In vitro Assessment of Different Antibacterials against Nanobacteria Isolated from Kidney Stones

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Abstract: Nanobacteria (NB) are implicated in stone formation in the urinary tract and antibacterials have been used with some success for the treatment of pathological calcification-related diseases, therefore, therapy to eliminate NB is major concern. Twelve antibacterial agents from various categories were tested against *in vitro* inhibition of NB, isolated from human kidney stones. The tested antibacterials were oxytetracycline, doxycycline, ampicillin, lincomycin, spectinomycin, trimethoprim, neomycin, erythromycin, florfenicol, streptomycin, gentamicin and colistin. A modified microdilution inhibitory assay was used to achieve the unique growth conditions and long multiplication times of NB. This modified microdilution method included inoculation of 96-well plates and determination of inhibition by periodic measurement of the absorbance for 30 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% gamma-irradiated fetal calf serum (γ-FBS) under cell culture conditions. Bactericidal or bacteriostatic effects were distinguished by subsequent subculture in drug-free media and monitoring for increasing absorbance. NB isolated from kidney stones were inhibited by trimethoprim, oxytetracycline, colistin, neomycin and doxycycline at levels achievable in serum and urine. The other antibacterials tested against NB exhibited an inhibitory concentration above clinically attainable levels. All tested antibacterials were bactericidal.

Key words: Nanobacteria, antibacterial agents, *in vitro* inhibition, bactericidal

INTRODUCTION

Modern medicine strives to get an efficient treatment for Nanobacteria (NB) as it highly treatment-resistant, persisters, dormant forms and biofilms containing hydroxyl apatite or carbonate (Abo-El-Sooud *et al.*, 2011a). NB are thought to play an important role in extraskeletal calcifying diseases including stones formation, urolithiasis and polycystic kidney disease (Kajander *et al.*, 2003), atherosclerosis (Jelic *et al.*, 2007), periodontal disease (Ciftcioglu *et al.*, 2003), rheumatoid arthritis (Cassell, 1998) and prostatitis (Bock *et al.*, 1989; Geramoutsos *et al.*, 2004). The stimuli for calcium salt deposition in patients with these conditions are unclear but nidi for precipitation and crystallization are needed even under supersaturation conditions (Carson, 1998). Two strains, one of Nanobacterium sanguineum and the other of Nanobacterium sp., were isolated from kidney stones and human and bovine sera, respectively (Kajander *et al.*, 1997). NB may also contribute directly to the primary pathogenesis of disease by acting as a system for the delivery of microbial and other toxins to tissues (Akerman *et al.*, 1997; Kajander *et al.*, 2001), a process that would require endocytosis (Ciftcioglu and Kajander,

1998). Silay and Miroglu (2007) demonstrated that the eradication of NB prevented the calcifications in coronary arteries and prostate with an acceptable level by performing a novel combination therapy which comprises a tetracycline antibiotic, nutraceutical and EDTA. They hypothesized that the risk of urolithiasis recurrence may be reduced with combined anti-nanobacterial therapy. In this regard, antibiotics (Vaessen *et al.*, 1997) have been used with some success for the treatment of pathological calcification-related diseases. The inhibitory effects of antibiotics on the calcifications of surgically implanted artificial materials have also been shown (Chandy *et al.*, 1996). In our previous study we have recently isolated NB from Egyptian patients with urolithiasis (Abo-EL-Sooud *et al.*, 2011b). Future research is required to determine the classical and potentially novel mechanism(s) by which drugs inhibit the growth of NB, alter the morphology of NB and affect the genesis of diverse types of microbial and tissue calcifications. The aim of our study was to assess the *in vitro* abilities of different antibacterial agents to inhibit NB. Moreover, to estimate the Minimal Inhibitory Concentration (MIC) of each tested drug.

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MATERIALS AND METHODS

Stones: Urinary tract stones were collected from male and female patients hospitalized in the Kasr El Aini, Cairo University, Egypt. Stones were demineralized in 1 M HCl and then neutralized (Folk, 1993), centrifuged at 14,000 X g for 15 min and the pellets used for immuno-fluorescence staining (IIFS) and Transmission Electronic Microscopy (TEM). Part of the pellets were suspended in DMEM, sterile-filtered and cultured in DMEM supplemented with γ-FBS (Sera-Lab, Crawley Down, Sussex, U.K.) under nanobacterial culture conditions.

