



In-vitro* and *in-vivo* antibacterial effect of five different sizes of silver nanoparticles (Ag-NPs) in *Labeo rohita

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ABSTRACT

The present study evaluated the efficacy of silver nanoparticles (Ag-NPs) on *in-vitro* antimicrobial activity via a battery of tests and *in-vivo* antimicrobial activity against *E. tarda* in Indian Major Carp, *Labeo rohita*. *In-vitro* antibacterial tests showed that Ag-NPs in the smaller size range exhibit better antibacterial activity as compared to larger size Ag-NPs. For *in-vivo* testing, fishes were challenged with *E. tarda* @ $6.7 \times 10^4/100\mu\text{l}$. The infected fishes were treated with 5 different sizes of Ag-NPs (27 nm, 46.1 nm, 71 nm, 96 nm and 103 nm) by 3 different routes of administration, i.e. by injection, feed and immersion @ 3.6 mg/kg body weight, 4.5 mg/kg body weight and 0.2 mg/litre, respectively, for 21 days. The *in-vivo* test showed that significantly higher protection was achieved in treatment with 27 nm Ag-NPs followed by 71 nm and 46.1 nm Ag-NPs. The 3 routes of administration increased the survival rate of *L. rohita* by 31%, 5% and 18%, respectively. Hence, it can be concluded that with further evaluation, nanoparticle based treatment technologies could overcome threats of multi-drug resistance syndrome either as an alternative or as supplementary to antibiotic therapy.

Key words: Antibacterial, Feed, Immersion, Injection, *Labeo rohita*, Lethal dose, Lethal concentration, Silver nanoparticles

Intensified aquaculture practices are in vogue to fulfil the fish consumption requirement of a rapidly growing global population. However, the occurrence of diseases in intensified aquaculture acts as a major impediment to socio-economic growth of the sector. Edwardsiellosis caused by *Edward siellatarida* (gram negative, motile, rod shaped bacteria) is a commonly reported disease across a variety of cultured fish in Asia, especially India and Japan (Herman *et al.* 1986), and is also common in reptiles, birds and mammals including humans (Rao *et al.* 2001). In fish, it is associated with acute to chronic infection of fry, fingerlings and adults resulting in large scale mortality (Mohanty *et al.* 2007). The disease spreads quickly because of the ability of *E. tarda* to survive in water without a host. Administration of antibiotics is proving to be a poor treatment strategy due to increasing appearance of multi-drug resistance syndrome in the pathogen. Hence, management of Edwardsiellosis in affected aquaculture systems is facing severe setbacks.

Silver nanoparticles (Ag-NPs) have emerged as a fast growing product in the industry mainly because of its antimicrobial activity (Kim *et al.* 2006, Ahamed *et al.* 2008). Ag-NPs application in disease management include nano-silvercoated filters to prevent fungal growth in shrimp

culture ponds (Johari *et al.* 2013); inhibition of important fish pathogen, *Aeromonas hydrophila* (Sarkar *et al.* 2012); and control of *Streptococcus iniae*, *Lactococcus garvieae*, *Yersinia ruckeri* and *Aeromonas hydrophila* (Soltani *et al.* 2009). The incorporation of nanotechnology in disease treatment could be more reliable as compared to the prevalent use of antibiotics. Hence, this study was carried out to provide possible solutions to emerging concerns in aquatic animal disease management with the objective to evaluate the potency of Ag-NPs in inhibition of *E. tarda in-vitro*, evaluating efficacy of Ag-NPs treatment in diseased *L. rohita in-vivo* besides resolving concerns regarding toxicity of Ag-NPs to treated organisms.

MATERIALS AND METHODS

The care and treatment of animals used in this study were in accordance with the guidelines of the CPCSEA [(Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Govt of India].

