

Nanobacteria: An infectious cause for kidney stone formation

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Background. Nanobacteria are cytotoxic, sterile-filterable, gram-negative, atypical bacteria detected in bovine and human blood. Nanobacteria produce carbonate apatite on their cell walls. Data on Randall's plaques suggest that apatite may initiate kidney stone formation. We assessed nanobacteria in 72 consecutively collected kidney stones from Finnish patients.

Methods. Nanobacteria and kidney stone units were compared using scanning electron microscopy (SEM). Demineralized kidney stones were screened for nanobacteria using a double-staining method and a specific culture method. Isolated nanobacteria were analyzed for mineral formation *in vitro* with Ca and ⁸⁵Sr incorporation tests.

Results. SEM highlighted the resemblance in size and morphology of nanobacteria and the smallest apatite units in the kidney stones. Nanobacterial antigens could be detected after the demineralization of the stones in 1 N HCl. Nanobacteria were surprisingly resistant to this treatment, and cultures could be established from 93.1% of the stones. Only struvite stones had common bacteria, in addition to the nanobacteria. When the results of all of the assays were combined, 70 of the 72 stones (that is, 97.2%) were nanobacteria positive. Although apatite stones indicated highest nanobacteria antigen signals, the overall nanobacteria positivity did not depend on the stone type. The isolated nanobacteria produced apatite stones *in vitro*, measured by Ca and ⁸⁵Sr incorporation.

Conclusions. We propose that kidney stone formation is a nanobacterial disease analogous to *Helicobacter pylori* infection and peptic ulcer disease. Both diseases are initiated by bacterial infection and subsequently endogenous and dietary factors influence their progression.

Kidney stone disease is common, affecting 12% of males and 5% of females in the Western world [1]. In addition, it is a major cause of morbidity involving the urinary tract [2]. Nonetheless, many aspects of this detrimental, complex phenomenon remain unclear. The physical chemistry of stone formation has been intensively

studied during the last decades. It has become clear that the pathophysiology of renal stone disease cannot be explained by crystallization processes alone [2]. The crystalline components of urinary tract stones can be classified into five types: calcium oxalate, calcium phosphate, bacterial related, purine, or cystine. The majority of urinary stones are mixtures of two or more of these components, with calcium oxalate combined with apatite being the most common [3, 4]. Calcific kidney stones in humans are located on renal papillary surfaces and consist of an organic matrix and crystals of calcium oxalate and/or calcium phosphate [5]. It has been stated that the core of 67% of calcium oxalate stones contains calcium phosphate [6]. In another study, approximately 90% of calcium oxalate calculi contained small amounts of phosphates at the calculi core [7]. Struvite stones, also called infection stones or triple-phosphate stones, account for approximately 10 to 15% of all kidney stones. Most struvite stones also contain apatite. The bacteria commonly present in these stones are the urease producers such as *Proteus* species, *Klebsiella*, *Pseudomonas*, *Corynebacterium* species, although urease-negative organisms, for example, *E. coli*, have also been found. Struvite stone formation has been thought to be due to the alkalization of urine by the urease and/or alkaline phosphatase activity of the organisms [8, 9]. Stone formation may be also affected by the modulation of the *in vivo* urokinase and sialidase activities of these organisms [10]. However, in many cases, the formation of phosphates was not a consequence of persistently elevated urinary pH values. Therefore, the presence of occasional papillary microinfections has been suspected [7].

Nanobacteria are carbonate apatite forming, cytotoxic bacteria recently discovered in human and bovine blood and blood products [11–13]. These bacteria have extraordinary properties [14, 15]. It has been speculated that nanobacteria may be the spherical deposits found in the kidneys of patients who suffer from kidney stones [16–18]. In this study, we describe new methods for detecting these novel organisms in kidney stones. Our results suggest that nanobacteria may act as a common nidus in kidney

Key words: bacteria, infection, kidney calculi, apatites, carbonate apatite, stone formation.

Received for publication January 28, 1999
and in revised form May 28, 1999

Accepted for publication June 16, 1999

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stone formation, and thus, their eradication could well represent a new approach to therapy for patients with kidney stones.

METHODS

Seventy-two consecutively collected human kidney stones arriving to K-SKS, Stone Analysis Central Laboratory, Jyväskylä, Finland, between October 1996 and March 1997 were screened for the presence of nanobacteria. The chemical structure of these stones was determined using Fourier transform infrared (IR) spectroscopy in K-SKS following the standard method for clinical use [19]. Patient identity was kept confidential, and no patient history or additional samples were available.

