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ETIOLOGY OF UROLITHIASIS FROM SOUTH INDIAN POPULATION: CORRELATION OF RECURRENCE AND ANTIBIOTIC RESISTANCE TO BIOFILM PRODUCTION CAPABILITIES OF UROPATHOGENIC MICROBES.

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ABSTRACT

Kidney stone formation is one of the most painful and widespread urological disorders. In this study, the prevalence of renal calculi in relation to urinary tract infections was investigated in 125 patients from Southern India. The chemical composition and bacteriological study of pre, post operative urine samples and stones was performed to clarify the relationship between urinary tract infections and stone formation. Commonest pathogens recovered by bacteriological analysis were identified as *E.coli* (38.1%) followed by *Pseudomonas aeruginosa* (33.3%), *Proteus mirabilis* (12%), *klebsiella pneumonia* (10%), *Enterococci* (4.5%) and coagulase negative *Staphylococcus* (2.4%) and were further confirmed by16S rDNA sequencing and phylogenetic analysis. The isolated organisms were tested for antibiotic sensitivity and the resistant strains were additionally screened for biofilm formation. We hypothesize that, recurrent renal calculi formation may be attributed to antibiotic resistant biofilm forming urinary tract infections along with environmental habitats.

KEYWORDS: **Urolithiasis, Renal calculi, Biofilm and Urinary tract infections.**

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INTRODUCTION

Urolithiasis, a multi-factorial recurrent renal disease is distributed worldwide in urban, rural, nonindustrial and industrial regions. Kidney stones were experiential with an annual incidence of 0.5- 1.9% and at least 10% of the population in the industrialized part of the world is afflicted by this disease[1]. Various risk factors contribute to the development of this disease including age, sex, diet, environment, Urinary Tract Infections (UTI), industrialization and socioeconomic status but not limited to any single factor[2]. The patient populations at risk for different locations of stones are disparate, with kidney stone prevalence most often in otherwise healthy individuals and bladder stone occurring in those with neurologic and/or anatomic abnormalities[3]. Urinary tract infections and urosepsis are complications which can precede or follow urolithiasis. Most cases of bacteriuria in patients with renal calculi represent asymptomatic bladder colonization and often the stones themselves can serve as source of infections[4]. Biofilm plays a very critical role in persistent and resistant renal and urinary tract infections[5]. Infectious (struvite) stones are known to be formed due to the interplay between infecting bacteria and mineral substrates derived from the urine. This interaction had resulted in a complex biofilm composed of bacteria, bacterial exoproducts and mineralized stone material[6]. Biofilm formation confers antibiotic resistance and facilitates gene transfer with the intestinal bacteria inside the host[7]. It was estimated that in India, five to seven million people suffer from stone disease. Renal calculi incidence shows wide regional variation in India and was reported in the Northern part like Maharashtra, Gujarat, Punjab, Haryana, Delhi and Rajasthan[1] whereas little was identified from Southern part. Therefore, the present study was undertaken to understand the epidemiology of renal calculi in South Indian (Andhra Pradesh) population and to evaluate their chemical composition and bacteriological spectrum of pre and post operative urine samples and renal calculi to clarify the relationship between urinary tract infections, their antibiotic sensitivity, nature and type of the stone formed.

METHODOLOGY

(i) Patients

The present study was conducted on 125 patients of urolithiasis (81 men and 44 women aged between 16-70 years) admitted in the Urology Department of NRI General Hospital, over a period of twelve months. A well documented history containing general and detailed information about the patient, associated risk factors, urinary symptoms were filed. Pre and post operative urine samples were collected aseptically for microscopic and macroscopic examination.

