The inhibitory effect of silver nanoparticles on the bacterial fish pathogens, *Streptococcus iniae*, *Lactococcus garvieae*, *Yersinia ruckeri* and *Aeromonas hydrophila*

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Abstract: The aim of this study was to assess the antibacterial effects of silver nanoparticles (Nanocid®) on 30 isolates of Gram-positive and Gram-negative bacteria that are pathogenic to fish, which consisted of strains of *Streptococcus iniae*, *Lactococcus garvieae*, *Yersinia ruckeri* and *Aeromonas hydrophila.* We used the liquid dilution method to find the minimum bactericidal concentration (MBC) using sub-culturing on the blood agar 30, 60 and 90 minutes after the inoculation of these bacteria. Isolates were prepared in sterile phosphate buffered serial dilutions of silver nanoparticles. For *S. iniae,* (14 isolates) the results showed that the MBCs were in a range of 5 to 0.15 µg/ml 30-90 minutes post-inoculation. For *L. garvieae* (14 isolates) the results showed that the MBCs were in a range from more than 10 µg/ml to 0.62 µg/ml 30-90 minutes post-inoculation. Also, the range of MBCs for *A. hydrophila* was 0.31 µg/ml to less than 0.15 µg/ml, while the range of MBCs for *Y. ruckeri* was from 2.5 to 0.62 µg/ml 30 to 90 minutes post-inoculation, respectively. Generally, the bacterial growth for these bacterial species was reduced from 1×10^1 cells/ml to no growth compared to initial inoculums of 10^7 cells/ml during 90 minutes after exposure to silver nanoparticles. No remarkable change was found in the bacterial count for the control samples (PBS without silver nanoparticles) during the experiment.

Keywords: silver nanoparticles, *Streptococcus iniae*, *Lactococcus garvieae*, *Yersinia ruckeri*, *Aeromonas hydrophila*, MBC.

Introduction

The emergence and increase in antibiotic resistance is an alarming concern in clinical diseases in both human medicine but veterinary medicine. The use of antimicrobial drugs in aquatic medicine can cause a serious problem in the environment because of the rapid spread of antibiotics through water. The use of metallic silver as an antimicrobial agent has been recognized for a long time (Lansdown 2002a) and several types of silver compounds are used today, including silver dressings, silver nitrate, silver zeolite and silver nanoparticles, for a variety of antimicrobial purposes (Kim *et al*., 2007; Rai *et al*., 2009).

Due to the increase in the outbreak of bacterial diseases in the aquaculture industry and the

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development of bacterial resistance, new antibacterial agents are required**.** One possibility is to use nanoparticles as antimicrobial drugs. Silver nanoparticles have proved to be one of the most effective metallic nanoparticles as they have a good antimicrobial efficacy against some bacteria, viruses and other eukaryotic microorganisms (Gong *et al*. 2007). However, more studies are required to evaluate the precise antimicrobial effects of such compounds. In particular, the assessment of the effects of different silver compositions on a range of pathogenic bacteria is important because of the risk of silver toxicity, which can cause agyrosis and argyria (Lansdown 2002b).

Therefore, the aim of this study was to evaluate the antibacterial effect of silver nanoparticles (Pars Nano Nasb Company, Tehran, Iran) against pathogenic isolates of *Streptococcus iniae* and *Lactococcus garvieae* that were recovered from diseased rainbow trout in different regions including Iran. The secondary aim was to find a suitable method to disinfect the waters used for trout farming in Iran, which are affected seriously by Streptococci and Lactococci (Soltani *et al*. 2005, 2008).

Materials and Methods

1.Preparation of silver nanoparticles

A water soluble form of colloidal, brown, silver nanoparticles called Nanocid® was used. It was concentrated at 4000 mg/L (stock solution) with an average nanoparticle size of 18 nm. This was a P-series powder product that was made by Nano Nasb Pars Company, Tehran, Iran for antimicrobial purposes.