Scanning electron microscopy (SEM) studies on nanobacteria cultured in serum-free (SF) Dulbecco's modified Eagle medium (DMEM) were performed as described previously [12]. The same method was applied to the kidney stones, but the fixation step was omitted.

We produced monoclonal antibodies Nb 5/2 and Nb 8/0, both IgG₁ class, against nanobacteria. The hybridoma clones were obtained from mouse splenocytes immunized with cultured nanobacteria of bovine origin, and fused with myeloma strain P3X63-Ag8.653 (ATCC CRL 1580; ATCC, Rockville, MD, USA). The hybridomas were cultured in the peritoneal cavity of Balb/c mice as ascites, and the monoclonal antibodies were purified with Prosep-A column affinity chromatography (Bioprocessing Ltd., Durham, Consett, UK).

Immunofluorescence staining (IFS) was performed on samples as described previously [13]. In a preliminary test, 30 stone samples were analyzed separately with Nb 8/0 and Nb 5/2 as follows: Powdered and demineralized samples were air dried on slides, heat fixed at 70°C for 10 minutes, and rehydrated by soaking in phosphate buffered saline (PBS) and blocked with Cas-Block (Zymed, South San Francisco, CA, USA) for 10 minutes. The slides were then covered with monoclonal antibody solution (10 µg/ml in PBS containing 5% rabbit serum). After a 30-minute incubation at room temperature, the antibody solution was drained, and the slides were washed three times (5 min each) with PBS and covered with FITC-conjugated rabbit antimouse IgG (Dako, Glostrup, Denmark) at a dilution of 1/50 in PBS. After incubation for 30 minutes at room temperature, the antibody was drained. The slides were washed three times (5 min each) in PBS, mounted with a medium containing 50% glycerin, 0.5% n-propyl gallate in PBS, and viewed under a Nikon Microphot-FXA microscope with fluorescence and differential interference contrast optics.

In a preliminary test, identical staining patterns were observed with both antibodies. Therefore, the analysis of the 72 stones was done using only Nb 8/0. 3T6 cells (ATCC CCL 96) were used as indicator cells for testing

the IFS of the nanobacteria cultivated from kidney stones using the method described earlier in this article, with exceptions as follows: (a) Fixation was with Carnoy's fixative (one part of acetic acid and three parts of methanol mixture) for 10 minutes, and (b) after fixation, the cells were permeabilized with 1% Triton X-100 in PBS for three minutes.

In the double-staining method, demineralized kidney stone samples and/or 3T6 cells infected with cultured material were fixed and stained by using IFS method, but Hoechst #33258 fluorochrome (0.5 µg/ml) was added to the FITC-conjugated antimouse IgG, secondary antibody. This DNA stain at the concentration used does not stain nanobacteria but does detect all known common bacteria [13]. The specific binding of mAb 5/2 was confirmed with a sodium borohydrate treatment [15]. Additionally, the specificity of the IFS was verified by using nonrelevant monoclonal antibodies (mAbs) and the double-staining method.

Standard control nanobacteria types, isolated from fetal bovine serum (FBS; Sera-lab 901045), were cultured as described previously [12, 13]. Nanobacteria, or the powdered kidney stone samples, were weighed (10 to 150 mg), demineralized in 1 N HCl for 10 minutes, neutralized, sterile filtered through 0.22 µm filter and cultured, either with or without γ-irradiated FBS, in 24-well plates in a volume of 2 ml, under the mammalian cell culture conditions described previously [15]. Negative controls were prepared similarly, but the sample was omitted. As positive controls, two types of Sera-lab nanobacteria cultures, less and highly mineralized cultures, were carried as samples throughout the procedure. The test cultures were observed weekly for four weeks using an inverted microscope. After a three-week culture period, the 3T6 cells in Chlamydia tubes were infected with 100 µl of the culture samples. Infected 3T6 cells were incubated for 24 hours and stained with the double-staining method.

