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Immunobiochemical Techniques for Detection and Identification of Nanobacteria Isolated from Renal Stones

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ABSTRACT

The present study is conducted to isolate on NB on solid media (Loeffler media) and to produce polyclonal antibodies for immunofluorescence assays. Moreover, to evaluate the re-isolation and molecular characterization of NB from kidney of experimentally infected mice. The culture of NB on solid media was visible macroscopically and microscopically as pin point at the bottom of the plat as stony like appearance with grayish to yellowish whit color after 6-8 weeks. While the result of DNA staining by Hoechst stain gave fluorescence appearance surrounding the NB which may be attributed to that the stain cannot penetrated the apatite layer on the surface of the NB. The histopathological examination showed the macroscopic enlargment and the inflamation of the kidney of infected mice while the microscopic examination revealed sever interstitial nephritis and thickening in parietal layer of Bowman's capsule indicating the pathological action of NB. Finally the result of DNA extraction and Polymerase Chain Reaction (PCR) was negative. Results proved the positive identification of NB involved specific stainability with Hoechst 33258 and with indirect immunofluorescence staining (IFS) on both types of media. These tools will be promising in diagnosis criterion of NB.

Key words: Nanobacteria, immunofluorescence, PCR, kidneys, histopathogy

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INTRODUCTION

"Nanobacteria" are nanometer-scale with dimensions of 20-200 nm in length have self-replicating nature and considered the smallest described bacteria to date (Kajander and Ciftcioglu, 1998). Additionally, these organisms produced a biofilm apatite, preventing their effective staining.

NB are thought to cause different calcifying diseases including stones formation, renal disease (Kajander *et al*., 2003), aortic valve stenosis (Jelic *et al*., 2007), periodontal disease (Ciftcioglu *et al*., 2003), chronic inflammatory diseases (Cassell, 1998) and prostatitis (Bock *et al*., 1989; Geramoutsos *et al*., 2004). Phylogenetic analysis based on comparison of 16S ribosomal DNA (rDNA) sequences has placed the NB isolated from fetal calf serum into the α_2 subgroup of *Proteobacteria* (Kajander *et al*., 1997)(plant associated bacteria). In our previous study we succeeded to isolate NB on liquid medium (DMEM) supplemented with 10% γ-irradiated FBS under cell culture conditions and detection was confirmed with Scanning Electronic Microscopy (SEM) and Transmission Electronic Microscopy (TEM).

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MATERIAL AND METHODS

Samples: Previously isolated NB from kidney stones of Egyptian patients with urolithiasis in laboratory were used (Abo-El-Sooud *et al*., 2011).

Cultivation of NB: Culturing will be conducted by using strict aseptic techniques with incubation at 37°C in humidified 5% CO_2 95% by culturing cultured in DMEM supplemented with 10% γ-irradiated fetal calf serum under cell culture conditions (Cartellieri *et al*., 2002). By negative staining, the sizes of the electron-dense particles of NB ranged from 0.2-0.3 upto 0.5 pm after 1 month in culture and they appear coccoid or coccobacillary in shape, either as single particles, in short chains, or predominantly as clusters. Needle-like apatite crystals areobserved on their surfaces by Transmission Electron Microscopy (TEM). Subcultures are made by passing small inoculums (1/10 of an old culture) into fresh DMEM supplemented with 10% of either the same serum or irradiated. Absence of classical microbes in cultures of NB is confirmed by sub culturing 200 μL aliquots in sheep blood agar and aerobic and anaerobic incubation at 37°C samples are also examined by TEM. Positive identification of NB include typical growth in

cell culture medium with a doubling time of 1-3 days, characteristic morphology by scanning electron microscopy SEM or TEM and measurement of the absorbance at 650 nm (Hjelle *et al*., 2000).

Culture in Hoyle's Medium LAB 027 (Lab M Limited Topley United Kingdom) or Loeffler media: Disperse 37 g in 1 L of deionized water mix well and sterilized by autoclaving, let for cooling and add 5% of lysis blood and 5 vials of X027. Mix well before pouring into petri dishes and dry the agar surface then inoculate the NB isolate.

Staining of NB with specific Hoechst Stain 33258 (UK) with the high concentration (5 μg mL^{-1}) is performed according to Schinke *et al*. (1996). Two samples of NB one from DMEM and the other from NB, cultured in petri dish were spread on two slides and stained by Hoechst stain 33258 after diluted by deionized water and examined after drying by microscope .