2.Bacterial isolates

Isolates of *S. iniae* (13 isolates), *L. garvieae*, (12 strains), *Y. ruckeri* (one isolate) and *A. hydrophila* (one isolate) were used. The *S. iniae* and *L. garvieae* strains were all isolated from the kidney or spleen of diseased rainbow trout from different parts of Iran during 2000-2008 (Soltani *et al*., 2008). These diseased fish showed the typical clinical signs of streptococcosis or lacotoccosis, which included bilateral exophthalmia in particular and a mortality rate of up to 50%. *A. hydrophila* was originally recovered from Persian sturgeon during a motile *Aeromonas* septicemia in northern Iran (Soltani and Kalbasi, 2001). *Y. ruckeri* (Danish isolate) was recovered from affected trout and was donated by Dr. K. Dalsgaard. Additionally, the isolates of *S. iniae* (Spanish isolate) and *L. garvieae* (Japanese isolates) were included. All of the bacterial isolates were characterized previously according to their phenotype and molecular analysis by polymerase chain reaction (PCR). A lyophilized ampoule of the second to fourth passages of each bacterial isolate was used for the study, with the exceptions of the Danish, Spanish and Japanese isolates that underwent several passages before use.

3.Preparation of bacterial cultures and evaluation of their inhibitory effect

Firstly, a lyophilized vial of each bacterial isolate was cultured on blood agar plates at 30°C for 48 hours and the bacterial suspension of these cultures was prepared in sterile phosphate buffered saline (PBS) that was adjusted to 1.0 McFarland standard (ca 3 x 10^8 cfu/ml). An aliquot of 5 µl of each bacterial suspension was added to separate tubes that contained serial dilutions of the silver nanoparticles (Nanocid L-series colloidal product that contained 4000 mg/L of silver nanoparticles; Pars Nano Nasb Company, Tehran). These consisted of 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10µg/ml prepared in sterile PBS. The tubes were then incubated at 30°C and aliquots of 10 µl of each sample in three replicates were spread onto blood agar plates at specific time intervals of 30, 60 and 90 minutes post-inoculation. The plates were incubated at 30°C overnight and the bacterial counts (cfu/ml) were then performed. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of silver nanoparticles at which no bacterial growth occurred on the blood agar plate **(**Lansdown, 2002a**).**

Results

The bacterial inhibition by silver nanoparticles

on strains of *S. inaie* and *L. garviae* are shown in Tables 1 and 2. Generally, the MBCs were decreased with increasing time-exposure of the isolates of both bacterial species to silver nanoparticles. The MBC against *S. inaie* isolates ranged from 0.15 to 5 µg /ml, while the range of MBC for *L. garviae* was from 0.62 to greater than >10 µg /ml. The minimum numbers of bacterial cells counted in the lowest concentration of silver nanoparticles at which bacterial growth was positive are shown in Tables 3 and 4.

In general, the bacterial growth for the isolates of both bacterial species of *S. inaie* and *L. garvieae* was reduced to 5 logs less than the initial inoculum during the time period of 30-90 minutes post-exposure to silver nanoparticles. No significant change was found in the bacterial count for the control samples (PBS without silver nanoparticles) during the experiment.

The MBCs against *A. hydrophila* were obtained at 0.31, <0.15 and <0.15 µg/ml at 30, 60 and 90 minutes after the exposure to the nanoparticles, respectively, while the equivalent values for *Y. ruckeri* were 2.5, 0.62 and 0.62 µg/ml, respectively. The minimum numbers of *A. hydrophila* and *Y. ruckeri* cells that were counted in the lowest silver concentration are shown in Table 5. In general, the bacterial growth for *A. hydrophila* was reduced to zero, while that of *Y. ruckeri* reduced to 6 logs less than the initial inoculums after 90 minutes from the exposure to silver nanoparticles. No significant change was found in the bacterial count for the control samples (PBS without silver nanoparticles) during any period measured in this set of experiments.

Table 1: Minimum bactericidal concentration (MBC) of silver nanoparticles against *S. iniae.*

	MBC (µg/ml)		
Bacterial strain	**After 30 minutes**	**After 60 minutes**	**After 90 minutes**
S. iniae 0198 Haraz	5	5	0.62
S. iniae 0183 Qom	1.25	0.62	0.62
S. iniae 0196A Tonekabon	2.5	2.5	1.25
S. iniae 0201A Haraz	5	5	5
S. iniae 199A Haraz	2.5	2.5	2.5
S. iniae 0191B Haraz	5	2.5	2.5
S. iniae LHK6 Shahrkord	<0.62	<0.62	<0.62
S. iniae VM10 Japanese	2.5	0.31	0.15
S. iniae 201C Haraz	1.25	0.62	0.62
S. iniae 195B Tonekabon	0.62	<0.62	<0.62
S. iniae LHK5 Shahrkord	2.5	0.31	0.15
S. iniae 172B Jajrod	5	1.25	1.25
S. iniae LG6 Langrod(Gilan)	2.5	1.25	0.62
S. iniae SH3 Haraz	0.15	0.15	0.15

