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# Identification of different bacterial species isolated from infected renal stones and evaluation of its uricolytic activity

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## Abstract

### Background

Nephrolithiasis remains a public health problem around the world, affecting up to 20% of the population. There are many risk factors that increase one's susceptibility to kidney stone formation. These include specific food, urinary tract infection, kidney disorders, and metabolic disorder. Also, some people with a family history of kidney stones may be more susceptible to develop stones.

### Objective

The aim was to isolate, purify, and identify bacteria from urinary stones. Also, determine the most bacterial isolate secreting uricase enzyme.

### Patients and methods

A total of 100 cases were included in the study (66 men, 34 women) at the National Institute of Urology and Nephrology, Nephrology Department. Urine and stone culture identification of bacteria and purification of uricase enzyme.

### Results

The study showed that there was the highest percentage of stones within the greater than 40 years age group and uric acid stones constitute the highest incidence (40%). The highest percentage of bacterial isolates was *Klebsiella* spp. (25%). *Klebsiella pneumonia* was the most potent uricase producer, out of all bacterial species isolated from human urine.

### Conclusion

The study reported that uric acid stones were significantly affected by the uricase enzyme.

**Keywords:** Uricase enzyme, bacterial species, kidney stones, *Klebsiella pneumonia*

## INTRODUCTION

Numerous risk factors responsible for stone formation have been identified, including environmental, metabolic, dietary, racial, sex, obstructive uropathology, and urinary tract infection [1]. In patients with urolithiasis, there is a high risk of recurrence; it is essential to identify the risk factors that have etiological importance and thus to predict the further course of the disease [2]. Bacterial infection may induce stone formation by increasing crystal adherence which facilitates tissue inflammation, production of an organic matrix and crystal–matrix interaction [3]. Persistent urinary tract infection with ureasplitting or nonsplitting bacteria may be the first factors in the synthesis of infected renal stones [4].

Renal stones are polycrystalline which are often associated with crystalluria and urinary tract infection. Crystalluria is the excretion of crystals in urine, is a marker of transient supersaturation of urine present both in normal physiological, and also in pathological conditions [5]. Crystals of size less than 5 mm pass out freely through the urinary tract, crystals of size more than 5 mm precipitate, leading to the calculus formation [6]. The most familiar type of stones is calcium

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oxalate and calcium phosphate which make at least 80% of all kidney stones [7]. Uric acid, cysteine, and mixed types of stones make up the rest of the urinary stones [8]. The sharp edges of oxalates calculi damage the urinary tract epithelium and encourage the growth of organisms by forming the nidus to the infectious agents [9].

Infected stones make up ~15% of whole urinary stone diseases [10]. Patients with infected stones have a high incidence of new stone growth and persistent infection, especially if residual stone fragments remain [11]. The importance of complete eradication of these organisms is a must in prophylaxis against new stone formation [12]. Uricase enzyme was firstly recorded in the genus *Pseudomonas* in 1909 [13]. Since that time, many investigators started the work on bacterial uricase, for example, *Bacillus subtilis* [14]. Microbial uricase (urate oxygen oxidoreductase) is a colorless protein, soluble in alkaline buffers with a molecular weight of about 10 000 kD [15]. It has been applied in the treatment of children with hyperuricemia [16] and the patients with primary gout [17]. Moreover, the purified uricase would have medical importance in the field of chemotherapy [18].

The present work aimed to identify different bacterial species isolated from urine and the extracted stones. Also, to determine the most potent bacterial isolates are secreting uricase enzyme and evaluating the possibility of its application as antistone treatment with future hope for the invention of new antistone medication.

## PATIENTS AND METHODS

A total of 100 participants at National Institute of Urology and Nephrology were included in the study suffering from renal stones, consisting of 66 men and 34 women (age:  $34.4 \pm 12.8$ , range: 15–65 years), were recruited with symptoms of renal colic and burning micturition, diagnosed as having renal stones and prepared for surgical extraction.

All individuals were subjected to the following:

- (1) Full history taking, exclusion criteria included patients with a history of renal failure, renal tumors, previous history of renal stones, and systemic diseases such as diabetes and autoimmune diseases.
- (2) Routine kidney function tests (urea, creatinine, and uric acid) were measured using a fully automated analyzer (Dimension RxL Max; Seimens USA).
- (3) Urine analysis by a commercially available reagent strip (Uric 3 V; Uricone Biotec, Korea).
- (4) Radiological examination: plain radiography, intravenous pyelogram, abdominal scan.
- (5) Culture and sensitivity of urine, antibiotics were stopped for at least 48 h before sampling. Midstream urine was collected in a sterilized wide-mouthed container after a rigorous cleaning of external genital organs and was cultured for 24 h on standardized blood agar and MacConkey agar, incubated at a temperature of 37°C for the growth of micro-organisms. Colony morphology

preliminarily characterized pure isolates of bacterial pathogens, Gram stain, and a standard biochemical reaction was used for full identification of Gram-positive and Gram-negative bacteria.