Cell cultivation: Isolated NB samples are cultured with 3T6 cells (ATTC CCL 96) in DMEM supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS). The 3T6 cells are incubated for 24 h before inoculation of NB and incubated at 37°C under 5% CO_2. The presence of bacteria is monitored weekly by Hoechst stain 33258 (Ciftcioglu *et al*., 2006). Two NB samples are inoculated in parallel in 5 mL of DMEM with L-glutamine (Gibco) supplemented with 10% FBS in T-25 culture flasks and incubated at 37°C in a 5% CO_2 environment for 4 weeks. Flasks are inspected macroscopically and microscopically weekly for biofilm formation and calcification.

Transmission Electron Microscopy (TEM) of infected cells is performed at the 4th week. The 3T6 cells cultured in DMEM with 10% FBS without inoculation of NB samples under the same culture conditions are used as negative control.

Production of polyclonal antibodies: Two six week old female Balb/C mice are inoculated intraperitoneally with 20 μg of nanons emulsified in Freund's complete adjuvant (1:1 v/v). Two booster doses are given in Freund's incomplete adjuvant at 14 day intervals. Bleeding is performed 2 weeks after the last immunization and serum separated by centrifugation and stored at 48°C until use. Also two pieces or tissue samples from kidney were taken one kept in 10% formalin for histopathological examination and the other for re isolation of NB.

Immunofluorescence assays: NB samples from DMEM and from infected 3T6 cell culture flasks after scraping are deposited on two slides with a pan nib then air dried and mixed in acetone for 2 min. Wells were saturated by 30 min incubation with PBS supplemented with 5% Bovine Serum Albumin (BSA) and overlaid with 30 μL serum diluted in PBS-BSA 3%. Bound antibodies are detected with anti-mouse conjugated (KLP-E USA) diluted 1:4 in PBS-BSA 3% containing 0.2% Evans blue (Sigma). All incubations are performed for 30 min in a moist chamber at 37°C and were followed by a washing in PBS two times for 10 min each, rinsing in demineralized water for 5 min and drying in air. Slides are examined under an Olympus BX51 immunofluorescence microscope at 3400 magnification. Sera of healthy mice are used as negative control. Also the same technique for 3T6 infected cell by NB isolates.

Histopathological examination: The kidneys were sliced and pieces were fixed in 10% buffered formaldehyde solution for histological study. The fixed tissues were processed by automated tissue processing machine.

Sections of 5 μm in thickness were prepared and then stained with hematoxylin and eosin (H and E). After that the sections were observed under the microscope for histopathological changes and their photomicrographs were captured.

Isolation of NB from kidneys of experimentally infected mice: Under sterile condition piece of kidney was taken and grinded well then filtered by a 0.22 μm filter and was prepared for TEM and also inoculated in 3T6 and examined by TEM.

Extraction of DNA from NB: Boiling single colony from each selected purified strain in 500 μL nuclease-free water for 90 sec, then cell debris collected by brief centrifugation then the supernatant use directly poured to the PCR reaction and also run in gel.

PCR amplification of 16S rDNA gene of selected isolates: The 16S rDNA gene of selected isolate is amplified by using universal primers pairs, 27f5-AGAGTTTGATCCTGGCTCAG-3 and 1492r 5-TACGGCTACCTTGTTACGACTT-3 to amplify 1500 bp (Weisburg *et al*., 1991). Amplification is carried out in 25 pi of PCR master mix kit (promega). The PCR amplification is carried out using Gene Amp PCR System 2400 Thermal cycler (Perkin Elmer), the reaction mixtures are amplified according to the following program: 94°C for 4 min as initial denaturation step, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min with a final extension at 72°C for 10 min. Then the PCR products were resolved by electrophoresis. The 5 μL of each PCR

products are run on 1% (w/v) agarose gel to confirm the size and purity of PCR products. (Gel is prepared by boiling typically a 1% (w/v) suspension of agarose in I×TBE buffer and casting it in a portable tray. The molten gel cool to below 50°C, before adding ethidium bromide to final concentration of 0.5 μg mL^{-1} pouring into the casting tray). The loading wells are formed by inserting Perspex combs into the molten gel and the gel left to be solidified, after-solidification the gel submerge in the electrophoresis tank by 1×TBE running buffer and the comb is carefully remove. Samples are mix with 25% (v/v) loading dye solution prior to loading into wells. The 1 kb plus DNA ladder is used as a marker. Electrophoresis is carrying out at 60 V, DNA is visualize under medium wave UV illumination and photographed by documentation system.

