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The Different Antibacterial Impact of Silver Nanoparticles Against *Legionella pneumophila* Compared to Other Microorganisms

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Abstract

Legionella pneumophila is the pathogen responsible for severe pneumonia known as Legionnaires' disease. *Legionella* can live under varied stress conditions, especially in cold environments, and is common in many artificial environments. In this study, the antimicrobial activity of biogenic silver nanoparticles, prepared using the culture supernatant of *Klebsiella pneumoniae,* was evaluated against different important microorganisms, especially *L. pneumophila*. Compared with all bacterial test strains, *L. pneumophila* showed lower susceptibility against silver nanoparticles (MIC 30 µg/mL). The MICs of silver nanoparticles were 10 µg/ml for *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Salmonella typhi*. *In addition, the MIC values of the* silver nanoparticles *for the fungal teststrain(Aspergillus niger* and *Candida albicans)* were 20 and 30 µg/ml, respectively. The time-kill course antibacterial assay of silver nanoparticles at its MIC concentration (30 µg/mL) was performed against *L. pneumophila* at cold (4 °C) and ambient (25 °C) temperatures. The results showed that a higher mortality rate was observed at 4 °C. In contrast, at 25 °C,a considerable tolerance was observed for *L. pneumophila* against 30 µg/ml of silver nanoparticles during the 24-h exposure time. Reserpine as a natural efflux inhibitor (10 µg/mL) was used in this study to reverse the resistance of *L. pneumophila* against silver nanoparticles at 25 °C. Higher anti-legionella activity was detected in the presence of reserpine at the mentioned temperature that indicated that efflux pumps may have an important role in the resistance of *L. pneumophila* against silver nanoparticles at 25 °C.

Keywords: Antimicrobial activity; efflux pump; hospital infection; Legionella; silver nanoparticles

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Introduction

The application of nanoparticles in health science has been increased during the past decades[1, 2, 24]. Silver nanoparticles (Ag NPs) have been well known to inhibit the growth of different pathogenic strains, making this nanomaterial a potent antimicrobial agent [5]. Recently, several reports have been published in the literature on the antimicrobial activity of Ag NPs prepared with different chemical or biological methods against a wide varities of pathogenic microorganisms [10, 11, 17,18, 21].However, to our knowledge, there is no study on the antibacterial effrct of Ag NPs against *Legionella pneumophila* as a life-threatening resistant bacteria, especially in immunocompromised and bacteremic patients [7]. There are several serious infectious diseasescaused by this bacterium such as a self-limited flu-like illness (Pontiac fever) and a severe pneumonia called Legionnaires' disease [8,9].

Legionella can survive under various stress conditions such as cold environments, primarily in hospital cooling towers, potable water systems, large central air conditioning systems, domestic hot water systems, fountains, and swimming pools. [14,16, 22]. The health care acquired infections caused by *Legionella* spp. have been mostly associated with water distributorsand/or air conditioner in health care facilities [14,16, 22]. Methods of controlling Legionella regardless of extensive research studies such as water source disinfection still is a hot topic in health care research. [6, 16,20, 25].

tThe antibacterial effect of biogenic Ag NPs, which itprepared using the culture supernatant of *K. pneumonia*, investigated against an isolate of *L. pneumophila* with the agar serial dilution method and the time-killcourse assay. In addition, the antibacterial activity of fresh prepared biogenic Ag NPs was further investigated with the agar serial dilution method against other test strains, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Salmonella typhi*, *Aspergillus niger,* and *Candida albicans*.

Materials and Methods

Bacterial test strain

L. pneumophila isolated from a fish pond near Ahwaz City, Khuzestan Province, Iran. A subculture of bacterium was grown on buffered charcoal yeast extract agar (BCYE-α, MAST Group Ltd., UK) supplemented with glycine (3g/L), anisomycin (0.08g/L), vancomycin (0.001g/L), and polymyxin B (50000 IU/L). These supplemental materials also purchased from MAST Group Ltd., UK. Plates incubated at 37 °C in humidified air with 2.5% CO_2 for 72-96 h. Gram staining, morphology of colonies on selected media and biochemical oxidase, and catalase tests selected for phenotype and physiological characterizations of the isolate according to the standard method [3, 23].