- (6) Stone examination and biochemical composition of renal calculi, which either passed through the urine if they are small or extracted through the cystoscope or by an operation. Processing of calculi for biochemical composition was done by a ready-to-use kit for semiquantitative determination of the essential components of urinary calculi (calcium oxalate, phosphate, magnesium, uric acid, and cysteine) (Merckognost; Merck KGaA, Germany).
- (7) Stone culture and sensitivity: processing of calculi for bacteriological culture was done as described by Ogata *et al.* [19]. The calculi were thoroughly rinsed in sterile physiological saline and then crushed with a sterile grinding mortar. The crushed calculi core was cultured in 5 ml thioglycolate broth, which was incubated at 37°C for 18–24 h. Then the subcultures were made on blood agar and MacConkey's agar plate for isolation of etiological agents.
- (8) Medium for uricase enzyme: nutrient broth medium (Manual of Microbiological Methods, 1957) contains (1 g): peptone (5 g); sodium chloride (5 g); beef extract (3 g); distilled water was completed up to 1.0 l. pH was adjusted at 7.0., then the medium was dispensed in tubes and autoclaved at 121°C for 15 min and kept in fridge until used. Precipitation of uricase enzyme produced by *Klebsiella pneumonia* by centrifugation at 4000 rpm in a cooling centrifuge, then the filtrate was used as the crude enzyme. Purification of the enzyme using ammonium sulfate to produce cell-free filtrate; then dialysis with borate buffer was carried out to remove traces of ammonium sulfate. Purification of the uricase by Sephadex G-100 gel chromatography technique was done and the active fraction was collected and tested for their uricase activity. A standard curve of uric acid was adopted from the spectrophotometric method of Itaya *et al.* [20]. The optical density was determined at 293 nm using Spectronic 21 spectrophotometers (Algomhorria, Germany). The difference between the control and the experiment was translated into units of enzyme activity from a uric acid standard curve. One unit of enzyme activity corresponds to the amount of enzyme, which oxidizes one micromole of uric acid to allantoin in 1 min.

## Statistical methods

Data were analyzed using Stata version 14.2 (StataCorp LLC, College Station, Texas, USA). Normality of numerical data distribution was examined using the Shapiro–Wilk test. Non-normally distributed numerical data were presented as a median and interquartile range, and intergroup differences were compared using the Wilcoxon rank sum test. Categorical data were presented as number and percentage and differences were compared using Fisher's exact test.

## RESULTS

After examination of different bacterial species isolated from urine and extracted stones, the results showed that the highest percentage of stone-related infection was within the greater than 40 years age groups (60%), followed by less than 20 years age group (37.5%), and lastly 20–40 years age group (20.8%). However, according to the total number of diseased patients, the highest percentage was within greater than 40 years of age group (49.9%), followed by 20–40 years age group (43.8%), and the lowest percentage was in less than 20 years age group (9.3%) (Table 1).

Uric acid stones constituted the highest incidence (40%), men (39.4%) and women (41.2%), followed by magnesium ammonium phosphate (27%), men (31.8%) and women (17.7%) and pure calcium oxalate (21%), men (21.2%) and women (20.5%). The lowest incidence was represented by cysteine stone (2.0%), men (1.5%), and women (2.9%) (Table 2).

The highest percentage of bacterial isolates was group 5 (25%), representing bacteria of *Klebsiella* spp., followed by group 8 (21.8%) containing bacteria of *Streptococcus* spp., and group 2 (18.8%) including bacteria of *Staphylococcus* spp. The lowest incidence (3.1%) include three groups (groups 6, 7, 9), representing the bacteria of *Citrobacter* spp., *Proteus* spp., and *Providencia* spp., respectively (Table 3).

Pure uric acid stones would be dissolved more rapidly by increasing the incubation period. However, the enzyme had little effect on conjugated stones through the second hour of incubation (Table 4).

## DISCUSSION

Nephrolithiasis remains a common health problem worldwide, affecting up to 20% of the adult population [21]. Of all types of renal stones, calcium oxalate is the most common [22]. In patients with nephrolithiasis, the urine is commonly supersaturated with calcium and oxalate ion [23], favoring

calcium oxalate crystal nucleation and growth [24]. Urinary stones are the third most common affliction of the urinary tract. They are exceeded only by urinary tract infections and pathologic conditions of the prostate. Despite modern antibiotic therapy, it is still a significant cause of morbidity and mortality. Patients with infection stones have a high incidence of new stone growth and persistent infection, especially if residual stone fragments remain. The importance of complete eradication of these organisms needs constant emphasis [12]. The cure is achieved by the removal of all foreign bodies (stones, matrices, and catheters) and by eradication of infection.

