The Role of Silver Nanoparticles in a Treatment Approach for Multidrug-Resistant Salmonella Species Isolates

Manar M Farouk, Amal El-Molla, Fayeza Salib, Yousef Soliman, Mohamed Shaalan

Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt; Central Laboratory for Evaluation of Veterinary Biologics (CLEVBI), Cairo, Egypt; Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.

Purpose: The main objective of this study is to investigate the antibacterial activity of silver nanoparticles (AgNPs) against multidrug-resistant Salmonella isolates recovered from diarrheic sheep and goats.

Methods: This study used chemical reduction synthesis of AgNPs to evaluate their antimicrobial effects by estimation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for each isolate using the microplate dilution method and tetrazolium salt reduction test to detect the viability percentage. In vivo treatment efficacy was assessed in mice by determining the viable count of Salmonella Enteritidis recovered from feces and by hematologic, biochemical and histopathologic examinations to confirm that use of AgNPs has no toxic or pathologic effects and to evaluate its ability in tissue regeneration following treatment.

Results: All recovered strains were identified as MDR with a prevalence of 4% and 3.6% in sheep and goats, respectively. The results of TEM, DLS, Zeta potential, and FTIR revealed typical characteristics of the synthesized AgNPs. Silver nanoparticles showed antibacterial activity against all recovered strains with MIC of ≤0.02–0.313 μg/mL (mean average 0.085 ± 0.126 μg/mL) and MBC of 0.078–1.250 μg/mL (average 0.508 ± 0.315 μg/mL). In vivo efficacy of AgNPs was observed by a reduction in the number of viable S. Enteritidis recovered from feces in an S. Enteritidis infected mouse model, with complete shedding stopping between treatment days 4 and 6. Hematologic, serum biochemical, and histopathologic analyses proved the ability of AgNPs to suppress inflammatory reaction caused by S. Enteritidis infection.

Conclusion: The study proved the effective ability of AgNPs to fight MDR Salmonella spp. in vitro and in vivo without adverse effects.

Keywords: multidrug resistance, silver nanoparticles, Salmonella, mice, minimum inhibitory concentration, minimum bactericidal concentration

Introduction

Sheep and goats are well known for their potential as livestock to transform feed into beneficial products such as fiber, milk, and meat. Salmonellosis is an infectious disease in animals that causes severe losses of livestock and serious public health problems worldwide. Salmonella is a key etiologic agent of infectious diarrhea and a major zoonotic pathogen globally. Antimicrobial agents are valuable tools for infectious diseases’ treatment and keeping animals healthy and productive. Simultaneously, antimicrobial-resistant pathogens pose serious and
costly problems for animal health. The main reason for development of multidrug resistance among Salmonella spp. in humans is the misuse of antibiotics resulting from lack of uniform policy and disregard for hospital infection control standards. This issue has sparked vigorous debate on the possibility that the use of antibiotics in animal feed will lead to a similar crisis; for example, antibiotic use as a growth stimulator is usually thought to promote the emergence and distribution of antibiotic-resistant strains in animal farms. However, veterinary data on the prevalence of antimicrobial resistance in animal pathogens are scarce, especially in developing countries, such as Egypt, where this study was conducted. Because of the threat of Salmonella spp. infections in Egypt, it is important to study the resistance mechanisms to antimicrobials in Salmonella spp. and their effect on livestock.

Infectious diseases represent one of the biggest health problems worldwide. Although, numerous antibiotics are available for commercial use, they mostly lack potency against newly evolved multidrug-resistant (MDR) pathogens, which gives rise to massive doses of antibiotics and the need to continuously develop new antibiotics. Recently the potency of metallic nanoparticles has been proven to control MDR pathogens, both independently and jointly with existing available antibiotics. Specifically, the promising properties of silver nanoparticles (AgNPs) have been shown to be suitable for multiple biomedical purposes, including their impressive ability for the formulation of novel antimicrobials. Recent studies regarding the antibacterial activities of AgNPs for MDR ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter sp.) showed impressive results for four of six pathogens in this group of bacteria. Nanosilver is one of the most commonly used nanomaterials, and its particle size ranges from 1–100 nm. The antimicrobial properties of silver (Ag+) have been well recognized for decades. Silver nanoparticles (AgNPs) have received considerable attention due to their biological, physical, and chemical properties based on their catalytic activity and bactericidal effects. One of the strengths of AgNPs versus traditional antibiotics is their interaction with various bacterial cellular reactions such that resistance to them is becoming less prevalent. The exact antibacterial modes of action for AgNPs are unclear. Nevertheless, various researchers have proposed several modes of action, including interference with the bacterial cell wall, production of reactive oxygen species, generation of Ag+ ions, and interaction with DNA. The key antibacterial mechanisms of AgNPs are either by liberation of Ag+ ions or by the intracellular deposition of nanoparticles. Moreover, the bactericidal activity is primarily due to inhibition of the synthesis of nucleic acid, protein, and the cell wall, as well as microbial cell disruption. Despite the steadily increasing presence of Ag-containing products in the market and extensive reports on the antimicrobial activity of AgNPs, insufficient data are currently available about the use of AgNPs as in vivo therapeutic agents. Different treatment methods have been proposed to eradicate salmonellosis; however, a critical consideration is that the use of antibiotics poses a risk of serious and costly animal health disorders and also leads to the rapid development of resistance. The emergence of antibiotic microbial resistance is one of the major challenges today, and there is an increasingly urgent need to promote safe approaches for the containment of MDR salmonellosis, such as the investigation of antimicrobial alternatives. According to the best of our knowledge, most of the previous studies did not investigate the antibacterial effect of AgNPs against the antibiotic-resistant Salmonella strains clinically isolated from small ruminants, especially the serovar Salmonella Enteritidis. Therefore, this study aims to determine the occurrence of MDR salmonellosis in diarrheic goats and sheep and to demonstrate antimicrobial efficacy of synthesized AgNPs both in vivo and in vitro for salmonellosis control. The present study was also designed to synthesize AgNPs through chemical co-reduction method with trisodium citrate and sodium borohydride. The synthesized AgNPs was coated with PVP and carrying a negative charge. AgNPs were characterized by transmission electron microscopy (TEM), DLS and zeta potential analysis, and Fourier transform infrared spectroscopy (FTIR) analysis.

Materials and Methods

Salmonella Species Isolation and Identification

A total number of 325 and 193 fecal swabs were collected from diarrheic sheep and goats, respectively, from different localities in Giza Governorate, Egypt. The swabs were microbiologically examined for Salmonella spp. isolation following ISO-6579 standards and biochemically identified according to Quin et al. Then the isolates were serotyped following the Kauffmann-White scheme using a commercially available antisera kit (SIFIN), as mentioned in Supplementary Appendix 1.
Molecular Confirmation of *Salmonella* Species Isolates

To extract DNA from the bacterial isolates, the boiling method by Wani et al.\textsuperscript{10} was used, and molecular verification was achieved by *Salmonella* spp. *invA* gene amplification.\textsuperscript{31} The primer sequence and thermal profile used is listed in Table 1.