Nanobacterial culture: The cultures were prepared using strict aseptic techniques in a cell culture facility. Nanobacterial samples were filtered through 0.2 mm filters before culturing. Subcultures were made using γ-FBS as a culture supplement. Subculturing of nanobacteria in Serum-Free (SF) DMEM was performed with monthly passages. SF nanobacteria attach firmly to the bottom of the culture vessel. These cultures were passaged or harvested with a rubber scraper. Cultures were established on Loeffler's medium supplemented with 10% conditioned medium from nanobacterial culture and DMEM replaced water in the formula (Nash and Krenz, 1991). The incubation period was 6 weeks under cell culture conditions. Only pure nanobacterial cultures were used. The samples were viewed under Light microscopy with Differential Interference Contrast (DIC) optics. The presence of nanoparticles in urinary tract stones was confirmed by morphological evidence with Scanning Electron Microscopy (SEM) and Transmission Electronic Microscopy (TEM) of inoculated 3T6 cell monolayer (Abo-EL-Sooud *et al.*, 2011b).

Nanobacteria culture in serum containing medium: Nanobacteria were cultured with 10% γ-FBS in DMEM medium (serum nanobacteria) for one month at 37°C in an atmosphere of 5% CO_2-95% air. The cultures were harvested by centrifugation and were suspended in phosphate buffered saline (PBS pH 7.4). Subculturing of the nanobacteria was made in 10% irradiated γ-FBS in DMEM medium. The growth of serum nanobacteria was followed by light microscopy and absorbance measurement with a spectrophotometer at 650 nm.

Antibacterial drugs: Authentic antibacterials were supplied as laboratory-grade powders of known potency, as follows: oxytetracycline, doxycycline, ampicillin, lincomycin, spectinomycin, trimethoprim, neomycin, erythromycin, florfenicol, streptomycin, gentamicin and colistin were obtained from the Arab Veterinary Industrial Co. (AVICO), Amman, Jordan. Most antibacterials were dissolved in DMEM to give a final concentration of 1 mg mL^{-1} and sterilized by membrane filtration (pore size, 0.2 μm; Millipore Corp., Bedford, Mass.). Antibacterial dilutions were prepared just prior to use in sterile plastic vials at double the required concentration in DMEM containing 10% irradiated γ-FBS to allow 1:1 dilution of the inoculum of NB. The isolates of NB were tested against each antibacterial at concentrations ranging from 500 to 0.48 μg mL^{-1}.

MIC and minimal bactericidal concentration (MBC) testing: Inhibitory tests were performed in 96-well, flat-bottom cell culture plates by a modification of the method described by Hannan *et al.* (1997) which involved (1) a longer incubation period, (2) drug dilutions from 500 to 0.48 μg mL^{-1} and (3) absorbance measurements at multiple time points. DMEM containing 10% γ-FBS was used as the culture medium in the tests and for preparation of dilutions of NB and test compounds. After completion of the twofold serial dilutions for each compound, 100 μL of a suspension of the culture of NB with a turbidity equivalent to that of a 0.5 McFarland standard (optical density at 650 nm, 15 mAbs) was added to each well in the microtiter plate. All compounds and each serial dilution were tested in triplicate; the duplicate experiments were run on different days. Each plate also contained four wells of uninoculated medium, which served as the sterility control; four inoculated wells of NB, devoid of any drug, were used as the growth controls. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2-95% air for a maximum of 14 days. Growth was monitored on days 0, 5, 10, 15, 20, 25 and 30 by measuring the absorbance at 650 nm. Prior to absorbance measurement, the plate lid was warmed with a preheated metal plate to eliminate condensation on the lid. Growth curves with standard deviations were determined for each drug treatment (Ciftcioglu *et al.*, 2002). The growth of NB from positive growth control wells was confirmed by Hoechst staining and with anti-NB monoclonal antibodies as described earlier (Ciftcioglu and Kajander, 1998). In an attempt to quantify indirectly the influences of the drugs on the slowly multiplying NB, an absorbance of 15 mAbs for the positive growth control on day 4 was used as the reference point for establishing growth or no growth for each test compound. Growth and inhibition curves for NB were determined for each drug treatment.