Table 2: Minimum bactericidal concentration (MBC) of silver nanoparticles against *L. garvieae*

	MBC (µg/ml)		
Bacterial strain	**After 30 minutes**	**After 60 minutes**	**After 90 minutes**
L. garvieae 0190A Tonekabon	1.25	1.25	0.62
L. garvieae 200A Haraz	5	1.25	1.25
L. garvieae VM8 Japanese	5	1.25	0.62
L. garvieae 0161B Lorstan	5	1.25	1.25
L. garvieae 170A Goharvar	1.25	0.62	0.62
L. garvieae VM2 Japanese	>10	>10	10
L. garvieae 167A Jajrod	>10	>10	10
L. garvieae ALS2 Lorstan	>10	>10	5
L. garvieae 165B Kermansha	>10	>10	5
L. garvieae 173B Jajrod	5	2.5	1.25
L. garvieae 194A	5	1.25	1.25
L. garvieae ALS4 Lorstan	>10	>10	10
L. garvieae 171 Goharvar	>10	>10	10
L. garvieae 141-3	2.5	1.25	1.25

Discussion

After a study of the available literature, no work has been done to evaluate the inhibitory effect of silver nanoparticles on bacterial fish pathogens, especially with regards to those that cause important disease outbreaks in the aquaculture industry that can have severe economic impacts. In particular, such information would be valuable for operation planning, and for the control and prevention of such fish pathogens entering into the water column. Therefore, emerging nanoparticle antimicrobial therapies are of great interest in the treatment of contaminated waters that are used for aquaculture activities.

In this study, the inhibitory effects of silver nanoparticles were evaluated on the growth of 30 isolates of both Gram-positive and Gram-negative bacteria that are pathogenic to fish. The results

Table 3: Number of *L. garveiae* cells counted in the lowest concentration of silver nanoparticles. The numbers in parentheses show the lowest concentration of nanosilver suspension at which the bacterial growth was positive.

Bacterial strain	Initial inoculation (CFU/ml)	Bacterial growth (CFU/ml)		
		After 30 minutes	After 60 minutes	After 90 minutes
L. garvieae VM8 Japanese strain	5.62×10^{7}	7×10^{2} (2.5)	2×10^{2} (0.62)	No growth (0.15)
L. garvieae 190A Tonekabon	4.0×10^{8}	2×10^{3} (0.62)	7.8×10^{2} (0.62)	1×10^{2} (0.31)
L. garvieae 161B Lorestan	1.2×10^{8}	1.4×10^{3} (2.5)	2×10^{2} (0.62)	1×10^{2} (0.62)
L. garvieae 165B Kermanshah	1.2×10^{8}	1.0×10^{3} (10)	6×10^{2} (5)	3×10^{2} (2.5)
L. garvieae 167B Jajrod	1.3×10^{8}	1.6×10^{3} (5)	9×10^{2} (5)	1×10^{2} (5)
L. garvieae 170A Goharvar	1.3×10^{8}	1.9×10^{3} (0.62)	1×10^{2} (0.31)	1×10 (0.15)
L. garvieae 173B Jajrod	1.4×10^{8}	4×10^{2} (2.5)	2×10^{2} (1.25)	2×10^{2} (0.62)
L. garvieae VM2 Japanese strain	9.0×10^{7}	3×10^{2} (10)	1×10^{2} (5)	1×10^{2} (2.5)
L. garvieae 194A Tonekabon	1.8×10^{7}	7×10^{2} (2.5)	2×10^{2} (0.62)	1×10^{2} (0.62)
L. garvieae 200A Haraz	2.2×10^{8}	2×10^{2} (5)	1×10^{2} (2.5)	1×10^{2} (0.25)
L. garvieae ALS2 Lorestan	8.7×10^{7}	6×10^{2} (10)	3×10^{2} (5)	2×10^{2} (5)
L. garvieae ALS4	1.2×10^{8}	1.9×10^{3} (10)	8×10^{2} (10)	2×10^{2} (5)
L. garvieae 141-3	1.2×10^{7}	2×10^{2} (1.25)	1×10^{2} (0.62)	1×10^{2} (0.62)

Table 4: Number of *S. iniae* cells counted in the lowest concentration of silver nanoparticles. The numbers in parentheses show the lowest concentration of nanosilver suspension at which the bacterial growth was positive.