Antimicrobial Susceptibility Test

The minimum inhibitory concentration (MIC) values for 10 antimicrobials were evaluated using broth microdilution assay in 96 well microtiter plates according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.\textsuperscript{32} Briefly, 100 μL of sterile Mueller Hinton broth was introduced into each well of a 96-well microtiter plate. Two rows were used as controls, a sterility control row containing 200 μL of sterile Mueller Hinton broth was used as a sterility indicator, and a culture control row containing 100 μL of sterile Mueller Hinton broth and 100 μL of subculture broth was used to verify the broth’s ability to support microbial growth. To determine MIC for antimicrobial agents, 100 μL of working solution of each agent at a specified concentration was introduced into the top row of the 96-well microtiter plate, and double-fold serial dilutions were performed according to the specified test range for each agent. Thereafter, 100 μL of subculture broth adjusted to the 0.5 McFarland standard was introduced to all wells except in the sterility control row, and all antimicrobials were tested in triplicate to ensure reproducibility of results. After confirming reproducibility, each plate was sealed with a sterile adhesive film and incubated at 37°C for 24 hours. Results were recorded when there was sufficient turbidity in the culture control row and no growth or turbidity in the sterility control row. The growth in each well was compared with that in the culture control, and the MIC was determined as the lowest concentration of the antimicrobial that completely inhibits growth. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) and EUCAST guidelines.\textsuperscript{33,34} The stock solution for each powdered antimicrobial agent was prepared, and the amount of weighed antimicrobial powder and the volume of diluent were calculated from the following equation:

\[
\text{Volume of diluent (mL)} = \frac{\text{Weight (mg)}}{\text{Potency (μg/mg)}}/\text{Concentration (mg/L)}
\]

The weighed antimicrobial powders were dissolved in 1 mL of dimethyl sulfoxide (DMSO) solvent and diluted to the final stock concentration with sterile distilled water. Working solutions were prepared according to the test range for each antimicrobial, which was chosen according to World Health Organization guidelines,\textsuperscript{35} as listed in Table 2.

Multiple Antimicrobial Resistance Index (MAR) Determination

The multiple antimicrobial resistance index (MAR) of each isolate was estimated using the Paul et al.\textsuperscript{36} formula, in which the number of antimicrobials to which isolates were resistant was divided by the total number (10) of antimicrobials to which the test isolate has been evaluated for susceptibility. The interpretation of MAR results was performed according to Christopher et al.\textsuperscript{37} as follows: isolates with an index <0.3 are considered to have narrow drug resistance (NDR); ≥0.3–0.7 are MDR, 0.8–0.9 are extensively drug-resistant (XDR); and pan drug resistant (PDR) isolates are resistant to all agents.

Synthesis of Silver Nanoparticles (AgNPs)

AgNPs synthesis was applied through a chemical reduction method according to El Mahdy et al.\textsuperscript{38} Briefly, a reducing solution of sodium borohydride (NaBH4) (research Lab®) was prepared by dissolving 1.89 g of NaBH4 in 50 mL distilled water and then refrigerated. The Ag source solution was prepared by dissolving 0.272 g of silver nitrate (AgNO3) in 344 mL of distilled water, followed by stirring for 15 minutes with a magnetic stirrer.

**Table 1** Oligonucleotide Primer Sequence and Thermal Profile for *invA* Gene

| Gene | Primer Sequence(5’-3’) | Thermal Profile | PCR Product (bp) | Reference |
|------|------------------------|----------------|------------------|-----------|
| invA | F: GGTGAAATTATCGCCACGTTCGG GCAA | • Initial denaturation at 95°C for 15 minutes(35 cycles):  
• Denaturation at 94°C for 30 seconds  
• Annealing at 64°C for 30 seconds  
• Extension at 72°C for 45 seconds  
• Final extension step at 72°C for10 minutes | 284 | [31] |
Table 2 Details of the Tested Antimicrobials and Their Antimicrobial Phenotypic Sensitivity Patterns, Results

| Antimicrobial     | Source (Company Name Reg. No and/or Batch No.) | Potency (µg/mg) | Test Range (µg/mL) | Sensitivity Pattern (Number)% |
|-------------------|----------------------------------------------|----------------|-------------------|------------------------------|
| Amikacin          | ATCO Pharma For Pharmaceutical Industries Reg. No. 3540/2014 | 250            | 0.5–4             | S 100% I 0% R 0%             |
| Amoxicillin       | UCCMA United Company For Chem. and Med. Preps. Reg. No. 1446/2009 B. No. 43218020 | 200            | 2–32              | S 25% I 5% R 70%             |
| Ciprofloxacin     | Apple Pharma B. No. 8541                      | 100            | 0.03–4            | S 10% I 30% R 60%           |
| Colistin          | PHARMA SWED-Egypt Reg. No. 1568/2012          | 20             | 4–64              | S 0% I 0% R 100%            |
| Doxycycline       | ARABCO MED Reg. No. 3094/2007 B. No. 0921/17   | 200            | 0.5–2             | S 0% I 0% R 100%            |
| Enrofloxacin      | VIGORA Reg. No. 9692/2014 B. No. 9101700      | 100            | 0.03–4            | S 100% I 0% R 0%            |
| Florfenicol       | PHARMA SWED-Egypt Reg. No. 2533/2015 B. No. 180341 | 100            | 2–64              | S 75% I 10% R 15%          |
| Gentamycin        | Medical Professions For Veterinary Products& Fodders Reg. No. 2335/2004 B. No. 751601 | 100            | 1–32              | S 90% I 5% R 5%            |
| Oxytetracycline   | PHARMA-SWEDE-Egypt B. No. 2099/2014            | 185.3          | 2–32              | S 0% I 0% R 100%           |
| Sulphamethoxazole | KELA, Belgium B. No. 26486A1a                  | 250/50         | 1–8               | S 5% I 0% R 95%            |

Abbreviations: S, sensitive; I, intermediate; R, resistant.

In 48 mL of distilled water, a stabilizing mixture of 0.504 g of polyvinyl pyrrolidone (PVP) (CDH®) and 2.912 g of trisodium citrate (ADWIC®) were dissolved, stirred for 15 minutes, and mixed with the prepared AgNO₃ solution. Then, 8 mL of the fresh, cold-reducing NaBH₄ solution was added to the mixture followed by stirring for 30 minutes. After that step, the reduction reaction was investigated for when the color of solution changed from colorless to a dark yellow or brown color. Finally, the prepared stock solution was kept in a dark glass bottle away from direct sunlight at 4°C. The yield of each step in the synthesis process is mentioned in Supplementary Appendix 2.

Silver Nanoparticles Characterization

Determination of the morphology and size of the integrated nanoparticles was made by using transmission electron microscopy (TEM) (JEM-1400-JEOL), which operated at an accelerating voltage of 80 kV. Briefly, one drop of AgNPs solution was deposited on the carbon-coated grid and left to evaporate at room temperature, thus forming a monolayer. The particle size was estimated using Image SP Viewer software. In addition, distribution of particle size and zeta potential analysis were measured at 25°C using a nano-zetasizer (Zetasizer Nano ZS, Malvern Paralytical) where deionized water was used as the dispersant. Briefly, the sample was diluted 1:10 and
then exposed to ultrasonication for 5 minutes for estimation of particle size distribution and it was diluted 1:1000 and three measurements were conducted for zeta potential analysis. Fourier transform infrared spectroscopy (FTIR) analysis was performed by FTIR spectroscopy (Hitachi Ltd., Tokyo, Japan) and recorded AgNPs in the frequency range of 4000–400 cm⁻¹ to identify the different functional groups in molecules responsible for the silver ion reduction and PVP-capping of AgNPs.

