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Research paper

Evaluation of the Bactericidal Effect of Nebulized Silver Nanoparticles on Common Respiratory Bacteria in Horses– *In Vitro* Studies

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ABSTRACT

Antimicrobial resistance is increasing in both human and veterinary medicine. Bacteria can be part of the etiology of respiratory disorders in horses. Bactericidal activity of silver has been largely described and silver is currently used in veterinary therapeutic applications such as wound dressings. The aim of this study was to assess the *in vitro* bactericidal effects of nebulized silver nanoparticles (AgNP) on 2 common equine respiratory bacteria, *Streptococcus equi* subsp. *zooepidemicus* and *Actinobacillus equuli* subsp. *equuli*. Firstly, antimicrobial susceptibility of AgNP was determined over time by turbidity assessment in liquid broth. Secondly, bacterial growth inhibition was tested after instillation or after nebulization of low (100 ppm) and high (500, 1,000 and 2,000 ppm) concentrations of AgNP on agar plate. Both bacteria were susceptible to AgNP, even at dilution 1:4 for *A. equuli* and 1:8 for *S. zooepidemicus* after 8 hours of incubation, and 1:256 for both bacteria after 24 hours of incubation. The bacterial growth was partially inhibited at low concentration and completely inhibited at high concentrations of instilled AgNP. The bacterial growth was completely inhibited after nebulization of low concentrations of AgNP for *A. equuli* and high concentrations of AgNP for *S. zooepidemicus*.

We concluded nebulized AgNP could be a candidate for innovative therapeutic way against bacterial respiratory disorders in horses. Nevertheless, further investigations are required to assess the *in vivo* potential and toxicity of nebulized AgNP.

1. Introduction

Poor performance is a multifactorial concern in horses. Respiratory disorders are considered as the most frequent cause of subclinical poor performance in race [1] and sport [2] horses, especially mild to moderate asthma [3]. Bacteria, namely gram-positive streptococci and gram-negative *Actinobacillus*/*Pasteurella* spp. could be part of the etiology of subclinical mild to moderate equine asthma [4,5] or clinical broncho- and pleuropneumonia [6]. Treatment of bacterial infections in equine airway may include antibiotics, either by systemic administration (sulphonamide/diaminopyrimidine associations [13], aminoglycosides [14], penicillin [15], tetracyclines [16] or third-[17] and fourth-generation [18] cephalosporins) or by aerosol therapy (aminoglycosides [14] or third-generation cephalosporins [17]).

The bacterial development of antimicrobial resistance is a worldwide issue, both in human [7] and veterinary [8] medicine, as it is occurring faster than the development of new antimicrobial drugs [7]. Antimicrobial resistance of bacteria is widely reported in horses with the most frequently used antibiotics in this species. In a New Zealand laboratory, 39.2% of the horses tested presented at least one multiple drug-resistant (MDR, means resistant to $\geq$ 3 antimicrobial classes) bacteria [19]. The prevalence of MDR streptococci increased in the UK within a decade while the susceptibility of bacteria to specific antibiotics decrease [20].

Conflict of interest statement: The authors declare no conflict of interest.

Animal welfare/Ethical statement: No live animals were used in this project; therefore, no approval by the Ethical Committee of the University of Liege was needed.

Author's contributions: TF, JND, JM and TA contributed to study design and writing-review and editing. CP and JND contributed to data collection. TF contributed to data interpretation and study supervision. CD contributed to writing-review and editing.

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Silver has been used as antimicrobial agent for millennia. More recently, the bactericidal action of silver nanoparticles (AgNP) has been shown against gram-positive and -negative bacteria [9]. In human and veterinary healthcare, AgNP is currently used in, among others, surgical sutures [10] and wound dressings [11]. The mechanism behind its biocidal effect is not totally defined, but could include damage to the cell membrane permeability, disturbance of the respiration function of the cell and possibly further damages on DNA [21]. Nebulized AgNP could represent an innovative treatment of bacterial disorders in equine airways. The aim of this study was to test the *in vitro* bactericidal effect of AgNP on 2 common equine respiratory bacteria, *Streptococcus equi* subsp. *zooepidemicus* and *Actinobacillus equuli* subsp. *equuli*, and to assess the preservation of its *in vitro* bactericidal activity after being aerosolized.

2. Materials and Methods

2.1. Antimicrobial susceptibility tests of AgNP

Ten tubes containing 200 μL of double concentrated Brain-Heart-Infusion (BHI) broth were prepared. AgNP at the concentration of 2,000 ppm (Wire2Wire Vet Products, Paris, Kentucky, USA) was diluted 1:10 in BHI and 200 μL were added in the first tube, obtaining 400 μL of AgNP 100 ppm. After shaking, 200 μL of the first tube were sampled and dropped in the next tube, obtaining a 1:2 dilution. The operation was repeated in order to obtain 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 dilutions. Gentamicin (500 μg/mL) and sterile saline were used as positive and negative controls, respectively.