RESULTS

Culture of NB on solid media take about 6-8 weeks indicating low rate of multiplication and appear at the bottom of the plate as pin point stony like appearance which due to its ability for mineral precipitation or biomineralization which lead to renal stone formation while under the microscope appear yellowish or grayish white stony like shape (Fig. 1).

The result of NB staining by Hoechst stain 33258 appear as fluorescence appearance surroundin g the NB colonies only which attributed to the apatite layer surrounding NB which prevent passage of the stain and so prevent DNA staining (Fig. 2).

While the results of IFS test of NB only in DMEM media revealed that there is fluorescence aggregations indicating positive reaction between NB and specific antibodies in serum compared with the control negative indicating specific antibodies attached to NB which is specific antigen as shown in (Fig. 3).

Moreover the result of IFS of NB-infected 3T6 cells showed the aggregation of antibodies on the surface

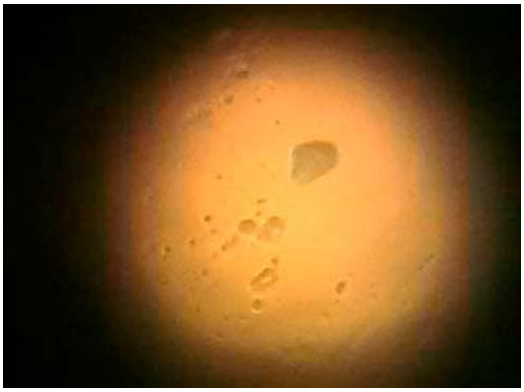

Fig. 1: Culture of NB on Loeffler media showing stony like appearance at the bottom of the plate

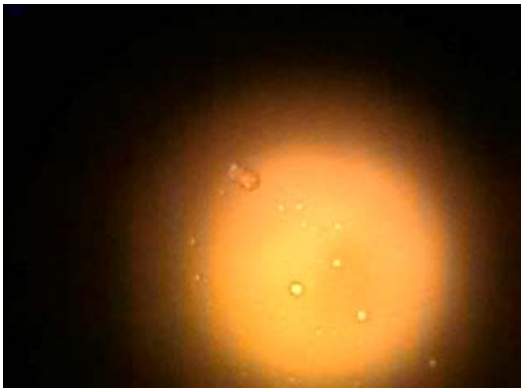

Fig. 2: DNA staining showing fluorescence appearance surrounding the NB colonies

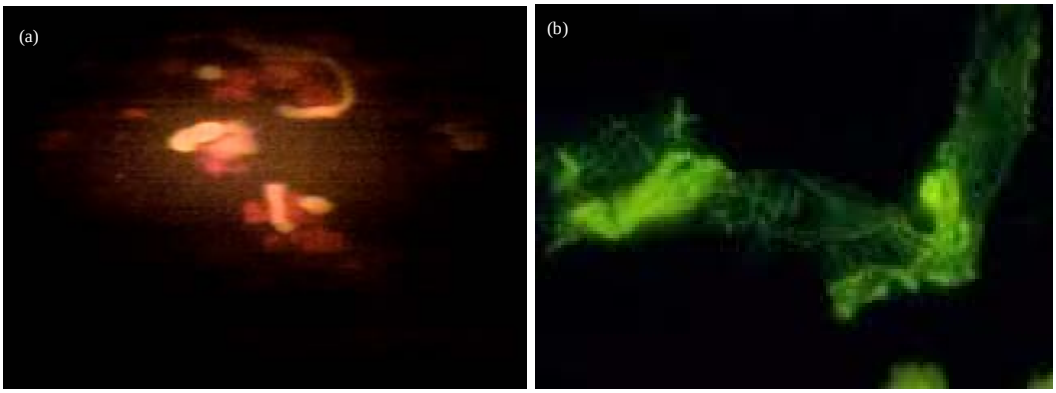

Fig. 3(a-b): (a) Negative and (b) Positive IF reaction between NB in DMEM and specific polyclonal antibodies

