African Journal of Biotechnology Vol. 10(52), pp. 10795-10797, 12 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1499
ISSN 1684–5315 © 2011 Academic Journals

Short communication

An *in vitro* evaluation of antibacterial effect of silver nanoparticles on *Staphylococcus aureus* isolated from bovine subclinical mastitis

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Accepted 18 August, 2011

Bovine mastitis is the most costly disease affecting dairy cows and milk production. As *Staphylococcus aureus* is considered as a major pathogen due to its prevalence in dairy herds, contagious nature of infection, economic impact of treatment and control and its resistance to antimicrobial agents is a well-documented challenge in dairy cows. Therefore, the purpose of this study was to investigate and determine the antibacterial activity of silver nanoparticles against *S. aureus* isolated from subclinical mastitis cows. The minimum inhibitory concentrations (MIC) distribution of the silver nanoparticles tested for *S. aureus* field isolates were determined by a broth dilution method. The results show MIC values ranging from 1.25 to 10 µg/ml. The MIC values that inhibited 50 and 90% of the population of the isolates tested of silver nanoparticles were 5 and 10 µg/ml for *S. aureus,* respectively. In addition, the results of this study demonstrated that the mean time of the antimicrobial action of silver nanoparticles against *S. aureus* is 7 min. This *in vitro* result clearly indicates that the silver nanoparticle might have a good activity against *S. aureus* with mastitis origin.

Key words: Bovine mastitis, silver nanoparticles, *Staphylococcus aureus.*

INTRODUCTION

Bovine mastitis is the most costly disease affecting dairy cows and milk production. This disease is one of the major causes of antibiotic use in dairy cows (Mitchell et al., 1998). *Staphylococcus aureus* is considered as a major mastitis pathogen due to its prevalence in dairy herds, contagious nature of infection and economic impact of treatment and control (Bradley, 2002). Its resistance to antimicrobial agents is a well-documented challenge in dairy cows. Because of resistance to antimicrobial agents in mastitis pathogens, there is a growing interest in using alternative antimicrobial agent.

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Abbreviations: CMT, California mastitis test; **MIC,** minimal inhibitory concentration; **TSB,** trypticase soy broth; **MBC,** minimal bactericidal concentration; **DNA,** deoxyribonucleic acid.

Silver is one of the non-toxic and safe antibacterial agents to the body, and can kill many harmful microorganisms (Dowling et al., 2003). It has been known since ancient times that silver and its compounds are effective antimicrobial agents. In particular, due to the recent advances in research on metal nanoparticles, silver nanoparticles have received special attention as a possible antimicrobial agent. When certain particles are very small size, nanosize, the number of particles may be increased in unit area. The total surface area of the nanosized silver particles is larger than that of large-sized silver in identity volume, then antibacterial ability of the nanosized silver particles is more effective than the large-sized silver (Yeo et al., 2003). Referring to the existing scientific literature, no data have been published in the onset time of the antimicrobial action of silver nanoparticles. Therefore, the objective of the present study was to investigate the antibacterial activity of silver nanoparticles against *S. aureus* isolated from subclinical.

Table 1. Determination of MIC, susceptibility and onset time of action of the silver nanoparticle against *S. aureus* (n = 7) isolated from subclinical mastitis cows.

Antimicrobial agent	Dilution range[1]	MIC range[1]	MIC_{50}[1]	MIC_{90}[1]	(%)S[2]	Average of onset time[3]	Mean of onset time[3]
Silver nanoparticle	0.039-160	1.25-10	5	10	100	3-10	7

1Dilution range, MIC range, MIC_{50} and MIC_{90} (in µg/ml); 2, % of sensitive isolates; 3, average and mean of onset time of action expressed per minute

mastitis in cow and to determine its antibacterial onset time.

MATERIALS AND METHODS

Nanoparticles

Nanosilver particles colloidal solution (Cat. No: PL-Ag-S10-10mg) was purchased from PlasmaChem GmbH, Germany. The average particle size was 10 nm.

Bacteria

Subclinical mastitis cows were defined as California mastitis test (CMT) positive (2+ or more) but absence of clinical signs of mastitis (Karimuribo et al., 2005). Milk samples were aseptically collected from each quarter of 50 cows with subclinical mastitis, briefly, immediately after the pre-milk hand-stripping, teat orifices were scrubbed with a cotton pledget saturated in 70% ethyl alcohol. Primary isolates were from milk samples that were streaked onto 5% sheep blood agar plates using a sterile cotton swab and incubated at 37°C for 18 to 24 h. Colony morphology, gram staining and catalase test were used to identify bacterial genus. The colonies identified as *S.* were submitted to coagulase test using rabbit plasma. The examinations were followed by examining the susceptibility profile of isolates towards bacitracin. The tests of sucrose, D-mannose, D-manitol, maltose, D-trehalose, raffinose fermentation, urease activity and acetoin production were also assayed. All tests were performed as described by Quinn et al. (1994). After identification, 7 isolates of S. aureus were inoculated in trypticase soy broth (TSB), and incubated at 37°C for 24 h. The bacterial concentration of TSB was estimated to be 3×10^8 cfu per ml using serial dilution method.