**Determination of Silver Nanoparticles Concentration**

An advanced microwave digestion system was used for digestion of the AgNPs sample, and the Ag⁺ concentration was determined by using elemental concentrations of the nanoparticle solution through inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Thermo Sci, Model: iCAP6000 series) in which argon gas was used for excitation of the element atom and the blank value for the element was deduced from the sample value.

**In vitro Antimicrobial Activity Assay**

**Bacterial Growth Inhibition**

One purified colony per each isolate was inoculated in brain heart infusion broth (Oxoid) and incubated at 37°C for 4 hours in a shaker incubator (225 rpm). Bacterial culture dilutions at a certain concentration of 1×10⁶ colony forming unit (CFU)/mL in brain heart infusion broth were prepared and confirmed by plate counting. One milliliter each of AgNPs solution and each diluted broth of the *Salmonella* spp. isolate diluted broth were mixed to obtain the final inoculum containing 5×10⁵ CFU/mL. In parallel, a control negative tube for each isolate was obtained by a mixture preparation containing the same volume of sterile distilled water and broth containing the bacteria, followed by overnight incubation of tubes at conditions just described. Finally, 0.1 mL from each tube was plated on the Mueller Hinton agar (MHA) plate (Oxoid) and incubated overnight in an ordinary incubator at 37°C, followed by colony counting according to Bresee et al.⁹

**MIC**

Serial microdilution assays were conducted to quantify the MIC values for AgNPs that inhibited bacterial growth, based on the method by Shaalan et al⁴⁰ with some modifications. Briefly, 100 µL of sterile Mueller-Hinton broth was introduced into each well of a 96-well microtiter plate, except for one well that was left as a blank according to microplate reader software instructions. Moreover, three rows from each plate were used as controls, as follows: one negative control row of AgNPs at starting concentration 5 µg/mL was used as blank values, which were deducted from the test values; one positive control row of ciprofloxacin at starting concentration of 0.1 mg/mL was included due to its antibacterial activity; and one culture control was included to verify that the broth was capable of supporting microbial growth. To test the antimicrobial properties of the AgNPs sample, 100 µL of AgNPs at a starting concentration of 5 mg/L were individually transferred into the top row of the micro-titer plate and double-fold serial dilutions were performed; all samples were tested in triplicate to ensure accurate and reproducible results. Before the addition of bacteria to the microtiter plates, all cultures used were subcultured into the suitable broth and diluted until just turbid (0.5 McFarland standard) to ensure an approximate concentration of 1.5×10⁵ CFU/mL. Hereafter 100 µL of subculture was added to all 96 wells, except in the negative control row, for which 100 µL of sterile Mueller-Hinton broth was added. Each plate was subsequently sealed with a sterile adhesive-sealing film to prevent evaporation of the test sample and incubated at 37°C for 24 hours.

**Tetrazolium Salt Reduction Assay**

Various colorimetric assays quantify the survival of cells through reducing salts of tetrazolium into formazan pigments, whereas the color change from colorless to pink can be quantified with a spectrophotometer.¹⁹ After incubation of microplates, 40 µL of indicator solution at a concentration of 5 mg/mL was added to each well of the micro-titer plates and incubated at 37°C for 15 minutes in a shaking incubator (150 rpm). Once an observable color change was observed within the culture control column after completion of the incubation period, the microplate plate reader (BioTek ELX-800) was used to measure the optical density of each plate at 570 nm. As defined by Shekar et al,⁴¹ MIC was the minimum concentration of AgNPs that inhibited 20% of the test microorganisms’ growth. The percentage of bacterial inhibition by AgNPs concentration was computed using the following equation for optical density (OD) after blank correction:

\[
\text{OD} = \frac{(\text{OD of Culture Control} - \text{OD of Test})}{\text{OD of Culture Control}} \times 100
\]

**Minimum Bactericidal Concentration (MBC)**

After measurement of ODs, 100 µL from each well was plated on an MHA plate (Oxoid) and incubated overnight.
in an ordinary incubator at 37°C to determine the least concentration that has no obvious growth (MBC) after 20 hours of incubation.

**In vivo Antimicrobial Activity Assay**  
**Experimental Design**

A total of 35 male mice of 25±3 g body weight were divided into five groups, as listed in Tables 3 and 4, and kept in plastic cages (7 mice/cage) in a well-ventilated room at ambient room temperature and humidity with natural light. Mice were acclimated for 1 week before starting the experiment and were reared with free access to commercial pelleted feed (21% protein) and water. After the acclimatization period, mice were immunosuppressed by intraperitoneal injection with a dose of 30 mg/kg/24 hours of cyclophosphamide (Endoxan®) for 3 successive days. On the last day of immunosuppression, all mice were fasted overnight followed by oral administration of 0.1 mL of 5% solution of sodium bicarbonate (NaHCO₃) to counteract the stomach pH immediately before oral infection. Infection with 1 mL of normal saline solution containing 1.5×10⁸ CFU/mL of the isolated *Salmonella Enteritidis* strain (isolate No.1) given by oral gavage to all infected groups; the other non-infected groups received normal saline only to exclude the stress factor. For verification of infection occurrence, the feces bacterial load of mice was estimated on the last day before and 2 days after infection; the onset of infection showed a gradual rise in bacterial load over the 2 days. The treatment regime was administered for 1 week with 10 μg/Kg body weight daily of AgNPs and 4 mg/Kg body weight daily of cefotaxime to the corresponding treated groups, in combination with administration of saline to other non-treated groups. Cefotaxime has been chosen for positive control group because the third-generation of cephalosporins, including cefotaxime, are successfully used in clinical practice as a therapeutic approach to salmonellosis. As well as the phenotypic sensitivity pattern of the used *S. Enteritidis* strain against cefotaxime was evaluated using the disc diffusion method according to the standards of CLSI, where the zone of inhibition equalled 30 mm which was interpreted according to CLSI, and it was identified as sensitive to cefotaxime (Farouk, unpublished work). All procedures for use of mice model in this research complied with ethics regulations of Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine-Cairo University (FVMCU), and received the approval number: VetCU20022020121.

**Bacteriologic Analyses**

Fecal samples were collected from all groups at the end of the acclimatization period and subjected to routine bacteriologic examination to confirm that mice were free of *Salmonella* spp. The *Salmonella* spp. were counted per gram of feces in all infected groups every day after beginning the treatment protocol. For the feces collected, 1 g was dissolved in 1 mL sterile normal saline, and then 100 μL aliquots from each fecal suspension were serially diluted (10-fold) in sterile saline. Viable counts were determined by plating 10 μL from each dilution on duplicate xylose lysine deoxycholate agar plates that were later incubated at 37°C overnight. The standard colonies were identified as well as counted on the plates. Moreover, the actual count was calculated using the following equation.

\[
\text{CFU/g} = 10^{-3} \times 1/\text{dilution} \times \text{average number of colonies on 2 plates}
\]

**Hematologic and Biochemical Parameters Analyses**

Before dissection, mice were anesthetized using chloroform vapor after the treatment regime ended. Blood samples were obtained into two separate plain and ethylenediaminetetraacetic acid (EDTA)-containing tubes by cardiac puncture. The blood in the EDTA tube was used to estimate parameters of hematology by an automated veterinary hematology analyzer. The serum separation was performed through centrifugation of a plain vacutainer at 3000 x g for 10 minutes and kept at −20°C till estimation of serum albumin, alanine aminotransferase (ALT), urea, and creatinine using dedicated test kits (Spectrum Diagnostics) according to manufacturer instructions.