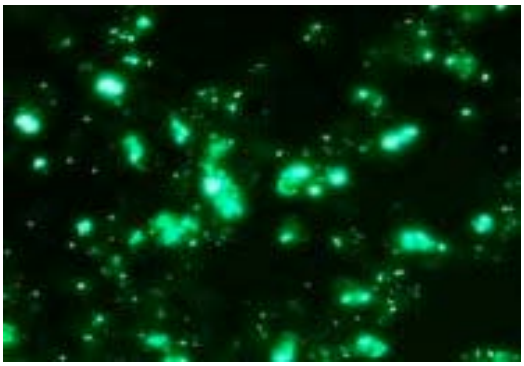

Fig. 4: Positive reaction of 3T6 cells infected by NB antigen and polyclonal antibodies

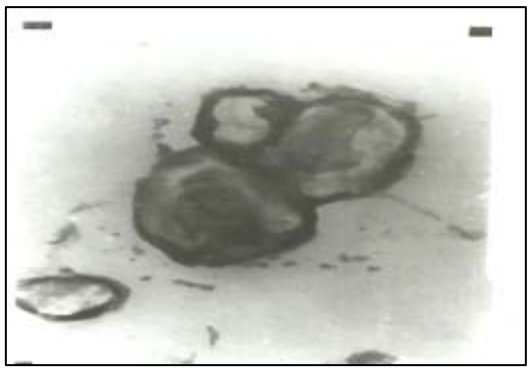

Fig. 5: TEM of infected 3T6 showed antibodies aggregations on the surface of the cell

of the cell indicating that NB has antigenic properties and its ability to initiate the immune system of mice to produce specific antibodies (Fig. 4).

While the result of TEM for sample of 3T6 cells infected by NB showed aggregations of antibodies on the surface of the cells as shown in Fig. 5, indicating positive reaction, also the result of TEM for sample of NB in DMEM media and polyclonal antibodies showed antigen and antibodies complexes and free antibodies molecules (Fig. 6).

Also the result of TEM for the reisolation of NB from the kidney of infected mice give positive result as it appear coccoid shape with apatite formation on the surface (Fig. 7) and also reculture on DMEM and 3T6 indicating the pathogenic ability of the NB isolate in kidney tissue and its ability or its rule in renal stone formation.

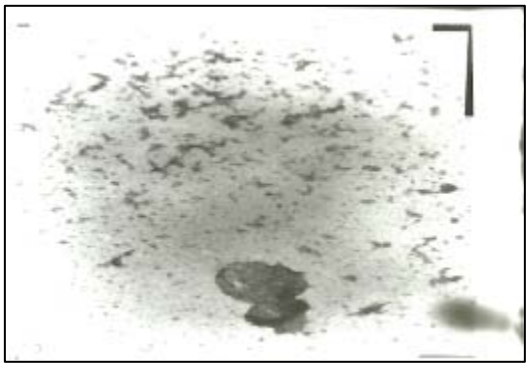

Fig. 6: TEM of NB in DMEM media and showed antigen and antibodies complexes and free antibodies molecules

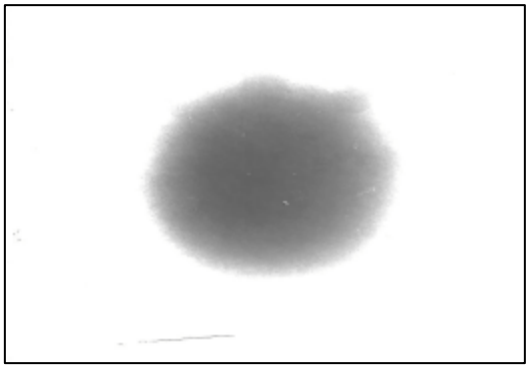

Fig. 7: TEM for the reisolation of NB from the kidney of infected mice give positive result as it appear coccoid shape with apatite formation on the surface

In accordance with previous results, the histopathological examination for kidney of infected mice during the production of polyclonal antibodies showed macroscopically marked inflammation and enlargement of the kidney compared to control negative as shown in (Fig. 8). While microscopically revealed sever interstitial nephritis and thickening in parietal layer of Bowman's capsule by (H and E 400X) indicating the pathogenic effect of NsB compared to the control negative (Fig. 9).

Finally the results of molecular characterization of NB isolate including DNA extraction showed negative result on gel (Fig. 10) which may be attributed to hydroxyappatite formation which may prevent DNA extraction and PCR as well.

