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Full Length Research Paper

In vitro antimicrobial effect of silver nanoparticles on *Lactococcus garvieae* and *Streptococcus iniae*

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Antimicrobial effect of silver nanoparticles on two fish pathogens, *Lactococcus garvieae* and *Streptococcus iniae* was investigated by microdilution method. A total of 16 isolates of *L. garvieae* and *S. iniae* that were obtained from diseased rainbow trout during 2009 to 2011 were examined. Antimicrobial effects of the compound were evaluated according to the broth microdilution method by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). According to the results MIC ranged from 1.12 to 5 µgml^{-1} for *L. garvieae* and 1.12 to 2.5 µgml^{-1} for *S. iniae* isolates. The mean MIC value was 2.59 and 2.1 for *L. garvieae* and S. iniae, respectively. The MBC was also calculated for the examined isolates as MBC ranged from 2.5 to 10 µgml^{-1} with the mean of 5.27 and 3.57 for *L. garvieae* and S. iniae, respectively. The results showed that *S. iniae* strains were more sensitive to silver nanoparticles than *L. garvieae* strains. This study demonstrated the in vitro antimicrobial effects of silver nanoparticles on the examined bacteria although the efficacy of silver nanoparticles in fish ponds in different conditions needs more investigations.

Key words: Silver nanoparticles, *Lactococcus garvieae*, *Streptococcus iniae*.

INTRODUCTION

The apparent increase of the occurrence of antibiotic resistance among bacteria from various areas of animal production during the recent years and its possible implications for public health (Aoki et al., 1999; Levy et al., 1998; Tollefson et al., 1999) have in many countries lead to an intensified surveillance of bacterial resistance. In the field of aquaculture, both therapeutic and environmental problems are important, as antibiotics and chemicals are released into the surrounding water, mostly rivers, during medical treatment of bacterial fish diseases (Aoki et al., 1999). The impact of these substances on the resident microflora is difficult to assess because of the complexity of the aquatic environment, while the resistance patterns of bacterial fish pathogens often reflect an intensive use of antimicrobial substances (Bruun et al., 2000; Smith et al., 1994a). The rapidly expanding aquaculture industry in Iran has suffered heavy economic losses due to bacterial pathogens, particularly *Lactococcus garvieae* and *Streptococcus iniae*, which are the most important Gram positive pathogenic bacteria causing *lactococcosis* and *streptococcosis* in rainbow trout farms. Both mentioned diseases have been reported in rainbow trout in several countries such as Australia, South Africa, Japan, Taiwan, England, Turkey, countries of the Mediterranean area and Iran (Ghittino and Prearo, 1992; Palacios et al., 1993; Chen et al., 2001; 2002; Chang et al., 2002; Soltani et al., 2008; Raissy and Ansari, 2011). They are responsible for high economical losses in rainbow trout farms.

Recently, the development of resistant or even multidrug resistant pathogens has become a major problem in rainbow trout culture industry in Iran and many countries, for instance *L. garvieae* resistance to different antibiotics should be mentioned (Raissy and Ansari, 2011). So, it seems that a new generation of antibacterial agents is required in order to prevent or reduce bacterial diseases in the aquaculture industry. It is well known that

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Table 1. Antimicrobial activity of silver nanoparticles on *L. garvieae* isolates.

Bacterial isolate	Silver nanoparticle	
	MIC (μgml^{-1})	MBC (μgml^{-1})
L. garvieae 02	2.5	5
L. garvieae 03	1.12	2.5
L. garvieae 04	2.5	2.5
L. garvieae 012	2.5	5
L. garvieae 017	5	10
L. garvieae 018	1.12	5
L. garvieae 019	1.12	2.5
L. garvieae 021	5	5
L. garvieae 022	2.5	10
Mean ± SD	2.59 ± 1.50	5.27 ± 2.91

silver ions and silver-based compounds are highly toxic to microorganisms. Antimicrobial mechanism of silver nanoparticles is related to accumulation in the bacterial cytoplasmic membrane, causing a significant increase in permeability and cell death (Sondi et al., 2004). Recently, it has been suggested that the antimicrobial mechanism of silver nanoparticles may also be related to membrane damage due to free radicals that are derived from the surface of the nanoparticles (Kim et al., 2007). Several salts of silver and their derivatives are commercially employed as antimicrobial agents. However, the antibacterial property of these nanoparticles depends on their stability in the growth medium as well as in fish ponds, since this imparts greater retention time for bacterium–nanoparticle interaction (Shrivastava et al., 2007). The aim of this study was to evaluate antibacterial effects of silver nanoparticles on fish pathogens *L. garvieae* and *S. iniae*.

MATERIALS AND METHODS

Bacterial isolates

A total of 16 isolates of *L. garvieae* and *S. iniae* were obtained from 13 rainbow trout farms between September 2009 and July 2011. The samples of the liver, kidneys and heart were placed on a 5% sheep blood agar with 1% yeast extract agar (Merck) plates and then incubated at 24 and 37°C for 2 to 3 days under aerobic conditions. Standard physiological and biochemical tests recommended by Austin and Austin (1999) were performed at 25°C. Identification of the isolates was confirmed by PCR assay as described by Zlotkin et al. (1998) when the expected 1100-bp PCR amplification product was observed. The isolates were stored at -70°C in tryptic soy broth (TSB) containing 10% glycerol until further use.