Synthesis of silver nanoparticles (Ag-NPs): Silver nanoparticles were synthesized as per Dragieva *et al.* (1999) with slight modification. A prepared 100 mM AgNO₃ aqueous solution was kept on an ice bath under continuous stirring using a magnetic stirrer for 20 min after which, 2 ml aqueous solution of 200 mM sodium borohydride (NaBH₄) was added to the AgNO₃ solution with continuous stirring. Once the solution turned light yellow, the magnetic

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stirrer was switched off and after 30 s, a drop of aqueous polyvinyl pyrrolidone (PVP) (0.3%) was added to stabilize and prevent aggregation of formed nanoparticles. The mean particle size, size distribution and zeta potential of silver nanoparticles were analyzed by nanoparticle analyzer. For surface analysis, the silver nanoparticles were imaged by Atomic Force Microscopy instrument.

Acute toxicity test: The test was carried out according to OECD Test Guideline 203: Fish Acute Toxicity Test. To estimate the lethal concentration₅₀ (LC₅₀) through immersion and lethal dose₅₀ (LD₅₀) value through injection and feed, fishes were exposed to Ag-NP at different concentrations. A blank control test (without Ag-NPs) was also performed under same exposure conditions. All the experiments were conducted with 10 fishes in each exposure tank. No water exchange was done during the test. Initially, the range finding test was conducted to ascertain the LC₅₀ and LD₅₀ followed by definitive test. The percentage mortality was recorded at 24, 48, 72, and 96 h interval. Data obtained from the experiment were processed by probit analysis.

In-vitro antibacterial effect

Antibacterial activity through total plate count (TPC):

To examine the bactericidal effect of silver nanoparticles on *E. tarda*, the bacteria was cultured on brain heart infusion (BHI) agar plates spiked with nanosized silver nanoparticles at 2 different concentrations of 20 µg and 10 µg / plate. Ag-NP free BHI plates cultured under the same conditions were used as control. All the plates were incubated for 24 h at 28°C and the numbers of colonies were counted. Total count was averaged from 3 repeat plates for each sample.

Growth curve analysis/time kill assay: In the presence of silver nanoparticles, *E. tarda* were grown in liquid BHI medium supplemented with 20 µg of AgNPs/cm³ of medium. Growth rates and bacterial concentrations were determined by measuring optical density (OD) at 600 nm at an interval of every 2 h.

Well diffusion method for nanoparticle susceptibility:

In well diffusion method, sterile molten Mueller Hinton Agar petriplates were swabbed with the overnight culture (52×10⁹ cells/ml) of *E. tarda*. The solid medium was gently punctured with the help of metallic borer to make a well. Finally the nanoparticles samples (20 µg, 10 µg and 5 µg) were added from the stock into each well and incubated for 24 h at 28°C. After 24 h, the zone of inhibition (diameter) was measured and expressed as millimeters.

Disk diffusion method for antibiotic susceptibility:

Kirby-Bauer disk diffusion susceptibility test was done by spreading the test organism evenly on the Mueller-Hinton Agar plate. Sterile antibiotic disks, one for each size of Ag-NP was manually placed using forceps on to the agar surface and to ensure the complete contact, the disk was pressed with the forceps. The plates were incubated at 28°C overnight and zone of inhibition was measured in mm.

Minimum inhibitory concentration (MIC) of Ag-NPs:

Fish pathogens, viz. *Edward siellatarda*, *Aeromonas*

hydrophila, *Aeromonas cavaei*, *Vibrio parahaemolyticus* and *Staphylococcus aureus* were used as test pathogens. MIC test was conducted according to National Commission for Clinical Laboratory Standard (NCCLS) using the reagent 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride. The yellow dye 2,3,5-triphenyltetrazolium chloride (TTC) was reduced in living cells by dehydrogenase to produce insoluble red TTC formazan crystals. Different concentrations (0.625, 1.25, 2.5, 5, 10 and 20 µg) of different sized nanoparticles were mixed with nutrient broth and 50 µl of 24 h old bacterial inoculum and allowed to grow overnight at 28°C for 24 h. AgNPs alone served as negative control and bacterial culture as positive control. MIC was determined as the lowest concentration of the nanoparticles that did not permit any visible growth of bacteria during 24 h of incubation on the basis of red colour formation.