Furthermore, the identification procedures confirmed with the 16s rDNA fragment amplification method using polymerase chain reaction (PCR). A single colony picked up and suspended in 50 µl of distilled water and lysed by heating at 95 °C for 5 min. Cell lysates, after centrifugation, used for PCR amplification. Amplification of 16S rDNA performed using forward and reverse primers as follows:

Forward 5' AgAgTTTgATCCTggCTCAg 3'
Reverse 5' TACggTTACCTTgTTACgACTT 3'

The reaction mixture composed of 1.5 mM $MgCl_2$, 10 mM PCR buffer, 200 µM dNTP (deoxynucleotide), 20 pmol of each forward and reverse primer, 2 µl of lysed cell suspension, and 1.25 U Super *Taq* DNA polymerase and distilled water up to 25 µl. All of the PCR reagents were purchased from Gen Fanavaran Co. (Tehran, Iran). The mixture was incubated at 94 °C for 2 min and then cycled 30 times as the following profile: 94 °C for 20s, 56 °C for 30s, 72 °C for 1.5 min, and then incubated for 7 min at 72 °C. The amplified DNA fragments were purified from agarose 1% gel using the QIAquick gel extraction kit (Qiagen, USA) according to the supplier's instructions and sent for automated sequencing using the above primers (Takapouzist Co., Iran). The obtained 16S rDNA sequence aligned with nucleotide databases using the BLAST program (National Center for Biotechnology Information), and the sequence submitted to GeneBank.

Biosynthesis of Ag NPs

Biogenic Ag NPs with particle size of < 100 nm prepared with the previously described method [15]. Briefly, Müller-Hinton Broth (MHB) prepared, sterilized, and inoculated with a fresh growth of *K. pneumonia*. The cultured flask was incubated at 37 °C for 24 h. After incubation, the culture centrifuged at 10,000 g, and the supernatant used to synthesize silver nanoparticles. Aqueous silver nitrate solution (10^{-3} M) separately added to reaction vessels containing supernatant of the test strain (1%, v/v), and the resulting mixture allowed to stand for 20 min under visible-light emission (1000 mmol/m^2s) generated by a 75W halogen lamp. The irradiated visible light's densities recorded using a DO9721K Datalogger Photo Radiometer. The properties of the freshly prepared Ag NPs confirmed using different instrumental devices. The UV-vis

spectrum of the freshly prepared silver colloid was monitored on a Labomed Model UVD-2950 UV-VIS Double Beam PC Scanning spectrophotometer, operated at a resolution of 2 nm. The samples' particle size and morphology studied with transmission electron microscopy (TEM, Philips model EM 208). For TEM micrography, an aqueous suspension containing the Ag NPs dispersed ultrasonically, and a drop of the suspension placed on carbon-coated copper TEM grids and dried. Micrographs obtained using the TEM device operated at an accelerating voltage of 80 kV. Moreover, the presence of elemental silver in the prepared biogenic Ag NPs confirmed with energy-dispersive spectroscopy (EDS) (model EM 208 Philips).

Antimicrobial assay

The susceptibility test studied with a conventional serial agar dilution method with some modification. BCYE-α agar medium (37.5 g/L) used as the culture medium instead of Müller-Hinton agar (MHA). The BCYE-α agar medium was further supplemented with serial concentrations of biogenic Ag NPs (5, 10, 15, 20 25, 30, 35, 40, 45 µg/mL). The inoculum was prepared from 72-96 h agar cultures of *L. pneumophila*, suspended in sterile distilled water to 0.08-0.1 optical density (measured at 600 nm), which corresponded to about 5×10^7 Cfu/mL. An inoculum of approximately 5×10^5 colony-forming units (Cfu) per milliliter of test strain (*L. pneumophila*)applied on the surface of the Ag NP supplemented BCYE-α agar plates. The data reported as minimum inhibitory concentrations (MICs), which was the lowest concentration of Ag NPs that inhibited visible growth after 72 h of incubation at 37 °C in humidified air with 2.5% CO_2. Moreover, in this study the antimicrobial activity of Ag NPs was also evaluated against other test strains, including *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. marcescens*, *S. typhi*, *A. niger,* and *C. albicans*. For the bacterial test strains, Müller-*Hinton* Agar (MHA) media inoculated with approximately 10^5 cells and incubated at 37 °C for 24 h. For fungus, *Sabouraud*Dextrose Agar (SDA) media were inoculated with 10^4 cells and incubated at 25 °C for 48 h. MICs were calculated as described for the above anti-*Legionella* test.

