

A Laboratory Diagnosis and Identification of *Bacillus subtilis* in Adult Urine Samples

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Abstract: This study aims to isolate and identify pathogens from a sample of adult urine obtained from humans suffering from urinary tract infection. In the subsequent study, aims to identify pathogens from a sample of adult urine obtained from patient are who suffering from kidney stones. It was found that *Bacillus subtilis* (RM01) was the most common isolated species. Uric acid crystals, calcium oxalate and muddy brown cast were observed in urine samples within 24 hours after collection. Clinical pathology and biochemistry tests were conducted to analyze the sample completely. The isolates were found to be antibiotic resistant. The study concluded that the isolates were resistant to chloramphenicol, tetracycline and streptomycin and could be diagnosed from urine samples. Increasing levels of infections indicated kidney stones.

Keywords: *Bacillus subtilis*, kidney stone, uric acid crystals, calcium oxalates

Introduction

Crystals, seen as 'a heap of rhomboical bricks', were the first element described when urine was investigated for the first time with a microscope in 1630 by the French scholar Fabricius Nicolaus De Peiresc (1580-1637) (1). Urinary calculi analysis is important to determine the possible etiology of stone formation and the pathophysiology of urolithiasis, as previously reported. Stone analysis plays a valuable role in the diagnosis of kidney stone disease, specifically in uncommon kidney stones such as uric acid, cystine, infection-induced, drug-induced, and NH₄-urate stones.

Stone formation usually results from an imbalance between factors that promote urinary crystallization, and those that inhibit crystal formation and growth (2). The main determinants of calcium oxalate (CaOx) supersaturation are oxalate and calcium concentration, while the latter associated to urinary pH determines calcium phosphate supersaturation. Urinary pH itself is the main determinant of uric acid super saturation (3).

Oxalate is a toxic compound abundant in the plant domain and extensively utilize in normal diets as a component of, vegetables, fruits, nuts, and grains. The regular consumption of oxalate ranges from 70 to 920 mg but extensively rises in vegetarians (4). Approximately 60 to 80 % of all kidney stones are composed primarily of calcium oxalate. The major factor of calcium oxalate crystal formation is the supersaturation of urinary calcium oxalate (5). Oxalate degrading enzymes are not present in humans (6). Urinary stone is one of the medical factors increasing the risk of complicating urinary tract infections (7). The stone can cause partial or complete obstruction to the flow of urine and permitting the bacterial growth in the urinary tract leading to infection (8). Also, the sharp edges of some urinary stones such as uric acid and calcium – oxalate damaging the epithelial layer of the urinary tract and encourage the bacterial growth (9).

B. subtilis is a Gram-positive, rod-shaped bacterium, usually found in soil. For a long, it has been considered as an obligative aerobic microorganism, though recently was characterized as also facultative anaerobe. *B. subtilis* has flagellates showing a decent capacity of motility in liquids, and under tough environmental settings (such as heat, cold, radiation, medical disinfectants) is also capable of forming endospores, which contributes significantly to its widespread existence in nature (10). *B. subtilis* inhabits frequently the human gastrointestinal tract in a carrier-state (11) and is considered non-harmful to humans.

Materials and Methods

Sample collection

The midstream urine samples were collected in sterile container and immediately taken into a microbiological laboratory.

Physical parameters

A urine samples was analyzed to determine appearance, volume, colour, pH, odour and sediment. The pH of the urine was 6.5.

Isolation and Identification of the organism

The purpose of this technique was to isolate, identify the microorganism by Gram staining method. The basic serial dilution procedure (Dubey and Maheswari) was used to isolate the bacteria Mac Conkey agar and MYP agar were used to confirm the isolate. The stock culture was maintained on nutrient broth under refrigerator condition.

Microscopic examination of urine sample

We observed uric acid crystals; calcium oxalate and muddy brown casts.