Minimum bactericidal concentration (MBC) of Ag-NPs:

To avoid the possibility of misinterpretation caused by negligible colour formation, MBC was also determined by sub-culturing the above (MIC) serial dilutions after 24 h in nutrient agar plates and incubation at 28°C for 24 h. MBC was regarded as the lowest concentration that prevents the growth of any bacterial colonies on nutrient agar medium.

In-vivo antibacterial effect

Fingerlings of *Labeo rohita* (10.60 to 14.20g) were challenged with *E. tarda* suspension of 0.10 ml in phosphate buffer saline (6.7×10⁴ CFU in 100µl) via intraperitoneal injection. After 24 h of infection, the fishes were treated with Ag-NPs through injection (3.6mg/kg body weight), feed (4.5mg/kg body weight) and immersion (0.2mg/kg body weight) for each size group of Ag-NPs forming 6 treatment groups of 10 fishes with 3 replicates each. Behavioural signs of disease in the fishes were observed and noted. After 21 days, cumulative survival percentage was calculated to assess the *in-vivo* antibacterial (*E. tarda*) effect of 5 different sized Ag-NPs.

Statistical analysis

The data were analyzed by statistical package SPSS by subjecting it to one-way ANOVA and Duncan's multiple range tests at 5% probability level.

RESULTS AND DISCUSSION

Characterization of silver nanoparticles: Nanoparticles of five different sizes were developed with different concentration of AgNO₃ and different stirring rate. The mean particle sizes of silver nanoparticles were found to be 27 nm, 46.1 nm, 71 nm, 96 nm and 103 nm with stable yellow colour. The characterization of 71 nm Ag-NPs showed presence of smaller sized NPs (1–10nm) along with majority 71 nm (Fig. 1c). The other four sizes were found to have a single peak for the respective frequency of size (Fig. 1). After size characterization, it was found that with increasing size of silver nanoparticles (Ag-NPs), zeta potential was in reverse order. Different properties of silver nanoparticles are presented in Table 1.