The control nanobacteria and isolated nanobacteria from 50 out of the 72 kidney stones were analyzed for mineral formation *in vitro* using Ca and ⁸⁵Sr incorporation tests. For Ca incorporation, the kidney stone cultures were subcultured (1/50) in SF-DMEM for periods of 8 and 21 days. Nanobacteria adhered to the culture vessel and produced mineral. The medium was removed and immediately used for the Ca measurement. The culture plates were washed three times with PBS, and then the nanobacterial biofilm was demineralized with 1 ml 1 N HCl. The stored media and HCl extracts were analyzed for Ca with a Perkin-Elmer 460 flame-atomic absorption spectrometer. Calcium incorporation into the biofilm was calculated as a percentage of the total Ca in the culture medium. For ⁸⁵Sr measurements, cultures were subcultured (1/50) in 1 ml DMEM containing 0.075 µCi/ml ⁸⁵Sr (MAP Medical Technologies Oy, Tikkakoski,

Finland) with specific radioactivity of approximately 100 Ci/mmol. Samples were treated in the same way as those for Ca analysis with the following modifications: The first PBS wash solution was combined with the medium, and after the HCl extract was removed, the plates were washed with 1 ml PBS, which was later added to the HCl extract. Samples were then measured in a gamma counter (Wallac Wizard; Wallac Oy, Turku, Finland), and the ^{85}Sr incorporation to the biofilm was calculated as a percentage of total ^{85}Sr . As a reference, bovine-originated nanobacteria were treated and cultured exactly as the kidney stone samples. As a negative control, the identical procedures were carried out using γ -irradiated serum culture as the starting material.

The SPSS 6.1 for Windows with the Kruskal–Wallis one-way analysis of variance, Mann–Whitney U–Wilcoxon rank sum W test, and Spearman correlation were used for statistical analyses of these results. The significance level was set at $P < 0.05$.

RESULTS

Scanning electron microscopy images of the spherical units in the carbonate apatite kidney stones were clearly similar in size and morphology to the mineralized forms of nanobacteria cultured under SF conditions (Fig. 1 a–c). Additionally, the same samples showed positive IFS with mAbs against nanobacteria (Fig. 1 d, e). IFS was positive also with demineralized nanobacteria (Fig. 2a) and with kidney stones. The chemical compositions and their percentage of distribution among the screened kidney stones and their IFS results are summarized in Table 1. There was a significantly higher positivity in the IFS performed on the apatite stones compared with those having a different chemical composition. According to the Spearman test, there was no significant correlation between stone weight and positivity of the IFS for the samples. Control IFS made by using nonrelevant mAbs yielded negative results. Nb 5/2 produced identical results to those of 8/0 in the 30 preliminary cases, and its positive signal could be removed specifically by sodium borohydrate treatment, which further confirmed the specificity of the staining.

Microscopic observations of the cultures after the three-week incubation period revealed tiny nanobacteria-like particles under $\times 400$ magnification. One-way analysis of variance showed no significant difference between the stone types, weights, and the cultural positivity by the microscopic observation (detailed data available upon request). The two struvite stones illustrated the presence of common bacteria when the double-staining method was performed after the demineralization steps. DNA-positive spots and IFS-positive spots were located in totally different positions in the parallel images, thus indicating the presence of both common bacteria and nanobacteria (data not shown). The double-staining method

indicated the absence of common bacteria in all of the nanobacteria and kidney stone cultures (Fig. 2 b, c). As shown in Table 1, the overall IFS was positive for 66.7% of the demineralized stones, but after culture and passage into the 3T6 indicator cell cultures, the positivity increased to 93.1%. Thus, the most sensitive detection method was the culture and passage into a mammalian fibroblast cell culture, followed by IFS (Fig. 2b). The 3T6 cells not infected with the samples were negative in the microscopy (Fig. 2d). Nucleic acid staining provided evidence for the absence of common bacteria in these cultures.

Forty-six nanobacteria isolates obtained from 50 kidney stones produced apatite that was measured by Ca and ^{85}Sr incorporation tests (Table 1). Ca and ^{85}Sr incorporation after a 21-day incubation period is shown in Figure 3 for two representative nanobacterial isolates from each major type of kidney stone along with results of the bovine-originated nanobacteria. Interestingly, Ca biomineralization takes place very rapidly. Most mineralization occurs during the first week, consuming half of the Ca and thus all of the phosphate present in the medium into apatite (Fig. 3). According to one-way analysis of variance results, there was no significant difference between the ^{85}Sr or Ca incorporation and the origin of the nanobacteria (stone types).