Following, 10 μL of BHI containing *S. zooepidemicus* (ATCC 700400, ATCC, Manassas, Virginia, USA) or *A. equuli* (ATCC 9346, ATCC, Manassas, Virginia, USA) were dropped in each tube. The BHI broth were prepared 24 hours before the experiment and showed a turbidity of 4 McFarland standards. Susceptibility to gentamicin at 25 μg/ml had been previously confirmed for both bacteria. Turbidity of each tube was measured using a densitometer (bioMérieux, Marcy-l'Étoile, France) ranging 0 to 4 McFarland standards at 620 nm wavelength [12], after 0, 2, 4, 6, 8 and 24 hours of incubation under shaking (400 rpm) at 37°C.

2.2. Bacterial growth inhibition by AgNP on agar media after instillation

Columbia blood agar plates were inoculated with 100 μL of BHI containing *S. zooepidemicus* or *A. equuli* using a spiral plater (Eddy Jet, Led techno nv, Heusden-Zolder, Belgium) and allowed to dry 5 minutes under a biosafety hood. The concentration of bacteria in the BHI was 2 to 2.5 CFU/μL, to obtain 200 to 250 colony forming units (CFU) per plate. Then, 1 mL of AgNP 100 ppm (SilvaPlex, Wire2Wire Vet Products, Paris, Kentucky, USA) or AgNP 2,000 ppm was spread on the plates using a rake. Gentamicin (500 μg/mL) and sterile saline were used as positive and negative controls, respectively. The plates were dried for 15 minutes under the hood before being incubated at 37°C. CFU were counted on the entire plate after 24 hours of incubation.

2.3. Bacterial growth inhibition by AgNP on agar media after nebulization

Columbia blood agar plates were inoculated with 100 μL of BHI containing *S. zooepidemicus* or *A. equuli* at the concentration 2-2.5 CFU/μL using the spiral plater, and placed 5 minutes to dry under turned off hood with a light airflow. Then, the plates were placed 15 cm under the nebulization source (Flexineb E2, Nortev Ltd, Galway, Ireland) of a nebulization chamber (Flexineb C2 Aerosol Box, Nortev Ltd, Galway, Ireland) (Fig. 1) and 2,5 mL of the AgNP were nebulized at the following concentrations: 100, 500, 1,000 and 2,000 ppm. Gentamicin (500 μg/mL) was used as positive control. A sealed untreated plate and a plate nebulized with sterile saline were used as negative controls. The plates were placed for 15 minutes under the hood before being incubated at 37°C. CFU were counted on the entire plate after 24 hours of incubation.

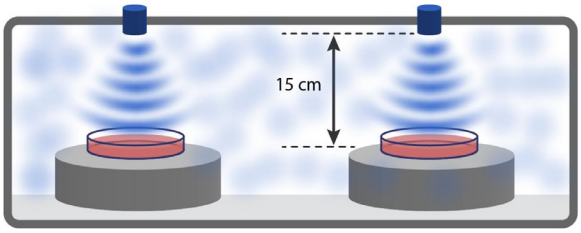

Fig. 1. Technical scheme of the nebulization chamber. The agar plates, inoculated with bacteria, were placed 15 cm under the nebulization source of a nebulization chamber and 2,5 ml of the solutions were nebulized.

2.4. Data collection and statistical analysis

All experiments have been realized in triplicate. The results of the triplicates are represented as mean ± standard deviation. Statistical analysis was realized using *t*-test for 2 independent means, and significance was obtained when $P < .05$.

3. Results

3.1. Antimicrobial susceptibility tests of AgNP

Turbidity increased in negative control after 6 hours of incubation for both bacteria, while the samples with all dilutions of AgNP showed no increase of turbidity (Fig. 2). After 8 hours of incubation, turbidity increased only for the dilutions of AgNP from 1:16 to 1:256 for *S. zooepidemicus* and from 1:8 to 1:256 for *A. equuli*. After 24 hours of incubation, no turbidity was observed for the dilutions of AgNP from 1:1 to 1:64 for both bacteria.

3.2. Bacterial growth inhibition by AgNP on agar media after instillation

Significant but incomplete inhibition of the bacterial growth was obtained with 1 mL of AgNP 100 ppm on both bacteria ($P = .04$). Complete inhibition of the bacterial growth was observed for both bacterial species when treated with AgNP 2,000 ppm (Fig. 3).