**Histopathologic Examination**

After treatment completion and blood collection, all mice were euthanized and dissected. Liver and intestine were removed carefully and fixed in formalin saline 10% solution. Cut sections of liver and intestine with 5 μm thickness were prepared and stained with hematoxylin and eosin dye, then microscopically inspected and photographed.

**Statistical Analysis**

All quantitative data obtained from the in vitro study were presented as mean±standard deviation, as shown in Supplementary Tables 1A–E). For the in vivo study, statistical analysis was performed with SAS® version 9.4. A statistical significance was regarded at *P*<0.05. Statistical significance between the first four groups was determined
using two-way ANOVA and one-way ANOVA between the three infected groups. All statistical analysis processes are illustrated in Supplementary Appendix 3.

Results
Isolation of Salmonella spp. From Diarrheic Sheep and Goats
Of the examined 325 and 193 fecal samples from diarrheic sheep and goats, respectively, Salmonella spp. was isolated from 13/325 (4%) and 7/193 (3.6%) samples, respectively (Table 5). All Salmonella spp. isolates were serotyped, as shown in Table 5. The identified isolates were as follows: nine Salmonella Mississipi, two Salmonella Durham, one Salmonella Ferruch, and one Salmonella ParatyphiA for sheep; two Salmonella Enteritidis, one Salmonella Allerton, one Salmonella Bonariensis, one Salmonella Kottbus, one Salmonella Mississipi, and one Salmonella Stanleyville for goats. In addition, agarose gel electrophoresis of polymerase chain reaction products revealed a specific amplicon size of 284 bp in all isolates, as shown in Figure 1. All recovered isolates complied with the typical biochemical identity of Salmonella spp., as shown in Supplementary Appendix 1.

Antimicrobial Phenotypic Resistance Pattern and MAR Indexing
All isolates were resistant to colistin, doxycycline, and oxytetracycline, and all were sensitive to amikacin and enrofloxacin, and, moreover, showed variable patterns to the other five antimicrobials (Table 2). The resistance rates were 95%, 70%, 60%, 15%, and 5% for sulfamethoxazole-trimethoprim, amoxicillin, ciprofloxacin, florfenicol, and gentamycin, respectively. All 20 isolates had an index range of 0.4–0.7 (Table 6), which was identified as MDR Salmonella spp.

Characterization and Concentration of AgNPs
Imaging on TEM showed spherically shaped AgNPs (Figure 2A) with a diameter mean of 6.8±2.28 nm (range 2.95–12.2 nm), the highest frequency of sizes was noted at

Table 3 Overall Mean of Selected Hematological and Serum Biochemical Parameters Following Treatment of Mice in the First Four Groups and Statistical Comparison Between Them

| Parameter/Unit | Mice Groups’ Results as (Mean±SEM) | Group (1) Neutral Control [Non-Infected and Non-Treated] | Group (2) AgNPs Control [Non-Infected and AgNPs-Treated] | Group (3) Negative Control [Infected and Non-Treated] | Group (4) Test Group [Infected and AgNPs-Treated] |
|----------------|------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| RBCs count (10⁹/μL) | 7.65±1.65 | 11.40±1.65 | 9.27±1.64 | 8.23±1.25 |
| PCV (%) | 39.20±5.16 | 42.95±5.16 | 44.70±5.16 | 41.20±3.90 |
| Hb content (g/dL) | 14.30±1.21 | 17.45±1.21 | 14.15±1.21 | 14.33±0.91 |
| WBCs count (10³/μL) | 11.07±3.51 | 13.27±3.51 | 14.82±3.51 | 13.75±2.65 |
| MCV (fl) | 51.31±3.22 | 37.74±2.22 | 48.29±2.22 | 50.13±1.68 |
| MCH (pg) | 18.76±0.38 | 13.00±0.38 | 15.33±0.38 | 17.4±0.29 |
| MCHC (g/dL) | 37.14±1.07 | 34.99±1.07 | 32.31±1.07 | 35.44±0.81 |
| Lymphocytes (10³/μL) | 5.5±3.01 | 5.3±1.01 | 7.56±1.01 | 6.93±0.01 |
| Monocytes (10³/μL) | 1.10±0.00 | 2.65±0.01 | 2.96±0.00 | 1.39±0.00 |
| Eosinophils (10³/μL) | 0.99±0.002 | 0.93±0.002 | 0.51±0.002 | 0.97±0.002 |
| Basophils (10³/μL) | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| Segmented Neutrophils (10³/μL) | 3.1±0.00 | 3.9β±0.00 | 2.97±0.00 | 4.15±0.00 |
| Band Neutrophils (10³/μL) | 0.33±0.001 | 0.40β±0.001 | 0.82β±0.001 | 0.31β±0.001 |
| Albumin (g/dL) | 3.34±0.24 | 3.20±1.08 | 3.07±1.08 | 2.91±1.08 |
| ALT (μL) | 98.00±19.54 | 103.83±11.28 | 138.33±15.95 | 130.8±12.36 |
| Urea (mg/dL) | 49.67±6.43 | 59.20±4.98 | 68.00±7.87 | 63.17±4.54 |
| Creatinine (μL) | 0.48±0.08 | 0.50±0.06 | 0.46±0.09 | 0.44±0.06 |

Note: Means within the same row with different letters are statistically significantly different, the other with common letters are not statistically different, and there is no evidence of significance in other variables without letters (at P<0.05).
Table 4 Overall Mean of Selected Hematological and Serum Biochemical Parameters Following Treatment of Mice in the Three Infected Groups and Statistical Comparison Between Them

| Parameter/Unit | Mice Groups’ Results (Mean±SEM) |
|----------------|----------------------------------|
|                | Group (3) Negative Control [Infected and Non-Treated] | Group (4) Test Group [Infected and AgNPs-Treated] | Group (5) Positive Control [Infected and Cefotaxime Treated] |
| RBCs count (10^6/μL) | 9.27±1.64 | 8.23±1.25 | 7.79±0.35 |
| PCV (%) | 44.70±5.16 | 41.20±3.90 | 37.53±1.45 |
| Hb content (g/dL) | 14.15±1.21 | 14.33±0.91 | 13.77±0.77 |
| WBCs count (10^3/μL) | 14.82±3.51 | 13.75±2.65 | 9.56±2.32 |
| MCV (fl) | 48.29±2.22 | 50.13±1.68 | 48.24±0.69 |
| MCH (pg) | 15.33±0.38 | 17.48±0.29 | 17.74±0.31 |
| MCHC | 32.31±1.07 | 35.44±0.81 | 37.35±0.09 |
| Lymphocytes (10^3/μL) | 7.56±0.01 | 6.93±0.00 | 5.66±0.00 |
| Monocytes (10^3/μL) | 2.96±0.00 | 1.39±0.00 | 1.89±0.00 |
| Eosinophils (10^3/μL) | 0.51±0.002 | 0.97±0.002 | 0.57±0.002 |
| Basophils (10^3/μL) | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| Segmented Neutrophils (10^3/μL) | 2.97±0.00 | 4.15±0.00 | 1.04±0.00 |
| Band Neutrophils (10^3/μL) | 0.02±0.001 | 0.31±0.00 | 0.40±0.001 |
| Albumin (g/dL) | 3.07±0.10 | 2.91±0.11 | 2.73±0.14 |
| ALT (μL) | 138.33±15.95 | 130.80±12.36 | 118.33±0.33 |
| Urea (mg/dL) | 68.00±7.87 | 63.17±4.54 | 46.33±4.67 |
| Creatinine (μ/L) | 0.46±0.09 | 0.44±0.06 | 0.47±0.07 |

Note: Means within the same row with different letters are statistically significant different and there is no evidence of significance in other variables without letters (at P<0.05).

