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Antibacterial effect of silver nanoparticles along with protein synthesis-inhibiting antibiotics on *Staphylococcus aureus* isolated from cattle mastitis

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Abstract

Introduction: *Staphylococcus aureus* is an opportunistic pathogen in dairy ruminants which is also found in healthy carriage and can be a major cause of mastitis. Various mastitis control programs have been used to combat the problem but have not always been efficient. In most countries, antibiotic resistance is extremely common. Silver nanoparticles have shown antimicrobial activity against *S. aureus*. In the present study the effect of silver nanoparticles on *S. aureus* isolated from cattle mastitis along with antibiotics of operative on protein bacterial synthesis investigated.

Materials and methods: Three hundred eleven milk samples were collected from the cow farms. Each milk sample was cultured on mannitol salt agar and was incubated. A total of 72 isolates of *S. aureus* were isolated from the bovine mastitis milk samples. *S. aureus* DNA extracted by DNA purification kit according to the manufacturer protocol. 58 isolates were confirmed as *S. aureus* by biochemical tests as well as *nuc* gene detection. MIC and MBC determined for silver nanoparticles with antibiotics on 50 isolates.

Results: The resistance of *S. aureus* isolates against erythromycin, gentamicin, streptomycin and doxycycline were 100, 22, 100 and 8%, respectively. 8 of all isolates were sensitive to 25 µg/ml concentration of silver nanoparticles. The 92% growth of the samples were inhibited at concentrations between 50-100 µg/ml.

Discussion and conclusion: The present study suggests that antibiotics which can inhibit protein synthesis have significant synergistic effect along with silver nanoparticles.

Key words: Mastitis, *Staphylococcus aureus*, Silver nanoparticle, Antibiotic

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Introduction

Staphylococcus aureus is an opportunistic pathogen in dairy ruminants where it is found in healthy carriage and can be a major cause of mastitis (1). Mastitis in dairy cattle is a persistent, inflammatory reaction of the udder tissue. Fatal mammary gland infection is common potential (2). This disease costs the US dairy industry about 1.7 to 2 billion dollar each year (3). Reported cure rates for *S. aureus* mastitis vary considerably. The probability of cure depends on cow, pathogen, and treatment factors (4). Various mastitis control programs have been used to combat the problem but have not always been efficient (5). In most countries, antibiotic resistance is common extremely. Combination therapy with penicillin and gentamicin may be used to treat serious infections; its use is controversial because of the high risk of damage to the kidneys (6).

Nanoparticles are sized between 1-100 nm (7). High surface area to volume ratios and unique chemicophysical properties of various nanomaterials are believed to contribute to effective antimicrobial activities (8). Metal nanoparticles, which have a high specific surface area and a high fraction of surface atoms, have been studied extensively due to their unique physicochemical characteristics such as catalytic activity, optical properties, electronic properties, magnetic properties, and antimicrobial activity (9). Antimicrobial mechanisms of nanomaterials include:

1- photocatalytic production of reactive oxygen species (ROS) that damage cellular and viral components,

2-compromising the bacterial cell wall/membrane,

3-interruption of energy transduction, and

4- inhibition of enzyme activity and DNA synthesis (10-11). Silver nanoparticles (AgNPs) have recently been synthesized and shown to exhibit antimicrobial activity against several species of bacteria including *S. aureus* (12). The antimicrobial activity of silver particles is influenced by the dimension of the particles, with smaller particles showing greater antimicrobial effect. Bacteria treated by metals including silver do not acquire resistance to the metals (13). The MIC (minimum inhibitory concentration) of AgNPs is one order lower than the one of silver ions (14). Therefore, the bactericidal metals have advantages over the conventional antibiotics which often cause the selection of antibiotic-resistant microorganism. The use of modern technology and the therapeutic properties of silver nanoparticles have requirements that had already been proven and it seems necessary. In the present study, the effect of silver nanoparticles has been investigated along with antibiotics of operation on protein bacterial synthesis.