(ii) Specimen collection and processing

Stone size was recorded arbitrarily from the X-ray films as small (longest diameter < 0.5 cms), medium (0.5-2 cms) and large (>2 cms) [8]. From patients, stones were recovered by Percutaneous Nephrolithotripsy (PCNL), Ureterorenoscopy (URS) and Cystolithotripsy (CLT) surgical procedures [9,10]. Under sterile conditions, the stone or the fragments of stones were collected from operation theatres in 5ml of physiological saline solution and processed for bacteriological studies as described by Ohkawa *et al.,*[11] . The stones were thoroughly rinsed in sterile physiological saline (pH 7.2) and then chemical analysis of crushed stones was performed as described by Winer, J.H and Hill B[12].

(iii) Bacterial Culture

The crushed stone core was cultured on blood agar and Mac Conkey's agar plates, incubated at 37^{o} C for isolation of etiological agents. The isolated organisms were microscopically examined and biochemical tests were performed[13].

(iv) PCR amplification and 16s rDNA sequencing

The PCR amplification was done by using the Forward FD1 **(**5′-AGAGTTTGATCCTGGCTCAG-3′) and Reverse RP2 (5′-ACGG CTACCTTGTTACGACTT-3′)[14] primers with the

following PCR conditions of initial denaturation 94^0 C for 5mins, 30 cycles of denaturation at 94^0 C for 30sec, annealing at 55^0 C for 30 sec, 72^0 C for 1min and final extension at 72^0 C for 10 mins. The amplified product was gel checked on 1% agarose and purified using GeneJET Gel Extraction Kit (Fermentas, India). The sequencing was performed in both the directions on ABI prism sequencer using the DNA primers 16SEQ2R, 16SEQ3F, INS16SREV, 16SEQ4F and 16SEQ4R. Sequenced data was analyzed by computer alignment program, Chromas Lite 2.01 (Technelysium Pty, Australia). The sequences were deposited to NCBI genbank and the accession numbers are: *E.coli_KLU*: KC211290, *P. aeruginosa_KLU:* KC211291 and *P. mirabilis_KLU:* KC211292.

(v) Phylogenetic Analysis

The BLAST database [15] of National Center for Biotechnology Information was used to compare 16S rDNA sequences of the *E.coli*_KLU, *P.mirabilis*_KLU, and *P.aeruginosa*_KLU with known 16S rDNA sequences. Multiple alignments were done in clustalW. Neighbour joining tree was constructed using Tree top soft ware (http://www.genebee.msu.su/services/phtree_reduced.html).

(vi) Detection of Antibiotic sensitivity and Biofilm producers:

Antibiotic sensitivity was done by using Kirby-Bauer disc diffusion method using Mueller-Hinton Agar Medium[16] . Inoculum was prepared using the direct colony suspension method.

(vii) Congo red Agar Method

Antibiotic resistant cultures were screened for biofilm formation using the method described by Freeman *et al.,*[17]. Medium composed of BHI (37gms/L), sucrose (50gms/L), Agar (20 gms/L) and Congo red stain (0.8 gms/L). Congo red was prepared separately as concentrated aqueous solution and autoclaved at 121^oC for 15minutes and added separately when the agar had cooled to 55^oC. Plates were inoculated and incubated at 37^oC for 2-3 days. Positive result was indicated by black colonies with a dry crystalline consistency. A non biofilm producer usually remained pink. The experiments were performed in triplicate and repeated for three times.

(viii) Tube Method

A qualitative and quantitative assessment of biofilm formation was done as described by Christensen *et al.,*[18]. In the present study, biofilm formation was evaluated in three different media, Tryptic soy broth, TSB with 1% glucose and TSB with 2.5% glucose. 1.8ml of the medium was inoculated with 0.2ml of fresh overnight grown microbial pure culture and incubated for 24hrs at 37^oC. Tubes containing only TSB were considered as negative controls. The tubes were decanted and washed with PBS (pH 7.2) and air dried. Dried tubes were stained with 1% crystal violet. Excess stain was washed with deionised water, dried in an inverted position for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Optical densities of the stained adherent cultures were determined with a UV-Visible Spectrophotometer (Genesis 10UV, Thermo scientific) at 595nm by dissolving the stain in 4ml of 95% ethanol. Experiments were performed in triplicate and repeated for three times. Bacteria were classified into three categories, non adherent, weakly, moderately and strongly adherent based upon the optical density of bacterial films produced in TSB with and without glucose [19, 20].