DISCUSSION

Two mechanisms have been proposed for calculi formation: the development of calculi attached to papillary epithelium and the development of calculi in cavities without any attachment to urothelium [20]. In 1936, Randall formulated a hypothesis concerning stone formation related to the renal papillae that was confirmed by Cifuentes-Delatte, Minon-Cifuentes and Medina in 1983 [21]. Papillary stones are small, rounded concretions, with one smooth convex face and one concave face, which corresponds to its implantation on the papilla [21]. Our study confirmed these findings (Fig. 1c). The small apatite units were observed in all kidney stones in different proportions. We also observed similar formations in *in vitro* nanobacteria culture under SF conditions (Fig. 1 a, b). Nanobacteria first adhere to the surface of the culture vessel and then create “cave-like” apatite fortresses with a concave face [13, 14]. Additionally, SEM images proved that nanobacterial mineralization takes place via the formation of several thin mineral layers (Fig. 1b), the same as in kidney stone formation.

Urolithiasis literature has accurately defined the composition and frequency of occurrence of human urinary tract stones. There have been many studies on the possible mechanisms of crystal aggregate formation following the initial nucleation of crystals from supersaturated urine

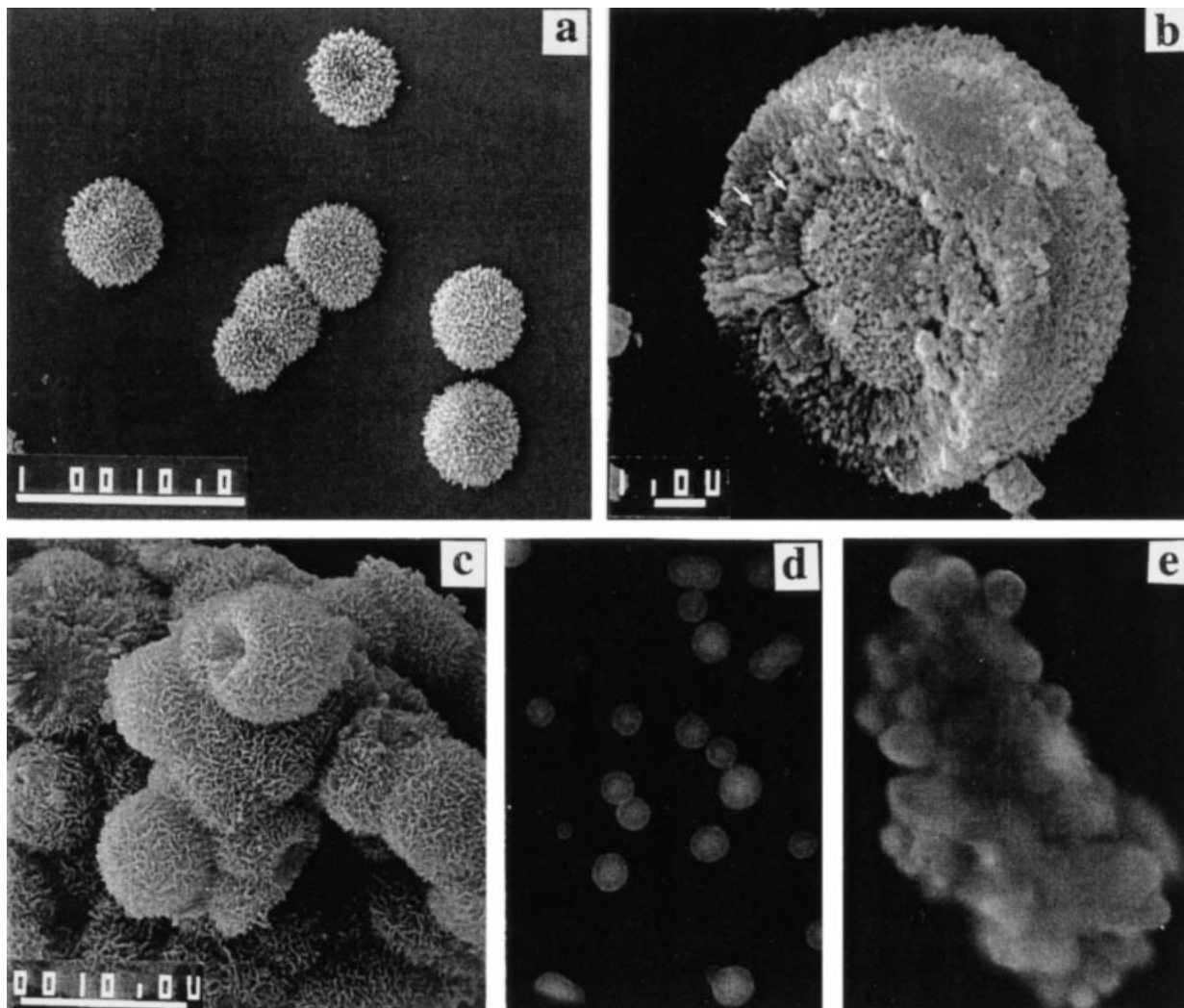


