

Phenotypic Characterization of *Nanobacterium Sp.* Isolated from Urinary Tract Calculi of Egyptian Patients

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Abstract: Isolation of Nanobacteria from different types of urinary tract stones from different populations all over the world raises the possibility that these microorganisms are etiological agents of these stones. The aim of this study was to isolate and identify the nanobacteria from different types of human urinary tract stones and serum from Egyptian population. We searched for nanobacteria from ten aseptically removed urinary tract (UT) stones by processing and subjecting the stones to mammalian cell culture conditions and also we searched for the bacteria from serum of the same patients. The isolated bacteria were identified using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Also we used SEM with eight fractured urinary tract stones. We observed the presence of apatite forming, ultra-filterable, coccoid microorganisms in 80% of the urinary tract stones and serum of patients. SEM studies revealed about 400 nm sized organisms with a rough surface. TEM images showed 200-600 nm sized nanobacteria with a distinct cell wall and a capsule covered with a hairy apatite layer. Nano-sized bacteria are present in human urinary tract stones and serum of the same patients suffering from stones and may play a crucial role in urinary tract stone formation in our bodies. However, further studies are required for detailed information about role of nanobacteria in initiating urinary tract stones.

Key words: Nanobacteria • Apatite • Urinary Tract Calculi • Calcification

INTRODUCTION

Nanobacteria are uncommon agents 100-fold smaller than common bacteria that can replicate apatite forming units [1]. They are the smallest cell-walled bacteria, only discovered at a recent time in human and cow blood and commercial cell culture serum [2].

In the few years since their discovery, nanobacteria have aroused equal parts of expected success and debate between scientists as possible stimulants of human calcific diseases [3]. They are the first calcium phosphate mineral containing particles isolated from human blood and were detected in numerous pathologic calcification related diseases. Ciftçioğlu and McKay [4] As in renal stones [2,5], black pigment gall stones [6], dental pulp stones [7] and salivary gland stones [8], also it was

attributed to calcification and fibrosis of gall bladder (cholecystolithiasis) [9], arterial calcification [10], atherosclerosis [11], pathological placental calcification [12], female interstitial cystitis/painful bladder syndrome [13], psammoma bodies (ovaries) [14,15], type III prostatitis [16], testicular microlithiasis [17], heart diseases (local calciphylaxis on the mitral valve) [18] and calcific aortic valve stenosis [19], periodontal diseases (gingivitis and periodontitis) [20], peripheral neuropathy in HIV patients [21] and may be linked to congenital rickets [22]. So nanobacteria play a crucial role in etiopathogenesis of many diseases and this association is independent from their nature.

Thus, emergence of nanobacteria in an organism was a pathological, not a physiological, process. Kutikhin *et al.* [23] and specifically the reported isolation of

nanobacteria from human kidney stones raises the curious possibility that these microorganisms are etiological agents of pathological extraskelatal calcification [2,24]. The present study was conducted to investigate the presence of nanobacteria in urinary tract calculi and to study their role in calculi formation.

MATERIALS AND METHODS

Surgically aseptically removed calculi from 10 Egyptian patients in El KasrEleiny hospital were collected. Serum was also collected from these patients in addition to three serum samples which were collected from healthy individuals. Each stone were divided into three fragments, One fragment of each stone were analyzed for their chemical composition by fourier- transformed infrared (IR) spectroscopy according to standard chemical analytical methods [25], other fragment was used for scanning electron microscopy and the last fragment was preserved for culture analysis. Also serum samples were preserved for culture analysis.

Culture of Nanobacteria from Kidney Stones: The stone samples were processed for the culture of nanobacteria according to the method of Ciftcioglu *et al.* [5]. The stones were manually ground, pulverized and demineralized in 1N HCl and neutralized with 0.5M Tris, (pH 10.5, Sigma) and the solutions were centrifuged at 20,000g for 30 min at 4°C in a Sorvall RC5B centrifuge. The pellet was suspended in serum free RPMI 1640 (Biowest), sterile filtered through 0.2um Millipore filters and the filtrate was cultured in flasks containing RPMI 1640 with 10% fetal calf serum (FCS, South America) and kept under tissue culture conditions (37°C, 5% CO₂ and 95% air). Also a control, RPMI was incubated with FCS but without stone filtrate. Subcultures were carried out in serum free RPMI after 4 weeks of initial inoculation and subsequently after every 15 days. The cultures were harvested by centrifugation at 20,000g for 45 min at 4°C, washed with phosphate buffered saline (PBS, pH7.2) and used for identification by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Culture of Nanobacteria from Serum Samples: The serum was processed for the culture of the Nanobacteria according to the method of Young *et al.* [26]. serum was filtered through 0.2 µm sterilizing filters, diluted 1:10 to 1:300 in DMEM (Sigma), followed by incubation at 37°C in cell culture conditions (37°C, 5% CO₂ and 95% air) for

several weeks (about 8 weeks). The cultures were harvested by centrifugation at 20,000g for 45 min at 4°C, washed with phosphate buffered saline (PBS, pH7.2) and used for identification by SEM and TEM.

