Research Article

Isolation and Characterization of Multiple Antibiotic Resistant *Aeromonas spp.* from Urinary Stone of Urolithiasis Patients

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ABSTRACT

Kidney stone disease also known as urolithiasis is condition when a solid piece of material occurs in the urinary tract. Kidney stones typically form in the kidney and leave the body in the urine stream. In present investigations bacteriological analysis of urinary stones of urolithiasis patients from hospital in the Barshi town was performed and antibiotic sensitivity of urinary bacterial isolates was studied. The bacteriological analysis of urinary stone of 66 patients of Urolithiasis was done. The urinary stones from the urolithiasis patients were collected, washed and crushed in normal saline and streaked on MacConkey's agar and Cysteine lactose electrolyte deficient medium and Mannitol salt agar. Morphological, cultural and biochemical characterization of the bacterial isolates was done as per Bergy's Manual of Systematic Bacteriology. Thirty three (33) samples showed presence of bacteria namely *Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Aeromonas spp. and Staphylococcus aureus*. All the isolates were further studied to determine their resistance/ sensitivity to various antibiotics such as Nitrofurantoin, Gentamicin, Ampicillin, Amoxycilin Amikacin, Norfloxacin, Cotrimoxazole, Cephatoxime, Ciprofloxacin, Chloramphenicol Tetracycline, and Nalidixic acid. The biochemical Identification was performed as per the standard methods and 16SRNA analysis was done for final identification. The Antibiogram of selected urinary isolates was studied by using disc diffusion technique and Kirby Bauer method. Among the urolithiasis isolates two isolates were identified as *Aeromonas spp* that exhibited resistance to several antibiotics. The proportion of other bacterial urinary isolates that were resistance to different antibiotics was also significant. These findings may offer help to clinicians and urosurgeons in deciding the appropriate empirical treatment for patients with Urolithiasis**.**

Keywords: Urolithiasis, *urinary tract infection*, Urinary stones, Antibiogram, multi-antibiotic resistance, *Aeromonas spp.*

INTRODUCTION

Historical and archaeological studies clearly reveal that ancient man suffered from urinary tract stone disease. The earliest evidence dates to before 4800 B.C. and was a bladder stone found among the pelvic bones of a young predynastic Egyptian. Another stone dating about 4200 B.C. was probably of renal origin and analysis revealed it to be composed of calcium carbonate, calcium phosphate and calcium oxalate. Hippocrates can be credited for being the first to explain the etiological factors of urinary calculi. He observed that many patients having calculi in the bladder had sandy sediment in their urine and suggested that the ingestion of muddy river water or water containing lime caused stone formation in the urinary tract. Galen, the great Roman physician also wrote about stone formation and recognized such risk factors as heredity, race, climate, diet, drinking water, the ingestion of alcohol, and metabolic abnormalities[3,5]. The cause and effect relationship between urinary infection and urinary stones has been known since antiquity[5]. Bladder stones that we now recognize as infection induced have been found in Egyptian mummies dating back to 5000 B.C. In the early twentieth century several clinicians noted the relationship between urinary stone disease and infection[1, 7].The first laboratory studies investigating the relationship of stone disease and infection was carried out by Rosenow and Meisser in 1923, which implanted *Streptococci* obtained from stone formers into the dental canal of dogs. Shortly thereafter, other investigators instilled bacteria into rabbit bladders and demonstrated development of bladder stones[10, 16, 17]. The association of urea splitting bacteria particularly of the *Proteus* species is well recognized but it is also noted that only few patients with *Proteus* infection had urinary stone[4].Recent report by Musher and Griffith indicate that the use of urease inhibitors can impair stone growth in the presence of persistent infection. Since greater than 50% of patients with infected stag horn stones have treatable metabolic disorders, it is imperative to fully evaluate these patients preoperatively in order to reduce the chances of recurrent stone formation[6]. Infection-induced stones refer to calculi composed of magnesium ammonium phosphate and carbonate appetite and urease producing microorganisms such as *Proteus*, *Klebsiella*, *Pseudomonas, Staphylococcus, E.coli*, *Enterococci*, *Streptococci*, *Lactobacilli*, *Bacteroides* etc. are known to be responsible for urinary stones [8,12].In Previous studies only the commonest pathogens recovered from pre-operative urine culture and stone culture were studied but information regarding uncommon bacteria exhibiting multiple antibiotic resistance is still inadequate. Therefore the present investigations were undertaken to study multidrug resistance of relatively uncommon bacterial uropathogens associated with urolithiasis or urine stones.