PCR Amplification 16s rRNA

The gene was amplified using oligonucleotide primers 16S rRNA.PA forward: (5' AGAGTTTGATCTGGCTCAG 3') and PH reverse: (5' TACGGTACCTTGTTACGACTT 3'). PCR conditions were as follows for the amplification of 16SrRNA from the obtained DNA: Add 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Perform PCR using the

following thermal cycling conditions. The program for PCR was as follows: 95o C for 2 min, 25 cycles of 95o C for 30 sec, 50o C for 30 sec, 72o C for 2 min and 72oC for 10 min, and extension at 72o C for 7 min, +4o C ∞ . Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Phylogenetic Analysis

The program MUSCLE 3.7 was used for multiple alignments of sequences (12). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (13). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model

Antibiotic sensitivity test

Antibiotic sensitivity test of *B. subtilis* was determined by growing on Mullern Hinton agar commercially available antibiotic discs of Chloramphenicol, Tetracyclin, and Streptomycin. Zones of inhibition were measured by aseptically placing the discs on agar medium, incubating at 28o C for 48 hours and recorded.

Results and Discussions

As a result of this investigation, we found a person suffering from kidney stone with a urinary infection of *Bacillus subtilis*. The patient complained about severe back pain, nausea, abdominal pain, skin allergy, and fatigue. Urinary parameters were recorded (Table 1). *Bacillus subtilis* was identified as a repeated pathogen through 16s rRNA sequencing, phylogenetic tree (Fig 1) and tests showed that it is more resistant to chloramphenicol than streptomycin and tetracycline. A microscopic examination of 24 hours urine samples suggests the formation of uric acid crystals, calcium oxalate and muddy cast. Clinical pathology and biochemistry tests suggested that further investigation of this study should take place (Table 2).

Conclusion

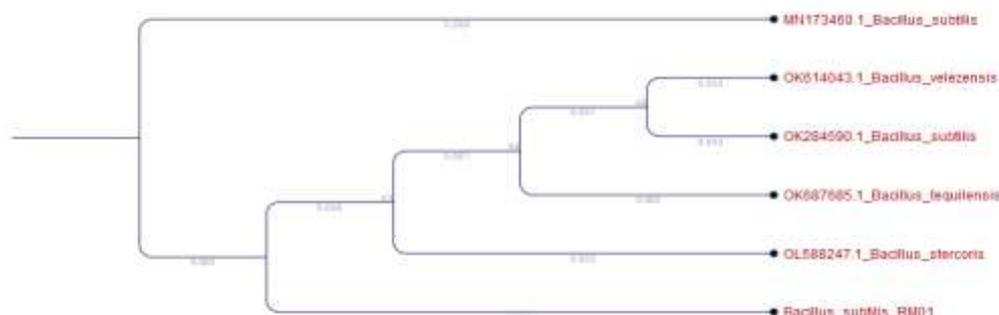
The study aims to isolate and identify the pathogen from adult urine samples to detect the urogenital tract infection, a threatening and common condition in both men and women. Kidney stones are the most painful and distressing conditions in men. *Bacillus subtilis* was isolated most frequently. During the first 24hours the urine sample showed uric acid crystals, muddy brown cast and calcium oxalate. Pathology tests were done for complete analysis. Antibiotic tests showed that isolates were resistant. As a result, isolates of chloramphenicol, tetracycline and streptomycin were resistant. Increased levels of infections led to the diagnosis of kidney stones. Future studies will examine the role of bacteria in developing renal stones.

Table 1. Parameters of urine sample

Test sample	Age	Gender	Colour of the urine	Appearance	pH	Pus cells	Volume
1	20	M	Pale yellow	Turbidity	Acidic	Packed with pus cells/ hpf	30
2	30	M	Pale yellow	Turbidity	Acidic	Packed with pus cells/ hpf	30

Table 2: Biochemistry and Pathology of urine samples.

Tests	mg/dL	Normal Range
Urine sample		
1. Urine sugar	Positive	-
2. Bile salt	Positive	-
3. Bile pigment	Negative	-
4. Urobilinogen	Positive	-
5. Urine albumin	Positive	-
Blood sample		
6. Blood sugar	112mg/dL	65-110 mg/dL.
7. Serum cholesterol	122mg/dL	150-250mg/dL
8. Blood urea	17.39mg/dL	5 to 20 mg/dL
9. Serum total protein	1.7	3.5 to 5.0 g/dl
10. Serum creatinine	0.7mg/dL	0.8-1.3 mg/dL.

Fig. 1 Phylogenetic tree (RM01)**Acknowledgment**

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