Scanning Electron Microscopy (SEM): A 30-45day old bacterial culture was centrifuged at 20,000g for 30 min at 4°C and washed with PBS [27]. Samples (Bacterial pellet) were fixed in 3% gluteraldehyde, dehydrated in series from ethyl alcohol and dried using the critical point procedure, then individually affixed using double-sided sticky tape and sputter coated with gold palladium.

Transmission Electron Microscopy (TEM): The bacterial pellet is processed for TEM by fixation in gluteraldehyde and osmium tetroxide, dehydrated in alcohol and embedded in an epoxy resin. Microtome sections prepared at approximately 500-1000 µm thickness with a Leica Ultracut UCT ultramicrotome. Thin sections were stained with tolodin blue (1X) then sections were examined by camera Lica ICC50 HD. Ultra-thin sections prepared at approximately 75-90µm thickness and were stained with uranyl acetate and lead citrate, then examined by transmission electron microscope JEOL (JEM-1400 TEM) at the candidate magnification.

RESULTS

All data obtained are summarized in Table 1. The chemical analysis study of urinary tract stones by Fourier Infrared spectroscopy indicated 50% as urates, 30% as oxalates and 20% as phosphates.

The culture analysis study revealed eight out of ten (80%) urinary tract stones showed a growth of nanobacteria. Also, the growths of nanobacteria were shown in serum samples from same patients. A pale white biofilm attached to the bottom of the culture flask was observed in 4 weeks old culture, while the control did not show any growth. The bacteria were slow growing and could be filtered through a 0.2 um filter. Standard microbiological techniques did not show the growth of any other micro-organism in the culture medium except nanobacteria species. The eight fractured UT stones examined by SEM showed similar characteristics. Spherical coccoid particles were observed, which were grouped in coarse clusters and bound together to a mineral structure (Fig.1). These particles were similar in size and morphology. The size of these particles varied from 200-500 nm.

Table 1: Summary of results include culture isolation of nanobacteria from urinary tract stones and from serum, SEM detection from urinary tract stones and their chemical analysis

NO.	Sex	Culture (stone)	Culture(Serum)	SEM	Stone analysis
1	female	N	N	N	Urates
2	male	P	P	P	Oxalate
3	male	P	P	P	Oxalate
4	male	P	P	P	Phosphate
5	female	P	P	P	Urates
6	male	P	P	P	Urates
7	male	P	P	P	Urates
8	male	P	P	P	Oxalate
9	male	N	N	N	Phosphate
10	female	P	P	P	Urates

P: Positive.

N: Negative.