MATERIALS AND METHODS

Collection of Sample

For isolation of uropathogens, urine and urine stone samples were collected from patients suffering from urolithiasis by using standard collection procedures. The patients were asked to pass the urine in clean container. Midstream urine samples were collected in sterile containers by taking all precautions to avoid the contaminations. The urine specimens were immediately brought to the laboratory and cultured within thirty minutes. Urinary stones were washed thrice with sterile normal saline and crushed in sterile pestle and mortal. Nidus was separated, crushed and emulsified in normal saline.

Isolation and identification of bacterial uropathogens

One loopful of un centrifuged urine samples was streaked on Mac Conkeys agar medium and Cysteine Lactose electrolyte deficient medium and Mannitol salt agar medium. The plates were incubated overnight at 37° C temperature and after incubation the colonies developed were processed as per standard methods to identify the bacterial isolates. Morphological, cultural and biochemical characteristics of well-isolated colonies were studied. Gram nature and motility were also studied. Biochemical tests were performed according to Bergy's Manual of determinative Bacteriology by using different solid and liquid media. Final identification was done according to16SrRNA analysis technique. The evolutionary history was inferred using the Neighbor-Joining method[15]. Phylogenetic analyses were conducted in MEGA4 [19]

Antibiogram studies by using Agar disc diffusion method

Standard antibiotic discs containing known concentrations of antibiotics were used for anti biogram determination. The antibiotic discs used were Nitrofurantoin, Gentamicin, Ampicillin, Amoxycilin Amikacin, Norfloxacin, Cotrimoxazole, Cephatoxime, Ciprofloxacin, Chloramphenicol, Tetracycline and Nalidixic acid (Table 3).Susceptibility or resistance of isolates to the antibiotics was tested with Kirby and Buayers agar diffusion method on Mac Conkeys agar plates. 0.1 ml suspension of the isolate was spread inoculated on sterile Mac Conkeys agar plate. Antibiotic polydisc each with 12 antibiotics was placed on the medium surface and the plates were kept for antibiotic diffusion at 4° C for 20 minutes. All the plates were incubated at 37° C for 24 hrs. After incubation zone of inhibition around each disc was observed and measured and the results were interpreted for resistance or susceptibility as per standards given by Clinical Laboratory Standards Institute (CLSI).

RESULTS AND DISCUSSION

Isolation and characterization of the uropathogens

Seven different bacterial isolates were selected on the basis of differences in cultural properties and those were further processed for their identification. Based on morphological, cultural and biochemical characteristics the isolates were identified as given in Table 1. Thus, the bacterial isolates were *E.coli* (n=2), *Proteus spp*. (n =2), *Klebsiella* (n=1), *Staphylococcus spp*. (n=1), *Pseudomonas spp.*(n=1) and *Aeromonas spp*(n=2). These isolates were derived from urine culture and urine stone culture and finally identified as *Escherichia coli, Proteus vulgaris Klebsiella pnumoniae*, *Staphylococcus aureus, Aeromonas spps.* and *Pseudomonas aeruginosa.* The *Aeromonas* species *and Staphylococcus aureus* were the only bacteria that could be isolated from crushed stones. Further study was focused on *Aeromonas spp.* because these organisms were relatively uncommon in urolithiasis conditions. The morphological, cultural and biochemical characteristics of *Aeromonas spp. SD BAR* and *Aeromonas spp*. *RCSR-NCCS-AM3* are presented in Table 2. The major distinguishing properties of these species are gram negative rod shaped, nonspore forming, actively motile, aerobic to facultative anaerobic, oxidase and catalase positive, absence of growth in 6% NaCl, positive HL test and positive Nitrate reduction tests. The other biochemical characteristics of the species are given in Table 2. The final identification of both *Aeromonas* isolates was done by 16S r-RNA sequence analysis. The dendrogram was obtained with 16S rRNA sequences (Fig.1 and Fig.2). The nucleotide sequences of these organisms viz *Aeromonas sp. SD BAR* and *Aeromonas sp. RCSR-NCCS-AM3* are accepted by DNA databank of Japan (DDBJ).The Accession numbers of these sequences are mentioned in respective figures.