The bactericidal or bacteriostatic effects of the drugs were determined. The drug-treated cultures of NB were subcultured (volume, 10 μL) into antibiotic-free medium and incubated for 30 days. Each positive and negative

control was handled in the same manner. When a subculture was negative for growth for any antibiotic or drug, the compound was classified as "NB-cidal"; if there was detectable growth as determined by a continuous increase in absorbance greater than that for the negative control, the compound was considered "NB-static." The growth was monitored weekly by measuring the absorbance at 650 nm for one month.

RESULTS

NB, isolated from human kidney stones was susceptible to trimethoprim (1.95 µg mL^{-1}), oxytetracycline (3.9 µg mL^{-1}), colistin (3.9 IU mL^{-1}), neomycin (31.25 IU mL^{-1}) and doxycycline 62.5 µg mL^{-1}) at clinically achievable levels in serum or urine of patients (Table 1) in the 30-day test. The other antibacterials tested against NB exhibited an inhibitory concentration above clinically attainable levels (125-500 µg mL^{-1}) (Table 1). All antibacterials were NB-cidal as subcultures were negative for growth as determined by non significant change in absorbance greater than that for the negative control wells. Growth curves showing the activities of potent antibacterials (trimethoprim, oxytetracycline, colistin,

doxycycline and neomycin) against kidney stone-derived isolates of NB are depicted in Fig. 1-5. Each antibacterial was plotted against the positive (drug-free) growth control negative control (growth medium only).

DISCUSSION

In our study NB isolated from kidney stones were inhibited by trimethoprim, oxytetracycline, colistin, neomycin and doxycycline at levels achievable in serum and urine. This finding was analogous to those reported by Ciftcioglu *et al.* (2002). They found that NB isolated from human kidney stones and kidney cyst fluids were

Table 1: Summary of MIC values for selected antibiotics (µg mL^{-1} or IU mL^{-1})

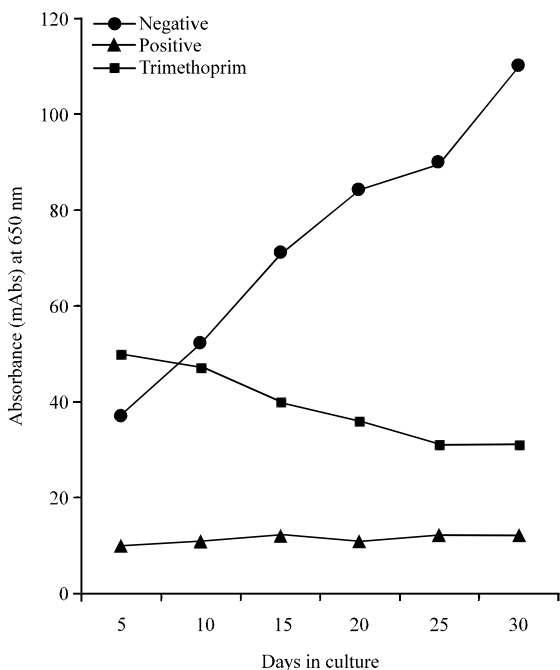| Antibacterial	MIC
Oxytetracycline	3.90
Doxycycline	62.50
Ampicillin	125.00
Lincomycin	500.00
Spectinomycin	500.00
Trimethoprim	1.95
Neomycin	31.25
Erythromycin	125.00
Florfenicol	500.00
Streptomycin	250.00
Gentamicin	250.00
Colistin	3.90

Fig. 1: Growth curves showing the activity of trimethoprim (1.95 µg mL^{-1}) against isolates of NB from kidney stones against the positive (drug-free) growth control and negative control (growth medium only)

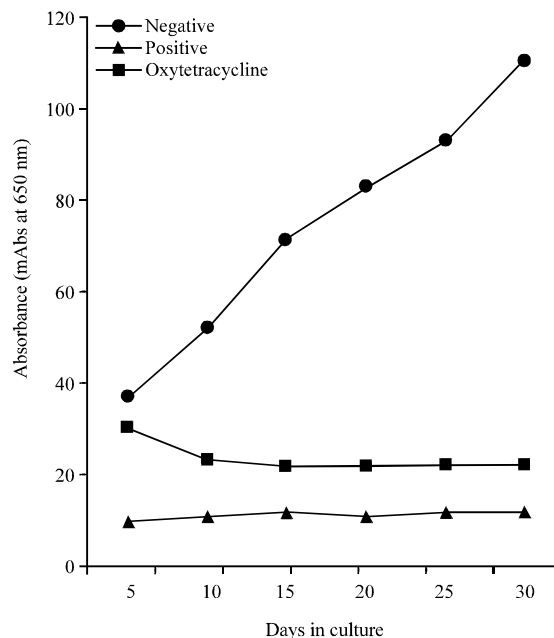Fig. 2: Growth curves showing the activity of oxytetracycline (3.9 µg mL^{-1}) against isolates of NB from kidney stones against the positive (drug-free) growth control and negative control (growth medium only)