Fig. 1. (a) Scanning electron microscopic (SEM) observation of calcified nanobacteria cultured in serum-free condition. (b) Detail from fractured nanobacteria showing mineral formation as layers shown by arrows. (c) SEM image of human apatite kidney stone showing apatite units. (d) Immunofluorescent (IF) staining of the nanobacteria similar to those shown in (a), and (e) the kidney stone shown in (c) with an anti-nanobacteria monoclonal antibody. Bars: a and c, 10 μm; b, 1 μm.

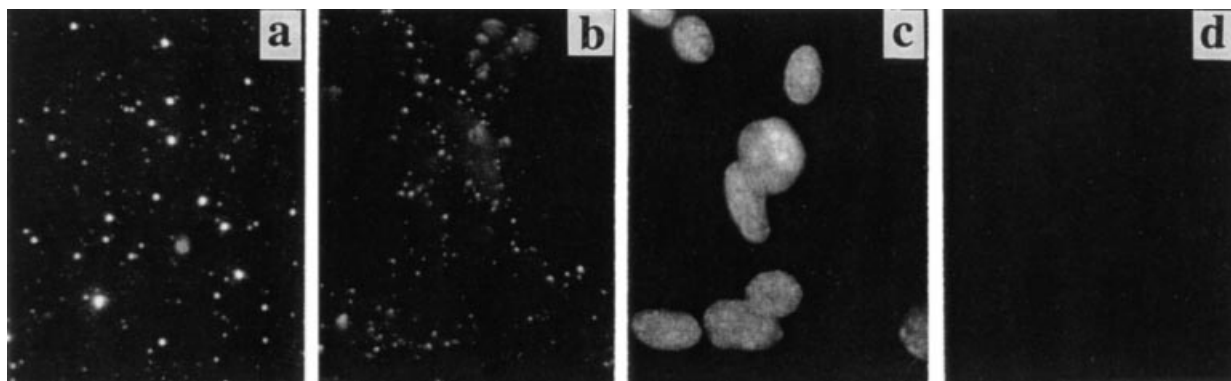


Fig. 2. (a) Immunofluorescent (IF) staining of demineralized nanobacteria. (b) Double staining of 3T6 cells infected with nanobacteria cultured from a kidney stone. (b) IF staining. (c) DNA staining. (d) Control IF staining of noninfected cells.

Table 1. Summary of the results from the nanobacterial detection methods obtained with the tested 72 human kidney stones grouped according to their chemical compositions

Stones	Cases		IF positivity of demineral. stones		Nanobacteria growth in culture		<i>In vitro</i> mineralization +/tested
	<i>N</i>	%	<i>N</i>	% ^a	<i>N</i>	% ^a	
Ca ox	34	47.2	16	47.1	32	94.1	18/22
Ca ox+CA	16	22.2	14	87.5	15	93.7	12/12
Ca ox+UA	2	2.7	1	50	2	100	2/2
CA	7	9.7	7	100	5	71.4	4/4
UA	6	8.3	3	50	6	100	6/6
CD	4	5.6	4	100	4	100	2/3
M	2	2.8	2	100	2	100	1/1
Cystine	1	1.4	1	100	1	100	ND
Total	72	100	48	66.7	67	93.1	46/50

Abbreviations are: Ca ox, calcium oxalate; CA, carbonate apatite; UA, uric acid; CD, calcium hydrogen phosphate-dihydrate; M, magnesium ammonium phosphate-hexahydrate; ND, not determined.