RESULTS AND DISCUSSION

(i) Correlation of Renal calculi with various risk factors

Out of 125 patients, the frequency of renal stone formation was more in males, 81 stones (65%) compared to females 44 stones (35%). 42 of 125 stones (33.6%) (Figure 1A) were culture positive and among 42 cases the infected stone incidence was more (24 stones) 57% in males while only 43% (18 stones) in females. Patients included in this study were above 15 years, with an age range between 16-70 years. In both the genders, the mean age where (48%) 20 infected stones were recovered is between 31-45 years;

followed by 16-30 years age where only 14 stones (33%) and from 46-90 years mean age patients, 8 stones (19%) were recovered. These results were analogous to the observations of M.Okuyama *et al.,*.[21]. Chemical analysis revealed that, among 125 stones, the incidence of calcium oxalate stones was 36.8% (46 stones), calcium phosphate 24% (30 stones), mixed 19.2% (24 stones), struvite 12% (15 stones), Magnesium phosphate 6.4% (8 stones) and uricacid+phosphate 1.6% (2 stones). In both males and females, the occurrence of calcium oxalate stones was common (37%) followed by calcium phosphate (24%) (Figure 1B). The occurrence of struvite stones was only 12% which was akin to the study by Sharma *et al.,*[22] and the high incidence of calcium oxalate stones (75%) was concurrent with the results of Rana gopal Singh *et al.,*[23]. The observed variations in chemical composition of calculi may be due to geographical variation and dietary habits of the patients which have a contributory influence on the type of calculi within a given area[23]. The higher incidence of renal stones in males (Figure 1B) probably could be due to increased serum testosterone level favouring increased endogenous oxalate production by liver, which in turn predisposes to oxalate stone formation[24]. Moreover, increased urinary citrate concentration in females may serve in protection against calcium urolithiasis. In both males and females, renal calculi were mostly recovered from the kidney (68.8%) followed by bladder (18.4%) and ureter (12.8%). This could be due to the fact that kidney acts as a first barrier filter for crystals thereby damaging tubular epithelium which acts as a nidus for the sterile stone formation. In males and females, the percentage of infected renal calculi was 66.6% and 55.5% in kidney, 20.8% and 33.3% in bladder, 12.5% and 11.11% in urethra respectively (Figure 2). When compared to males, it was observed that in female's infection rate was higher in bladder (33.3%) followed by kidney (55.5%). This might be due to close proximity of urethra to bladder[8] and shorter in length in females .

Figure1

Urolithiasis patient's distribution (%) based on age, gender and chemical composition