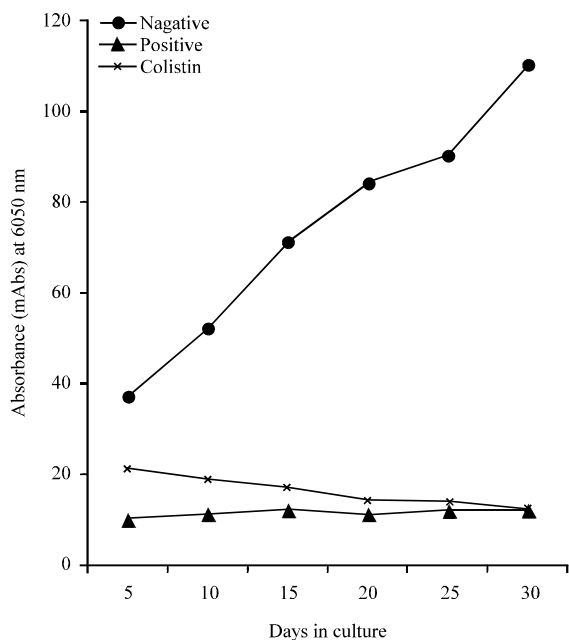Fig. 3: Growth curves showing the activity of colistin (3.9 IU mL^{-1}) against isolates of NB from kidney stones against the positive (drug-free) growth control and negative control (growth medium only)

Fig. 5: Growth curves showing the activity of neomycin (31.25 IU mL^{-1}) against isolates of NB from kidney stones against the positive (drug-free) growth control and negative control (growth medium only)
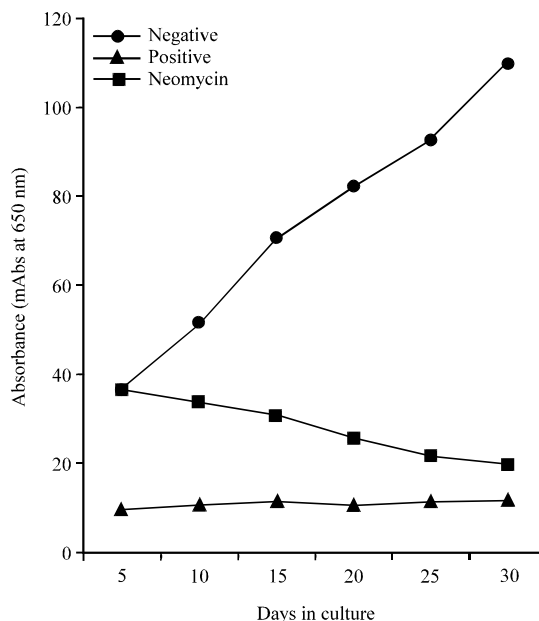

