Title:

Pathogens in exacerbations of chronic rhinosinusitis differ in sensitivity to silver nanoparticles

Running title:

AECRS pathogens – sensitivity to silver nanoparticles

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Abstract

Introduction. The significance of the microbiome in chronic rhinosinusitis (CRS) is not clear. Antimicrobials are recommended in acute exacerbations of the disease (AECRS). Increasing rates of antibiotic resistance stimulate research on alternative therapeutic options including silver nanoparticles (AgNPs), sometimes referred to as “colloidal silver”. However, there are concerns regarding the safety of silver administration and the emergence of silver resistance. In this cross-sectional observational study, we assessed the sensitivity of sinonasal pathogens to AgNPs and compared it with the toxicity of AgNPs for nasal epithelial cells.

Method. Negatively charged AgNPs (13±5 nm) were obtained with the use of tannic acid. Minimal inhibitory concentrations (MIC) of the AgNPs were determined for pathogens isolated from patients with AECRS. Cytotoxicity was tested on human nasal epithelial cells line *in vitro*.

Results. 48 clinical isolates and 4 reference strains were included in the study (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Acinetobacter baumanii, Serratia marcescens, Enterobacter cloacae*). The MIC values differed between isolates, even within the same species. All of the strains were sensitive to AgNPs in concentrations nontoxic to human cells during 24 hours exposition. However, 48h exposition to AgNPs increased toxicity to human cells, narrowing their therapeutic window and enabling 19% of pathogens to resist the AgNPs biocidal action.

Conclusions. AgNPs can potentially be used in intranasal drugs to treat most episodes of AECRS. Sensitivity testing may be necessary before application. Results of sensitivity testing for reference strains cannot be extrapolated to other strains of the same species.

Importance

Chronic rhinosinusitis is characterized by nasal congestion, discharge and facial pain. Bacterial infections cause acute exacerbations of the disease when the symptoms temporarily get worse. Exacerbations are usually treated with antibiotics. Increasing rates of antibiotic resistance stimulate research on alternative antibacterial agents. Various forms of silver, including silver nanoparticles, are suggested as intranasal antibacterial drugs. However, there are concerns regarding the safety of silver administration and reports of silver-resistant bacteria in various clinical settings. In our study, we attempted to assess whether silver nanoparticles could be used to treat patients with exacerbations of chronic rhinosinusitis. We found out that most of the bacteria isolated from the patients were sensitive to silver nanoparticles at concentrations that were still safe for the nasal epithelium. It suggests that silver nanoparticles could potentially be used as intranasal drugs but sensitivity testing should be obligatory before their administration to prevent the development of silver resistance.

Introduction

Chronic rhinosinusitis (CRS) is an inflammatory disease of the sinonasal mucosa. Understanding of the complex relationships between the inflammatory process and the microbiota of the sinuses is still in its infancy. The role of bacteria seems to be more evident in acute exacerbations of chronic rhinosinusitis (AECRS). If sudden worsening of symptoms is accompanied by purulence in the sinuses, the incident is attributed to bacterial infection and antibiotic treatment is recommended (1, 2). Increasing antibiotic resistance of sinonasal pathogens is one of the most important factors that contribute to treatment failures in AECRS. As a result, there is growing interest in novel therapeutic options.

Intranasal preparations of silver nanoparticles (AgNPs) are proposed as an alternative treatment of sinonasal infections if antibiotic therapy is ineffective. Silver nanoparticles (AgNPs) are objects of any shape and dimensions in the range from 10^{-9} m to 10^{-7} m built from silver atoms. Depending on the methods and stabilizing agents used to synthesize the AgNPs, they differ significantly in physicochemical and biological properties (3). The AgNPs dispersed in liquid media are sometimes referred to as “colloidal silver” (4). However, this term is not precise and may lead to misunderstandings. Many over-the-counter preparations marketed as “colloidal silver” contain various unspecified forms of silver, but no well-characterized silver nanoparticles (5).

Potential medical applications of AgNPs evoke either enthusiasm or serious concerns. The antimicrobial properties of silver have been known for millennia. Silver has been shown to be effective against a wide variety of microbes, including Gram-positive and Gram-negative bacteria, yeast and fungi (6). It damages many vital structures in the cells and therefore it was believed that resistance to silver was very unlikely to emerge (7, 8). Unfortunately, recent studies prove that bacteria can develop manifold mechanisms of silver resistance, some of which additionally result in cross-resistance to antibiotics (9). Research on

AgNPs toxicity provides contradictory results. Some studies report excellent safety of AgNPs preparations (4, 10, 11), while others provide alarming data on cytotoxicity and accumulation in many organs including the brain after intranasal administration (12). The toxicity of silver preparations probably depends on dosing and formulation but reliable data on the subject is lacking.