The size of 10 nm, as shown in the histogram (Figure 2B), the average particle size distribution of the prepared AgNPs by volume is shown in Figure 3A, and the highest peak of size distribution was observed at 24.77 nm. Zeta potential analysis results showed that AgNPs were carrying a negative charge of −37.3±5.7 mV (Figure 3B), the FT-IR spectrum confirmed AgNPs synthesis and stability due to the reduction and capping by PVP and showed seven peaks (Figure 4) at the following wave numbers: 3463, 2852, 2072, 1638, 1391, 1294, and 550 cm⁻¹. Moreover, measurement by ICP-AES demonstrated 5.096 μg/mL of silver ion concentration.

Table 5 Isolation Rate and Serotyping of Salmonella Species Isolates

| Isolate Origin | No. Examined | Positive Samples for Salmonella Species | Isolation Rate (%) |
|----------------|--------------|----------------------------------------|-------------------|
|                |              | Serotype Name (Number of Isolates)      | Total Number      |
| Diarrheic sheep | 325          | S. Mississippi (9) S. Durham (2) S. Ferruch (1) S. ParatyphiA (1) | 13 | 4% |
|                |              | S. Enteritis (2) S. Allerton (1) S. Bonariensis (1) S. Kottbus (1) S. Mississippi (1) S. Stanleyville (1) | 7 | 3.6% |

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In vitro Antibacterial Activity of AgNPs

Bacterial Growth Inhibition

The synthesized AgNPs exhibited growth inhibiting activity against all 20 tested strains of MDR Salmonella spp., and the bacterial count was less than the initial count before incubation (≤5×10⁵ CFU/mL) for all tested isolates.

MIC and Viability % of Salmonella spp

The MICs of the synthesized AgNPs ranged from ≤0.002–0.313 μg/mL, and the mean average was 0.085±0.126 μg/mL (Table 6). The AgNPs concentrations ranged from 5 μg/mL to 0.002 μg/mL, which were assessed for their efficacy in the present study, as detailed in Tables 7–9.

MBC

The initial MBCs were recorded as the lowest AgNPs concentration to show no change in color when the color of the culture control row matches that of the pH endpoint control (Figure 5). Final readings were recorded after incubation of inoculated MHA plates, which was determined by no obvious growth of the corresponding dilution comparison with obvious growth of culture control inoculated plates. The MBCs ranged from 0.078–1.250 μg/mL with an average mean of 0.508±0.315 μg/mL (Table 6).

In vivo Antibacterial Activity

Treatment Efficacy

The experimental infection of mice was confirmed by noticing a steady increase in the viable count of Salmonella Enteritidis in feces within 2 days of infection. Oral administration of AgNPs was found to induce noticeable decreases in the count of viable Salmonella Enteritidis retrieved from feces, as shown in Figure 6, where the numbers of viable S. Enteritidis were 4.4×10⁴, 2.16×10⁴, and 1.507×10⁴ CFU/g feces in the first, second, and third day of treatment, respectively. Oral treatment with cefotaxime resulted in quite a decrease in the viable count in comparison to the AgNPs treated group, where the viable S. Enteritidis counts were 6.9×10³, 3.6×10³, and 3.4×10³ CFU/g feces in the first, second, and third day of treatment, respectively. Salmonella Enteritidis shedding in feces stopped during days 4 and 6 of the treatment protocol in both the AgNPs and cefotaxime treated groups. Also, the viable counts of S. Enteritidis decreased in the feces of the other infected control group, and shedding stopped 2 days later in comparison with the other two treated groups.

Effect of the Synthesized AgNPs on Hemato-Biochemical Parameters

In comparison between the first four groups, as shown in Table 3, a significant difference was evident in some parameters, including band neutrophils count, mean corpuscular hemoglobin concentration (MCHC), serum albumin, and ALT. Moreover, there was no evidence of significance for other parameters. Regarding band neutrophils count, there was a significant increase in the infected and non-treated group compared with the other three groups, which did not differ from each other due to significant effects of infection (F₁,₁₅=4.61, P=0.0485), treatment (F₁,₁₅=5.90, P=0.0281), and infection-treatment interaction (F₁,₁₅=5.90, P=0.0281). In the case of MCHC, serum albumin, and ALT parameters, there was a significant decrease in albumin and an increase in MCHC and ALT in both infected groups versus the non-infected, and no evidence of difference between both the infected groups and both the non-infected groups, however, differences were noted due to the infection effect for albumin (F₁,₁₅=5.93, P=0.0301), ALT (F₁,₁₅=5.31, P=0.0383), and MCHC (F₁,₁₅=5.35, P=0.0353). In comparing the three infected groups, however, the only significant evidence was in band neutrophils count, and the three groups were completely different from each other, as shown in Table 4. The count was the highest in the infected and nontreated group, followed by the count of the infected and cefotaxime-treated group, then the infected and AgNPs-treated group (F₂,₁₅=4.91, P=0.0299).

Effect of the Synthesized AgNPs on the Histology of Liver and Intestine

Histopathologic lesions were mainly observed in the negative control group of S. Enteritidis-infected and non-treated mice. The inflammatory and degenerative lesions were predominant in liver, including congestion of hepatic blood vessels, acute inflammatory response with...
infiltration of mononuclear cells, dilatation of the hepatic sinusoids, perivascular cuffing with inflammatory cells, and subcapsular edema (Figure 7A–F). The intestine samples showed necrobiotic changes and acute inflammatory reaction (Figure 7G). In contrast, the infected and AgNPs-treated group expressed a mild inflammatory reaction that was less than that for the non-treated groups and maintained the integrity and structure of hepatocytes, whereas the intestine showed a normal histologic pattern (Figure 8A–C). A similar normal histological pattern with mild pathological changes was noted in the AgNPs-treated non-infected group (Figure 8D–F).