Material and methods

Sampling

For detection of clinical and subclinical mastitis, California mastitis test (CMT) carried out. Three hundred and eleven milk samples were collected from the cow farms of Tabriz and Urmia, Iran. The breasts of cows were washed and disinfected by 70% alcohol and then were dried using sterile cotton and disposable towel. First milking of the teat was discarded and 10 ml of milk

were collected in a sterile BHI bottle. All samples were kept at 4°C and transported immediately to the lab for latter examination.

Isolation and identification of *S. aureus*

Each milk sample (100 µl) was cultured on the surface of mannitol salt agar (Merck, Germany) and was incubated at 37°C for 24 h. Colonies suspected as *S. aureus* were selected and transferred to 5% sheep blood agar (Difco, USA). Gram stain, culture characteristics, and coagulase test using fresh rabbit plasma (tube method) were used for the presumptive identification of all isolates (15). Out of the 311 bovine mastitis milk samples 72 isolates of *S. aureus* were studied.

Molecular diagnosis of *S. aureus*

S. aureus DNA extracted from the 24-hour culture of *S. aureus* in BHI medium according to DNA purification kit (Fermentas, Germany) manufacturer protocol. To accurately identify of *S. aureus*, *nuc* gene was amplified by PCR (16). The primers of F (5'-GCGATTGATGGTGATACGGGT-3') and R (5'-AGCCAAGCCTTGACGAACTAAAGC-3') were used for *nuc* amplification. PCR reaction was taken using the PCR kit (CinnaGen PCR master kit) in a final volume of 25µl, containing 12.5µl of master mix (with 2X concentration), 0.4 mM of each primer and 2 µl of DNA sample. Volume of the mixture with deionized distilled water adjusted to 25 µl. For negative control, sterile water was used instead of DNA and the extracted DNA of *S. aureus* (ATCC 29213) used as a positive control. DNA replication with pattern of initial denaturation temperature of 94°C for 3 min, 35 thermal cycles each consisting of denaturation at 94°C temperature for 1 minute, binding stage at 55°C for 30 seconds, and along step at 72°C for 1.5 min were performed. The final step to complete the reaction at 72°C for 3.5 min was performed. A PCR product was obtained with equal size of all *S. aureus* isolates. The size of PCR products was determined using 1.2% garose gel electrophoresis and marker of GeneRuler^{TM} 100 bp DNA ladder (Frementas, Germany).

Susceptibility test antibiotics

Bacterial samples were incubated in Mueller Hinton broth medium (Merck, Germany) and were cultured at 37°C for 24 h. After growth, the samples were compared with a turbidity tube of 0.5 McFarland (number of bacteria 10^8-10^9). The 48 wells of micro plate were considered with four wells each in one row. To determine the pattern of isolates resistance against Erythromycin, Gentamicin, Streptomycin and Doxycycline, Disk Agar Diffusion method was carried out on Muller Hinton Agar and results were reported as resistance percentage (17).

Measurement of minimum inhibitory concentration and minimum bactericidal concentration for silver nanoparticles

Eight dilutions (0, 25, 50, 60, 70, 80, 90 and 100 µg/ml) of silver nanoparticles with 97 nm in diameter (Malvern instrument, UK) were prepared using saline normal. In each well, 900 µl of bacterial suspension was added and then the 100 µl of different concentrations of silver nanoparticles were

added in the wells. To establish the antimicrobial activity of silver nanoparticles on the bacterial growth, the minimum inhibitory concentration and minimum bactericidal concentration of silver nanoparticles were determined for *S. aureus* using optical density of the bacterial culture solution containing different concentration of silver nanoparticles after 24h. All of the experiments were triplicated, on three different days.

Measurement of MIC and MBC for silver nanoparticles along with antibiotics

To determine the MIC and MBC of silver nanoparticles in combination with antibiotics, 50 µl of each antibiotic and 50 µl of different concentrations of silver nanoparticles were added into each well containing 30 µl Hinton medium (Merck, Germany) then incubated for 24 h at 37°C (18). Finally, the rate of bacterial growth on culture plates containing bacterial suspensions with silver nanoparticles and various antibiotics were tested and the MIC and MBC of silver nanoparticles were determined along with antibiotics.