Antibiotic resistance studies of *Aeromonas species*

For determination of sensitivity/resistance of *Aeromonas* isolates to the antibiotics used, Kirby-Bauer disc diffusion method was used. The zones of inhibition, if any, around the discs were compared with the standard recommended zones as per CLSI. The resistance pattern of *Aeromonas spp*. is as shown in Table 3. The *Aeromonas* strain viz. *Aeromonas sp. RCSR-NCCS-AM3* exhibited resistance to Gentamicin, Ampicillin, Amoxycilin Norfloxacin, Cotrimoxazole, Cephatoxime, Ciprofloxacin, Chloramphenicol, Tetracycline and Nalidixic acid. Thus, The *Aeromonas* strain viz. *Aeromonas sp. RCSR-NCCS-AM3* was found to show resistance to all the antibiotics used except Amikacin and Nitrofurantoin while the strain *Aeromonas sp. SD BAR* exhibited resistance to Nitrofurantoin, Gentamicin, Ampicillin, Amoxycilin, Norfloxacin, Cotrimoxazole, Cephatoxime, Ciprofloxacin, Chloramphenicol, Tetracycline and Nalidixic acid. *Thus, Aeromonas sp. SD BAR* also showed resistance to all antibiotics except Amikacin only (Table 3 and Fig.4). Thus both the *Aeromonas* strains viz *Aeromonas sp. SD BAR and Aeromonas sp. RCSR-NCCS-AM3* showed multiple antibiotic resistances to number of commonly used antibiotics for treatment of urinary infections. As both the strains are isolated from crushed urine stones they might have role in stone formation and infection. *Aeromonas* strains are not as common as other species

such as *E.coli*, *Pseudomonas* etc. and therefore their multiple antibiotic resistances has significance. Fig.3 represents the multiple antibiotic resistances in *Aeromonas sp. RCSR-NCCS-AM3*. The multiple antibiotic resistances in *Aeromonas spp.* may be due to repeated exposure to various antibiotics during treatment. Multiple antibiotic resistance study of different isolates showed that *Aeromonas sp. SD BAR and Aeromonas sp. RCSR-NCCS-AM3* were more resistant to number of antibiotics than the other common urinary isolates (results not given here).In fact, *Aeromonas* species are commonly present in the aquatic environments and have been isolated from various food products such as fish, shellfish, raw meat and vegetables. Recently *Aeromonas* species have been reported as causative agents of human diseases such as gastroenteritis and wound infections[9, 13]. As per our literature search, this is probably the first report indicating association of *Aeromonas species* with urinary stones or urolithiasis conditions. Role of these pathogens in urinary infection and urine stone formation has to be elucidated further. Isolation of these organisms directly from urinary stone and their multi antibiotic resistance have led to more concern with the role of these organisms in pathogenesis of urolithiasis as well as possibility of emergence of resistant variety from the nonpathogenic ones by genetic transfer mechanisms in nature. The *Aeromonas* species isolated in present investigations exhibited resistance to β-lactums such as Ampicillin, Amoxycillin and third generation Cephalosporins such as Cefotoxime and the broad spectrum antibiotic Tetracycline and non beta lactams including fluoroquinolones and gentamycin. The urinary infections involving antibiotic resistant bacteria are increasing recently. The prevalence of extended spectrum beta-lactamases (ESBL) producing bacterial pathogens have increased the problems further due to transfer of resistance traits to generally nonpathogenic commensals rendering them resistant to antibiotics. Whether the antibiotic resistance trait of *Aeromonas* species is chromosomal or extra chromosomal is to be determined by further genetic characterization. The present investigations indicate the possibilities of emergence of antibiotic resistant bacteria species from seemingly nonpathogenic commensals like *Aeromonas*. Such bacteria may cause complications in management of urolithiasis conditions. Therefore it is suggested that systematically planned programs be undertaken to control the emergence of antibiotic resistance in bacterial pathogens and the nonpathogenic commensals also. Emphasis must be given to regulated and appropriate use of antibiotics by doctors and development of awareness among the people also through social counseling. Complete unraveling of association of commensal bacteria with urinary stones, their role in stone formation and pathogenesis and development of antibiotic resistance in such bacteria needs further investigation.

Aeromonas sp. RCSR-NCCS-AM3. Strain 2 is the strain from our patient. *Aeromonas punctata strain 3K1C15* was used as an out group.

The evolutionary history was inferred using the Neighbor-Joining method (Helen Phillips, 1998). The optimal tree with the sum of branch length = 30.39356665 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches[15]The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 355 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4[19].