Bacterial strain	Initial inoculation (CFU/ml)	Bacterial growth (CFU/ml)		
		After 30 minutes	After 60 minutes	After 90 minutes
S. iniae 201A Haraz	1.2×10^{8}	8×10^{2} (2.5)	2×10^{2} (2.5)	2×10^{2} (2.5)
S. iniae 199A Haraz	1.2×10^{8}	5×10^{2} (1.25)	4×10^{2} (1.25)	1×10^{2} (1.25)
S. iniae 183 Qom	1.8×10^{7}	4×10^{2} (0.62)	4×10^{2} (0.31)	1×10^{2} (0.31)
S. iniae VM10 Spanish strain	1.7×10^{7}	1.2×10^{3} (1.25)	11×10^{2} (0.15)	No growth (0.15)
S. iniae 172B Jajrod	4.3×10^{7}	2.0×10^{3} (2.5)	1×10^{2} (0.62)	1×10^{2} (0.62)
S. iniae 191B Haraz	1.0×10^{8}	1.2×10^{4} (5)	8×10^{2} (2.5)	2×10^{2} (2.5)
S. iniae 195B Tonekabon	1.2×10^{8}	3×10^{2} (0.31)	2×10^{2} (0.31)	1×10^{2} (0.15)
S. iniae 201C Haraz	9.3×10^{7}	2×10^{2} (0.62)	1×10^{2} (0.31)	No growth (0.15)
S. iniae 196A Tonekabon	2.9×10^{8}	6×10^{2} (1.25)	2×10^{2} (1.25)	1×10^{1} (0.62)
S. iniae LHK5 Shahrkord	1.4×10^{8}	1×10^{2} (1.25)	1×10^{2} (0.62)	0 (0.15)
S. iniae LHK6 Shahrkord	3.2×10^{7}	4.3×10^{3} (0.15)	8×10^{2} (0.15)	1×10^{2} (0.15)
S. iniae LG6 Langrod (Gilan)	9.5×10^{7}	3×10^{2} (1.25)	1×10^{2} (1.25)	No growth (0.15)
S. iniae Sh3 Haraz	1.8×10^{8}	No growth (0.15)	No growth (0.15)	No growth (0.15)

Table 5: Number of *A. hydrophila* and *Y. ruckeri* cells counted in the lowest concentration of silver nanoparticles. The numbers in parentheses show the lowest concentration of nanosilver suspension at which the bacterial growth was positive.

Bacterial strain	Initial inoculation (CFU/ml)	Bacterial growth (CFU/ml)		
		After 30 minutes	After 60 minutes	After 90 minutes
A. hydrophila 04 Iran isolate	3.1×10^{7}	1×10^{2} (0.31)	No growth (0.15)	No growth (0.15)
Y. ruckeri Danish isolate	3.8×10^{7}	3×10^{2} (1.25)	1×10^{2} (0.31)	1×10 (0.31)

obtained showed that the MBC of silver nanoparticles to *S. iniae* isolates was generally lower than for *L. garvieae* isolates. In addition, this study showed that *S. iniae* strains were more sensitive to silver nanoparticles than *L. garvieae* strains. According to our results, both the Gram-negative bacterial species of *A. hydrophila* and *Y. ruckeri* showed lower MBCs than these isolates of *S. iniae* and *L. garvieae*. The lowest MBC was obtained for *A. hydrophila*.

Kim *et al*. (2007) showed that silver nanoparticles have more inhibitory effects on *E. coli* than *S. aurous, which is* probably because of the different cell wall membrane structures between Gram-positive and Gram-negative bacteria. The exact mechanism of the inhibitory effects of silver nanoparticles on microorganisms is unknown. Some studies have reported that the positive charge of the silver ion is crucial for its antimicrobial activity through the electrostatic attraction between the negatively charged cell membrane of the microorganisms and the positively charged nanoparticles (Dibrov *et al*., 2002). In contrast, other researchers have reported that the antimicrobial activity of silver nanoparticles on Gram-negative bacteria was dependent on the concentration of silver nanoparticles, and this was closely associated with the formation of "pits" in the cell walls of bacteria, which results in permeable bacterial membranes and consequent bacterial cell death (Sondi and Salopek-Sondi, 2004).