Recent studies show that bacteria in clinical settings sometimes harbour silver resistance genes and occasionally the clinical isolates can tolerate exceedingly high silver concentrations (13). These findings undermine the conviction that silver is a universal antimicrobial and can be used for any infection without previous sensitivity testing. However, due to the lack of routine screening, the incidence of silver resistance in human infections is unknown.

The objective of our study was to assess if AgNPs could be effectively applied to fight pathogens that cause exacerbations of CRS. The AgNPs in the study were obtained with the use of tannic acid. We tested the susceptibility to AgNPs in 48 pathogens isolated from 50 patients with AECRS. To our knowledge, to date, this is the largest survey on AgNPs susceptibility in CRS patients. In parallel experiments, we evaluated the toxicity of AgNPs for nasal epithelial cells *in vitro*. Our purpose was to determine if there is a potential therapeutic window, that is a concentration of AgNPs that is already effective against the pathogens but still safe for the host's epithelium. The secondary goal was to evaluate the incidence of bacterial strains that were resistant to AgNPs at concentrations non-toxic for human cells.

Results

Swabs from the sinonasal cavity were obtained from 50 patients with acute exacerbations of chronic rhinosinusitis (AECRS). The group included 27 women and 23 men aged 25-80

(mean age 51). All of the patients have undergone endoscopic sinus surgery in the past, so their sinuses were accessible for sampling. Most of the participants had chronic rhinosinusitis with nasal polyps (90%) and presented with comorbidities such as asthma (54%), aspirin-exacerbated respiratory disease (10%) or allergy (38%).

As presented in the flow chart (Fig. 1), the sensitivity to AgNPs was tested in 48 out of 97 isolates. Species considered nonpathogenic and bacteria that require media that inhibit the AgNPs were excluded.

The minimal inhibitory concentration (MIC) values of AgNPs for the clinical isolates and reference strains are presented in Table 1. Higher MIC values indicated lower sensitivity to AgNPs. The MIC values for the clinical isolates ranged from 5 to 40 mg/L with the median value of 10 mg/L. However, for each of the identified species, the sensitivity to AgNPs varied between isolates.

The lowest median MIC value was noted for *P. aeruginosa* followed by *S. aureus* and *A. baumanii* while the highest values were observed for the other Gram-negative rods. However, due to high variability within each species, the differences in sensitivity between species were not statistically significant. There were no significant differences between Gram-positive and Gram-negative bacteria. It is also apparent that the MIC values for reference strains cannot be considered representative of the clinical isolates. The reference strain of *P. aeruginosa* ATCC 27857 used in the study was less sensitive to AgNPs than *S. aureus* ATCC 29213, *E. coli* ATCC 25922 or *K. pneumoniae* ATCC 31488, while the results obtained for clinical isolates showed a reversed trend.

Median MIC values for antibiotic-resistant strains were higher than for antibiotic sensitive bacteria, but this difference was not statistically significant (Table 2). There was no significant correlation between decreased sensitivity to AgNPs and any of the resistance

mechanisms or resistance to any particular antibiotic. However, the number of antibiotic-resistant bacteria in the study was probably too low to observe possible more subtle relationships.

The cytotoxicity of silver nanoparticles was assessed in the *in vitro* culture of human primary nasal epithelial cells (HNEpC). Incubation in the presence of AgNPs reduced the viability of epithelial cells in a dose-dependent manner. However, high concentrations of the nanoparticles and prolonged incubation time were required to achieve cytotoxic effects (Fig. 2). At 24 hours of incubation, cell viability remained above 80% for up to 75 mg/L AgNPs, and no morphological changes implicating cell death were observed. At the highest concentration tested (100 mg/L), cell viability measured with the MTT assay was 65.11% +/- 4.86, suggesting that human nasal epithelial cells are much less sensitive to silver nanoparticles than any of the tested bacterial strains. The half-maximal inhibitory concentration (IC50) for the 48-hour time point was determined at 60.03 mg/L, which was also above MIC for 100% of the tested pathogens. 80% viability after 48 hours was maintained at 25 mg/L. This concentration of AgNPs was effective against 39 (81%) of the isolates (Fig. 3).

The findings of the colorimetric assay were confirmed with a cell death assay based on cell membrane integrity (trypan blue exclusion assay). Results obtained using both methods were comparable, which indicates that the lower absorbance values observed in the MTT assay are due to the changes in cell viability and not to changes in cell mitochondrial activity or cytostatic effects of the nanoparticles.

Discussion

Despite the growing number of studies on the antimicrobial properties of AgNPs, it is very difficult to draw universal conclusions. Practically every research group use their own

method to produce the AgNPs which results in varied physicochemical and biological properties of the final product. Previously, we showed that the antibacterial and antifungal properties of AgNPs differ significantly depending on the stabilizing agent used to obtain them (14). Furthermore, the bacterial strains differ between studies. Frequently only one reference strain is selected to represent a species. Therefore, MIC values reported for pathogens such as *S. aureus*, *Escherichia coli* or *Pseudomonas aeruginosa* vary between studies (6, 15-18).

