

ORIGINAL ARTICLE

Evaluation of the Bactericidal Effect of Silver Nanoparticles Against Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Sensitive *Staphylococcus aureus* (MSSA) Strains Isolated from Mastitic Milk of Small Ruminants and their Surrounding Environment in Aswan, Egypt

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Abstract

In the recent years, emergence of infectious diseases caused by drug resistant pathogens had been increased; therefore there is an urgent need to search for new alternative and effective antimicrobial agents to overcome the drug resistance. In the present investigation, the study group consisted of 90 sheep and 90 goats with clinical evidences of mastitis in 17(18.89%) goats and 5 sheep (5.56%) that manifested swollen udder with or without systemic signs of illness related to mastitis. Standard bacteriology was performed on pretreatment milk samples from the 17 goats and 5 mastitic ewes as well as 60 soil samples and 60 pail water samples. The bacteria isolated were identified as *Staphylococcus aureus* 12 (70.6%) from goats and 5 (100%) from sheep. In addition, *S. aureus* could also be identified in 41 (68.3%) soil samples and 42 (70%) water samples. The current study aimed to explore the bactericidal effect of silver nanoparticles (AgNPs) on methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) strains isolated from mastitic milk and their surrounding environment (water and soil) in Aswan, Egypt. AgNPs solution was synthesized by typical one-step synthesis protocol using soluble starch and was characterized using transmission electron microscopy and atomic absorption spectrophotometer. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AgNPs were examined to evaluate the bactericidal efficiency. In the present study, no significant discrepancy was observed between the influence of AgNPs on MRSA and MSSA ($P > 0.05$). It can be concluded that Ag-NPs have strong bactericidal effect against both MRSA and MSSA strains.

Keywords MBC, MIC, MRSA, MSSA, Silver nanoparticles (AgNPs)

1. Introduction

Staphylococcus aureus is one of the most pathogenic microorganisms for humans and animals since it causes severe infections, such as, wound infection, endocarditis, osteomyelitis, pneumonia, abscesses and food poisoning (Tauxe 2002; Deurenberg and Stobberingh 2008; Chambers and DeLeo 2009; Sowash et al. 2014). *Staphylococcus aureus* is capable of acquiring *mecA*; a gene responsible for the production of penicillin binding protein 2a. This protein offers lower binding affinity for methicillin and other similar β -lactam antibiotics (Katayama et al. 2005; Bustos-Martinez et al. 2006; Kinnevey et al. 2013). The emergence of Methicillin Resistant *Staphylococcus aureus* (MRSA) becomes a worldwide problem (David et al. 2008). MRSA was first isolated in human in the United Kingdom in 1961 (Barber 1961), while MRSA was first isolated from mastitic cow milk by Devriese et al. (1972) in 1972. In the last few years, (MRSA) has been considered as a critical public health problem threatening humans and animals (Petinaki and Spiliopoulou 2012). According to the aforementioned, researchers commence to search for new alternative antimicrobial agents for treatment of MRSA.

Recently, Nanoparticles (NPs) have been used as antimicrobial agent against drug resistant bacterial strains (Koo et al. 2005; Gong et al. 2007; Lopez Goerne et al. 2012; Mahmoudi and Serpooshan 2012; Rai et al. 2012; Franci et al. 2015; Kim et al. 2017; Wang et al. 2017; Yuan et al. 2017; Kumar et al. 2018). Because of their unusual chemical and physical properties such as small size with large surface area and high reactivity, nanoparticles easily penetrate microorganisms and render their antimicrobial activity (Morones et al. 2004). Due to their high antimicrobial activity against viruses, bacteria and other eukaryotic micro-organisms, safety and cost effectiveness, silver nanoparticles have been recognized to be the most effective nanoparticles (Gong et al. 2007; Szmackinski et al. 2008; Lazar 2011; Taraszkievicz et al. 2013; Salomoni et al. 2015; Patra and Baek 2017). Roy et al. (2007) reported that the antimicrobial action of Ag-NPs is more effective than that of broad-spectrum antibiotics used worldwide. Therefore, AgNPs have been commonly used as antimicrobial agent against drug resistant bacterial strains (Kvítek et al. 2008; Jones and Hoek 2010; Lara et al. 2010; Ansari et al. 2011). AgNPs can also be applied in cases of burn, traumatic wound dressings, diabetic ulcers, coating of catheters, dental works, medical devices and water

filtration (Jain and Pradeep 2005; Silver et al. 2006; Gong et al. 2007; Kim et al. 2007; Thomas et al. 2007; Rai et al. 2009; Martinez-Gutierrez et al. 2010; Dosoky et al. 2015; Ibrahim et al. 2020). In the present study, the bactericidal effect of silver nanoparticles against MRSA and MSSA strains isolated from mastitic milk of small ruminants and their surrounding environment in Aswan was investigated.