Aeromonas salmonicida sub sp. pectinol was used as an out group: Strain 12 is the strain from our patient ***Aeromonas spp*. SD BAR**

Table 1: Biochemical characteristics of bacterial isolates from urinary stones

Test	Isolate1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7
Indole	+	+	+	+	+	+	+
Methyl Red	+	-	-	+	-	-	+
Vogues Proskauer	+	-	-	-	+	+	-
Citrate	+	-	-	-	+	+	+
Urease	-	+	+	-	+	-	+
Phenylalaninedeamination	+	+	+	-	-	-	+
H_2S in TSI	+	+	+	-	-	-	-
Inositol	-	-	-	-	-	-	-
Mannitol	+/-	+/-	-	+/+	+/+	+	+
Dulcitol	+/-	+/-	+/-	+/-	+/-	-	-
Salicin	-	-	-	-	-	+	+

Isolate 1: *Staphylococcus spp*. Isolate 2: *Proteus spp*. Isolate 3: *Pseudomonas spp* Isolate 4*: E. coli* Isolate 5: *Klebsiella*, Isolate 6 : *Aeromonas spp.*1 and Isolate 7: *Aeromonas spp.2*

Table 2: Morphological, cultural and biochemical characteristics of Aeromonas species isolated from urinary stones:

Characteristics	*Aeromonas sp. SD BAR*	*Aeromonas sp. RCSR-NCCS-AM3*
Gram nature	Gram negative	Gram negative
Shape	rod shaped	rod shaped
Motility	motile	motile
Indol	+	+
Methyl red	-	+
VogusPrausker	+	-
Citrate	+	+
Urease	-	+
Nitrate reduction	+	+
Oxidase	+	+
HL test	+/-	+/-
Catalase	+	+
Growth at 0% Nacl	+	+
Growth at 6% Nacl	-	-
Starch hydrolysis	+	+
Gelatin hydrolysis	+	-
Glucose	+/+	+/+
Lactose	+	+
Mannitol	+/+	+/+
Inositol	-	-
Arabinose	+	-
H_2S	-	-
Salicin	+	+

Note: + indicates positive test**, -** indicates negative test, **+/-** indicates sugar fermentation with acid but no gas, **+/+** indicate sugar fermentation acid and gas

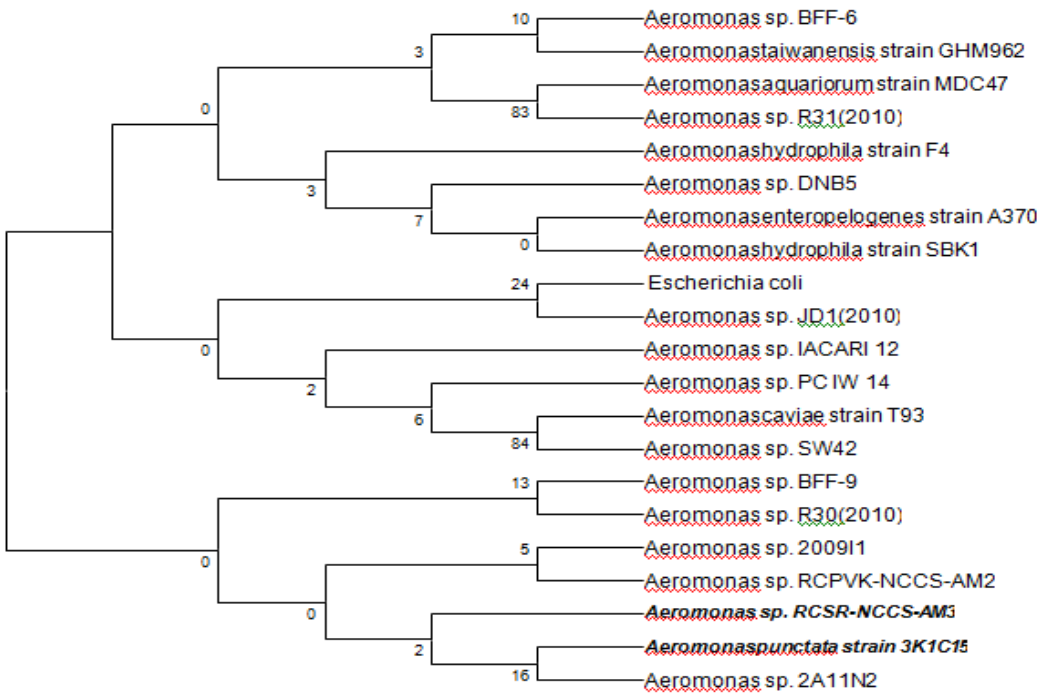

Figure 1: Evolutionary relationships of 21 taxa

Table 3: Multiple antibiotic resistance in *Aeromonas sp. SD BAR and Aeromonas sp. RCSNCCS-AM3*