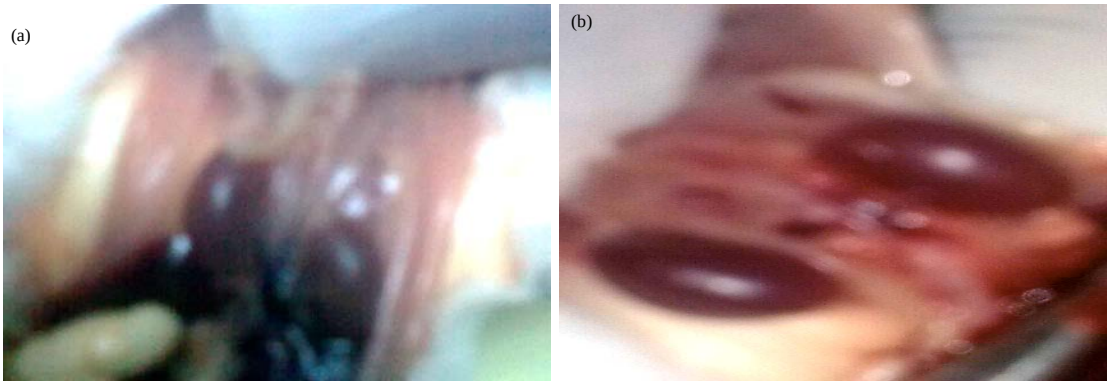

Fig. 8(a-b): (a) Kidney of a normal mice and (b) Kidney of infected mice showed enlargement and sever inflammation

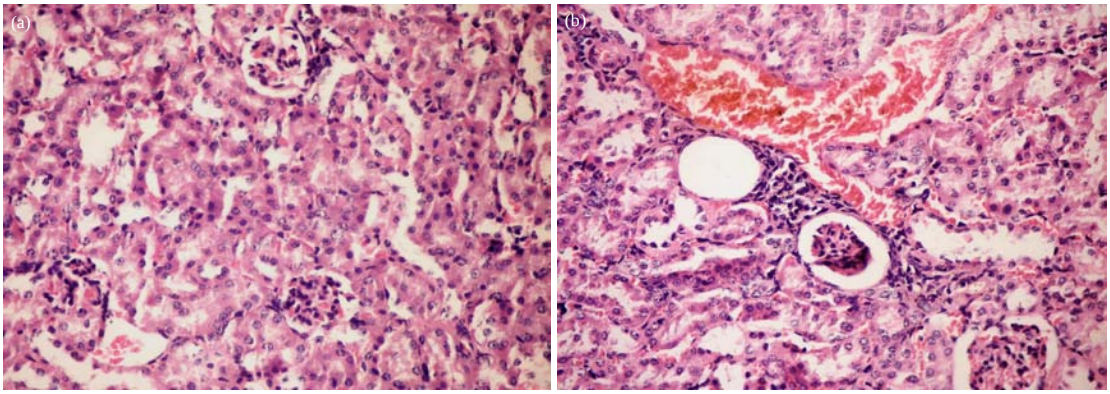

Fig. 9(a-b): Kidney of (a) Normal mice and (b) Infected mice showed sever interstitial nephritis and thickening in parietal layer of Bowman's capsule by (H and E 400X)

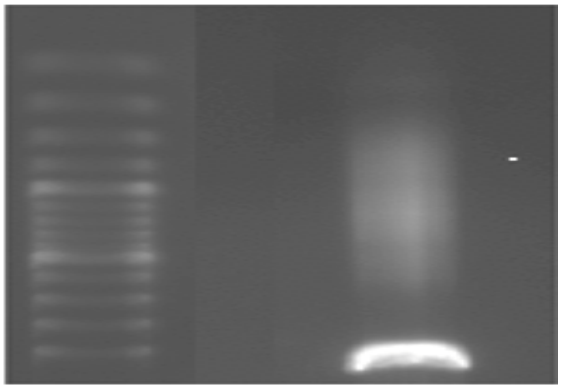

Fig. 10: Negative DNA extraction on polymerase chain (PCR) reaction for nanobacteria bp: Base pair

DISCUSSION

In our previous study we succeeded to isolate liquid medium (DMEM) supplemented with 10% γ-irradiated FBS under cell culture conditions and detection of NB was confirmed with SEM and TEM (Abo-El-Sooud *et al*., 2011).

In this studys results of culture of NB on solid media showed that grayish to yellowish white stony like and needle like colonies penetrated through the media forming deposits on the bottom of the plate appeared by naked eye and by inverted microscope after 6-8 weeks indicating low rate of replication and also its ability for biomineralization and crystal formation. This result is in agreement with that recorded by Ciftcioglu *et al*. (1999). They found that colonies on modified Loeffler medium forming stony pillars, needle-like crystal deposits in the plate similar to the hydroxyapatite crystals.