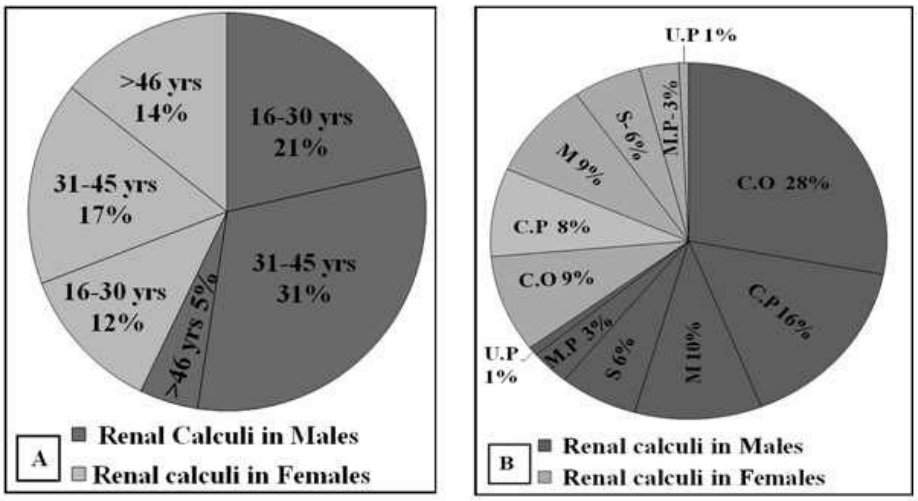

Figure 1 A) Age and Gender wise percentage distribution of renal calculi B) % distribution of types of renal calculi in Males and Females based on chemical composition. C.O - Calcium oxalate, C.P - Calcium phosphate, M - Mixed, S - Struvite, M.P - Magnesium phosphate, U.P - Uricacid+Phoshate calculi.

Among 125 patients, urolithiasis was more frequent in people drinking bore or ground water 43 stones (34.4%) followed by municipal tap water 58 calculi (46.4%), ponds and lakes 24 calculi (19.2%) and of 42 infected calculi, the ratios were 50% (21 stones), 38% (16 stones) and 12% (5 stones) respectively. This specifies that calculi were frequent in people drinking public municipal tap water and infected calculi in people consuming bore water.

Figure 2

Gender based stone prevalence % in different sites of urinary track

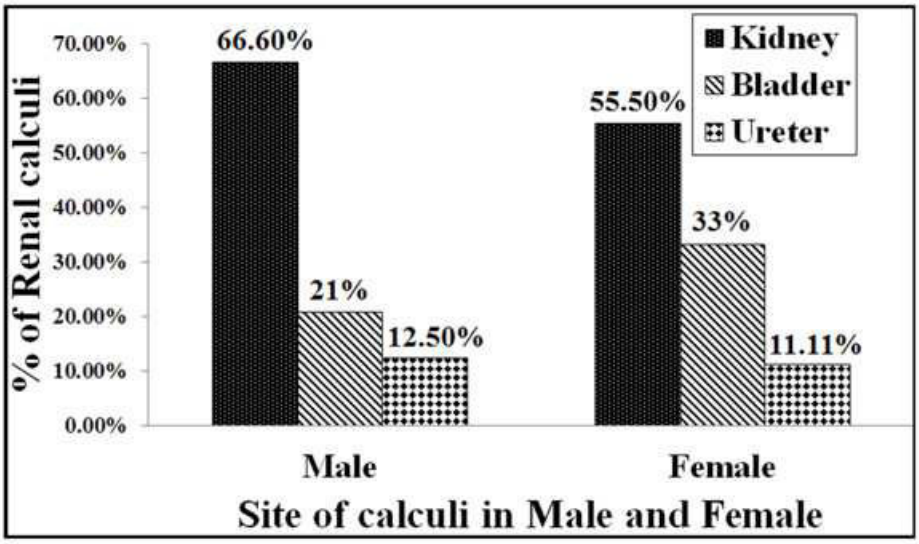

Figure 2 Gender based percentage wise distribution of renal calculi in kidney, bladder and Ureter. In both genders, kidney stones were common where as prevalence of stones in bladder was more in females when compared to females.

In the present study, 82.4% of stones were recovered from people on a mixed diet as compared to those on a vegetarian diet (17.6%) whereas infected calculi percentages were 81% (34 stones), 19% (8 stones) respectively. This is in concurrent with studies done by Lemann *et al*.,[25]. Among 42 culture positive stones, 25 were radio opaque and 9 were radiolucent stones. Infectious stones were more common in radio opaque variety due to matrix formation in which microorganisms can reside.