^a Percentage in the particular kidney stone group

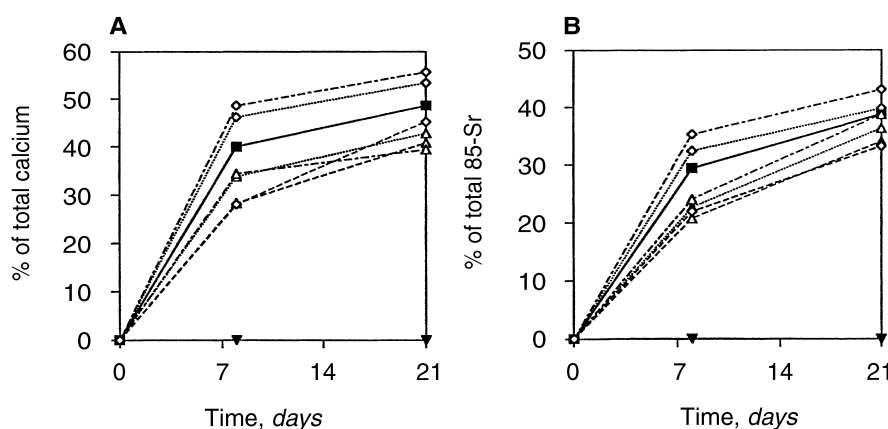


Fig. 3. Biomineralization of Ca (a) and incorporation of ⁸⁵Sr (b) by the nanobacteria isolates of human kidney stones and by the controls. Altogether, 46 of the 50 stones analyzed indicated incorporation. Data from two representative nanobacterial isolates from each major type of kidney stone are shown.

[3]. Renal tubular fluid in the distal nephron is supersaturated with calcium and oxalate ions that nucleate to form the most common crystal, calcium oxalate monohydrate, in renal stones. Urine supersaturation values correlated well with stone composition [22, 23]. It is not known how these nascent crystals are retained in the nephron to form calculi in certain individuals.

In our previous work, we have proven that all kinds of nanobacterial forms are internalized by many types of mammalian cells, and once internalized, they are cytotoxic [12]. In a rabbit experiment, we showed that ^{99m}Tc-labeled nanobacteria, injected intravenously, had a tissue specific distribution with a major accumulation in the kidneys and subsequently in urine. The presence of live excreted nanobacteria in urine is proof that these organisms may be involved in the kidney stone formation [24]. However, kidney stone formation is a complex phenomenon involving several endogenous factors. Adherence of crystals to renal epithelial cells is inhibited by specific urinary components such as glycosaminoglycans, uropontin, nephrocalcin, and citrate, each of which binds to the crystalline surface. Thus, competition for the crys-

tal surface between soluble anions in the tubular fluid and anions on the apical cell surface could determine whether or not a crystal binds to the cell [25]. Recently, it has been shown that the Tamm-Horsfall protein inhibits urinary crystal aggregation [26]. A renal-specific gene, which is associated with kidney stone formation, has also been identified [27]. The importance of hereditary factors in kidney stone formation has gained interest recently [1, 28, 29]. The relationship between the existence of these kinds of additional promoting and/or inhibiting factors and nanobacteria positivity in kidney stone patients should be evaluated.

We propose a new theory to explain the formation of human kidney stones: Nanobacteria may act as nidi for kidney stone formation. This theory is supported by the following findings: (a) 97.2% of the analyzed kidney stones contained nanobacteria. (b) Almost all kidney stones have apatite as a component [3]. (c) Nanobacteria are the only known organisms in the human body that produce apatite and accumulate in the kidney [24]. (d) Nanobacteria isolated from human kidney stones produced stones in culture. (e) An organism cytotoxic to mammalian cells

in vitro [15] and causing apoptosis in kidney tissue (our unpublished data) is unlikely to be a mere bystander in the development of kidney stones. (f) Contamination was ruled out because the control cultures remained negative. (g) Our unpublished pilot work could also detect nanobacteria in kidney stones collected from patients in United States, indicating that our observations are not related to a phenomenon unique to Finland.

These previously mentioned promoters and inhibitors of stone formation may have important roles in the progress of kidney stone formation on the initial nidus, nanobacteria. Further work is needed to fulfill Koch's postulates.

ACKNOWLEDGMENTS

We thank Dr. Kalle Willman for his help in obtaining the stone samples, Dr. Alpo Pelttari for his expertise in electron microscopy, and Dr. Marjatta Kantola (University of Kuopio) for help in the AAS analysis.

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