Time-kill course assay

The effect of biogenic Ag NPs on the MIC concentration (30 µg/mL) was evaluated with the conventional time-kill course assay. For this purpose, sterile sodium chloride solutions (0.9% w/v) were supplemented with biogenic Ag NPs (30 µg/mL), inoculated with *L. pneumophila* (1×10^8 Cfu/mL), and incubated at different temperatures 4 and 25 °C in humidified air with 2.5% CO_2. The viable cell counts for each vessel monitored at different intervals (4, 8, 12, 24 h) with the Cfu quantification method using BCYE-α agar plates.

Results and Discussions

Identification of isolate

In this study, BCYE-α agar medium supplemented with glycine, anisomycin, vancomycin and polymyxin B used to isolate the *Legionella* bacterium. Figure 1 shows the test strain isolated from the fishpond near Ahwaz City (Iran) that as grown on the BCYE-α agar medium (plate A) and Feeley Gorman Agar (FG agar) (plate B). A ground glass appearance around the bacterial cell mass observed for this isolate on the FG Agar medium (plate B). However, no ground glass appearance and no pigmentation observed for colonies of this test strain on the BCYE-α agar medium. In microbiological examinations, the strain had Gram-negative rods, and showed no growth on sheep blood agar. In addition, this strain was positive in the oxidase and catalase tests. All of the above results were in agreement with *Legionella* spp. based on the standard diagnostic tests for identifying bacteria [3, 23]. After the bacterial 16S rDNA was amplified with PCR, an amplified fragment of 1410 bp developed. It aligned to sequences in GenBank, and the alignment further confirmed that the

Table 1. Antimicrobial effect of biogenic silver nanoparticles against different test strains

Minimum inhibitory concentration (µg/ml)	Type	Test strains
30	*Legionellaceae*	*Legionella pneumophila*
10	*Enterobacteriaceae*	*Escherichia coli*
10	*Enterobacteriaceae*	*Klebsiella pneumoniae*
10	*Enterobacteriaceae*	*Pseudomonas aeruginosa*
10	*Enterobacteriaceae*	*Serratia marcescens*
10	*Enterobacteriaceae*	*Salmonella typhi*
10	*Staphylococcaceae*	*Staphylococcus aureus*
20	-	*Candida albicans*
10	-	*Aspergillus niger*

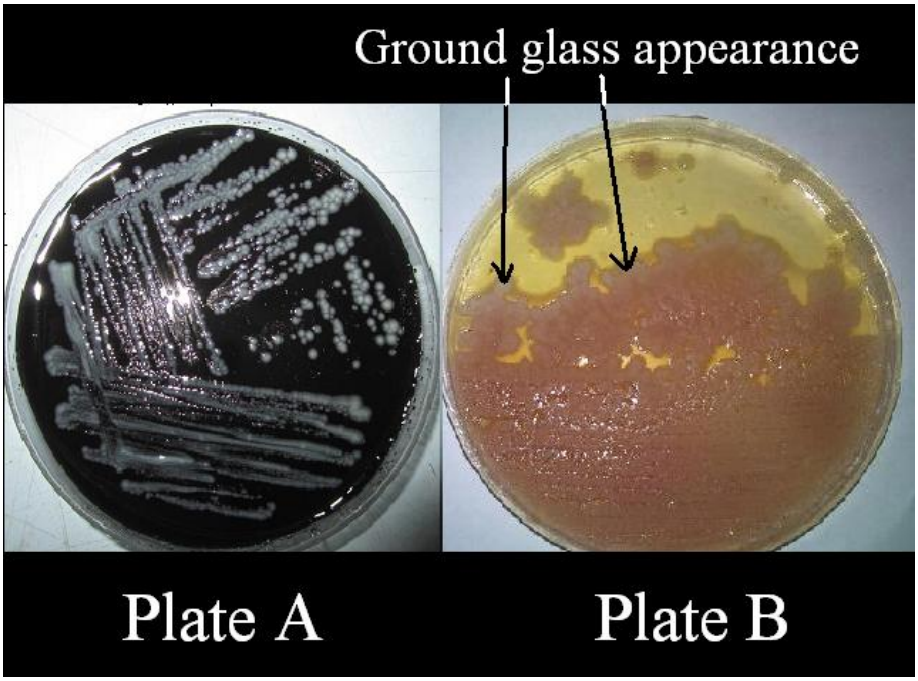

Figure1. Culture plate showing the *Legionella pneumophila* growth on the BCYE-α agar medium (plate A) and Feeley Gorman Agar (plate B).

isolated bacterium was representative of *L. pneumophila*. The sequence submitted to GenBank and assigned the following accession number: HQ840733. Alignment results revealed 99% identity with the *L. pneumophila* bacterium.