Results

Molecular Identification of *S. aureus* isolates

A total of 311 milk samples were cultured, 72 *S. aureus* isolates identified using cultural and biochemical tests. Specific molecular diagnosis carried out by *nuc* gene amplification. The primers amplified the expected size of 279 bp in 58 *S. aureus* isolates which 50 of them used in the next stages of the research. (Fig. 1)

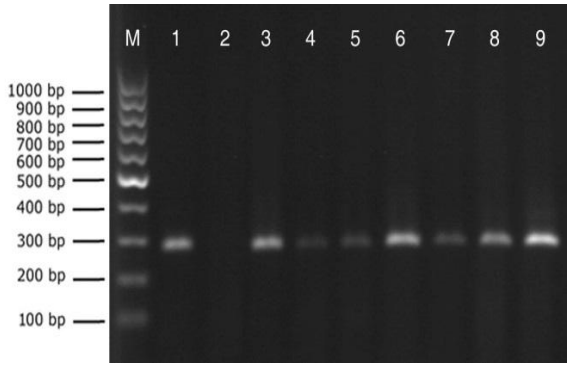

Fig 1- electrophoresis of PCR products of *nuc* gene.
M: 100 bp DNA ladder (fermentas-Germany)
1: positive control (*S. aureus* ATCC 29213).
2: Negative control (reaction without DNA).
3-9: PCR products of the expected size of 279 bp.

Antibiotic Resistance

All of 50 isolates cultured in a concentration of 50µg/ml of the operative antibiotics separately. The resistance of *S. aureus* isolates against erythromycin, gentamicin, streptomycin and doxycycline were 100, 22, 100 and 8%, respectively. The MIC and MBC were determined along with silver nanoparticles only in resistant isolates.

Measurement of MIC and MBC

In this study, 50 isolates of *S. aureus* with eight different dilutions of silver nanoparticles were examined; the growth of four isolates was inhibited at 25 µg/ml concentration of silver nanoparticles, which was recorded as MBC. Eight percent of all isolates were sensitive to 25 µg/ml concentration of silver nanoparticles. Nighty eight percent of the samples at concentrations between 50-100 µg/ml were inhibited. The sensitive isolates to 25 µg/ml silver nanoparticles in next tests were excluded. The results of MIC and MBC for silver nanoparticles and antibiotics along with silver nanoparticles on 46 remained isolates are shown in Table 1. All tests performed in triplicate.

Table 1- Results of interaction of silver nanoparticles and antibiotics.

Treatment / Resistant sample	AgNPs MIC,MBC (µg/ml)	AgNPs + DoxycyclineMIC ,MBC (µg/ml)		AgNPs + Streptomycin MIC,MBC (µg/ml)		AgNPs +GentamicinMIC, MBC (µg/ml)		AgNpS + Erythromycin MIC,MBC (µg/ml)	
1	70/80	-	-	A	>100	-	*-	S	25/50
2	-	S	<25	A	>100	-	-	I	70/80
3	70/80	-	-	S	90/100	-	-	S	60/70
4	100<	-	-	A	>100	-	-	S	50/60
5	-	-	-	I	>100	-	-	S	90/100
6	100<	-	-	A	90/100	-	-	S	70/80
7	80/90	-	-	I	>100	-	-	S	70/80
8	100<	-	-	A	>100	-	-	A	70/80
9	-	-	-	I	>100	-	-	S	70/80
10	100<	-	-	A	70/80	-	-	A	90/100
11	100<	-	-	I	>100	-	-	S	50/60
12	100<	-	-	A	70/80	S	<25	S	50/60
13	60/70	S	<25	A	>100	-	-	I	25/50
14	25/50	S	<25	A	>100	-	-	S	70/80
15	80/90	-	-	A	90/100	S	<25	S	70/80
16	80/90	-	-	A	>100	-	-	A	80/90
17	60/70	-	-	A	>100	-	-	A	90/100
18	80/90	-	-	I	>100	S	<25	S	50/60
19	100<	-	-	A	>100	-	-	S	25/50
20	90/100	-	-	A	70/80	S	<25	S	<25
21	50/60	-	-	A	90/100	-	-	A	70/80
22	-	-	-	A	>100	-	-	A	80/90
23	25/50	-	-	A	90/100	S	<25	I	25/50
24	25/50	-	-	A	>100	-	-	I	90/100
25	-	-	-	S	80/90	-	-	S	25/50
26	-	-	-	A	90/100	-	-	A	60/70
27	50/60	-	-	A	80/90	S	<25	A	80/90
28	25/50	-	-	A	>100	-	-	A	90/100
29	80/90	-	-	I	25/50	-	-	A	80/90
30	25/50	-	-	A	90/100	-	-	S	25/50
31	60/70	-	-	I	>100	S	<25	S	70/80
32	100<	-	-	A	80/90	-	-	S	50/60
33	60/70	-	-	A	80/90	S	<25	S	60/70
34	60/70	-	-	A	90/100	-	-	A	80/90
35	-	S	<25	A	90/100	-	-	S	60/70
36	70/80	-	-	S	90/100	S	<25	S	70/80
37	100<	-	-	A	>100	-	-	S	50/60
38	80/90	-	-	A	70/80	-	-	S	50/60
39	60/70	-	-	I	80/90	-	-	A	90/100
40	80/90	-	-	I	60/70	-	-	A	90/100
41	-	-	-	A	>100	-	-	A	90/100
42	70/80	-	-	A	90/100	-	-	A	90/100
43	-	-	-	A	50/60	-	-	S	60/70
44	70/80	-	-	A	>100	-	-	A	70/80
45	25/50	-	-	A	>100	-	-	S	50/60
46	80/90	-	-	I	50/60	-	-	S	<25