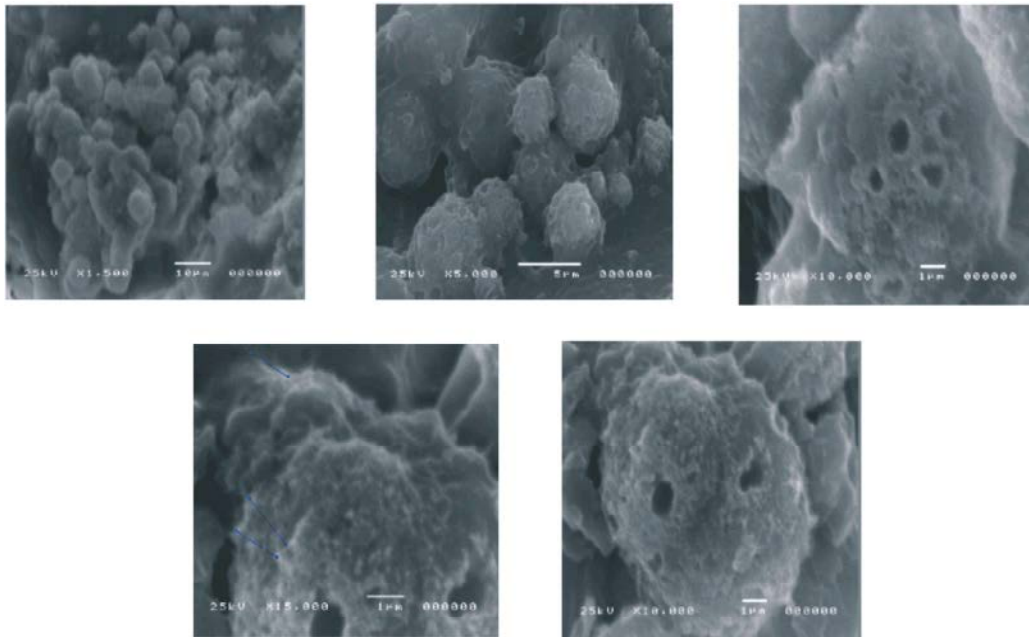


Fig. 1: The figure shows SEM picture of different magnifications of fractured urinary tract stones containing nanobacteria.

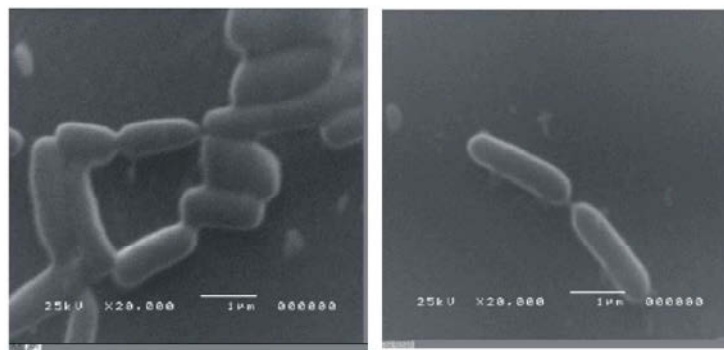


Fig. 2: The figure shows SEM picture of a 45 day old culture from urinary tract stones at 20,000X magnification showing nanobacteria of between 200-500 nm (bar=1µm)

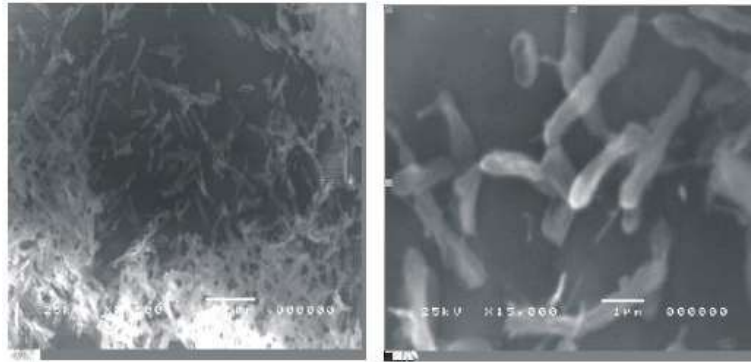


Fig. 3: The figure shows SEM picture of a 60 day old culture from serum of same patients of urinary tract stones at different magnifications showing nanobacteria with a rough surface.