In our study, we showed that the sensitivity to AgNPs in reference strains was not representative of the values noted for clinical isolates of the same species. The MIC values for the reference strains of *S. aureus E. coli* and *K. pneumoniae* were two to five times lower than the median values for the clinical isolates. In contrast, the reference *P. aeruginosa* was characterized by MIC values that were twice higher than the median value for clinical isolates of the same species.

Among the clinical isolates, there were considerable differences in the MIC values between strains. These differences were not species-specific. For most species, we found strains that were highly sensitive to AgNPs (MIC 5 mg/L) as well as strains that were inhibited only by much higher concentrations of the antimicrobial (40 mg/L).

In general, Gram-positive bacteria are believed to be less sensitive to AgNPs than Gram-negative bacteria because of their thicker cell wall. Indeed, in several studies, *E. coli* was more susceptible than *S. aureus* (19) with 2-10 times lower MIC values depending on the study (15, 16). Our study did not support this hypothesis. There were no statistically significant differences in MIC values between Gram-positive and Gram-negative bacteria. The mean MIC value for Gram-negative strains was even lower than for the Gram-positive isolates.

Previously, the efficacy of AgNPs against clinical isolates from patients with CRS and otitis was assessed by Feizi *et al.* (4). Eighteen bacterial strains were included in the study (5 MRSA, 5 *P. aeruginosa*, 5 *H. influenzae*, 3 *S. pneumoniae*). *P. aeruginosa* was more sensitive to AgNPs than other species, but in general Gramm-positive isolates were not universally more resistant than Gramm-negative bacteria. The authors also noted that MIC values were different among strains for each of the species. The study was conducted in Australia and utilized different AgNPs than the ones used in our research, but the conclusions are strikingly similar to our observations carried out in Europe.

These observations show that the sensitivity of sinonasal pathogens to AgNPs is not easily predictable. If AgNPs are used in therapy, each strain isolated from a patient needs to be tested individually to determine possible resistance.

Recent observations suggest that uncontrolled application of silver without prior knowledge about the pathogen's sensitivity may result in exposition to sublethal concentrations and promote resistance development. Bacteria developed multiple resistance mechanisms that protect them from silver and AgNPs including expression of substances that cause sequestration of AgNPs or reduction of Ag^+ to nontoxic Ag^0, ion efflux pumps, modifications of the cell membrane permeability or even changes in the bacteria's surface electrical charge which results in electrostatic repulsion of the AgNPs (7, 8). It is particularly alarming that the development of silver resistance as a result of silver exposure seems to be very probable. In an experiment conducted by Blanco Massani *et al.* silver resistance was rapidly induced in *E. coli* exposed to increasing concentrations of $AgNO_3$ (9). This result suggests that silver preparations should be applied with caution. Just like antibiotics, they need to be used only when antimicrobial treatment is clearly indicated and the dosage must be adequate to achieve concentrations above the MIC values of the targeted pathogen.

Alarmingly, resistance to silver was shown to promote cross-resistance to antibiotics. Li *et al.* observed that silver resistant mutants were deficient in porins which resulted in decreased sensitivity to cephalosporins, tetracycline and chloramphenicol (20). Blanco Massani *et al.* noted tolerance to ciprofloxacin and ampicillin after induction of silver resistance in *E. coli* (9). Apparently, the mechanisms that prevent the accumulation of silver may also transport antibiotics from the cytoplasm. Reduced susceptibility to silver observed in methicillin-resistant *S. aureus* (MRSA) in comparison to methicillin-sensitive strains was explained by the induction of efflux pumps (21). In our study, 37.5% of the isolates had mechanisms of antibiotic resistance. We observed higher MIC values for these isolates compared to antibiotic sensitive bacteria but the differences did not reach statistical significance.

Studies reporting the prevalence of silver resistance in clinical settings are scarce. However, their results to date seem to be fairly optimistic. Most data on the subject is related to bacteria isolated from burns, ulcers and other wounds that are frequently treated with dressings containing silver. Although silver resistance genes are encountered in 2-6% of clinical isolates, genetic resistance usually does not translate into phenotypic resistance (13, 22-24). Nevertheless, the first strains with alarmingly high silver resistance are occasionally found in environments where exposition to silver is more frequent. In 2006, Ip *et al.* reported the prevalence of silver resistance among bacterial isolates from patients in a Burns unit (25). Only one out of 117 isolates was actually resistant to silver. It is noteworthy that it was a multidrug-resistant strain of *Enterobacter cloacae* probably selected after prolonged antibiotic and silver therapy. In 2013, Finley *et al.* tested 130 clinical isolates for phenotypic resistance and found that silver was bactericidal for all of them (26). In 2015 the same author reported screening of 859 isolates (13). Silver resistance genes were found in 31 strains. Alarmingly, 2 isolates (*Klebsiella pneumoniae* and *Enterobacter cloacae*) tolerated silver ion concentrations

up to 5500 uM and were resistant to many commercially available burn and wound dressings. Moreover, Elkrewi *et al.* showed that bacteria that do not display overt Ag-resistance frequently harbor genes that cause cryptic resistance which is readily activated upon silver challenge (27). These results indicate that silver resistance, although currently rare, may become an increasing problem in the future in case of unlimited overuse of silver preparations.