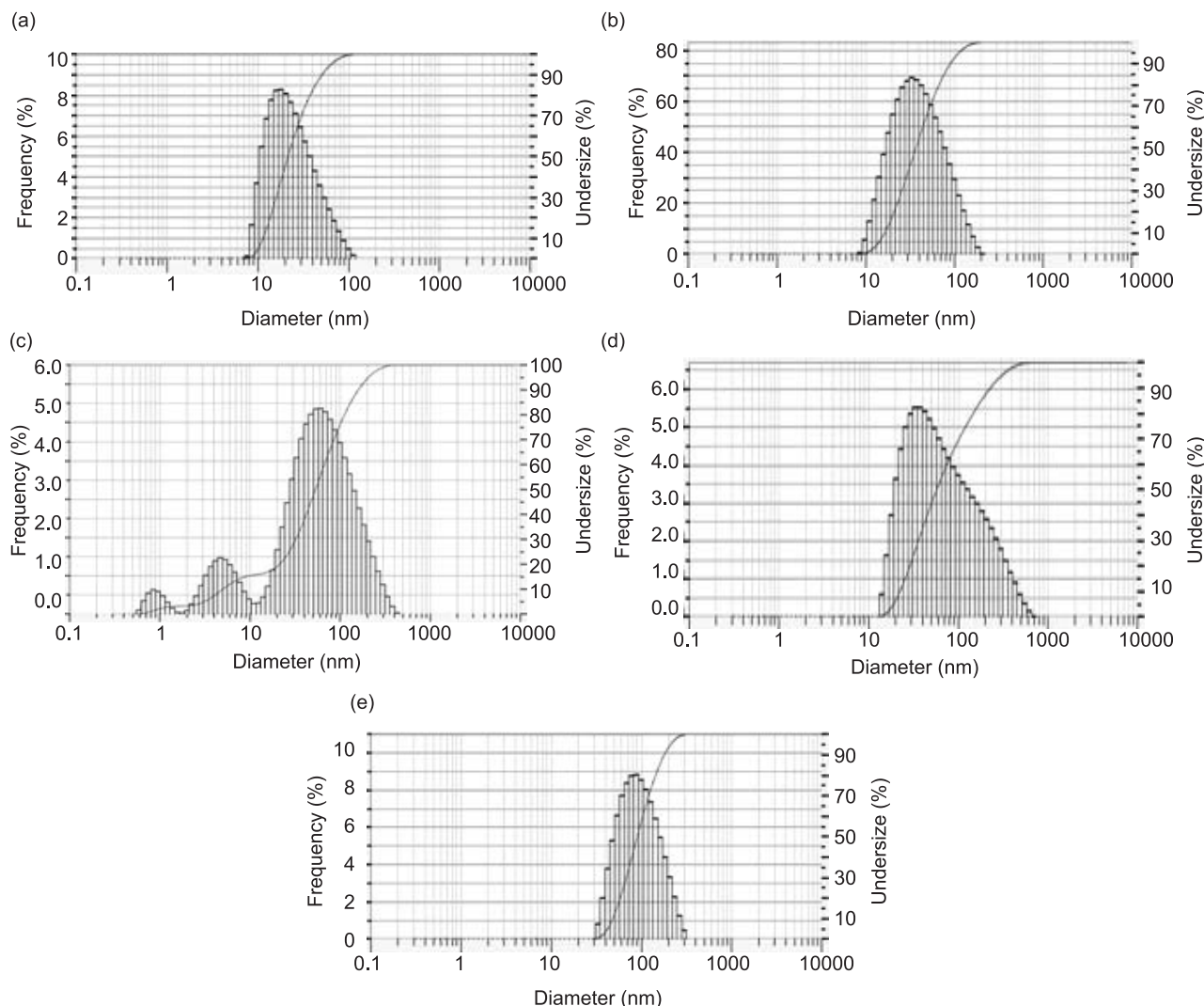


Fig. 1(a-e). Mean particle size and distribution of AgNPs. a, 27 nm; b, 46.1 nm; c, 71 nm; d, 96 nm; e, 103 nm.

Table 1. Properties of five different sizes of silver nanoparticles

| Parameter | 27 nm | 46.1 nm | 71 nm | 96 nm | 103 nm |
|--|-----------|-----------|-----------|-----------|-----------|
| Temperature (°c) | 25 | 25 | 25 | 25 | 25 |
| Diluents name | Water | Water | Water | Water | Water |
| Polydispersity index | 0.264 | 0.545 | 0.648 | 0.414 | 0.525 |
| Z average (nm) | 58.4 | 17.3 | 33.3 | 262.7 | 62.2 |
| Zeta potential (Mv) | -40.5 | -39.1 | -26.8 | -26.3 | -18.3 |
| Electrophoretic mobility (Cm ² /Vs) | -0.000314 | -0.000302 | -0.000208 | -0.000204 | -0.000145 |

Morphology study of the nanoparticles along with E. tarda through atomic force microscopy: The corresponding 2D and 3D image (AFM) are shown in Fig. 2 where rod shaped bacteria surrounded by nanoparticles were displayed.

Acute toxicity test: The results of 96h LC₅₀ of Ag-NPs through immersion and 96h LD₅₀ of Ag-NPs through injection and feed are tabulated in Table 2. Large sized Ag-NPs were found to be more toxic as compared to smaller

sized Ag-NPs which was reflected in their LC₅₀ doses (2.334 mg/L for 103 nm Ag-NPs and 3.474 mg/L for 27 nm Ag-NPs). Similarly, the LD₅₀ for larger Ag-NPs (103 nm) was 40.164 mg/kg and 53.630 mg/kg and the LD₅₀ for small Ag-NPs (27nm) was 68.631 mg/kg and 80.439 mg/kg respectively via injection and feed respectively.

Acute toxicity (LC₅₀ and LD₅₀ test): There are concerns to both fish and human health on the use of nanoparticles as drugs. Acute toxicity and trophic transfer are of concern.