Biosynthesis of silver nanoparticles

Ag NPs colloid (inset in the left illustration in Figure 2) prepared using culture supernatant of *K. pneumoniae* under light emission (1000 mmol/m^2s) and used for the antimicrobial assays. The Ag NPs characterized with UV-visible spectroscopy (the left illustration in Figure 2). The technique outlined above has proved to be very useful for the analysis of nanoparticles [15].As illustrated in the left illustration in Figure 2, a strong and broad surface plasmon peak (λ_{max} 425 nm) was observed for the Ag NPs prepared using the *K. pneumoniae*. Observation of this strong but broad surface plasmon peak has been well documented for various metal nanoparticles with sizes ranging widely from 2 to 100 nm [19].The strong surface plasmon resonance of *K. pneumonia* was centered at ca. 425 and well matched with our previously reported works [15,19]. The upper right illustration in Figure 2 shows representative TEM images recorded from the drop-coated film of the Ag NPs synthesized by treating the silver nitrate solution with culture supernatants of *K. pneumonia* under light emission (1000 mmol/m^2s). The Ag NPs that formed were spherical and had a diameter of less than 100 nm. The silver nanocrystallites display an optical absorption band peak at approximately 3 KeV, which is typical of the absorption of metallic silver nanocrystallites due to surface plasmon resonance (the lower right-hand picture in Figure 2) [13].

Antimicrobial activity of silver nanoparticles

The antimicrobial activity of Ag NPs was evaluated with the agar dilution method against *L. pneumophila* and other test strains, which are listed in Table 1. The test strains showed different susceptibility against Ag NPs (Table 1). The lowest antibacterial activity (MIC 30 µg/ml) was observed against *L. pneumophila*. The MICs of Ag NPs were 10 µg/ml for other bacterial test strains, including *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. marcescens*, and *S. typhi. In addition, the MICs values of* Ag NPs *for A. niger and C .albicans*were 20 and 30 µg/ml, respectively.

Time-kill course assay

The time-kill course study selected for further investigation of the antibacterial activity of Ag NPs against *L. pneumophila* at different temperatures (4, 25 °C). The result of this experiment, which carried out with Ag NPs against *L. pneumophila*at MIC concentration (30 µg/mL), is shown in Figure 3.