(ii) Infectious Renal calculi

The bacteriological study of renal calculi revealed stone culture positive in 42 cases, pre-operative urine positive in 40 cases and post-operative urine positive in 17 cases. In all three cultures, *Escherichia coli* was the predominant microorganism followed by *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterococci* and coagulase negative *Staphylococci* (Figure 3). In two cases, even though the pre-operative urine is negative, stone culture was positive which clearly indicates that the culture resides inside the central core of the stone. Both pre-operative urine and stone culture were positive in 22 cases, where same organism was isolated from 18 cases (82%) and different organisms from 4 cases (18%). It has been reported [26] that initiation of urolithiasis is done by bacterial infections and its progression depends on endogenous and dietary habits. Out of 42 infectious stones, infections were more commonly seen in struvite stones 31% (13 stones) followed by calcium oxalate 21.4% (9 stones), mixed 14.3% (6 stones), Magnesium phosphate 14.3% (6 stones), calcium phosphate 14.3% (6stones) and uricacid+phosphate 4.7% (2 stones) (Figure 3). Prevalence of infected renal calculi depends mainly on urease and non urease producing bacteria. *Pseudomonas aeruginosa* was the predominant organism isolated from calcium oxalate and struvite stones whereas *E.coli* from struvite, calcium oxalate and calcium phosphate stones, while *Proteus* and *Klebsiella* species from mixed and struvite stones[27,28]. In the present study done for the first time in south Indian population (A.P), *E.coli* a non-urease producing microorganism was observed as the predominant organism from infected stones and indicates that non-urease producing organisms probably also contribute to calculi formation at some stage of their development [29].

Figure 3
Microbial load associated with pre and post operative urine and renal stones.

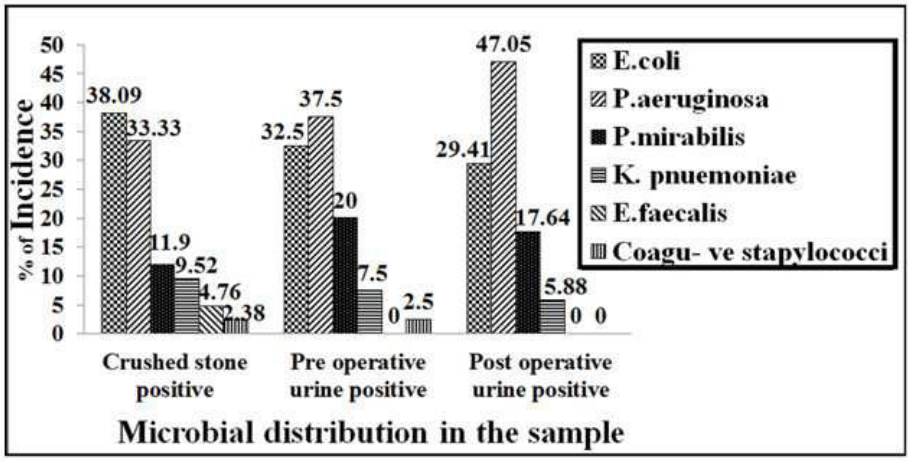

Figure 3: Different microorganisms isolated from pre and post operative urine samples and crushed stone cultures. E. Coli was the highest incident organism in crushed stone cultures whereas Pseudomonas was predominant in both pre and post operative urine samples. Coagulase negative staphylococci was found only in preoperative urine and crushed stone analysis.

(iii)16s rDNA sequencing and Phylogenetic analysis

The 16s rDNA sequencing was performed for the three predominant culture isolates from calculi and resulted sequences were BLAST analyzed and identified as *E.Coli, Pseudomonas aeruginosa* and *Proteus mirabilis.* The phylogenetic tree for *E.coli*_KLU formed two groups (Figure 4A) from the node, the *E.coli*_KLU isolate showed close relationship to the strain SMS-3-5 a multi drug resistance strain[17]. *Escherichia coli* is the most predominant uropathogen (80%) isolated in acute community-acquired uncomplicated infections[30]. *P.aeruginosa*_KLU showed close relativeness to the *P.aeruginosa* AS2 strain (Figure 4B) which is a clinical isolate[31]. *P. aeruginosa* is the third most common pathogen associated with hospital-acquired catheter associated UTIs[32]. Though the virulence factors of *P. aeruginosa* in causing urinary tract infections are still unclear, *P. aeruginosa* strains isolated from urinary tract infections are identified as high producers of elastase and protease enzymes[33]. *P.mirabilis*_KLU had a close relationship with *P. mirabilis* strain HI4320 (Figure 4C), a prototypical and common isolate worldwide from urinary tract infections[34]. Clinically, *P. mirabilis* is known to possess the best ability to form stones in the bladder and kidney as well as crystalline biofilms[35]. *P. mirabilis* infection is linked with urolithiasis and is caused by the induction of urease enzyme, which hydrolyzes urea to ammonia causing the local pH to rise and subsequent precipitation of magnesium ammonium phosphate (struvite) and calcium phosphate (apatite) crystals[36-38].