The question of whether AgNPs can be safely used as antimicrobial drugs in humans is under debate. Silver preparations are available over-the-counter despite a lack of adequate regulatory approval (5). The toxicity levels reported for eukaryotic cells vary and depend on the properties of the AgNPs used in the experiments and the type of cells under consideration (15 mg/L for alveolar epithelial cells, 30 mg/L for monocytes, 80 mg/L for HeLa epithelial cells) (28-30).

The toxicity of AgNPs for human cells was compared with the toxicity for sinonasal pathogens in two studies. Feizi *et al.* studied 18 clinical isolates from patients with CRS and otitis media. The AgNPs for this study were produced using *Corymbia maculate* leaf extracts. The AgNPs were not toxic to human immortalized bronchial epithelial cells Nuli-1in concentrations equal to MIC for the sinonasal pathogens and up to 175 ppm after 1 hour of exposure (4). Chen *et al.* studied the antibacterial properties of commercially available 10 nm AgNPs that were effective against reference strains of *E. coli* and *S. aureus* at the concentration of 5 ppm. They found that human nasal squamous cell carcinoma cells (RPMI2650) maintained >80% viability after 24 hours of exposure to 5 ppm of AgNPs (31).

In our study, to assess the cytotoxicity of silver nanoparticles, we used primary HNEpC cells, which are the most reliable two-dimensional *in vitro* model of the nasal epithelium (32). We observed that the AgNPs were nontoxic to epithelial cells in concentrations that inhibited the growth of all bacterial strains tested when incubated with the cells for 24 hours. At 48

hours of incubation, silver nanoparticles were not cytotoxic up to 25 mg/L, which is above the median MIC value for the tested bacteria. However, 9 (19%) of the clinical isolates were not sensitive to AgNPs at this concentration.

It is debatable what time of exposure in cytotoxicity experiments is representative of the real-life pharmacokinetics of the AgNPs in the nose and the sinuses. In the study conducted by Feizi *et al.* the incubation time was very short (1 hour). The authors explained that the mucociliary clearance eliminates the AgNPs within about 15 minutes when applied in a nasal solution (4). However, to fight an infection, it will probably be necessary to maintain the drug concentration above the MIC value for a longer period. For antibiotics, the recommended time of treatment of sinonasal infections is between 5 days and 3 weeks. Therefore, it is reasonable to assume that AgNPs need to be used in a formulation that would increase the retention time of the nanoparticles in the nasal cavity or a dressing that would stay in contact with the nasal mucosa for prolonged periods of time.

We showed that the AgNPs were not toxic to HNEpC cells at concentrations above MIC for the sinonasal pathogens in the first 24 hours of the experiments, but the cytotoxicity increased when the time of exposure was longer. This observation suggests that the real-life toxicity of AgNPs may be higher than assumed from short-time experiments on cell lines.

On the other hand, several studies indicate that standard two-dimensional *in vitro* cell culture models may overestimate the cytotoxicity of different agents, including silver nanoparticles (33, 34). Differentiated 3D *in vitro* models might more closely resemble what occurs *in vivo* due to barrier properties that reduce absorption across the stratified epithelium. Zavala *et al.* (2016) showed that higher concentrations and longer exposure times of air pollutants are needed to induce cytotoxic effects in a 3D *in vitro* model of airway epithelium compared to the 2D epithelial cell culture. Chen *et al.* (2019) found no toxicity of AgNPs in a 3D model of a stratified epidermis while observing a pronounced cytotoxic effect of

equivalent doses of AgNPs in a 2D culture of keratinocytes. Similar 3D models of differentiated nasal epithelium cultured at the air-liquid interface, containing cilia and mucus-producing cells, have been described (35, 36).

Local toxicity for nasal epithelial cells is not the only factor that limits the potential topical application of AgNPs in patients with CRS. Systemic distribution of AgNPs was observed after intranasal administration in rodent models. Aggregation of AgNPs was noted in the spleen, lung, kidney and nasal airway (12). This resulted in enhanced destruction of erythrocytes in the spleen, but no other microscopic changes were associated with the AgNPs depositions. It has to be noted that the doses of AgNPs used in the study were exceedingly high (100-500 mg/kg). However, repeated inhalation of AgNPs for 28 days at concentrations of 10^6 particles/cm^3 caused no significant changes in the nasal cavity or lungs of rats, only an increase in the number and size of goblet cells. Moreover, direct nose-do-brain transport of AgNPs is suspected to bypass the blood-brain barrier (12, 37, 38), but not all of the studies support this observation (5). Importantly, the application of AgNPs results in significantly lower concentrations of silver in blood than the delivery of $AgNO_3$ (5).