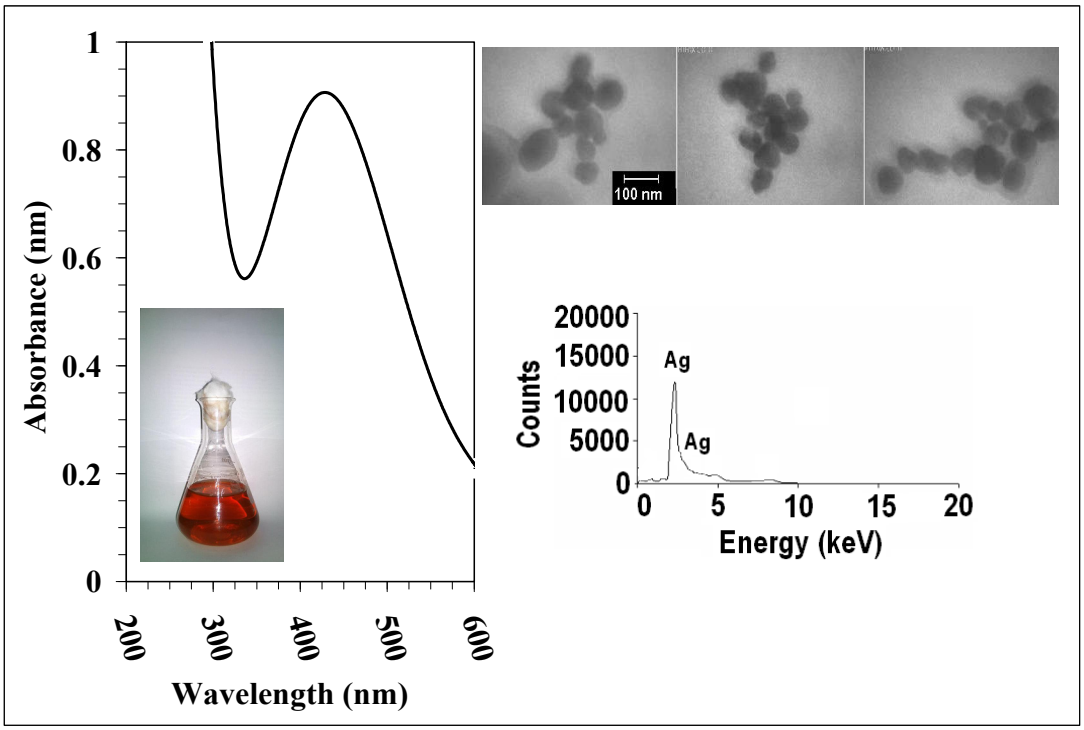

Figure 2. The characterization of silver nanoparticles prepared by *Klebsiella pneumoniae* under visible-light emission (1000 mmol/m^2s) generated by a 75W halogen lamp. The left illustration shows the UV-visible spectrum of prepared silver nanoparticles, and the inset in this illustration depicts a flask contain silver colloid prepared with the biological method. The right illustrations show transmission electron micrographs (upper pictures) and the energy-dispersive spectrum of silver nanoparticles (lower figure).

Microbiological examination of the inoculated suspensions containing Ag NPs using conventional the Cfu determination method revealed no viable cells after 24 h at 4 °C. In contrast, we detected considerable numbers of viable *legionella* cells (about 1.2×10^5 to 5.5×10^5 Cfu/mL) in inoculated vessels and in control tubes (without Ag NPs) that were inoculated at 25 °C for 24 h. The presence ofstrong heavy-metal efflux pumps has been established and reported for *L. pneumophila* [12].These heavy-metal efflux pumps may be involved inthe resistance mechanisms of *L. pneumophila*against Ag NPsduring 24-h exposure time at 25 °C. The capability of efflux pumps in *L. pneumophila*may be decreased at 4 °C and lead to accumulation of toxic Ag NPs inside *L. pneumophila*. The silver accumulation in the cells may explain the enhanced antimicrobial activity of Ag NPsagainst *L. pneumophila* at 4 °C compared to ambient temperature. In the presence of an efflux pump inhibitor, the antibacterial effect of Ag NPs should be enhanced against *L. pneumophila*. To investigate this hypothesis, the antibacterial activity of Ag NPs at MIC concentration (30 µg/mL) was further evaluated using the time-kill course assay in the presence and absence of reserpine (Sigma Aldrich) (10 µg/mL) against *L. pneumophila*. A higher mortality rate for Ag NPs was observed in the presence of reserpine against *L. pneumophila* at 25 °C (Figure 4).

In this study, biogenic silver nanoparticles were prepared with culture supernatant of *K. pneumoniae,and their antimicrobial activity investigated against different microorganisms, especially* against an isolate of the *Legionella* spp. *bacterium. This test strain was isolated from a fishpond near Ahwaz City (Iran) and carefully identified as L. pneumophila*. Comparison of the MIC values indicated that Ag NPs could inhibit visible growth of the *L. pneumophila* and *C. albicans* at a higher concentration (30µg/ml). Lower concentrations of Ag NPs showed a similar toxicity effect against other test strains. The time-kill course study demonstrated that *L. pneumophila* survives in the presence of the MIC level of Ag NPs (30 µg/ml) during 24 h at 25 °C (Figure 3). Reserpine, as a well-known alkaloid, can inhibit the efflux pumps in the different microorganisms [4]. Efflux pumps may be involved in the bacterial resistance of *L. pneumophila* against Ag NPs at 25°C. For the first time