Table 2. Probit analysis of LC₅₀ (through immersion) and LD₅₀ (through injection and feed) of two different sizes silver nanoparticles (AgNPs)

| 103 nm | | | | | | | | | | | | | | | |
|-------------------------------|----------------------|-------|--------------|-------------------------------|--------------|--------------|--------------------------|---------------|--------------|----------------------|---------------|--------------|-----|--------------|--------|
| Injection (mg/kg body weight) | Immersion (mg/litre) | | | Injection (mg/kg body weight) | | | Feed (mg/kg body weight) | | | Immersion (mg/litre) | | | | | |
| | Probit | Conc. | Log of conc. | Probit | Conc. | Log of Conc. | Probit | Conc. | Log of Conc. | Probit | Conc. | Log of Conc. | | | |
| 0.1 | 63.765 | 1.805 | 1.883 | 0.1 | 1.389 | 0.143 | 0.1 | 35.548 | 1.551 | 0.1 | 49.519 | 1.695 | 0.1 | 0.826 | -0.083 |
| 0.2 | 65.395 | 1.816 | 1.891 | 0.2 | 1.903 | 0.279 | 0.2 | 37.070 | 1.569 | 0.2 | 50.894 | 1.707 | 0.2 | 1.179 | 0.072 |
| 0.3 | 66.597 | 1.823 | 1.896 | 0.3 | 2.388 | 0.378 | 0.3 | 38.207 | 1.582 | 0.3 | 51.908 | 1.715 | 0.3 | 1.526 | 0.183 |
| 0.4 | 67.641 | 1.830 | 1.901 | 0.4 | 2.898 | 0.462 | 0.4 | 39.206 | 1.593 | 0.4 | 52.791 | 1.723 | 0.4 | 1.901 | 0.279 |
| 0.5 | 68.631 | 1.837 | 1.905 | 0.5 | 3.474 | 0.541 | 0.5 | 40.164 | 1.604 | 0.5 | 53.630 | 1.729 | 0.5 | 2.334 | 0.368 |
| 0.6 | 69.636 | 1.843 | 1.910 | 0.6 | 4.164 | 0.620 | 0.6 | 41.145 | 1.614 | 0.6 | 54.482 | 1.736 | 0.6 | 2.866 | 0.457 |
| 0.7 | 70.727 | 1.850 | 1.915 | 0.7 | 5.055 | 0.704 | 0.7 | 42.222 | 1.626 | 0.7 | 55.409 | 1.744 | 0.7 | 3.571 | 0.553 |
| 0.8 | 72.027 | 1.857 | 1.920 | 0.8 | 6.342 | 0.802 | 0.8 | 43.517 | 1.639 | 0.8 | 56.513 | 1.752 | 0.8 | 4.618 | 0.664 |
| 0.9 | 73.868 | 1.868 | 1.928 | 0.9 | 8.687 | 0.939 | 0.9 | 45.380 | 1.657 | 0.9 | 58.082 | 1.764 | 0.9 | 6.598 | 0.819 |