S: Synergistic, A: Antagonist, I: Inactive

*: In the samples that were susceptible to antibiotics, MIC and MBC of antibiotics along with silver nanoparticles were not investigated.

Discussion and conclusion

More noticeably, the increase in bacterial resistance to antimicrobial agents poses a serious problem in the treatment of infectious diseases as well as in epidemiological practice. Increasingly new bacterial strains have emerged with dangerous levels of resistance, including Gram-positive and Gram-negative bacteria. Dealing with bacterial resistance requires precautions that can lead to the prevention of the emergence and spread of multiresistant bacterial strains and the development of new antimicrobial substances. The results of this study cleared that the infection rate of cows with *S. aureus* is about 16.07%. The high rate of resistance was to Streptomycin and erythromycin. All isolates showed high sensitivity to doxycycline and gentamicin. In a recent study, minimum inhibitory concentration of silver nanoparticles on *S. aureus* was 100 µg/ml, which corresponds to the current study (19). In the study of Shahverdi et al, effect of silver nanoparticles and Fourteen antibiotics on *S. aureus* and *E. coli* investigated using disk diffusion method. The effect of penicillin G, amoxicillin, erythromycin, clindamycin and vancomycin antibiotics on *S. aureus* was better than the rest. Erythromycin on *S. aureus* was the most effective than other antibiotics (20). In the present study the antibiotics which effect on 30S ribosomal subunit (doxycycline and gentamicin) had a higher synergistic effect on *S. aureus* in combination with silver nanoparticles.

The silver nanoparticles first attach to the surface of the cell membrane and penetrate further inside the bacteria. The cytoplasm destroys as the Ag NPs penetrated the cell (21). So antibiotics that affect on protein translation may influence on ribosomes and stop them, resulting in the inhibition of bacterial cell growth and multiplication. Streptidine ring of Streptomycin (as one of the major drug rings) causes of neutralizing effect on antibacterial silver nanoparticles (22). In this study, erythromycin (effective on 50S ribosomal subunit) showed synergistic effect with silver nanoparticles that was concord with studies of Fayaz et al and shahverdi et al (23-24).