Fig. 4: Growth curves showing the activity of doxycycline (62.5 μg mL^{-1}) against isolates of NB from kidney stones against the positive (drug-free) growth control and negative control (growth medium only)
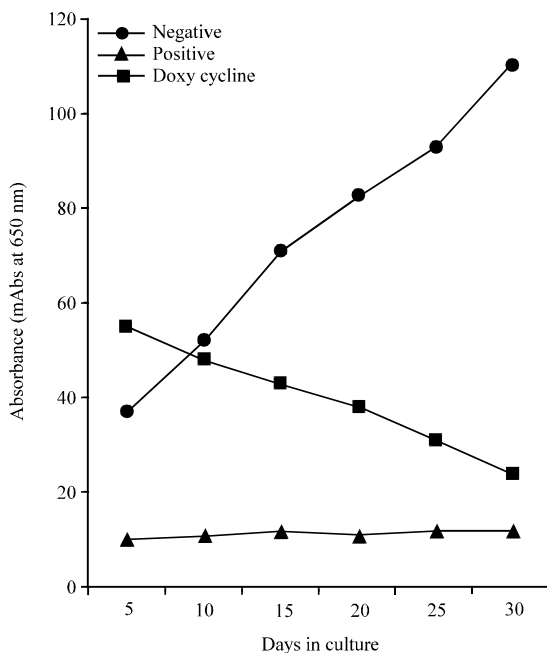
in vitro inhibited by tetracycline HCl, nitrofurantoin, trimethoprim, trimethoprim-sulfamethoxazole and ampicillin at levels achievable in serum and urine. We found that trimethoprim is the most potent cidal tested antibacterial agent with MIC of 1.95 μg mL^{-1}. In this respect, Ciftcioglu *et al.* (2002) found that the trimethoprim-sulfamethoxazole combination was not as effective as trimethoprim alone. This was somewhat surprising in view of the fact that this combination is widely used in the treatment of urinary tract infections. Trimethoprim, is reported to inhibit protein and DNA synthesis; we did not find reports of calcium chelation activities for these drugs. Trimethoprim is a very potential anti-nanobacterial drug for human and animal therapy. Oxytetracycline is highly effective and was bactericidal in the *in vitro* test. Kidney stone patients treated with 500 mg day^{-1} initially had nanobacteria-positive urine culture results but began to have negative urine cultures during the treatment (Shoskes *et al.*, 2005). This indicates that tetracycline treatment can be effective in human and animal treatments for nanobacteria eradication. As stated previously, tetracycline is bound and concentrated to the mineral surface of nanobacteria even short exposure periods, chelate calcium and inhibit metalloproteinases, a property of potential use in the treatment of osteoarthritis, periodontitis and cancer (Hartzen *et al.*, 1997).

Tetracycline is already used in the treatment of some periodontal diseases and dental stone formation (Ryan *et al.*, 1996). This may explain why tetracycline has bactericidal effect on nanobacteria. This bactericidal effect is unique to nanobacteria: other bacteria show only bacteriostatic effect. Tetracyclines are thus potential drugs for eliminating nanobacteria from cell cultures and biological products. There was a difference in the *in vitro* activity of oxytetracycline (MIC 3.9 μg mL^{-1}) and that of doxycycline (MIC 62.5 μg mL^{-1}) against NB. Although doxycycline is more highly protein bound and approximately 10 times more lipophilic than oxytetracycline (Cunha *et al.*, 1982), their activities against NB observed *in vitro* correlated with their comparative levels of calcium binding. The level of chelation of tetracycline to calcium (40%) is reported to be twice that for doxycycline (19%) (Von Wittenau, 1968). It should be noted that tetracyclines have been used in the treatment of pathological calcifications and autoimmune diseases with often remarkably good results. The aminoglycoside antibiotics neomycin, gentamicin and streptomycin all show anti-nanobacterial effects at relatively high antibiotic concentrations. This class of antibiotic is primarily known as inhibitor of protein synthesis but more recently it has been recognized that they displace cell biofilm-associated calcium and magnesium that link polysaccharides of lipopolysaccharide molecules (Peterson *et al.*, 1985). Gentamicin and neomycin did not block the multiplication of NB. NB are positive by the differential Limulus amoebocyte lysate assay (Hjelle *et al.*, 2000) but the lipopolysaccharide of NB has not been sufficiently characterized to allow further speculation regarding the observed lack of activity of these antibiotics. It is commonly known that ampicillin inhibits bacterial cell wall synthesis but like some other penicillins, it is also a calcium chelator (Crossland, 1970). The inhibition by ampicillin may also have been influenced by the lack of detectable β-lactamase in NB and the somewhat zwitterionic nature of ampicillin that enables it to penetrate the cell walls of gram-negative bacteria (Livermore and Williams, 1996). Since ampicillin and related drugs are administered at very high doses and are concentrated into urine at levels exceeding the observed MIC values, they can be useful in the treatment of urinary tract nanobacterial infection. Other tested antibiotics were found to have MIC values exceeding 500 μg mL^{-1}. These antibiotics are thus unlikely to be effective in eradication of nanobacteria when used in monodrug therapy, although they may be effectively employed in conjunction with other antibiotics in a multidrug treatment regimen.

CONCLUSION

NB isolated from kidney stones were inhibited *in vitro* by trimethoprim, oxytetracycline, colistin, neomycin and doxycycline at levels achievable in serum and urine. Future research is required to evaluate the *in vivo* effects of the tested antibacterials in patients with urolithiasis.

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