Sr. no	Antibiotic used	Disc Antibiotic Concentration(mcg)	*Aeromonas sp. SD BAR*	*Aeromonas sp. RCSR-NCCS-AM3*
1	Amikacin (Ak)	30	S	S
2	Amoxycilin(Am)	30	R	R
3	Ampicillin(A)	10	R	R
4	Cephotaxime(Ce)	30	R	R
5	Chloramphenicol(C)	30	R	R
6	Ciprofloxacin(Cf)	5	R	R
7	Co-trimoxazole(Co)	25	R	R
8	Gentamicin(g)	10	R	R
9	Nalidixic Acid(Na)	30	R	R
10	Nitrofurantoin(Nf)	300	R	S
11	Norfloaxacin(Nx)	10	R	R
12	Tetracycline(T)	30	R	R

R= Resistant, S = Sensitive, mcg = microgram

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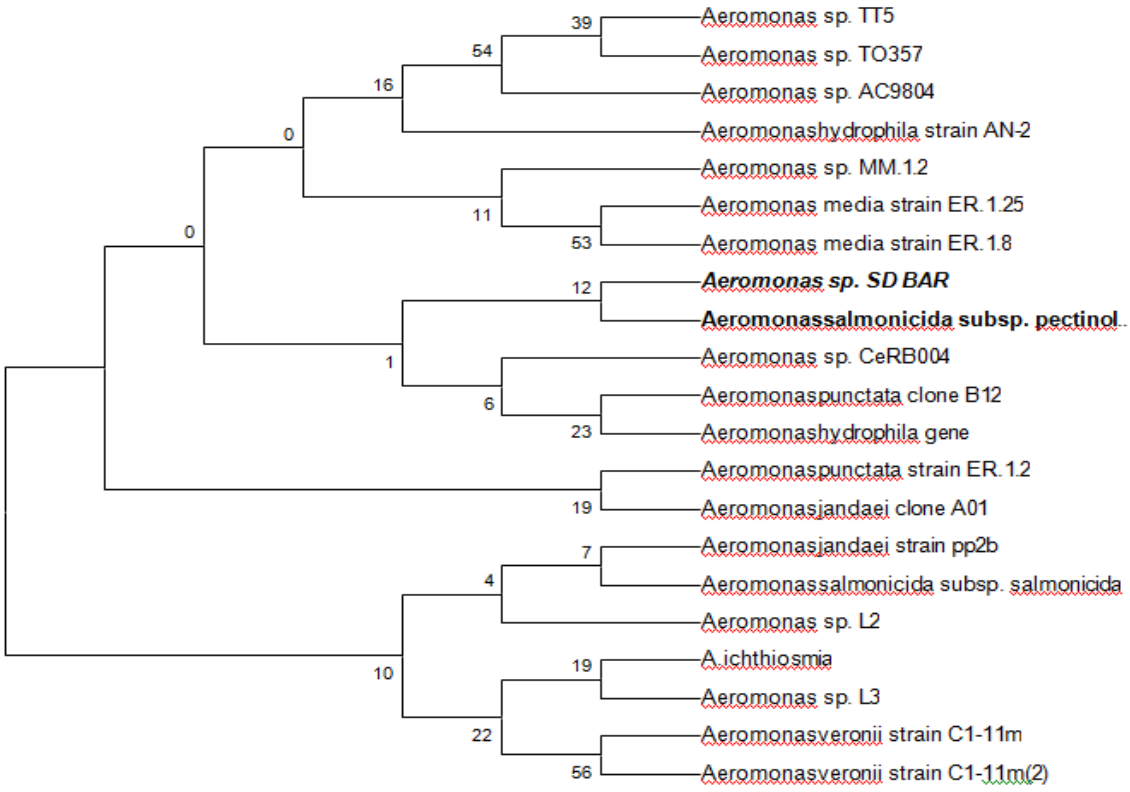