Antimicrobial susceptibility tests

A stock solution (4000 mgL^{-1}) of silver nanoparticle with an average size of 18 nm was prepared. Antimicrobial susceptibility tests were performed according to the broth microdilution methods described by the Clinical and Laboratory Standards Institute (CLSI, 2006). The solution was serially diluted in cation-adjusted Mueller-Hinton broth (CAMHB) containing 5% lysed horse blood as the concentrations ranged from 0.56 to 80 μgL^{-1}. In preparation for inoculation, the 0.5 McFarland suspensions of the isolates were diluted 10 fold with MHB. Diluted inocula (5 ml) were added to each tube and incubated at 25°C for 24 h. A negative control was also considered without silver nanoparticle or inoculum. The lowest concentration of nanoparticle that visibly inhibited bacterial growth was considered as the MIC (CLSI, 2006). The isolates were divided into resistant, intermediate and susceptible groups, according to the CLSI criteria for animal isolates (CLSI, 2008). The MBC was also defined as the lowest concentration of silver nanoparticle with no bacterial growth on the blood agar medium (Soltani et al., 2009).

RESULTS

A total of 16 bacterial strains including 9 isolates of *L. garvieae* and 7 isolates of *S. iniae* were used for evaluation of antimicrobial effect of silver nanoparticles. The isolates were collected from diseased fish from rainbow trout fish farms in Chaharmahal va Bakhtyari Province in west Iran. The identity of the isolates was confirmed by biochemical tests and also PCR. The results of antimicrobial activity of silver nanoparticles are presented in Tables 1 and 2. According to the results MIC ranged from 1.12 to 5 μgml^{-1} for *L. garvieae* and 1.12 to 2.5 μgml^{-1} for *S. iniae* isolates. The mean MIC value was 2.59 and 2.1 for *L. garvieae* and *S. iniae*, respectively. The MBC was also calculated for the examined isolates as MBC ranged from 2.5 to 10 μgml^{-1} with the mean of 5.27 and 3.57 for *L. garvieae* and *S. iniae*, respectively.

DISCUSSION

The majority of the trout fish farms along the river release their effluents after passage of sedimentation ponds and without further treatment. Various amounts of antibiotic may still be present in the effluent water following antibiotic therapy on the farms (Smith et al., 1994b;

Table 2. Antimicrobial activity of silver nanoparticles on *S. iniae* isolates.

Bacterial isolate	Silver nanoparticle	
	MIC (μgml^{-1})	MBC (μgml^{-1})
S. iniae 07	2.5	2.5
S. iniae 011	2.5	5
S. iniae 014	1.12	5
S. iniae 015	2.5	2.5
S. iniae 016	2.5	5
S. iniae 017	2.5	5
S. iniae 018	1.12	2.5
Mean ± SD	2.1 ± 0.67	3.57 ± 1.33

Vaughan et al., 1996) and persist on and around fish farms (Halling-Sorensen et al., 1998; Hektoen et al., 1995). Numerous studies suggest a correlation between findings of increased bacterial resistance levels on and around inland fish farms and the antimicrobial agents used at the farms (DePaola et al., 1988; Guardabassi et al., 2000; McPhearson et al., 1991; Spanggaard et al., 1993). The development of resistant or even multidrug resistant pathogens in recent years has become a major problem in rainbow trout culture industry in Iran and many countries, for instance *L. garvieae* resistance to different antibiotics should be mentioned (Raissy and Ansari, 2011). So, it seems that a new generation of antibacterial agents is required in order to prevent or reduce bacterial diseases in the aquaculture industry. In this investigation, antimicrobial effects of silver nanoparticles on two important fish pathogenic bacteria *L. garvieae* and *S. iniae* was studied.

The antibacterial efficiency of the silver nanoparticles has been investigated by many researchers by introducing the particles into a media containing bacteria. Antibacterial activity of the silver particles at low concentrations has been proven before (Kim et al., 2007; Rhim, 2006; Shrivastava et al., 2007; Soltani et al., 2008).

In a previous study the concentration of 25 μgml^{-1} silver nanoparticles was found to be strongly inhibitory for *Escherichia coli* and silver nanoparticles were found to have a less significant effect on the growth of Gram-positive bacteria (Shrivastava et al., 2007). The fact that Gram-positive bacteria are less susceptible to the antimicrobial activity of silver nanoparticles has been emphasized by some authors (Kawahara et al., 2000; Kim et al., 2007; Rhim et al., 2006). It was speculated that this may be due to differences in the cell wall structure (Kawahara et al., 2000). The cell wall of Gram-positive bacteria contains multiple layers of peptidoglycan compared to the cell wall of Gram-negative bacteria. Peptidoglycan is a complex structure and often contains teichoic acids or lipoteichoic acids which have a strong negative charge, which may contribute to sequestration of free Ag ions. Thus, Gram-positive bacteria may allow less Ag to reach the cytoplasmic membrane than Gram negative bacteria (Kawahara et al., 2000) and may therefore be less susceptible to these compounds. Soltani et al. (2009) indicated that MBC of silver nanoparticles ranged from 0.15 to 5 μgml^{-1} for *S. iniae* and from 0.62 to >10 μgml^{-1} for *L. garvieae*. In this study MIC and MBC was in the range of 1.12 to 2.5 μgml^{-1} and 2.5 to 5 μgml^{-1} for *S. iniae* and 1.12 to 5 and 2.5 to 10 μgml^{-1} for *L. garvieae*. The mean MIC and MBC was 2.1 and 3.5 μgml^{-1} for *S. iniae*; 2.5 and 5.2 μgml^{-1} for *L. garvieae*. The results showed that the mean MIC and MBC of silver nanoparticles to *S. iniae* isolates was 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. The results found in this study were in good agreement with the findings of Soltani et al. (2009). This study demonstrated the *in vitro* antimicrobial effects of silver nanoparticles on pathogenic bacteria although possibility of use of synthesized silver nanoparticles in fish ponds needs more investigations.

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