Furthermore, the staining of NB culture with Hoechst 33258 stain gives positive result showed fluorescence appearance surrounding the NB colonies. Results are in agree with that obtained with Schinke *et al*. (1996). Moreover, the results of IF for NB sample obtained from DMEM media culture and 3T6 cell culture infected by NB are confirmatory and indicating the positive reaction of both antigen and antibodies, the reaction of polyclonal antibodies with sample of NB free in DMEM and also with the intracellular NB inside the infected 3T6 sample, which is specific reaction for both NB and antibodies obtained from serum of infected mice. These results indicated positive identification of NB and are in accordance with that obtained by Kucers *et al*. (2007) who reported that indirect IFS with specific monoclonal anti-NB antibodies are methods to diagnose NB in biologics, cells, tissues, blood and urine with immune detection with NB-specific monoclonal antibodies, electron microscopy and culture techniques (Ciftcioglu *et al*., 1999).

Also the results of TEM for NB with serum contain antibodies which showed antigen-antibodies complexes and free antibodies are also appearing. While the TEM for infected 3T6 cells with polyclonal antibodies showed the reaction and the aggregation of antibodies on the surface of the cells, the results are similar to that obtained by Zeng *et al*. (2006) and Hjelle *et al*. (2000) who recorded that positive identification of NB include typical growth of NB in cell culture medium with a doubling time of 1-3 days, characteristic morphology by Scanning Electron Microscopy (SEM) or TEM by measuring absorbance at 650 nm.

The result of re-isolation of NB from kidney of infected mice during production of polyclonal antibodies indicating that our isolate is a pathogenic isolate and can produce crystal formation and these confirmed by culturing in DMEM media and 3T6 cell culture.

The histopathological examination showed grossly enlargment and inflamation of the kidney of infected mice. Microscopical examination revealed sever interstitial nephritis and thickening in parietal layer of Bowman's capsule. These finding confirmed that NB isolate is renal pathogenic isolate. These results are in accordance with that obtained by Sohshang *et al*. (2000), who reported those bacteria or other agents producing such nidi, if present in blood and in urine accelerate pathologic calcification *in vivo*. Also this is clinically important because blood contains phosphate near its saturation level. Also Driessens *et al*. (1989) showed that NB may thus participate in activation-inhibition processes regulating a large number of responses inside and outside cells. Thus, NB could have multiple pathologic actions in the body. Giachelli *et al*. (2005) reported that when apatite is found in soft tissue considered being pathological calcification. Among various hypotheses proposed for pathological tissue calcification, recent evidence supports the possibility that self-replicating calcifying nanoparticles (CNPs) can contribute to such calcification. These CNPs have been detected and isolated from calcified human tissues, including blood vessels and kidney stones and are referred to as NB. Also apatite on NB produces carbonate on their cell walls which may initiate kidney stone formation (Ciftcioglu *et al*., 1999). In this respect, Shen *et al*. (2010) concluded that NB may be an important etiological factor for type III prostatitis in Sprague-Dawley rats. The results of DNA extraction is negative that may be attributed to hydroxyapatite formation which is calcium, phosphate and carbonate precipitation on NB cell wall which prevent action of lytic buffer on the cell wall so prevent DNA extraction.

Cisar *et al*. (2000) reported that the 16S rDNA sequences previously ascribed to *Nanobacterium sanguineum* and *Nanobacterium* sp., were found to be indistinguishable from those of an environmental microorganism, *Phyllobacterium mysinacearum*, that has been previously detected as a contaminant in PCR. So, they provide evidence that biomineralization previously attributed to NB may be initiated by nonliving macromolecules and transferred by self-propagating microcrystalline apatite.

Furthermore, detection of co-cultivated microorganisms on cell cultures is routinely performed by PCR with universal 16S rDNA primers and direct immunofluorescence is routinely performed with specific antibodies and these approaches have never led to the identification of *Nanobacterium* sp. microorganisms. However, transient Gimenez-positive inclusions are frequently observed in our experience. The structures have been observed routinely, considered as artifacts, as are the structures we observed when using TEM on UUT stone material-inoculated 3T6 cells.

CONCLUSION

Results proved the positive identification of NB involved typical growth rates and optical properties, specific stainability with Hoechst 33258 and with indirect immunofluorescence staining (IFS) on both types of media. These tools will be promising in diagnosis criterion of NB.

ACKNOWLEDGMENT

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