3.3. Bacterial growth inhibition by AgNP on agar media after nebulization

Nebulization of all concentrations of AgNP was performed without technical issue. No growth of *A. equuli* was present 24 hours after nebulization with 2,5 mL of AgNP 100 ppm (Fig. 4). Therefore, treatment of *A. equuli* with higher concentrations (500, 1,000 and 2,000 ppm) of AgNP was not performed. Inhibition of *S. zooepidemicus* growth was observed 24 hours after nebulization of 2,5 mL with AgNP 500, 1,000 and 2,000 ppm but not after nebulization of AgNP 100 ppm ($P = .96$).

4. Discussion

Gram-positive *S. zooepidemicus* and gram-negative *A. equuli* have been used in this study as these 2 bacteria are the most frequently isolated from horses suffering of (sub)clinical respiratory

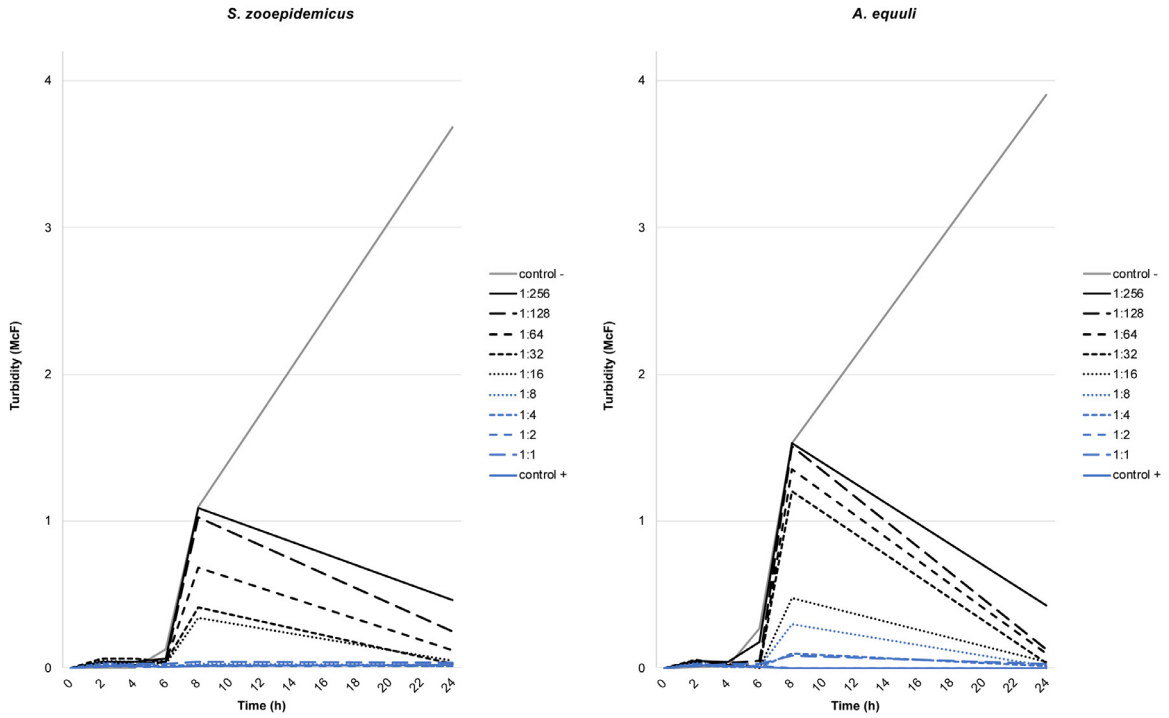

Fig. 2. The antimicrobial susceptibility tests obtained by broth microdilution showed no turbidity for silver nanoparticles diluted 1:1 to 1:8 on *S. zooepidemicus* and 1:1 to 1:4 on *A. equuli* after 8 hours of incubation. Gentamicin and saline were used as control positive (+) and negative (-), respectively. Notice all dilutions had a bactericidal effect at 24 hours of incubation, while the higher dilutions did not significantly inhibit the bacterial growth after 8 hours of incubation.