2. Materials and methods

2.1. Animals and samples

Animals:

A total of 90 sheep and 90 goats were subjected for clinical examination where 17(18.89%) goats and 5 (5.56%) sheep showed obvious signs of clinical mastitis including swollen udder with or without systemic signs of illness related to mastitis based on the criteria described by Dirksen et al. (1993).

Samples:

a. Milk

Milk samples were collected from 17 goats and 5 sheep mastitic milk samples out of 90 sheep and 90 goats. About 10 ml of milk were hygienically drawn from each quarter into a sterile 50 ml falcon tubes. Milk sampling was done according to the recommendation of National Mastitis Council (1999), immediately transported to the laboratory in ice-cooled containers and analyzed within 24 to 48 h after collection.

b. Soil

Sixty soil samples were collected according to Clegg et al. (1983).

c. Water

Sixty pail water samples were taken from pail used for watering of animals in clean sterile transparent 50 ml falcon tubes.

2.2. Silver nitrate (Ag NO₃) crystal, soluble starch

2.3. Cultures

MRSA and MSSA strains were isolated from milk of mastitic sheep and goats and their surrounding environment.

2.4. Isolation and identification of *S. aureus*

It was done according to **Melter et al. (1999)** and **Lee (2003)**. The samples were immediately suspended in Tryptone Soya Broth (TSB, Oxoid) containing 10% NaCl and then incubated at 37°C for 24h for selective enrichment of Staphylococci. Enrichment cultures were then streaked out on mannitol salt agar (MSA, Oxoid) and incubated at 37°C for 24h. The presumptive isolates were phenotypically characterized by yellow fermentation of mannitol.

2.5. Phenotypic identification of Methicillin Resistant *S. aureus* (MRSA)

The positive *S. aureus* samples were re-streaked on mannitol salt agar (MSA, Oxoid) supplemented with 6 mg/L of oxacillin (Sigma, St. Louis, Mo.) for selective isolation of MRSA. A sample was positive for MRSA if one or more colonies were identified and one representative colony was selected from each sample for further testing. The isolates were further confirmed as MRSA by using DrySpot Staphytest Plus latex agglutination test kit (Oxoid, United Kingdom).

2.6. Inoculum preparation for minimal inhibitory concentrations (MIC)

Preparation of bacterial suspension: Colonies from MRSA and MSSA isolates were transferred to 5 ml nutrient broth and incubated at 37°C for 17 h. Tenfold serial dilution were prepared using sterile nutrient broth as a diluent, by mixing 9 ml nutrient broth and 1 ml bacterial suspension and 1ml was transferred from the first tube to the second tube till 10^{-7} concentrations and 1 ml quantity from each dilution was spread on plate count agar and incubated at 37°C for 24 h. About 4-5 colonies from plate count agar contain 20-300 colonies were transferred to 5 ml nutrient broth then the broth culture was incubated at 35-37°C, until it achieved turbidity of the 0.5 McFarland standards. The turbidity of broth culture was adjusted with sterile broth to obtain turbidity comparable to that of the 0.5 McFarland standards. This suspension was containing about $1-2 \times 10^6$ CFU / ml.

2.7. Synthesis of silver nanoparticles (Ag-NPs)

Stable AgNPs less than 100 nm were synthesized according to (**Vigneshwaran et al. 2006**). Briefly, 1gm of soluble starch was added to 100 ml of deionized water and heated till complete dissolution

and cooling and 0.08 gm silver nitrate (AgNO_3) was added to 1ml of deionized water in dark glass bottle. Soluble starch solution was mixed with silver nitrate solution. This mixture was put in dark glass bottle, wrapped in aluminum foil and kept in autoclave at 121°C for 5 minutes. Clear yellow color solution indicated to the formation of AgNPs. Graphite Furnace Atomic Absorption (Model 210VGP) was used for determining the concentration of AgNPs stock solution. The size of AgNPs was measured by transmission electron microscopy (TEM) (JEOL-JEM- 100CX) at Electron Microscopy Unit, Assiut University, Egypt.

2.8. Determination of minimum inhibitory concentration (MIC)

MIC technique was performed according to **CLSI (2009)** using successive serial 2 fold dilution technique. The original concentration of stock AgNPs solution was 100 μg / ml. The MIC technique was done in triplicate to confirm the MIC value for the tested organism.