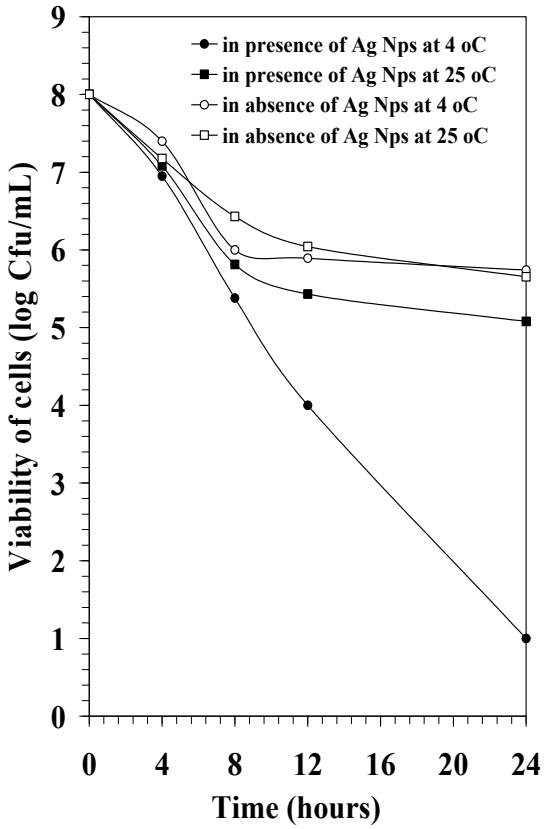

Figure 3. Time-kill kinetics of *Legionella pneumophila*in the presence of silver nanoparticles at the minimum inhibitory concentration level (30 µg/mL) at 4 and 25 °C.

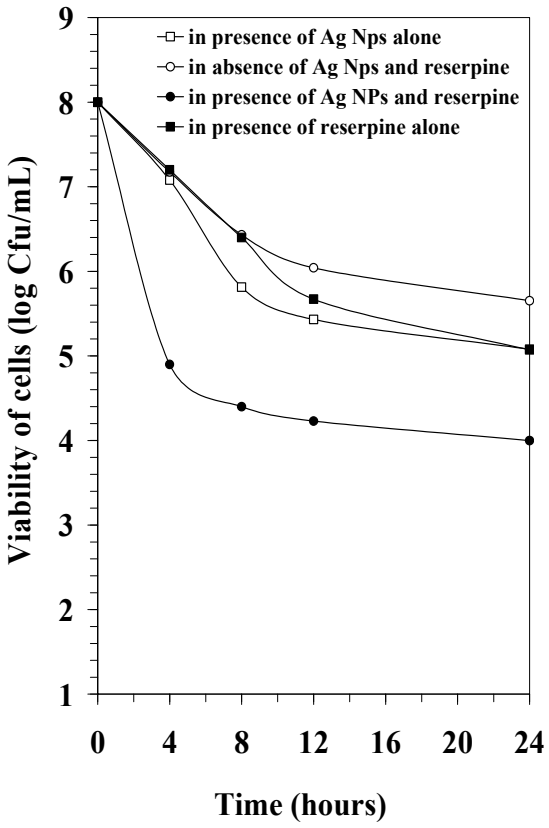

Figure 4. The effect of reserpine on the mortality of *Legionella pneumophila* in the presence and absence of silver nanoparticles (30 µg/mL) at 25 °C evaluated with the time-kill course assay.

in this investigation, we demonstrated that using an efflux inhibitor (reserpine) with a combination of Ag NPs (Figure 4) can significantly increase the anti-*legionella* activity of Ag NPs at the mentioned temperature (25 °C).

In contrast, the highest mortality rate in the absence of an efflux pump inhibitor was observed for Ag NPs against *L. pneumophila* at 4°C (Figure 3). This bacterium has been found with increasing frequency in both natural and artificial environments, especially in air-conditioning cooling towers [7-9]. In addition, the microorganism shows a considerable resistance to different disinfected agents [14, 16, 22].

Conclusion

Different reports on the antibacterial activity of Ag NPs against a wide variety of organisms have been published in the literature, but the antibacterial effect of Ag NPs against *Legionella* spp. has not yet been investigated and should be understood [10, 11, 12, 17, 18,21]. Howerver, this bacterium causes severeal serious infectious disease, is resistant against cold shock, and is common in many artificial environments such as hospital cooling towers and large central air conditioning systems [7, 8, 9, 14, 22, 16]. , We studied the antibacterial activity of Ag Nps against *Legionella* bacteriumfor the first time. Although this bacterium showed a considerable resistance against Ag Nps at ambient temperature but a good antibacterial activity observed for Ag Nps against *Legionella* at 4 °C. As a conclusion, Ag NPs can be added to the cooling water systems to kill *L. pneumophila* at cold conditions.

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