The safety of AgNP sinonasal rinses (15 mg/L) in 11 patients with CRS was assessed by Ooi *et al.* (10). Four patients had elevated serum silver levels but no adverse events were reported and the silver levels did not reach the threshold for argyria. In our study, only 27 (56%) of pathogens were sensitive to AgNPs at concentrations of 15mg/L However, Ooi *et al.* applied different AgNPs than the ones used in our study, which excludes any direct comparisons.

In conclusion, the results of our study suggest that most episodes of AECRS could be safely and effectively treated with AgNPs obtained with the use of tannic acid because most pathogens are sensitive to AgNPs in concentrations safe for the nasal epithelium. However, *in vitro* experiments may under- or overestimate the toxicity of antimicrobials. Therefore, further *in vivo* studies on the toxicity, pharmacokinetics and pharmacodynamics

are required before AgNPs are approved as intranasal antimicrobials in humans. Due to the unpredictability and significant differences in the MIC values between strains, testing of sensitivity to AgNPs should be indicated before their application in every case. This approach may prevent exposition to suboptimal drug concentrations that promotes the development of silver resistance.

Limitations

The study group included only patients with postoperative AECRS so the results presented in this paper may not apply to bacterial exacerbations of CRS in patients who did not undergo sinus surgery.

In this study, we used one type of AgNPs. The properties of AgNPs obtained with different methods may vary.

This observational study was designed to estimate the prevalence of phenotypic resistance while the prevalence of resistance genes or cryptic resistance and determination of resistance mechanisms requires more detailed exploration in future studies.

The results of *in vitro* tests of AgNPs efficacy are always affected by the media in which the experiment is conducted due to their interaction with ions, macromolecules and blood (8). Therefore further research on the interactions between the AgNPs and the secretions in the sinonasal cavities is necessary before their application *in vivo*.

Methods

Sample collection. The samples for this cross-sectional observational study were collected in the outpatient clinic of the Department of Otolaryngology of the University Hospital in Krakow in 2018 and 2019 (Jagiellonian University Medical College Bioethics Committee approval no. 1072.6120.208.2017). The eligibility criteria included: diagnosis of CRS according to EPOS 2012 diagnostic criteria (39), prior endoscopic sinus surgery (ESS), signs and symptoms of bacterial exacerbation (40) (worsening of symptoms, purulence in the sinonasal cavity), no antibiotic therapy for at least a week. Swabs were collected under endoscopic guidance from the pathological secretions from the middle nasal meatus or the infected sinuses. Bacterial strains were stored at -80 ^{o}C and thawed for experiments. Then bacteria were inoculated on Columbia Blood Agar to obtain full cell activity. Isolation and identification of bacteria and antibiotic susceptibility testing were performed as previously described (41). Reference strains were obtained from the American Type Culture Collection (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27857, *Klebsiella pneumoniae* ATCC 31488).

Synthesis of silver nanoparticles. The silver nanoparticles (AgNPs) were obtained using tannic acid as a reducing and stabilizing agent. An aqueous solution of tannic acid (40 mL, 0.5 mM) was introduced to silver nitrate solution (320 mL, 11 mM). After dynamic stirring ammonia solution (30 µL, 25 wt %) was added to the reaction mixture and subsequently stirred for 30 minutes. The AgNPs were purified using the ultrafiltration method and procedure described previously (14). The AgNPs obtained in this process were characterized by a diffusion coefficient of 5.37 x 10^{-7} cm^2s^{-1}, hydrodynamic diameter 12±1 nm, electrophoretic mobility -3.30±0.23 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$ and zeta potential -52±3 mV (the values determined at pH 5.8 and temperature of 37°C, the ionic strength of suspension was not

regulated). This type of AgNPs was selected due to their promising antibacterial properties observed in our preliminary experiments (14).

Determination of the minimal inhibitory concentration (MIC) of the AgNPs. To determine the minimum inhibitory concentrations (MIC) of AgNPs, the suspension of the tested microorganisms 0.5 on the MacFarland optical density scale was diluted with saline 100 times to obtain a cell density of 106 CFU mL-1 (colony forming unit) and inoculated on the surface of Müller–Hinton agar (OXOID). Next, a sterilized pipette tip was used to form wells on the agar surface with a diameter suitable for a 200 μL pipette. Each well was filled with 100 μL of the AgNP suspensions in concentrations ranging from 5 to 100 mg L^{-1} (in a triplet for each concentration). The AgNP concentration was increased by 5 mg L^{-1} in subsequent wells. Pure medium without AgNPs was used as a control sample. After pre-incubation (4 h, 4°C), the plates were incubated at 37° C over 18 h. The zone of inhibition (ZI) was measured with Skjutmatt Digital calliper (Limit). MIC was defined as the lowest concentration of AgNPs for which the IZ of microorganisms around the well was observed. All experiments were performed in triplicate. This method was not applicable for *Streptococcus pneumoniae* and *Haemophilus influenzae* because of the interactions between AgNPs and the growth media suitable for these species. Therefore, the results obtained for these bacteria were considered unreliable and were excluded from the analyses.