### Discussion
Recently the epidemiology of *Salmonella* spp. and recently evolved resistant strains have caused concern in both human and veterinary medicine because of their zoonotic impact. Although the existence and spread of MDR strains in seemingly healthy slaughtered sheep and goats have previously been recorded, there is less information about its prevalence in diseased sheep and goats. In the current study of diarrheic sheep and goats all within the Giza Governorate in Egypt, *Salmonella* spp. were isolated in 4% (13/325) of fecal samples in sheep and from 3.6% (7/193) of the sample in goats (Table 5). Nearly similar isolation rates were reported in other studies of diarrheic lambs, sheep, and goats: 3.6% in diarrheic lambs, 4.5% among diarrheic sheep, and 5.26% in both diarrheic sheep and goats. The concern of antibiotic resistance bacteria in Africa has been gaining particular interest throughout the previous decade. Furthermore, very little has been understood concerning the significant extent of the threat, because monitoring for antibiotics resistance to date has been only implemented within a limited number of countries. In the current study, all recovered isolates were detected as MDR based on their MAR range of >0.3 to <0.8. However, these isolates showed different phenotypic resistant patterns to the 10 tested antimicrobial agents. The most evident pattern of complete resistance (100%) was seen for colistin, doxycycline, and oxytetracycline, followed by 95%, 70%, and 60% for sulfamethoxazole–trimethoprim, amoxicillin, and ciprofloxacin, respectively. Moreover, the most effective

### Table 6 MDR Index and Overall Antimicrobial Activity of the Synthesized Silver-Nanoparticles on Tested MDR *Salmonella* Species Strains (MIC, MBC, and Inhibition % at MIC)

| Serotype Name (Isolate Number) | No. of Antimicrobials to Which are Resistance | MAR Index | Antimicrobial Activity of AgNPs Inhibition % at Which MIC Calculated | MIC (μg/mL) | MBC (μg/mL) | MBC/MIC Ratio |
|--------------------------------|---------------------------------------------|------------|---------------------------------------------------------------|------------|------------|---------------|
| S. ParasyphiA                  | 6                                           | 0.6        |                                                               | 26.14      | ≤0.002     | 312.5         |
| S. Farcomm                  | 4                                           | 0.4        |                                                               | 23.13      | 0.313      | 75            |
| S. Durham (No.1)             | 7                                           | 0.7        |                                                               | 32.89      | 0.005      | 654           |
| S. Durham (No.2)             | 6                                           | 0.6        |                                                               | 20.96      | 0.010      | 2096          |
| S. Mississippi (No.1)        | 5                                           | 0.5        |                                                               | 32.13      | 0.313      | 102           |
| S. Mississippi (No.2)        | 6                                           | 0.6        |                                                               | 20.27      | 0.010      | 2027          |
| S. Mississippi (No.3)        | 6                                           | 0.6        |                                                               | 21.39      | ≤0.002     | 2139          |
| S. Mississippi (No.4)        | 5                                           | 0.5        |                                                               | 34.27      | ≤0.002     | 3427          |
| S. Mississippi (No.5)        | 5                                           | 0.5        |                                                               | 29.10      | 0.010      | 2910          |
| S. Mississippi (No.6)        | 5                                           | 0.5        |                                                               | 25.85      | 0.005      | 2585          |
| S. Mississippi (No.7)        | 4                                           | 0.4        |                                                               | 22.24      | 0.010      | 2224          |
| S. Mississippi (No.8)        | 4                                           | 0.4        |                                                               | 25.39      | 0.020      | 2539          |
| S. Mississippi (No.9)        | 5                                           | 0.5        |                                                               | 20.23      | 0.313      | 64.17         |
| S. Stanleyville            | 6                                           | 0.6        |                                                               | 21.66      | 0.313      | 68.67         |
| S. Mississippi (No.10)       | 6                                           | 0.6        |                                                               | 20.09      | 0.005      | 2009          |
| S. Kortbus                 | 5                                           | 0.5        |                                                               | 29.85      | ≤0.002     | 2985          |
| S. Bonariensis              | 7                                           | 0.7        |                                                               | 22.65      | 0.078      | 286.09        |
| S. Allerton                | 6                                           | 0.6        |                                                               | 34.32      | ≤0.002     | 3432          |
| S. Enteritidis (No.1)        | 5                                           | 0.5        |                                                               | 27.60      | ≤0.002     | 2760          |
| S. Enteritidis (No.2)        | 6                                           | 0.6        |                                                               | 21.90      | 0.020      | 1.09          |
agents were amikacin, enrofloxacin, gentamicin, and florfenicol (Table 2).

The emergence of such MDR serovars acts as a key factor in therapeutic failure for treatment of infections in both animals and humans, constitutes a great challenge for this pathogen control, and advocates for the need to investigate antimicrobial alternatives as a method of control. Owing to their antimicrobial activity, metallic nanoparticles may serve as potential alternatives for some traditional antibiotics. This study integrated AgNPs through a chemical reduction reaction. For this integration, AgNO3, NaBH4, and PVP were used as a source for Ag+, a reducing agent, and stabilizing agent to prevent agglomeration of particles, respectively. Trisodium citrate acted simultaneously as both stabilizing and reducing agent. Furthermore, the use of PVP and sodium citrate improved the stability of newer nanoparticles.

In the current study, AgNPs were integrated through chemical reduction reaction to clarify its ability to overcome all clinically recovered MDR Salmonella spp. isolates and it showed efficient antimicrobial activities against all of them. Furthermore, it showed efficient inhibitory activities on all isolates ranging from 84.81–99.92% at concentration 5 μg/mL, MIC range (≤0.002–0.313 μg/mL) with an average mean of 0.085±0.126 μg/mL, and MBC range (0.078–1.250 μg/mL) with an average of 0.508±0.315 μg/mL. These findings are compatible with results of a previous study that reported the antibacterial activity of AgNPs against Salmonella Typhimurium. The MBC/MIC (Table 6) ratio of AgNPs for different isolates showed variant bacteriostatic to bactericidal effects of AgNPs, which may be justified by the different resistance patterns of the tested isolates. The bactericidal effect of AgNPs is attributed to the reaction between the charges of bacterial cell wall and metal ions. Moreover, the main factors affecting bactericidal properties are stability, size, surface area, and shape of AgNPs.

The FTIR spectrum
Table 7 Percentage of Bacterial Inhibition of S. ParatyphiiA, S. Ferruch, S. Durham (No. 1 and 2) and S. Mississippi (No. 1-3) Isolates at Different Serial Dilutions of Silver Nanoparticles

| Conc. (µg/mL) | Inhibition % |
|--------------|--------------|
|              | S. ParatyphiiA | S. Ferruch | S. Durham (No. 1) | S. Durham (No. 2) | S. Mississippi (No. 1) | S. Mississippi (No. 2) | S. Mississippi (No. 3) |
| 5.000        | 99.34         | 98.89      | 82.23             | 86.35             | 97.21                | 92.78                  | 94.87                   |
| 2.500        | 92.12         | 97.93      | 66.02             | 79.69             | 97.16                | 89.22                  | 71.20                   |
| 1.250        | 85.51         | 29.57      | 60.10             | 76.57             | 45.26                | 56.93                  | 64.16                   |
| 0.625        | 65.41         | 27.77      | 51.27             | 64.65             | 41.16                | 53.48                  | 56.08                   |
| 0.313        | 33.88         | 23.13      | 45.59             | 63.76             | 32.13                | 50.70                  | 35.27                   |
| 0.156        | 31.90         | 19.97      | 44.14             | 47.37             | 19.72                | 40.43                  | 34.41                   |
| 0.078        | 30.72         | 19.27      | 43.17             | 43.41             | 18.60                | 26.43                  | 31.18                   |
| 0.039        | 29.11         | 17.57      | 41.35             | 35.44             | 13.43                | 23.28                  | 30.42                   |
| 0.020        | 28.31         | 14.26      | 39.18             | 32.56             | 11.10                | 20.49                  | 30.04                   |
| 0.010        | 26.62         | 13.52      | 38.81             | 20.96             | 5.42                 | 20.27                  | 26.62                   |
| 0.005        | 26.61         | 11.76      | 32.39             | 18.21             | 4.82                 | 19.76                  | 26.05                   |
| 0.002        | 26.14         | 11.17      | 16.44             | 11.50             | 3.70                 | 14.88                  | 21.39                   |

Note: Concentration inhibiting at least 20% of bacterial growth is considered MIC.