2.9. MIC technique

Sterile capped test tubes were numbered from 1 to 6, one positive (MHB+ bacterial inoculation) and one negative (MHB+ AgNPs) tube. Two ml Muller Hinton Broth (MHB, Oxoid) were added to each tube, then 2 ml from AgNPs stock was added to the first tube and to the negative tube. The first tube was shaken and 2ml were transferred to the second tube. The contents of the second tube were mixed and then transferred 2ml to the third tube. Dilutions were continued in this manner till tube 6 and finally 2 ml were discarded. Finally, 0.1 ml of bacterial inoculum was added to all 6 tubes and to positive tube. All tubes were incubated at 37°C for 24 h.

2.10. Determination of minimum bactericidal concentration (MBC)

Five grams / L of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) were added to Ag-NPs to halt the antimicrobial reaction between Ag-NPs and bacteria as described in the European quality standards (**Nen 1997**). MBC was determined by subculturing the content of all positive MIC tubes on mannitol salt agar (MSA) plates and incubated at 37°C for 24h. The MBC was determined as the lowest concentrations of silver nanoparticles that killed 100% of bacteria. Disinfection activity of AgNPs was measured by MBC/MIC ratio.

2.11. Statistical analysis

MIC and MBC tests were performed in triplicate, and the results were expressed as the mean \pm the standard

errors of the mean. SPSS was used to compare these results.

3. Results

Table (1): Prevalence of isolated MRSA and MSSA from mastitic milk of goats and sheep, soil and water

Sample	Samples	<i>S. aureus</i>	MRSA	MSSA
Goats milk	17	12 (70.6%)	5 (29.4%)	7 (41.2)
Sheep milk	5	5 (100%)	3 (60%)	2 (40%)
Soil	60	41 (68.3%)	21 (35%)	20 (33.3%)
Water	60	42 (70%)	21 (35%)	21 (35%)
Total	142	100 (70.4%)	50 (35.2%)	50 (35.2%)

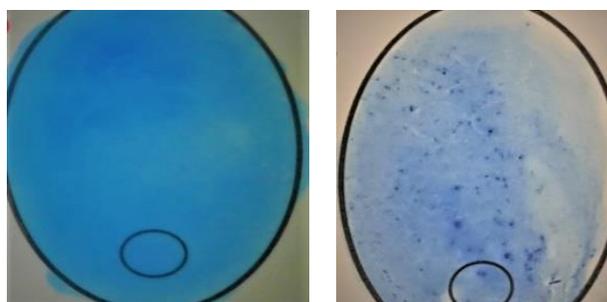


Fig. 1. Negative and positive DrySpot Staphytest plus Latex agglutination test-kit

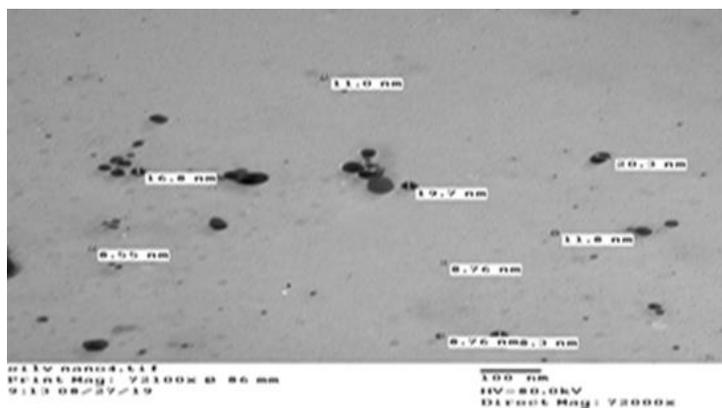


Fig. 2. Transmission Electron Microscopy (TEM) image of Ag-NPs with spherical shapes (sizes ranged from 8.55 to 20.3nm).