Figure 4

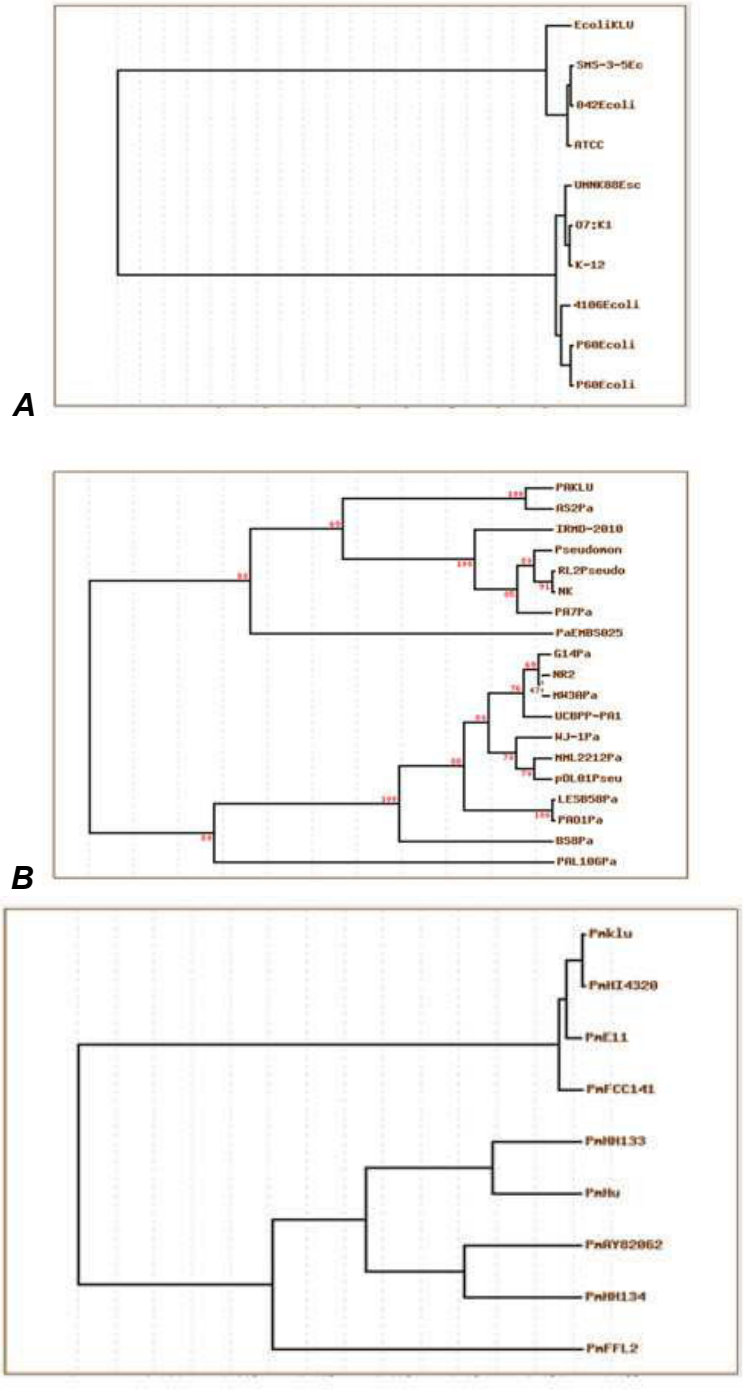

Figure 4 Phylogenetic analyses of 16s rDNA sequences of Urolithiasis isolates of (A) the sequences retrieved from NCBI. Accession numbers CP000970, FN554766 and CP000946 were used for E.coli phylogenetic analysis. (B) For P.aeruginosa GU447238, JF708942, HQ625018, NK 2 EU352760 and CP000744 accession numbered sequences were used for comparison (C) AM942759, HM585375 and JF772100 were used for P.mirabilis phylogenetic analysis.