Determination of the minimum inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC)

The MIC determinations of the silver nanoparticles for *S. aureus* field isolates were performed using the serial dilution method. The dilutions tested were 0.0195 to 160 µg/mL. The MIC was determined after 24 h of incubation at 37°C. The first dilution with no visible growth was considered as the MIC for each isolate. The concentration of the silver nanoparticles able to inhibit the visible growth of 50% of a population of microorganisms (MIC_{50}) and the concentration of the silver nanoparticles able to inhibit the visible growth of 90% of a population of microorganisms (MIC_{90}) were calculated for *S. aureus* field isolates as well as the minimum and maximum MIC (Table 1).

Determination of the onset of the action of nanosilver particles

To examine the onset of the action of nanosilver particles, 0.1 ml of TSB media containing 3×10^8 cfu/ml from each isolate were inoculated into 12 sterile tubes each containing 20 ml of TSB plus 10 µg/ml nanosilver particles (the concentration of nanosilver particles used in this experiment was the same as MIC_{90} obtained in the previous experiment). The media were incubated at 37°C for 0, 1, 3, 7, 10, 15, 30, 45, 60, 120, 180 and 240 min (for tubes no. 1 to 12, respectively). Using a sterile swab, samples from these tubes were sub-cultured onto plates containing 8 ml of Blood agar and incubated at 37°C for 72 h. The study was repeated twice.

RESULTS AND DISCUSSION

Based on the results of CMT, 50 cows were diagnosed with subclinical mastitis. Then the samples of milk cows were cultured on the blood agar media and colonies suspected to *S. aureus* were selected. Characteristics such as white, opaque colonies and positive coagulase, catalase, hemolysis, mannitol fermentation, fermentation of maltose and VP as well as growth on the specific media such as Baird Parker (black colonies) and observation of Gram positive clusters colonies in Gram staining examination confirmed our diagnosis of 7 *S. aureus* isolates. Many investigators have used the same methods to identify, separation and purification of *S. aureus* (Nunes et al., 2007; Schneider et al., 2004).

The present study is the first one to evaluate the effect of silver nanoparticles on *S. aureus* isolated from subclinical mastitis. Using serial dilution method, the MIC distribution and MIC_{50} and MIC_{90} values (MIC that inhibited 50 and 90% of the isolates tested, respectively) of the silver nanoparticles for *S. aureus* field isolates are summarized in Table 1. MICs being in the range of 1.25-10 µg/ml with a MIC_{90} of 10 µg/ml. All isolates were susceptible to silver nanoparticles. None of the isolates were considered tolerant as assessed by the general rule that tolerance is indicated if the sum of the MIC is greater than 32.

The results of the study performed to determination of the onset time of the antimicrobial action of silver nanoparticles are demonstrated in Table 1. The range of the onset time was 3-10 min and its mean was approximately 7 min.

The result of the present study showed that nanoparticles of silver at a concentration of 10 µg/mL after

approximately 7 min are able to kill *S. aureus* isolates. In agreement with the results of the present study, it has been reported by other investigators that nanoparticles of silver have antibacterial effects (Grier, 1983). Lee et al. (2003) reported that nanosilver particles in colloidal solution at a concentration of 5 ppm have excellent antibacterial effect against *S. aureus* and *Klebsiella pneumonia*. Cho et al. (2005) reported also that the MIC of silver nanoparticles against *S. aureus* is 5ppm. However, due to the use of: different methods of production, different nanoparticles size, and different nanoparticles shape, many products commonly summarized under the name nanoparticles can be physically and chemically completely different preparations (Pal et al., 2007). Depending on these factors, nanoparticles can have highly variable antibacterial properties (Merisko-Liversidge et al., 2003), which result in controversial results. Pal et al. (2007) reported that the MIC of silver nanoparticles against *Escherichia coli* is 12.5 µg/mL. Furthermore, it has been reported by Ugur and Ceylan (2003) that MIC for silver nitrate against *S. aureous* is in the range of 8-80 µg/ml.

To author's knowledge, there is no study investigating the onset time of antibacterial activity of nanosilver particles. The mechanism of bactericidal action of silver nanoparticles is still not well understood. However, it was shown that the interaction between silver nanoparticles and constituents of the bacterial membrane caused structural changes in and damage to membranes, finally leading to cell death (Grier, 1983).

Pal et al. (2007) speculated that the action of silver nanoparticles is broadly similar to that of silver ion. It reacts with the thiol group and inhibits respiratory enzymes, facilitating the generation of reactive oxygen species and consequently damaging the cell.

Silver nanoparticles have also intensive tendency to react with sulfur and phosphorus groups. Thus, the cell membrane proteins containing sulfur and compounds containing phosphorus such as deoxyribonucleic acid (DNA) are likely to be the preferential sites for silver nanoparticle (McDonnell and Russell, 1999). Based on result obtained in this study and from literature review, it can be concluded that antibacterial activity of nanoparticles of silver commerce relatively soon after inoculation of *S. aureus*. Furthermore, using nanosilver particles as an alternative to antibiotics in subclinical mastitis can be considered. Further work should be performed to investigate different aspects of this issue.

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