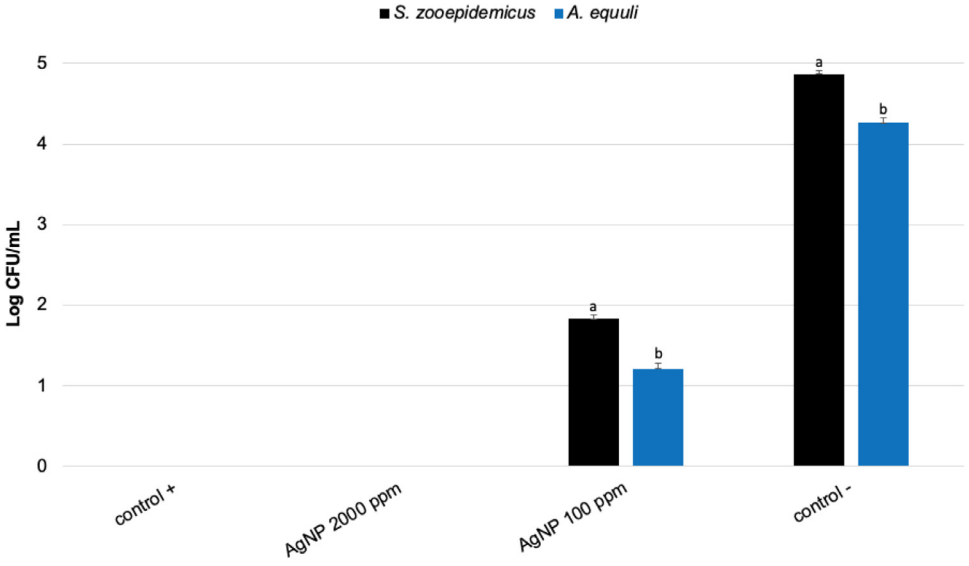

Fig. 3. Colony forming units (CFU) of *S. zooepidemicus* and *A. equuli* observed 24 hours after instillation of silver nanoparticles (AgNP) at different concentrations. Gentamicin and saline were used as control positive (+) and negative (-), respectively. AgNP at 2,000 ppm concentration showed complete inhibition of bacterial growth (no CFU observable). AgNP at 100 ppm concentration showed incomplete but significant inhibition of bacterial growth. Different superscript letters indicate significant difference between treatments ([a] *P*-value is .037; [b] *P*-value is .043).

infections [4,5]. Antibiotics are commonly included in the treatment of bacterial respiratory disorders. Unfortunately, an increase in antimicrobial resistance of bacteria is observed worldwide, both in humans [7] and animals [8], and represent nowadays a challenge for the practitioners. In order to decrease the use of antibiotics and the development of antimicrobial resistances, there is a need to develop alternatives to antibiotics in the treatment of bacterial infections, in particular secondary or subclinical ones. In this context, the bactericidal properties of AgNP have been used in human and veterinary healthcare [10,11]. The tested AgNP preparation is chelated to citric acid in order to make this metal soluble, which is requested for aerosolization. The chelation is a chemical formation between a metal ion and a ligand, the formed complex being more stable than the isolated ion [22]. Citric acid is currently used in food industry as antimicrobial agent [23]. Due to the chelation of AgNP with citric acid, pH of the soluble chelated AgNP is varying between 2.40 and 2.50, depending on the AgNP concentration.

The aim of this study was to show the bactericidal activity of AgNP on 2 common equine respiratory bacteria, when AgNP is