(iv) Antibiotic sensitivity

The isolated cultures of *E.coli* showed resistance to Nalidixic acid, amoxycillin+clavunicacid and Norfloxacin, *Pseudomonas* was resistant to ciprofloxacin, gentamicin, amikacin and piperacilin+tazobactum, *Proteus mirabilis* to ceftazidime+ tazobactum, amoxycillin+clavunicacid and co-trimoxazole, *klebsiella* to ceftazidime+ tazobactum, ciprofloxacin and amoxycillin+clavunicacid, *coagulase negative staphylococcus* was resistant to pencillin and methicillin (Table1).

E.coli, *Pseudomonas and klebsiella's* resistance to fluoroquinolone (Norfloxacin and ciprofloxacin) antibiotics is attributed to mutations that alter the quinolone-binding site on two target enzymes—DNA gyrase and topoisomerase IV and also the mutations that decrease fluoroquinolone accumulation inside the bacterial cells by either decreasing the bacterial cell's outer membrane permeability, which limits fluoroquinolone entry, or increase the activity of the efflux pumps [39]. Several mechanisms of aminoglycoside antibiotic (Amikacin, Gentamicin) resistance are reported with *Pseudomonas aeruginosa.* One mechanism is increased impermeability across the cell wall. This has been evident in strains that lack modifying enzymes and those do not exhibit cross-resistance with other classes of antibiotics. The actual mechanism for this type of resistance is not known. More recent data suggest that up-regulation of the efflux system MexXY-OprM, affects the aminoglycoside antibiotics. Lastly, *Pseudomonas aeruginosa* isolates have been shown to contain aminoglycoside modifying enzymes including AAC(6')-I, APH(2"), APH(3')-VI, and AAC(3)-II [15-18]. From the present study, it was observed that Levoflaxcin, Nitrofuratoin, Tobramycin, Imipinem, Ofloxacin and Cefoperazone in combination with sulbactum serve as better antibiotics for acute UTI's.

Table1
Antibiotic sensitivity results.

SNO	Antibiotics	Name of the Organism				
		E.coli	*Pseudomonas*	*Proteus mirabilis*	*Klebsiella*	*Coagulase negative Staphylococcus*
1	**Levofloxacin**	**S**	**S**	**S**	**S**	**S**
2	**Nitrofuratoin**	**S**	**S**	**S**	**S**	**S**
3	Ceftazidime+tazobactum	S	S	R	R	S
4	Ciprofloxacin	S	R	S	R	S
5	**Cefoperazone+sulbactum**	**S**	**S**	**S**	**S**	**S**
6	Nalixidic acid	R	S	S	S	S
7	Amoxyclav	R	S	R	R	S
8	Norfloxacin	R	S	S	S	S
9	Gentamicin	S	R	S	S	S
10	**Tobramycin**	**S**	**S**	**S**	**S**	**S**
11	Amikacin	S	R	S	S	S
12	Co-trimoxazole	S	S	R	S	S
13	Piperacilin+tazobactum	S	R	S	S	S
14	**Imipinem**	**S**	**S**	**S**	**S**	**S**
15	**Ofloxacin**	**S**	**S**	**S**	**S**	**S**
16	Vancomycin	NA	NA	NA	NA	S
17	Methicillin	NA	NA	NA	NA	R
18	Pencillin	NA	NA	NA	NA	R

a. List of 18 antibiotics screened along with antibiotic sensitivity and resistance of five microbes E.coli, Pseudomonas, Proteus, Klebsiella and Staphylococcus isolated from renal calculi. R- Resistant, S- Sensitive, NA- Not applicable.

