNA and phylogenetic analysis

16S rRNA gene sequences of all the bacterial isolates were found and analyzed for taxonomical classification against a database from NCBI containing 16S rRNA gene sequences using BLAST algorithm [26,27]. Ten close matches were aligned with query sequences using CLC sequence viewer. Phylogenetic tree was constructed for the alignments using Neighborhood Joining method [28].

2.7. Antibiotic susceptibility tests

The four isolates were tested for antibiotic susceptibility pattern by disk diffusion (Kirby Bauer's technique) on Mueller-Hinton (MH) agar according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [29]. An aliquot of 0.1 ml 0.5 McFarland equivalent fresh bacterial culture was spread on MH agar and allowed to dry for 5–10 min. Antibiotic discs were then placed and incubated at 37 °C for 24 h [30,31]. The tested antibiotics with their concentrations are as following: Ampicillin (AMP, 10 mcg), Ciprofloxacin (CIP, 30 mcg), Gentamicin (GEN, 10 mcg), Nalidixic acid (NA, 30 mcg), Chloramphenicol (C, 30 mcg), Amikacin (AK, 30 mcg), Kanamycin (K, 30 mcg), Erythromycin (E, 15 mcg), Rifampicin (RIF, 5 mcg), Ampicillin/Cloxacillin (AX, 10 mcg), Tobramycin (TOB, 10 mcg), Netillin (NET, 30 mcg), Levofloxacin (LE, 5 mcg), Meropenem (MRP, 10 mcg), Ceftazidime (CAZ, 10 mcg), Gentamicin (GEN, 10 mcg), Ticarcillin (TI, 75 mcg), Piperacillin (PI, 100 mcg), Cefepime (CPM, 30 mcg), Cefoperazone (CPZ, 75 mcg), Nitrofurantoin (NIT, 300 mcg), Cefotaxime (CTX, 30 mcg), Imipenem (IPM, 10 mcg), Amoxyclav (AMC, 30 mcg), Tetracycline (TE, 30 mcg), Co-Trimoxazole (COT, 25 mcg), Penicillin G (P, 1 unit), Streptomycin (S, 10 mcg), Sulphatriad (S3, 300 mcg). All the antibiotics were obtained from Himedia, India.