Fig. 3. Percentages of MRSA isolates showing values of MIC and MBC

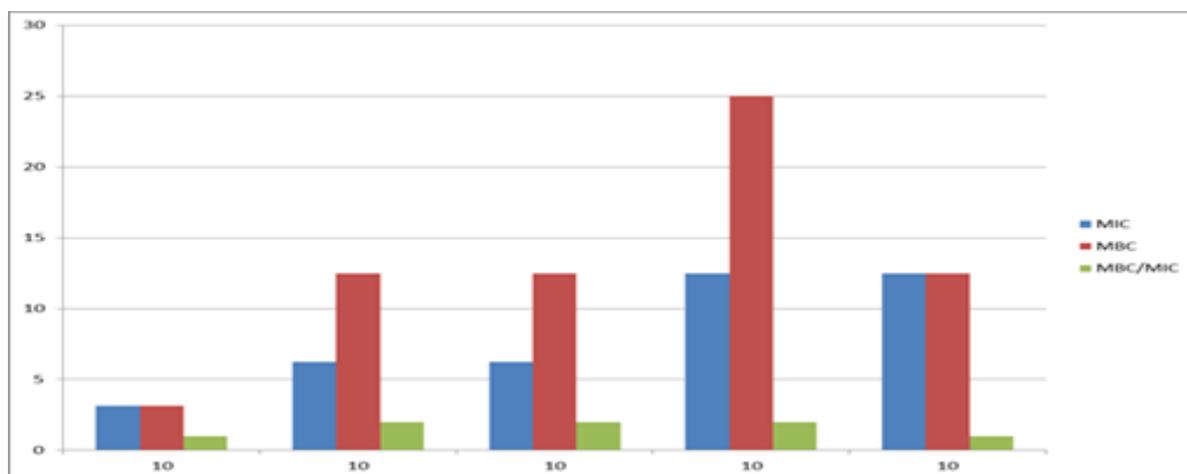


Fig. (4). Percentages of MSSA isolates showing values of MIC and MBC

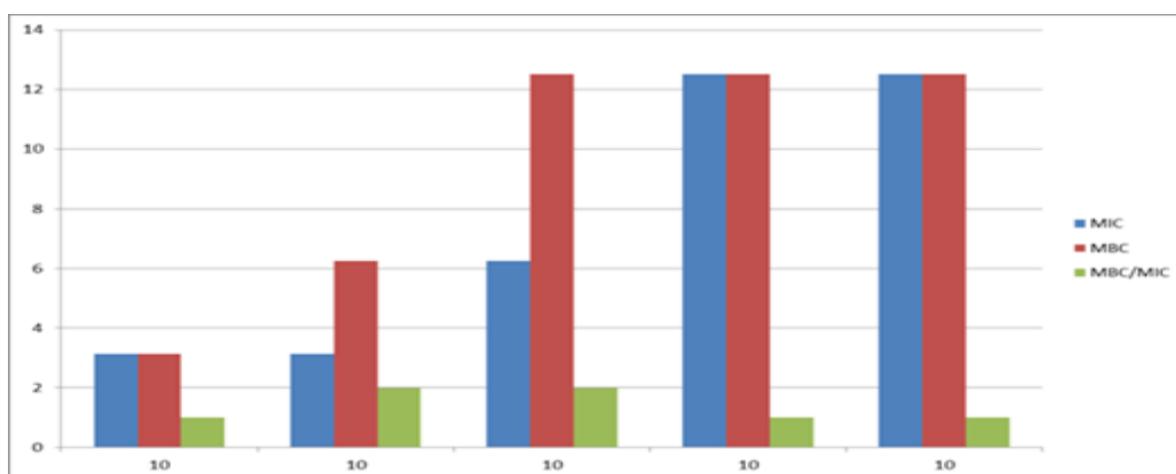


Fig. 5. Results of MIC test: Concentration of AgNPs (tube1=100 µg/ml; tube2=50 µg/ml; tube3=25 µg/ml; tube4=12.5 µg/ml; tube5=6.25 µg/ml; tube6= 3.125 µg/ml; P=positive tube and N=negative tube). Tube 4 is considered as MIC tube as it is the lowest concentration inhibits MRSA.

Table (2): MIC and MBC and MBC/MIC ratio of Ag-NPs tested against MRSA and MSSA.

MRSA (no=50)				MSSA (no=50)			
Isolate Numbers (%)	MIC value (µg / ml)	MBC value (µg / ml)	MBC/MIC Ratio	Isolate Numbers (%)	MIC value (µg / ml)	MBC value (µg / ml)	MBC/MIC ratio
10	3.125	3.125	1	10	3.125	3.125	1
10	6.25	12.5	2	10	3.125	6.25	2
10	6.25	12.5	2	10	6.25	12.5	2
10	12.5	25	2	10	12.5	12.5	1
10	12.5	12.5	1	10	12.5	12.5	1
Mean	8.125±0.19^a	13.125±0.78^a	1.6	Mean	7.5±0.73^a	9.375±0.41^a	1.4

^a: non-significant correlation (p > 0.05)