(iii)Biofilm Formation

Among the 42 infected renal calculi, *E.coli* was recovered from 16 stones, pseudomonas from 14, *Proteus* from 5, *Klebsiella* from 4, *Enterococci* from 2 and *Coagulase negative Staphylococcus* from a single stone respectively. All these microorganisms were screened for antibiotic sensitivity. Among these, seven microorganisms which exhibited antibiotic resistance was further screened for biofilm formation. *E.coli* is the most common organism recovered from renal calculi followed by *Pseudomonas, Proteus* and *Klebsiella* subsequent to antibiotic treatment. It was observed that these are the most common bacteria that are associated with urinary tract infections (UTI) [40]. All seven cultures (*E.coli, Pseudomonas, Proteus, Klebsiella, Staphylococcus, and Enterococcus)* were identified as biofilm producers by Congo Red Agar and tube method (Table 2). *Proteus* and *Pseudomonas* showed strong biofilm formation followed by *E.coli, klebsiella, Enterococcus and Staphylococcus*. It was clearly evident that apart

from *klebsiella* all other strains showed a significant enhancement in biofilm formation with addition of glucose. Biofilm formation by *pseudomonas* and *Staphylococcus* is concomitant with recurrent acute pyelonephritis [41]. Enterococci are more frequently isolated from asymptomatic infections and are associated with lower levels of pyuria [42]. In our study, out of 42 infected calculi, seven (17%) showed antibiotic resistance in patients even after treatment with antibiotics for UTI infections. Among seven, three were identified as strong biofilm producers, four as moderate producers. Recurrence of renal calculi is due to the ability of microorganisms to produce biofilm which increases resistance to antibiotics that are normally prescribed. Bacteria play a vital role in (renal calculi) formation of calcium phosphate shell as a crystallization centre [4]. Such stones become secondarily infected, acts as adhesion sites there by promoting biofilm [5,43] which can alter the urinary environment making more crystallization happen and there by facilitating novel lithogenesis.

Table 2
Biofilm formation by organisms isolated from renal calculi.

S.No	Microorganisms	Colony morphology on Congo Red Agar Plates	Biofilm formation by Tube Method
1	*E.coli KLU1*	Black colonies	++
2	*E.coli KLU2*	Black colonies	++
3	*Klebsiella*	Pink colonies	++
4	*Proteus*	Black colonies	+++
5	*Pseudomonas*	Pink colonies	+++
6	*Enterococcus*	Black colonies	+++
7	*Coagulase negative Staphylococcus*	Pink colonies with black background	++

b.Biofilm formation by microorganism's isolated from renal calculi was screened by colony morphology on congo red agar plates. Balck colonies categorize strong biofilm producers; pink colony with black background indicates weak or moderate biofilm formation whereas pink colonies specify non-biofilm produces. Biofilm formation by tube method was also confirmed by using Tryptic soy broth medium with and without glucose. + (weak biofilm producer), ++ (Moderate), +++ (strong biofilm producer).

CONCLUSION

Urolithiais, a multifactorial disease, is the most common ailment in the world with increasing incidence in the recent years. Extensive epidemiological studies are necessary to understand the pattern of the disease, providing evidence of molecular pathogenesis. In our study, we observed that some of the common uropathogenic bacteria associated with renal calculi are able to produce biofilm enhancing their resistance to antibiotics, which could be attributed to the recurrence of the disease. In order to understand the correlation between biofilm formation and recurrent infections, it is essential to characterize these microbes directly from kidney stones. This will provide better insight of the microbial virulence factors allowing us to target them with drugs specific for biofilm penetration. Clinicians can then make better use of such epidemiological susceptibility data to refer to the institution's antibiogram allowing personalized selection of antibiotics to target patient specific microbial culture.

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