Epidemiologically, the causative agents of streptococcosis, lactococcosis, yersiniosis, and motile *Aeromonas* septicemia are wildly scattered in the environment because of the wide range of bacterial sources and reservoirs (Brown, 1993; Vendrell *et al*., 2006). Disease transmission occurs mainly through the column of contaminated waters. Because of this, water treatment is therefore very important. In this study, silver nanoparticles were able to reduce the growth of all of the tested isolates of *S. iniae*, *L. garveiae, A. hydrophila* and *Y. ruckeri* significantly after 30-90 minutes of exposure at a concentration up to 5 µg/ml. Therefore, it could be possible to use such a nanomaterial as a water treatment to control or at least reduce the outbreaks of disease due to these bacterial fish pathogens. This is particularly applicable in the regions where rivers are commonly used as the water source for fisheries. However, such nanomaterials should be used within a mesh or filter system in the inlet water column, rather than in the form of soluble suspension to avoid dispersal into the wider environment. Other researchers have suggested that the use of filters containing silver nanoparticles could be an effective and sustainable point-of-use water treatment technology (Sharma *et al*., 2009).

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References

1. Brown, L. (1993). Aquaculture for Veterinarians: Fish Husbandry and Medicine. Pergamon Press. New York, USA. pp: 447.
2. Dibrov, P., Dzioba, J., Gosink, K. K., Hase, C. C. (2002) Chemiosmotic mechanism of antimicrobial activity of Ag(+) in *Vibrio cholerae*. Antimicrobial Agents Chemotherapy.

46: 2668-2670.

3. Gong, P., Li, H., He, X., Wang, K., Hu, J., Tan, W., Zhang, S., Yang, X. (2007) Preparation and antibacterial activity of Fe_3 O_4@Ag nanoparticles. Nanotechnology. 18: 604-611.
4. Kim, J. S., Kuk, E., Yu, K. N., Kim, J. H., Park, S. J., Lee, H. I., Kim, S. H., Park, S. J., Park, Y. H., Hwang, C. Y., Kim, Y. K., Lee, Y. S., Jeong, D. H., Cho, M. H. (2007) Antimicrobial effects of silver nanopaticles. Nanomedicine, Nanotechnology, Biology, and Medicine. 3: 95-101.
5. Lansdown, A. B. (2002a) Silver 1. Its antibacterial properties and mechanism of action. J. Wound Care. 11: 125-130.
6. Lansdown, A. B. (2002b) Silver 2. Toxicity in mammals and how its products aid wound repair. J. Wound Care. 11: 173-177.
7. Rai, M., Yadav, A., Gade, A. (2009) Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances. 27: 76-83.
8. Sharma, V. K., Yngard, R. A., Lin, Y. (2009) Silver nanoparticles: Green synthesis and their antimicrobial activities. Advances in Colloid and Interface Sci. 145: 83-96.
9. Soltani, M., Kalbasi, M. R. (2001) Protection of Persian sturgeon (*Acipenser persicus*) fingerling against *Aeromonas hydrophila* septicemia using three different antigens. European Association of Fish Pathologists. 21: 235-240.
10. Soltani, M., Jamshidi, S., Sharipour, I. (2005) Streptococcosis caused by *Streptococcus iniae* in farmed rainbow trout in Iran: biophysical characteristics and pathogenesis. European Association of Fish Pathologists. 25: 95-106.
11. Soltani, M., Nikbakht, G., Mousavi-Ebrahimzadeh, H. A., Ahmadzadeh, N. (2008) Epizootic outbreaks of lactococcosis caused by *Lactococcus garvieae* in farmed rainbow trout (*Oncorhynchus mykiss)* in Iran. European Association of Fish Pathologists. 28: 207-213.
12. Sondi, I., Salopek-Sondi, B. (2004) Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. Advances in Colloid Interface Sci. 275: 177-182.
13. Vendrell, D., Balcazar, J. L., Ruiz-Zarzuela, I., Blas, I. B., Muzquiz, J. L. (2006) *Lactococcus garvieae* in fish: a review. Comparative Immunology and Microbiology and Infectious Diseases. 29: 177-198.