Postoperatively, long-term antimicrobial therapy with agents known to be effective against the specific organism involved was needed in most cases to eradicate infection [11]. High resistance rates may be a result of abuse of antimicrobials, which leads to the development of resistant strains. There is no much investigation tracing the pathogenesis and follow-up of the treatment of this challenging case. Regarding age distribution, our results showed that the most affected age was between 20 and 40 years age group followed by over 40 years and the least age group affected was under 20 years. This might be related to the age of activity and youth, where they would be highly exposed to several factors that predispose to stone formation. This result is in agreement with other studies which show urolithiasis occurs more frequently in the third decade of life [25].

Ogata *et al.* [19] performed a study in which renal stones were mostly seen in the third and fourth decades of life. Other studies were done by Baker *et al.* [26] who found that the peak age for the development of renal infection stones most commonly in women was between the ages of 20 and 55 years and the second peak is seen, particularly in men, between 55 and 70 years of age. As regards sex, the present study showed that the male to female ratio was 1.94, which is slightly higher than that detected by Dall'era *et al.* [27] (1.48). This also

**Table 1: Distribution of infected renal stones among different age groups**

| Stone-related infection to their total number (%) | Stone-related infection within each group (%) | Number of infected renal stones (n=32) | Stone-related and nonrelated infection (n=100) | Age group |
|---|---|--|--|-----------|
| 9.3   | 37.5  | 3                                      | 8  | <20       |
| 43.75   | 20.8  | 14                                     | 67   | 20-40     |
| 46.87   | 60  | 15                                     | 25   | >40       |

**Table 2: Frequency of renal stones composition and distribution of sex**

| Composition of stones             | Total (n=100) | Men                          |  | Women                        |
|-----------------------------------|---------------|------------------------------|--|------------------------------|
|                                   |               | Number of samples (%) (n=66) |  | Number of samples (%) (n=34) |
| Pure calcium oxalate              | 21            | 14 (21.2)                    |  | 7 (20.5)                     |
| Calcium oxalate/calcium phosphate | 10            | 4 (6.1)                      |  | 6 (17.7)                     |
| Magnesium ammonium phosphate      | 27            | 21 (31.6)                    |  | 6 (17.7)                     |
| Uric acid                         | 40            | 26 (39.4)                    |  | 14 (41.2)                    |
| Cysteine                          | 2             | 1 (1.5)                      |  | 1 (2.9)                      |

**Table 3: Incidence of different species of bacterial isolates among stone-related infection**

| Preliminary identification           | Number of each isolate (incidence %) (n=32) |
|--------------------------------------|---|
| <i>Enterobacter</i> Spp. (group 1)   | 2 (6.3)                                     |
| <i>Staphylococcus</i> Spp. (group 2) | 6 (18.8)                                    |
| <i>Pseudomonas</i> Spp. (group 3)    | 2 (6.3)                                     |
| <i>Escherichia coli</i> (group 4)    | 4 (12.5)                                    |
| <i>Klebsiella</i> spp. (group 5)     | 8 (25)                                      |
| <i>Citrobacter</i> spp. (group 6)    | 1 (3.1)                                     |
| <i>Proteus</i> spp. (group 7)        | 1 (3.1)                                     |
| <i>Streptococcus</i> spp. (group 8)  | 7 (21.8)                                    |
| <i>Providencia</i> spp.              | 1 (3.1)                                     |

**Table 4: Application of uricase enzyme on renal stones under laboratory scale**

| Stone content                | Uric acid (µl/ml) | Uric acid (µl/ml) residual | Time |
|------------------------------|-------------------|----------------------------|------|
| Pure uric acid               |                   | 2.44                       | 1 h  |
|                              | 10                | 1.72                       | 2 h  |
| Uric acid                    |                   | 2.2                        | 1 h  |
| Calcium oxalate              | 8.2               | 2.2                        | 2 h  |
| Uric acid and                |                   | 2.8                        | 1 h  |
| Magnesium ammonium phosphate | 9.8               | 2.8                        | 2 h  |
| Control                      | 10                | 10                         | 1 h  |
|                              |                   |                            | 2 h  |

results in higher than that detected by Qaader *et al.* [28] (1.30). This may be related to hormonal effects with high inhibitory activity, lower food intake, and lower body size. Also, it has been postulated that the increased citrate concentration in urine of women, may aid in protecting women from calcium urolithiasis since citrate inhibits the nucleation of calcium oxalate crystals [29].