***In vitro* primary cell culture and cell viability assays.** Human nasal epithelial cells (HNEpC; PromoCell) were cultured in Airway Epithelial Cell Growth Medium (basal medium supplemented with BPE, EGF, insulin, hydrocortisone, epinephrine, triiodo-L-thyronine, transferrin and retinoic acid; PromoCell) at 37 °C in a 5% CO_2 atmosphere. HNEpC cells at passage 5 were used in the experiments. Cells were plated in 48-well plates (BD Falcon) coated with type I collagen (Corning) at the initial density of 1×10^4 cells/cm^2, 24 hours prior to the experiments. The growth medium was then changed to the experimental cell culture medium

containing nanoparticles or vehicle control (water). The experimental medium consisted of phenol red-free DMEM, prepared from powder using water or a mixture of water and 214 mg/L tannic acid-modified silver nanoparticles (AgNPs) suspension in water, and supplemented with 1 g/L glucose, $NaHCO_3$ (Gibco), sodium pyruvate (Lonza), 1% bovine serum albumin (BSA), L-glutamine, and P/S (50 units/mL of penicillin and 50 µg/mL of streptomycin). A 48-hour incubation in the experimental medium had no negative effects on the viability of the cells compared to the PromoCell culture medium. Cell culture reagents were from Sigma unless otherwise specified.

A) MTT assay

MTT assay was performed after the incubation in the presence of AgNPs, as previously described (42). Following 2-hour incubation with MTT, formazan crystals were dissolved in 5 mM HCl in isopropanol and absorbance read at 540 nm. Experiments were performed in triplicate for each experimental condition. Data represent mean values expressed as a percentage of the vehicle control ± SD from three independent experiments.

B) Trypan blue exclusion

After the 48-hour incubation in the presence of AgNPs, cells were harvested with StemPro Accutase (Gibco), centrifuged, resuspended in DPBS. The cell suspension was diluted 1:1 with 0,4% trypan blue and counted in a hemocytometer (43).

Statistical analysis. The normality of distribution of the microbiological data was tested using the Kolmogorov-Smirnov test, Mann-Whitney test was used for comparisons of two groups and Kruskal-Wallis test was used to compare multiple groups (Statistica 13.0). A p-value of < 0.05 was considered statistically significant. For the HNEpC viability assays, statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism 8.0).

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Contributor roles

Joanna Szaleniec – conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing – original draft, writing – review and editing

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Tables

Table 1. Minimal inhibitory concentration (MIC) of AgNPs for the clinical isolates and reference species [mg/L]. Higher MIC values indicate lower sensitivity to the AgNPs.

Species (no of isolates)	Reference strain	Clinical isolates			
		median	min	max	standard deviation
Staphylococcus aureus (30)	5	10	5	40	11.78
Pseudomonas aeruginosa (5)	10	5	5	20	6.51
Escherichia coli (4)	5	25	10	25	7.50
Klebsiella pneumoniae (3)	5	25	10	30	10.40
Klebsiella oxytoca (2)		25	10	40	21.21
Acinetobacter baumanii (2)		10	10	10	0

Serratia marcescens (1)		25	25	25	0
Enterobacter cloacae (1)		30	30	30	0
All clinical isolates (48)		10	5	40	11.23
All Gram-positive clinical isolates (30)		10	5	40	11.78
All Gram-negative clinical isolates (18)		15	5	40	10.60

Table 2. Minimal inhibitory concentration (MIC) of AgNPs for clinical isolates with and without mechanisms of antibiotic resistance [mg/L]. Higher MIC values indicate lower sensitivity to the AgNPs. ESBL - extended-spectrum beta-lactamases; MRSA - methicillin-resistant *Staphylococcus aureus*; MLS - resistance to macrolides-lincosamides and streptogramins.