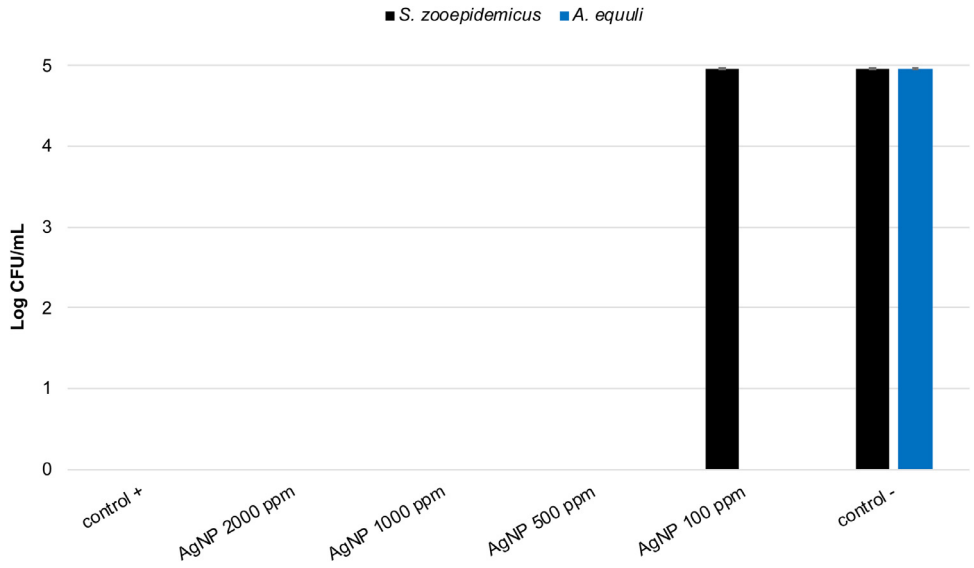

Fig. 4. Colony forming units (CFU) of *S. zooepidemicus* and *A. equuli* observed 24 hours after instillation of silver nanoparticles (AgNP) at different concentrations. All concentrations of AgNP were nebulized on *S. zooepidemicus*, but only AgNP 100 ppm was nebulized on *A. equuli*. Gentamicin and saline were used as control positive (+) and negative (-), respectively. AgNP 100 ppm showed no inhibition of growth on *S. zooepidemicus*. All other treatments with nebulized AgNP showed complete inhibition of bacterial growth (no CFU observable).

nebulized. In a first time, we determined the antimicrobial susceptibility of the bacteria to AgNP. After 24 hours of incubation, no turbidity was observed when AgNP was added at dilutions 1:1 to 1:128 on both *S. zooepidemicus* and *A. equuli*. Nevertheless, after 8 hours of incubation, the observations were different, with no turbidity for dilutions of AgNP 1:1 to 1:8 dilution for *S. zooepidemicus* and 1:1 to 1:4 for *A. equuli*. This could suggest a time-dependent bactericidal effect of AgNP. Following, bactericidal effect was determined on inoculated agar plates. When instilled on the agar plates, AgNP showed a significant antimicrobial effect at both 100 and 2,000 ppm concentrations. Nevertheless, bacterial growth was not completely inhibited using AgNP 100 ppm concentration, while it was using AgNP 2,000 ppm concentration. This could suggest a concentration-dependent bactericidal effect of AgNP.

Aerosol therapy has been used since a long time in veterinary medicine for the administration of, among others, corticosteroids, bronchodilators and antibiotics, directly in the equine airway. It allows the administration of lower drug doses to horses, with subsequently lesser side effects. Nevertheless, this method of treatment requisites instructed users and adequate equipment, as proper use and size of the particles determine the correct drug deposit in the airway. Different types of equipment are available, most of them are able to aerosolize any solution without denaturalization. No *in vitro* nebulization model is described for testing antimicrobial effect of drugs. A nebulization chamber model was used in this study, with the agar plates being placed at sufficient distance to be in contact between the aerosol flow without direct impaction of it on the plates.

In the second part of this study, we aimed to show AgNP maintained its bactericidal activity after nebulization on inoculated agar plates. The experiment was first realized using AgNP 100 ppm concentration, the commercial concentration which is used empirically in the veterinary field. As *A. equuli* growth was completely inhibited using this concentration, higher concentrations were not tested on this bacteria. The 100 ppm concentration of AgNP did not show effect on *S. zooepidemicus* growth, and higher concentrations were further tested on this bacteria. All higher concentrations of AgNP did completely inhibit its growth. The difference in bactericidal effect on both bacteria could be explained by a different interaction of AgNP with the outer membrane of the gram-negative species. A practical consequence could be the need to adapt the dosage of the treatment to the bacterial species identified in the respiratory samples. Beside the way of treatment, different results between instillation and nebulization of AgNP 100 ppm on *A. equuli* may be due to different used volumes, 1 mL and 2,5 mL respectively. A higher volume has been used with nebulization to compensate the loss of product in the nebulization chamber.

In vivo use of AgNP in horses based on the presented *in vitro* results should be made carefully, as the efficacy and toxicity *in vivo* is not shown yet. Although the volume of the nebulization chamber model is circa the same as the tidal volume in healthy adult horses, the model does not reproduce the respiratory physiology and anatomy, such as airflow, rhythm, frequency, presence of mucus and epithelial cells. On the other side, absence of toxicity of AgNP on host cells and organism remains to be proved. Some toxicological studies have shown the clearance and effects of short-term inhalation of AgNP in murine lungs [24–26]. At the authors' knowledge, toxicity for long-term inhalation of AgNP in horses has not been studied yet. The results of the present study suggest a possible time- and concentration-dependent bactericidal effect of AgNP.

5. Conclusions

Considering these results, AgNP presents an *in vitro* bactericidal action on 2 common equine respiratory bacteria and its action is preserved after nebulization. AgNP could therefore possibly be a candidate for a new way of treatment against respiratory bacterial infections in horses. At the authors' knowledge, AgNP is not currently registered as human or veterinary medicine. However, the clinical relevance may be limited at this stage. Toxicity of AgNP on the horse's airway and long-term *in vivo* use have to be further investigated before *in vivo* use.

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