Table 8 Percentage of Bacterial Inhibition of S. Mississippi (No. 4-9) and S. Stanleyville Isolates at Different Serial Dilutions of Silver Nanoparticles

| Conc. (µg/mL) | Inhibition % |
|--------------|--------------|
|              | S. Mississippi (No. 4) | S. Mississippi (No. 5) | S. Mississippi (No. 6) | S. Mississippi (No. 7) | S. Mississippi (No. 8) | S. Mississippi (No. 9) | S. Stanleyville |
| 5.000        | 92.45         | 99.77         | 99.91             | 92.53             | 84.81                 | 99.66                  | 93.47                   |
| 2.500        | 86.73         | 84.21         | 72.91             | 85.11             | 67.08                 | 36.61                  | 87.86                   |
| 1.250        | 72.27         | 80.21         | 68.08             | 79.98             | 65.80                 | 33.67                  | 75.26                   |
| 0.625        | 48.82         | 80.17         | 57.87             | 70.75             | 61.01                 | 29.44                  | 69.08                   |
| 0.313        | 40.18         | 76.17         | 57.50             | 69.68             | 59.08                 | 20.23                  | 64.87                   |
| 0.156        | 39.36         | 65.35         | 57.50             | 68.53             | 47.68                 | 16.16                  | 43.21                   |
| 0.078        | 37.64         | 59.02         | 51.66             | 60.87             | 28.98                 | 15.20                  | 21.66                   |
| 0.039        | 35.64         | 40.51         | 35.11             | 46.13             | 26.97                 | 13.67                  | 17.03                   |
| 0.020        | 35.00         | 38.11         | 27.86             | 27.40             | 25.39                 | 8.42                   | 16.92                   |
| 0.010        | 34.55         | 29.10         | 28.80             | 22.24             | 15.38                 | 6.84                   | 16.82                   |
| 0.005        | 34.45         | 15.41         | 25.85             | 15.85             | 2.16                  | 2.43                   | 14.97                   |
| 0.002        | 34.27         | 15.79         | 13.45             | 14.24             | 1.04                  | 2.32                   | 14.40                   |

Note: Concentration inhibiting at least 20% of bacterial growth is considered MIC.

reveals the stability of the synthesized AgNPs where the silver ion reduction and PVP-capping of AgNPs occur simultaneously; the FT-IR spectrum shows strong bonding at peak 1 at wavenumber 3463 cm⁻¹, which corresponds to the N-H stretches with the amine group, and peak 2 at 2852 cm⁻¹, which corresponds to C–H stretches with the alkenes group. A strong absorption peak at 1638 cm⁻¹ is corresponding to carbonyl group stretching of PVP, indicating the PVP capping for AgNPs which prevents its reaggregation and agglomeration. The two peaks at 1391 and 1294 cm⁻¹ are reported for bond vibrations of N–H–O complex and NO₃ group, respectively. The last band at 550 cm⁻¹ corresponds to carbonyl stretch. The synthesized AgNPs in this study are spherical in shape and this finding may be attributed to its wider antibacterial activity in inhibiting all isolates and they are carrying a negative charge of −37.3±5.7 mV, which indicates good stability of the synthesized particles. The negative charge of AgNPs is attributed to the interaction between the PVP and metallic nanoparticles surface. Moreover, the bactericidal efficacy of AgNPs against gram-negative bacteria depends on AgNPs concentration. It was observed that the percent of inhibition and bactericidal efficacy of
Table 9 Percentage of Bacterial Inhibition of S. Mississippi (No. 10), S. Kottbus, S. Bonariensis, S. Allerton and S. Enteritidis (No. 1 and 2) Isolates at Different Serial Dilutions of Silver Nanoparticles

| Conc. (µg/mL) | Inhibition % |
|----------------|-------------|
|                | S. Mississippi (No. 10) | S. Kottbus | S. Bonariensis | S. Allerton | S. Enteritidis (No. 1) | S. Enteritidis (No. 2) |
| 5.000          | 89.48        | 99.19      | 97.57         | 99.81        | 99.92                  | 99.01                      |
| 2.500          | 81.14        | 95.20      | 94.33         | 99.42        | 96.96                  | 88.55                      |
| 1.250          | 75.81        | 51.95      | 31.64         | 88.48        | 91.27                  | 54.18                      |
| 0.625          | 60.96        | 51.77      | 30.47         | 77.02        | 78.99                  | 53.51                      |
| 0.313          | 59.45        | 45.63      | 28.89         | 70.27        | 70.22                  | 48.69                      |
| 0.156          | 53.85        | 43.74      | 24.55         | 60.37        | 66.06                  | 44.68                      |
| 0.078          | 50.57        | 32.54      | 22.65         | 56.56        | 42.01                  | 42.91                      |
| 0.039          | 49.66        | 32.31      | 19.07         | 40.93        | 33.68                  | 38.63                      |
| 0.020          | 42.87        | 31.46      | 17.09         | 40.47        | 33.48                  | 21.90                      |
| 0.010          | 30.71        | 30.84      | 16.46         | 38.23        | 32.56                  | 19.67                      |
| 0.005          | 20.09        | 30.84      | 10.85         | 37.74        | 29.12                  | 18.50                      |
| 0.002          | 11.03        | 29.85      | 10.26         | 34.32        | 27.60                  | 6.73                       |

Note: Concentration inhibiting at least 20% of bacterial growth is considered MIC.

The synthesized AgNPs were in a direct proportional relationship with AgNPs concentration (Tables 7–9), as shown in a previous report by Swarnavalli et al.58 which noted that the higher antibacterial effects were observed with increasing the AgNPs concentration. Furthermore, the bactericidal effects of AgNPs increased by decreasing its size.58,61 The size range of synthesized AgNPs is 2.95–12.2 nm, based on TEM measurements, while the DLS analysis showed an average of particle size distribution (at 24.77 nm). DLS measurements are affected by dispersants, which results in higher size values than obtained by TEM analyses,62 which is also similar to the previous findings.58,61 and is considered a key factor responsible for the observed antimicrobial activity. This finding can be justified as follows: the maximum contact area, surface area-to-volume ratio, and AgNPs relative concentration will be increased when AgNPs size decreases.58,61 This relationship can be the result of the powerful penetration of the nanoparticles and a wide area of interaction between the nanoparticles and microorganisms.58,61 The previous in vivo studies did not declare the phenotypic resistance characteristics of the challenge strain used, so in the present study in vivo antimicrobial activity of integrated AgNPs was visualized against MDR S. Enteritidis, which was recovered from a diarrheic goat with MAR value of 0.5. It was obvious that this strain was pathogenic to mice based on histopathologic and hemato-biochemical evidence in infected mice, as well as a marked decrease of the number of viable S. Enteritidis isolates recovered from feces of the treated groups, with shedding stopped completely between days 4 to 6 of treatment. In addition, the viable count gradually decreased during the treatment period, and there was a difference in cefotaxime-treated and AgNPs-treated groups, which are findings compatible with those of Abd-Elhakeem et al.26 Also, the therapeutic dose of AgNPs was less than that of the cefotaxime dose, and the efficacy of AgNPs versus cefotaxime was greater in decreasing bacterial count. Therefore, our study