As regards composition of stones, in the present study, the infected stones (magnesium ammonium phosphate) accounted for 27%, pure calcium oxalate stones 21%, uric acid stones 40%, and only 12% included calcium oxalate/calcium phosphate and cystine stones. However, Jungers *et al.* [30] claimed that infection (struvite) stones accounted for 42.2%, calcium stones 26.7%, uric acid stones 17.8%, and hereditary diseases 13.3% of cases. On the other hand, Fredic *et al.* [31] observed 80% calcium oxalate and calcium phosphate stones, 10% struvite, 9% uric acid, and the remaining 1% was cysteine. The infected stones were predominant in men (31.8%) and women (17.7), whereas uric acid stones were in women (41.2%) and men (39.4%). Pure calcium oxalate stones were in men (21.2%) and women (20.5%). On the other hand, Jungers *et al.* [30] results showed that women were predominant among patients with infection and calcium stones. Men were predominant with uric acid or hereditary stone disease. Also, Fredic *et al.* [31] observed that about 5% American women and 12% of men would develop a renal stone at some time of their life and

its prevalence has been rising in both sexes. The percentage of infected renal stone in the present study was 32%. This rate may be higher when compared with that of Mariappan *et al.* [32] (11.1%), while it may be comparable to the results of Mariappan and Loong [33] which was 28.08%. This might be related to geographical distribution, race, individual variation, socioeconomic status, and dietary influences. In our study, it was found that infection of the urinary tract by *Klebsiella* spp. accounted for 25%, *Streptococcus* spp. constituted for 21.8%, *Staphylococcus* spp. constituted for 18.8%, *Escherichia coli* accounted for 12.5%, *Pseudomonas* spp. accounted for 6.3%, which indicates persistent urinary tract infection with ureasplitting or nonsplitting bacteria. These bacteria may be the primary factors in the synthesis of infected renal stones. A similar finding was also reported by Machavi *et al.* [10] who found that urinary tract infection with certain bacteria plays an essential role in the synthesis of renal stones. *E. coli* is not a urease-producing microorganism. Because of that, it is having the ability to produce calculus. However, the current study showed that *E. coli* was the bacteria that cause mixed calculi. The present finding is consistent with the study of Sakhaee [34]. Moreover, urinary tract infection with *E. coli* shows the conversion of a commensal population into pathogenic organisms. This may be due to decreased intake of water leading to the concentration of urine and also injury caused by the peculiar characteristics of the calculi to the urinary tract epithelium; thus forming a nidus for the growth of bacteria and acting as a good media for the pathogenic organisms to grow.

Uricase from microbial origin has been found to be of many important applications, which is the treatment of children suffering from hyperuricemia and gout in humans [35]. Accumulation of uric acid causes life-threatening conditions including lymphoid malignancies and/or deposition of uric acid in connective tissues [36]. Intramuscular or intravenous administrate uricase of *Aspergillus flavus* causes a rapid reduction in the serum and urinary levels of uric acid [37]. Also, the oral administration of actinomycetes cell suspension might have resulted in stimulating kidney tubules [38] to increase clearance of uric acid from the blood. More to this point, the purified uricase enzyme was found to have exhibited a remarkable decomposition of uric acid in the blood of domestic pigeons. It reached to 38% within the a duration of 3 days due to intramuscular injection, while it resulted in decomposition of 29% after 3 days and increased to 31% after 7 days due to the oral administration of purified enzyme.

The application of this enzyme from a laboratory point of view was established by Knauf *et al.* [39], who found the suitability of adapting uricase for determination of uric acid levels in serum with a recovery ratio ranging from 98 to 100%. The present study was directed toward the study of microbial uricase produced by *K. pneumonia*, the most potent uricase producer, out of all bacterial species isolated from human urine. The most important of which is *K. pneumonia* as it shows high uricolytic activity. Production and activity of uricase

were found to be affected by cultural and physical factors. The partially purified *K. pneumoniae uricase* was used as a decomposer for uric acid by applying it on grained stones of different compositions, uric acid stone, and conjugated stones. Uric acid stone affected significantly by the enzyme while conjugated stones had little effect through the second hour of incubation.

## CONCLUSION

Urolithiasis, involving the upper urinary tract, is a disease of multiple factors. A variety of intrinsic and extrinsic factors influence the incidence of disease in individuals and all population. Persistent urinary tract infection with urea-splitting or nonsplitting bacteria may be the first factors in the synthesis of infected renal stones. In metabolic stones, bacterial superimposition may be responsible for the recurrent urinary tract infections; controlling urinary tract infection, metabolic causes, and other risk factors can lead to a considerable decrease in the incidence of nephrolithiasis in this area. The application of enzyme technologies to pharmaceutical research, development, and manufacturing is a growing field and is the participant of many articles, reviews, and books.

## Recommendations

Lifestyle changes help to reduce recurrent stone diseases. Renal calculi can be prevented by drinking plenty of water daily (2–4 l), make sure you avoid getting dehydrated. Further studies are recommended to establish a novel usage of uricase enzyme as an antistone treatment.

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## Conflicts of interest

There are no conflicts of interest.

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