Silver nanoparticles have been widely used for development of biological and pharmaceutical processes, products, and applications such as coating material for medical devices, orthopedic or dental graft materials, topical aids for wound repair, clothing, underwear and socks, textile products, and even washing machines (25). It is well known that silver, whether in an ionic or nanoparticle form, is highly toxic to microorganisms (26). The synergistic effect of silver nanoparticles under the influence of various factors such as particle size, dose and duration of use, shape, temperature, and pH dependent, few studies have investigated the role of synergistic effects of silver nanoparticles along with antibiotics. Increasingly new bacterial strains have emerged with dangerous levels of resistance, including *S. aureus*. Dealing with bacterial resistance requires precautions that can lead to the prevention of the emergence and spread of multiresistant bacterial strains and the development of new antimicrobial substances. The excellent antibacterial activity against the *S. aureus* bacterium even at a low silver loading makes silver nanoparticles very ideal for a highly cost-effective antimicrobial solution with long-lasting effect in green industrial applications (27).

Present study suggests that antibiotics which can inhibit protein synthesis have significant synergistic effect along with silver nanoparticles. This research provides helpful insight into the development of new antimicrobial agents. To elucidate the mechanism of this synergistic effect, more elaborate experimental evidence will be

needed. Using silver nanoparticles with different shapes and sizes, an affordable way to increase the antimicrobial effect, but it is essential to have an attention on its toxicity in eukaryotic cells. Thus study of the toxicity, characteristics and mechanisms of effect of silver nanoparticles on the bacteria is required.

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تاثیر ضد باکتریایی نانو ذرات نقره همراه با آنتی بیوتیک‌های مهار کننده سنتز پروتئین بر *استافیلوکوکوس اورئوس* جداشده از موارد ورم پستان گاو

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چکیده

مقدمه: *استافیلوکوکوس اورئوس* یک باکتری بیماری‌زا در نشخوارکنندگان شیروار است که در گله‌های سالم یافت شده و می‌تواند علت اصلی ورم پستان باشد. برنامه‌های کنترلی متعددی به منظور مبارزه با این مشکل به کار رفته اما همیشه کار آمد نبوده است. در بیشتر کشورها، مقاومت آنتی بیوتیکی بسیار رایج شده است. نانو ذرات نقره فعالیت ضد میکروبی را علیه *استافیلوکوکوس اورئوس* نشان داده اند. در مطالعه حاضر، تاثیر نانو ذرات نقره همراه با آنتی‌بیوتیک‌های موثر بر سنتز پروتئین، بر روی *استافیلوکوکوس اورئوس‌های* جدا شده از ورم پستان گاو بررسی شده است.

مواد و روش‌ها: تعداد ۳۱۱ نمونه شیر از دام‌داری جمع‌آوری شد. هر نمونه شیر بر روی محیط مانیتول سالت آگار کشت و انکوبه شد. تعداد ۷۲ جدایه *استافیلوکوکوس اورئوس* از نمونه‌های شیر ورم پستان گاوی جدا شد. استخراج DNA *استافیلوکوکوس اورئوس* طبق دستور شرکت سازنده کیت خالص‌سازی انجام شد. ۵۸ جدایه *استافیلوکوکوس اورئوس* به وسیله آزمایش‌های بیوشیمیایی و تعیین ژن *nuc* تایید شدند. حداقل غلظت مهارکنندگی رشد و حداقل غلظت کشندگی رشد برای نانو ذرات نقره و نانو ذرات نقره همراه با آنتی‌بیوتیک در مورد ۵۰ جدایه تعیین شد.

نتایج: مقاومت جدایه‌های *استافیلوکوکوس اورئوس* به اریترومایسین، جنتامایسین، استرپتومایسین و داکسی سایکلین به ترتیب ۱۰۰، ۲۲، ۱۰۰ و ۸ درصد بودند. ۸ در صد جدایه‌ها نسبت به غلظت ۲۵ میکروگرم در میلی لیتر نانوذرات نقره حساسیت داشتند. رشد ۹۸ درصد نمونه‌ها در غلظت‌های مابین ۵۰ تا ۱۰۰ میکروگرم در میلی لیتر مهار شدند.

بحث و نتیجه‌گیری: مطالعه حاضر پیشنهاد می‌کند که آنتی‌بیوتیک‌های مهار کننده سنتز پروتئین همراه با نانو ذرات نقره در اغلب نمونه‌ها دارای اثر سینرژیستی هستند.

واژه‌های کلیدی: ورم پستان، *استافیلوکوکوس اورئوس*، نانو ذرات نقره، آنتی بیوتیک

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