Figure 5 A microtiter plate showing the colorimetric assay for detection of the viability percent using tetrazolium salt reduction test, yellow color indicates no viable bacteria; pink color indicates viability of bacteria. Well (A1): left empty as blank according to microplate reader software instructions; Culture control wells (A2–A12) contain 100 µL of sterile Mueller-Hinton broth and 100 µL of subculture broth containing 1.5×10⁵ CFU/mL; Control negative row (B) contains double-fold, serially diluted AgNPs at a starting concentration of 5 µg/mL in 100 µL of sterile Mueller-Hinton broth and 100 µL of subculture broth containing 1.5×10⁵ CFU/mL; Control negative row (C) contains double-fold, serially diluted ciprofloxacin at starting concentration of 0.1 mg/mL in 100 µL of sterile Mueller-Hinton broth and 100 µL of subculture broth containing 1.5×10⁵ CFU/mL; Test rows (D, E, and F): contain double-fold, serially diluted AgNPs at starting concentration of 5 µg/mL in 100 µL of sterile Mueller-Hinton broth and 100 µL of subculture broth containing 1.5×10⁵ CFU/mL where the isolate tested in triplicate. Wells in rectangles (D5, E5, and F5) indicate the MBC against the isolate where there was no change in color when the color of the culture control row matches that of the pH endpoint control.
spotlights the in vivo efficacy of AgNPs on MDR S. Enteritidis strain in comparison to cefotaxime use as the conventional antibiotic.

Hematologic, biochemical, and histopathologic examinations in different groups were used to ascertain whether AgNPs had no toxic or pathologic effect in the AgNPs control group and to evaluate its ability in the tissue healing process following treatment in the test group. Regarding the hematologic findings, as shown in Table 3, there was a significant increase in band neutrophils count in the infected and non-treated group versus the other three groups due to the significant effects of infection, treatment, and infection–treatment interaction. These findings may be attributed to infection that resulted in an increased band neutrophils count. Treatment decreased band neutrophils count, and, with regard to the treatment effect as shown in Table 4, the count was significantly different between three groups and the count was higher in infected and non-treated group than in both other groups. Also, it was observed that count decreased in both treated groups compared with the non-treated group. However, the treatment effect of AgNPs was more evident in decreasing the count than in the cefotaxime-treated group. Furthermore, with regard to the infection effect, there was a significant decrease in albumin and an increase in MCHC and ALT in both infected groups versus the other non-infected groups, and no evidence of difference between both infected groups and between both non-infected groups. The increase of liver enzyme ALT and decrease in albumin is supported by previous studies, which reported that Salmonella spp. infection results in hepatic granulomas or paratyphoid nodules, causing release of liver enzymes in serum and decreasing its ability to produce albumin. In addition, previous studies found that the significant difference in the increased band neutrophils count in the non-treated versus treated groups may be attributed to the contributions of neutrophils in the effort for salmonellosis control. The response to strong inflammatory stimulus leads to an increased demand for neutrophils production and thus results in left shift neutrophilia because of the liberation of immature band neutrophils into circulation. Although the significant decrease in neutrophils may be attributed to the ability of mice to respond to treatment and the efficacy of AgNPs and cefotaxime in eliminating infection and decreasing inflammatory reactions, the effect of AgNPs is more evident than that for cefotaxime. So the hematobiochemical findings provided evidence for the clinical pathologic effect of the challenge of the S. Enteritidis strain in infected groups and the ability of AgNPs to suppress the developed inflammatory reaction. Moreover, there was no evidence of significant adverse toxic or pathologic effects of AgNPs on these test subjects, especially liver and kidney function tests which act as valuable indicators for toxicity; liver and kidneys are responsible for detoxification and excretion.

Mice are considered an ideal model for studying Salmonella spp. pathogenesis because they are a natural host. However, the limitation in this model is the absence of diarrhea in mice after oral infection. Oral streptomycin was administered before experimental infection with S. Typhimurium to create the ideal clinical signs of diarrhea. Nevertheless, because the aim was to evaluate the therapeutic effect of AgNPs, the use of streptomycin may have altered the results. Alternatively, a 3-day regime of I/P cyclophosphamide can be used as an immunosuppressive agent before

![Figure 6 Effects of treatment with AgNPs and cefotaxime on fecal shedding of Salmonella Enteritidis (CFU/g) in comparison to the infected and non-treated group.](image-url)
Figure 7 Microphotograph of histopathology lesions from Salmonella enteritidis-infected and nontreated mice (HE stain). (A) Liver shows congestion of central and portal veins (arrows) and focal aggregations of mononuclear inflammatory cells, in addition to the presence of inflammatory cells surrounding the bile duct in the portal area. (B) Subcapsular edema in the liver and dilatation of hepatic sinusoids (arrow). (C) Perivascular cuffing of dilated central vein of the liver with inflammatory cells (arrow). (D) Vacuolar degeneration and necrotic changes of hepatocytes with mononuclear cell infiltration between the hepatic cords. (E) Focal aggregation of mononuclear inflammatory cells, mainly histiocytes, between the degenerated hepatocytes (arrow). (F) View of the central vein of the liver surrounded completely by mononuclear inflammatory cells (arrow). (G) Intestine with necrotic enteritis, vasculature, and destruction of enterocytes and diffuse inflammatory cell response.
oral infection.\textsuperscript{42} The pathologies induced by the infection of S. Enteritidis are mainly inflammatory, with the inflammatory findings in liver similar to those in previous studies on mice,\textsuperscript{42,68} including congestion, edema, and inflammatory cells infiltrations. The necrotic changes in intestine are in line with Lee et al.\textsuperscript{69} The findings of this current study showed that in the groups treated with AgNPs, the severity of inflammatory response is greatly reduced. This finding is attributed partially to killing activity of AgNPs toward gram-negative microorganisms.\textsuperscript{70–72} The other action of AgNPs is an anti-inflammatory effect,\textsuperscript{73} which helps reduce the severity of inflammation induced by salmonellosis. Finally, the in vivo study declared the safety and potency of the integrated AgNPs in the treatment of MDR salmonellosis so it can serve as an alternative antimicrobial, especially at the occurrence of antibiotic-resistant bacterial infections.\textsuperscript{74}

**Conclusion**

This study focused on MDR *Salmonella* spp. in diarrheal sheep and goats in Egypt for which conventional antimicrobial therapy may fail to provide a response, the ability of AgNPs in their inhibition of *Salmonella* spp. both in vitro and in vivo without adverse effects, and an estimation of MIC to determine an effective concentration for when a commercial product can be prepared. The study findings support that AgNPs may be used as a therapeutic agent. However, further studies are needed to develop and design a safe and effective delivery system of AgNPs for small ruminants.
Abbreviations
Ag⁺ ions, silver ions; AgNPs, silver nanoparticles; ALT, alanine aminotransferase; CFU, colony forming unit; ICP-AES, inductively coupled plasma atomic emission spectroscopy; MAR, multiple antimicrobial resistance; MBC, minimum bactericidal concentration; MCHC, mean corpuscular hemoglobin concentration; MDR, multidrug resistant; MHA, Mueller-Hinton agar; MIC, minimum inhibitory concentration; OD, optical density; PVP, polyvinyl pyrrolidone; TEM, transmission electron microscope.

Data Sharing Statement
All data used to generate these results is available in the main text.

Ethics Approval and Consent to Participate
The study was approved by Ethics of Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine-Cairo University (FVMCU) and received the approval number: VetCU20022020121.

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Author Contributions
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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