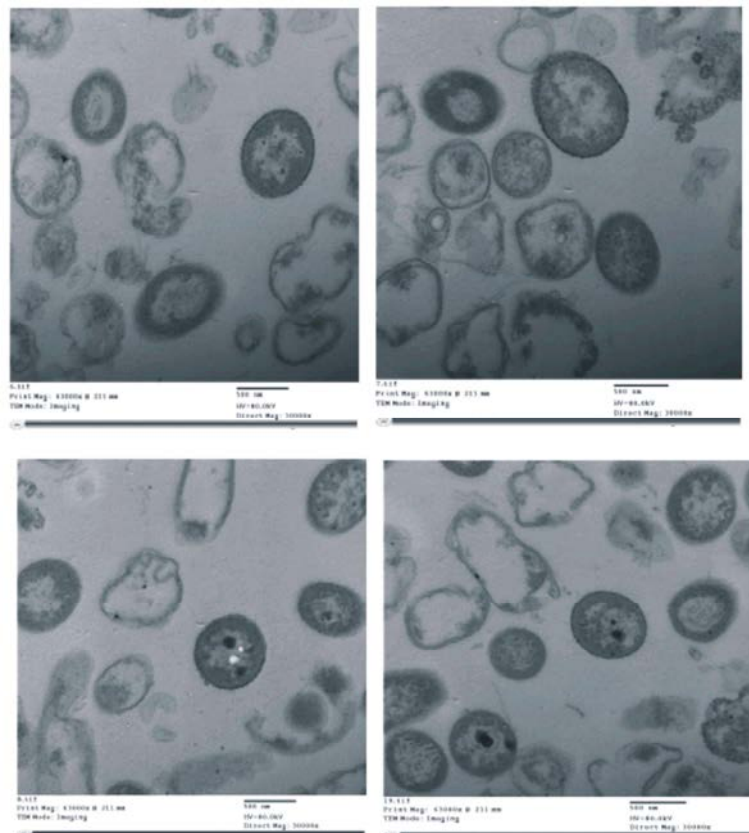


Fig. 4: The figure shows TEM picture of a 45 day old culture cultured from serum of same patients of urinary tract stones at 30000X magnification showing nanobacteria of between 200-600 nm and surrounded by a hairy apatite (Bar=500nm).

SEM of the biofilm showed coccoid particles with a diameter ranging between 200-500 nm. The organisms were prokaryotic in shape and had a rough surface (Fig2). TEM of biofilm showed coccoid with thick cell walled structures. Cell wall and capsule were distinct and surrounding the organism hairy apatite structure with a diameter ranging between 200 and 600 nm (Fig.3). We also

noticed that serum of healthy individuals show no growth of nanobacteria.

DISCUSSION

Nanobacteria, agents that intermediate apatite nucleation and crystal growth, remain a debatable topic of

discussion [28-30]. The scientists who first discovered nanobacteria have referred that nanobacteria are the Helicobacter pylori of kidney stone disease and that urinary tract calculi is a nanobacterial disease [2,27]. Nanobacteria were demonstrated as the smallest described bacteria to date, with dimensions of 0.08 to 0.5 μm . Moreover, these organisms were found to produce a biofilm containing hydroxyl apatite or carbonate, preventing their effective staining [2]. In our study we succeeded in isolation of nanobacteria from urinary tract calculi by using an extraordinary level of aseptic cultural technique which was needed in dealing with nanobacteria other than any other bacteria and we demonstrated its presence by using SEM and TEM analysis of the cultured bacteria. These results were in agreement with previously reported success for corresponding approaches. Kajander *et al.* [1], Kajander and Çiftçioglu [2], Ciftcioglu *et al.* [5] and Khullar *et al.* [27] otherwise, other scientists as Cisar *et al.* [24] declared that living or non-living nature of these bacteria is not clear and suggested that their apparent replication may be due to crystallization from culture medium and that the so-called nanobacteria are non-living self-propagating mineral compounds. The difference in the chemical structure of the stones between urates, oxalates and phosphates has no relationship with the presence or absence of nanobacteria, moreover difference of patients sex as males or females from which the stone is removed doesnot indicate any clear explanation in association with the isolation of nanobacteria from such stones. The fractured UT stones examined by SEM coccoid particles, which were grouped in clusters and bound together to a mineral structure like that which were described by Ciftcioglu *et al.* [5] and Drancourt *et al.* [31]

The cultured bacteria were apatite forming ultrafilterable self-replicating coccoid microorganisms of a diameter ranging from 100-600 nm which shows a variable sized nanobacteria with a distinct cell wall and capsule and which is covered by a "hairy" apatite layer and this description of the nanobacteria had similarities with the previously described nanobacteria with many other scientists who work on it as Khullar *et al.* [27] and this gives us a great support to our results which shows the presence of nanobacteria in urinary tract calculi. We observed the growth of nanobacteria in 8 out of 10 (80%) of urinary tract stones from Egyptian patients that raise the intriguing possibility of the causation of urinary tract stones by nanobacteria, which may promotes the view of Kajander [32] that nanobacteria are infectious pathogens responsible for a global health risk. Thus, the present

study was initiated to confirm the presence of nanobacteria from Egyptian patients and to study their role in urinary tract stone formation. Our results indicate the presence of atypical mineral forming ultra-filterable self-replicating nanosized nanobacteria in the urinary tract stones. Our findings indicate that these are living organisms capable of self-propagation. However, there is a crucial need for further studies to characterize and to prove the existence of these bacteria and their specific role in urinary tract stone formation mechanism and in many other extra pathological calcifications all over our bodies.

CONCLUSION

In our conducted study, we concluded that Nanobacteria have a significant role in initiation of urinary tract calculi in our bodies with a high percentage (80%). As we succeeded in isolation of Nanobacteria from different types of human urinary tract stones and serum of same patients by using a unique methodology specific for culture of Nanobacteria and also we succeeded in identification of the bacteria by using SEM and TEM analysis. So, we suppose that nanobacteria could be one of the important causes of urinary tract stones.

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