Mechanisms of antibiotic resistance (number of bacterial isolates)	median	min	max	standard deviation
no mechanisms of antibiotic resistance (34)	10	5	40	11.55
with mechanisms of antibiotic resistance (14)	17.5	5	40	10.85
ESBL (4)	25	25	30	2.50
MRSA+MLS (2)	15	5	25	14.14
MLS (7)	10	10	40	11.80

Figures

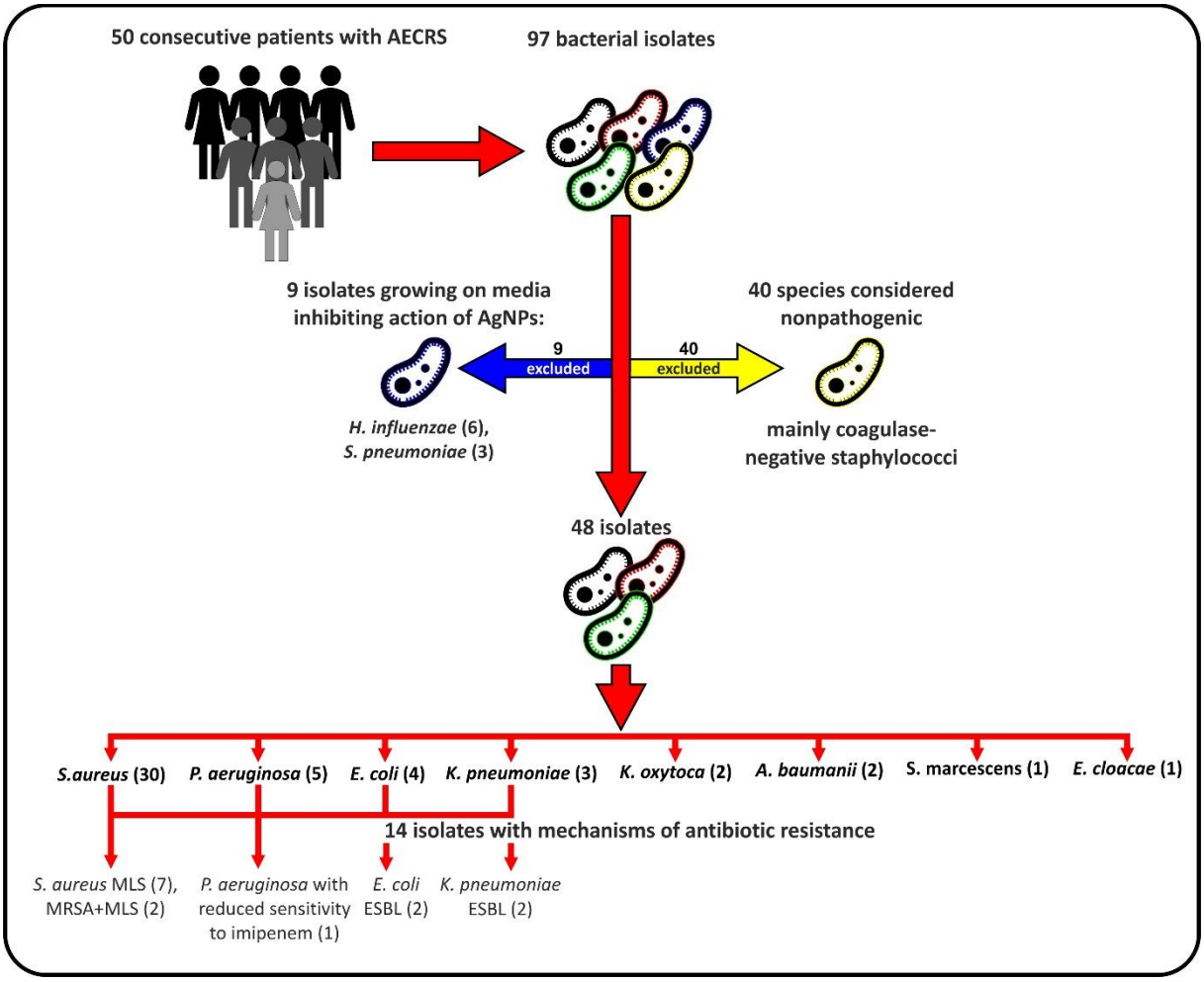

Fig. 1. Flow chart detailing the selection of bacterial isolates for the study.