Figure 2: Evolutionary relationships of 9 taxa

The evolutionary history was inferred using the Neighbor-Joining method.[6] The optimal tree with the sum of branch length = 32.06258237 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches[15].The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 458 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4[19]

Figure 3: Multiple antibiotic resistance in *Aeromonas sp. RCSR-NCCS-AM3*

Figure 4: Resistance in *Aeromonas spp*. SD BAR

CONCLUSION

The niche of *Aeromonas* is mainly marine water. In our patients, we did not look for this epidemiological link. Although it is an uncommon uropathogen, the likelihood of its isolation from unusual sites, especially in Urolithiasis is alarming. It is interesting to note that both the resistant isolates were resistant to most of the antibiotics. *Aeromonas sp. SD BAR* shows resistant to 12 antibiotics and *Aeromonas sp. RCSR-NCCS-AM3* shows resistant to 10 antibiotics. The surveillance of *Aeromonas* strains and their characterization will enhance the ability of the public health and health care system to rapidly recognize and aggressively contain infection and colonization due to these antibiotics resistant pathogens. There is evidence to suggest that the prevalence of *Aeromonas* infections may be dramatically underestimated in developing nations. A variety of demographical, societal, environmental, and physiological emergence factors likely play critical roles in enhancing the frequency of transmission of pathogens to hosts and the increasing trend in antibiotics resistance in the bacteria make extended studies on the bacteria imperative and this is subject of investigation in our group.

REFERENCES

1. Brown T.R., on the relation between the variety of microorganisms and the composition of stone in calculus pyenephritis. JAMA, 36, 1901, 1395-1397.
2. Butt A.J., Historical survey: In: Etiologic factors in renal lithiases, ed. Butt, A. J., 1965 pp3-47,.
3. Curhan G.C., Willett W.C., Knight E.L. and Stampfer M.J., Dietary factors and the risk of incident kidney stones in younger women: Nurses' Health Study II. *Arch Intern Med*.; 164, 2004, 885-891.
4. Dalton J.R. and Bergquist E.J.Urinary tract infection and stone disease. Series in clinical epidemiology of UTI, 1987, 201-211.
5. Finkielstein V. A., Strategies for preventing calcium oxalate stones. *CMAJ*10, 2006, 1407-1409.
6. Helen Phillips. Could bacteria make kidney stones? Published online 23 July 1998 Nature, doi:10.1038/news980723-8
7. Hellstorm J., The significance of S*taphylococcus* in the development and treatment of renal and urethral stones. Br. J. Urol: 10, 1938, 348-372.
8. Hugosson, J., Grenabo, L., Hedelin, H., Pettersson, S. and Seeberg, S., Bacteriology of upper urinary tract stones. J Urol.; 143, 1990, 965–968.
9. Janda, J.M., Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. Clin.Microbiol. Rev., 4, 1991, 397-410.
10. Krambeck A.E., Gettman M.T., Rohlinger A.L., Lohse C.M., Patterson D.E. and Segura J.W., Diabetes mellitus and hypertension associated with shock wave lithotripsy of renal and proximal ureteral stones at 19 years of follow up. *J. Urol*., 5, 2006, 1742-7.
11. Mushar D.M. and Griffith D.P., Urease inhibition: Alternative to antibacterial treatment in urolithiasis research ed; 1984, 483-486, Network, Plenum press.
12. Norman J., M. D.Nemoy. A.Thomas and M.D. Stamey, Surgical, bacteriological and biochemical management in infection stones.JAMA ; 245, 1971, 1470-1476.
13. Palumbo S.A., the *Aeromonas hydrophila* group in food. In: Austin B, Altwegg M, Gosling PJ, Joseph S. eds. The Genus *Aeromonas*. Wiley and Sons; pp., 1996, 287-31
14. Saitou N. and Nei M., The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*4, 1987, 406-425.
15. Sinha M.K., Collazo-Clavell M.L. and Rule A. Hyperoxaluric nephrolithiasis is a complication of Roux-en-Y gastric bypass surgery. *Kidney International,* 72:2007, 100-107.
16. Straub M., Hautmann R., Developments in stone prevention. *Curr.Opin. Urol*., 2, 2005, 119-126.
17. Resenow E.C. and Meisser J.G., The production of urinary calculi by devitallization and infection of teeth in dogs with streptococci from cases of nephrolithiasis. Arch. Int. Med. 31, 1923, 807-829.
18. Tamura K, Nei M. & Kumar S., Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (*USA*) 101, 2004, 11030-11035.

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