4. Discussion

In this study, clinical examination of a total of 90 goats and 90 sheep revealed the presence of clinical signs suggesting mastitis in 17 (18.89%) goats and 5 (5.56%) sheep. Results illustrated in **Table (1)** revealed isolation of *Staph aureus* from 12/17 (70.6%) of goat milk and in 5/5 (100%) of sheep milk, which is considered the most pathogenic microorganism for humans and animals. In this study, *S. aureus* was also present in 41/60(68.3%) soil samples and in 42/60 (70%) water samples. Interestingly, such findings refer to the importance of soil and water as potential sources of infection to susceptible goats and sheep. MRSA was isolated from 5/17 (29.4%), 3/5 (60%), 21/60 (35%) and 21/60 (35%) of goat milk, sheep milk, soil and water samples respectively. Data presented in **Table (2)** showed that the mean values of MIC and MBC of AgNPs against MRSA were 8.125 ± 0.19 and 13.125 ± 0.78 $\mu\text{g} / \text{ml}$, respectively. While, the mean values of MIC and MBC of Ag-NPs against MSSA were 7.5 ± 0.73 and 9.375 ± 0.41 $\mu\text{g} / \text{ml}$, respectively.

The obtained MIC value for MRSA nearly agreed with the results recorded by (**Das et al. 2015; Asghar et al. 2018**) who found that the MIC for MRSA was $8 \mu\text{g}/\text{ml}$. Whereas, MBC value for MRSA nearly agreed with the findings recorded by (**Surwade et al. 2019**) who found that MBC for MRSA was $12.5 \mu\text{g}/\text{ml}$. Our data disagreed with the findings of (**Ayala-Nunez et al. 2009**) who found that the MIC and MBC against MRSA and MSSA were 1.35 and 2.7 mg/ml, respectively and (**Wady et al. 2014**) who found that the MIC and MBC against MRSA were $1.95 \mu\text{g}/\text{ml}$ and $3.91 \mu\text{g}/\text{ml}$, respectively. On the other hand, agreed with (**Kotb and Sayed 2015**) who found that mean values of MIC and MBC of Ag-NPs against MRSA were 23.44 ± 0.21 and 31.25 ± 0.26 $\mu\text{g} / \text{ml}$, respectively; while the mean values of MIC and MBC of Ag-NPs against MSSA were 11.33 ± 0.14 and 13.28 ± 0.17 $\mu\text{g}/\text{ml}$, respectively. The bactericidal effect of Ag-NPs against MRSA was higher than data of commercial antibiotic. For example, the MIC of gentamicin antibiotic against MRSA was $64 \mu\text{g}/\text{ml}$ (**Ayala-Nunez et al. 2009**). The significant difference in MIC and MBC values might be attributed to the size and shape of nanoparticles, the methods of preparation, type of reducing and stabilizing agent and the genetic variation of the isolated organisms (**Ansari et al. 2011; Wady et al. 2014**).

The MBC/MIC value is a parameter referring to the bactericidal efficiency of Ag-NPs. The obtained results in **Table (2)** showed that Ag-NPs inhibited the growth of both of MRSA and MSSA in a bactericidal

effect rather than a bacteriostatic effect (MBC/MIC ratio < 4). The bactericidal activity agents are preferable than bacteriostatic as if the bacteria are killed rather than inhibited, this will lead to rapid combating of infection and reducing of bacterial resistance (**French 2006**).

The mode of bactericidal action of AgNPs is still not clear. Several researchers have suggested that Ag-NPs bind to the surface of the cell membrane and changed their cellular permeability and the respiration (**Lok et al. 2006; Panacek et al. 2006; Choi et al. 2008; Li et al. 2013**). It is also proposed that Ag-NPs penetrate inside the bacteria and inactivate DNA replication, producing reactive oxygen species (ROS) causing death of the cell (**Park et al. 2011; Gurunathan 2015; Wang et al. 2017; Yuan et al. 2017**).

It is also obvious that there was no significant difference between the effect of Ag-NPs on MRSA and MSSA ($p > 0.05$). This result proved that the impact of AgNPs on both MRSA and MSSA was similar; explaining that resistant proteins that make bacteria resist antibiotics not influenced by nanoparticles and AgNPs not only act on PBP2a but act on a broad range of targets, such as cell membrane and cytoplasmic proteins and genomic DNA. This results agreed with (**Kong and Jang 2008; Petica et al. 2008; Ayala-Nunez et al. 2009; Lara et al. 2010; Ansari et al. 2011; Cavassin et al. 2015; Kotb and Sayed 2015; Rangari et al. 2015**).

5. Conclusion

Results of the current study revealed that the effect of AgNPs on MRSA and MSSA did not vary substantially. The bactericidal effect of Ag-NPs against MRSA was higher than that of the commercial antibiotics

6. Conflict of interest statement

No conflicts of interest.

7. References

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