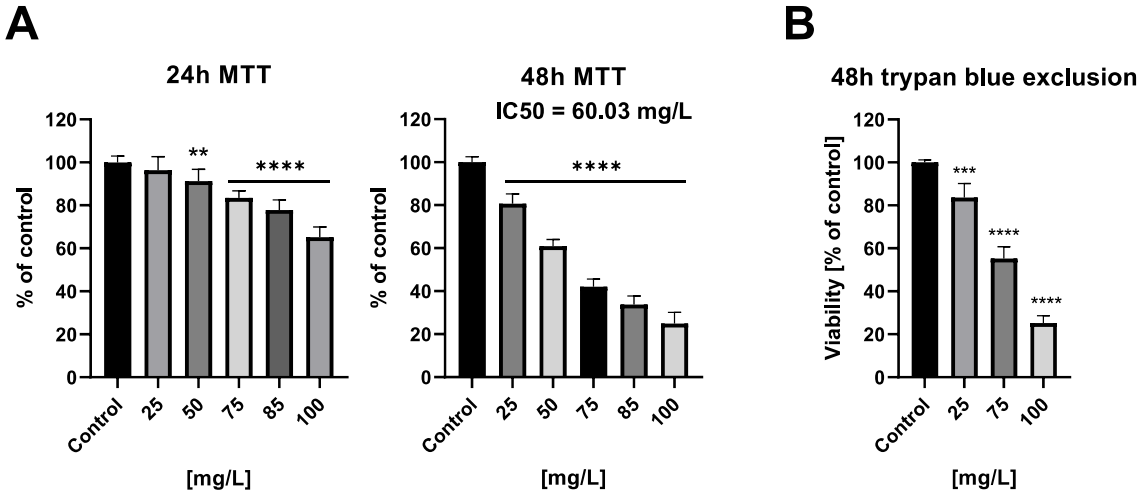

Fig. 2. Cell viability of human primary nasal epithelial cells. (A) MTT assay following exposure of cells to AgNps at the indicated concentrations for 24 and 48 hours. Data represent mean values of three independent experiments performed in triplicate (n=9), expressed as % of control ± SD. * indicates values significantly different from the control. (B) Trypan blue exclusion assay. The percentage of viable cells was determined after a 48-hour incubation in the presence of AgNPs. Data represent mean values ± SD. Statistical significance compared to the control.

** $P \leq 0.01$; *** $P \leq 0.001$; P **** $P \leq 0.0001$.

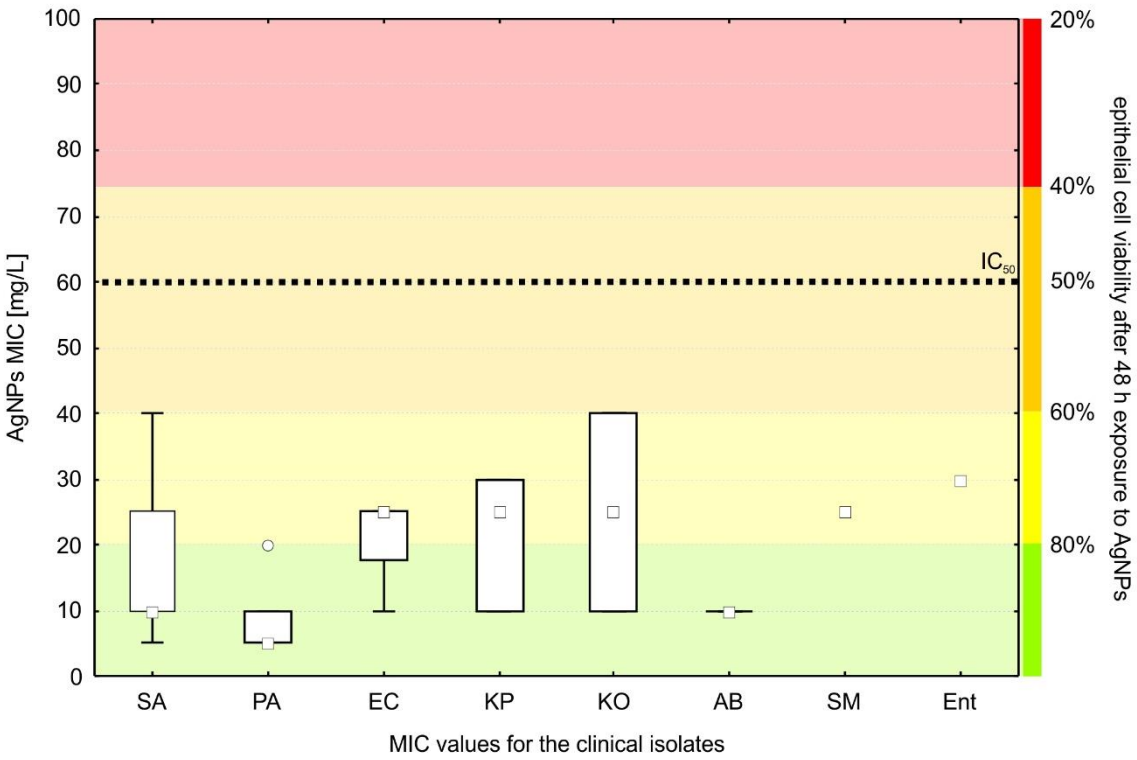

Fig. 3. MIC values for the clinical isolates compared to the nasal epithelial cell viability after 48 h exposure to AgNPs (IC_{50} - the half-maximal inhibitory concentration). SA – *Staphylococcus aureus*, PA – *Pseudomonas aeruginosa*, EC – *Escherichia coli*, KP – *Klebsiella pneumoniae*, KO – *Klebsiella oxytoca*, AB – *Acinetobacter baumanii*, SM – *Serratia marcescens*, Ent - *Enterobacter cloacae).* The boxes represent the interquartile range, the whiskers extend to the highest and lowest values, the squares represent the median values, the circle represents an outlier.