27 nm

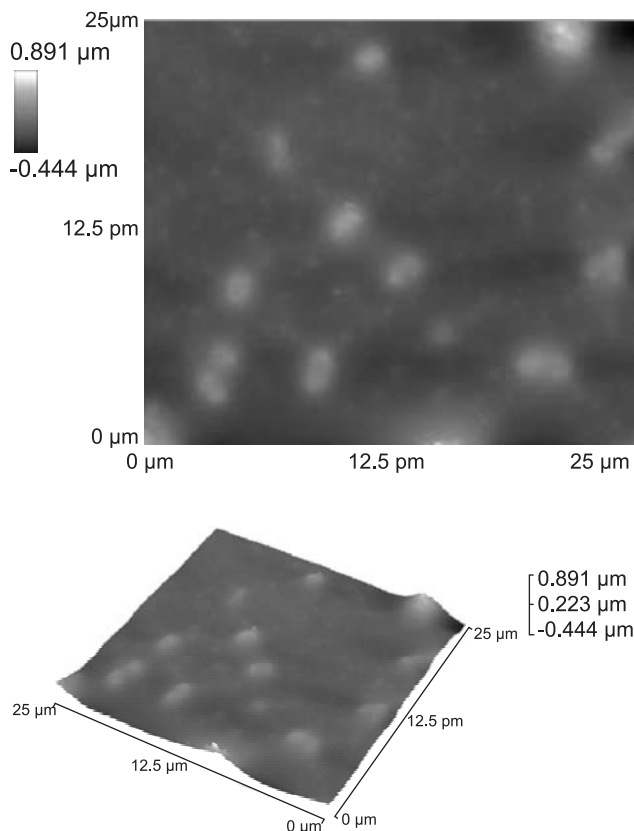


Fig. 2. AFM of silver nanoparticles along with *E. tarda* (Left-2D image, Right-3D image).

Hence, prior to use, it is important to know the toxicity of a substance in order to determine its safe dose to organisms. Jahanbakshi *et al.* (2012) reported LC₅₀ in goldfish and silver carp to be 83.9 ppm and 66.4 ppm, respectively, to commercial NanosilR. In zebra fish, the LC₅₀ values of nanosilver of 81 nm and Ag ion was found to be 84 μg/l and 25 μg/l, respectively in 48 hours (Bilberg *et al.* 2010). Shaluei *et al.* 2013 reported LC₅₀ value of 61 nm size commercial Ag-NPs (NanocidR) on silver carp (*Hypophthalmichthys molitrix*) for 96 h as 0.810, 0.648, 0.383 and 0.202 mg/l. In this study, larger sized Ag-NPs were found to be more toxic as compared to smaller sized Ag-NPs. Overall, the obtained LC₅₀ of the present study was found to vary from other existing reports on LC₅₀ studies. Intercomparability of results is hindered by the difference in size ranges used amongst various studies. The differences in toxicity may be due to variation in the particle properties at different sizes, differences in the species under study including age, stock and condition of the tested species along with experimental factors. The difference may also be due to changes in water quality and the test species (Shaluei *et al.* 2013, Rathore *et al.* 2002). Hence, it is evident that silver nanoparticles of different sizes give different degrees of toxicity as the test organisms were different, and also exposure times and experimental conditions differed.

In-vitro Antibacterial Effect

Antibacterial assay through total plate count (TPC): The

Table 3. *In-vitro* antibacterial effect of silver nanoparticles (20 µg and 10 µg) on BHI Agar

| Size of silver nanoparticles (nm) | Conc. of silver nanoparticles(µg) | CFU |
|-----------------------------------|-----------------------------------|-----|
| Control | 0 | 93 |
| 27 | 2010 | 25 |
| 46.1 | 2010 | 311 |
| 71 | 2010 | 05 |
| 96 | 2010 | 517 |
| 103 | 2010 | 712 |

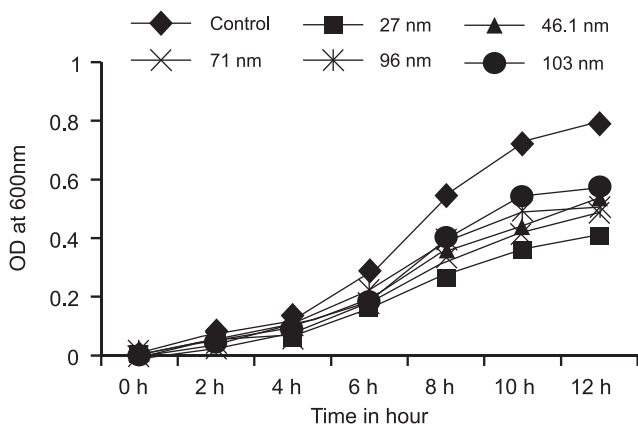


Fig. 3. Growth curve of *Edwarsiella tarda*.

antibacterial effects of five different sized silver nanoparticles with decreasing colony forming units (CFUs) in the presence of 20 µg and 10 µg of silver nanoparticles are displayed in Table 3. It was observed that 71 nm Ag-NPs at 20 µg concentration resulted in complete inhibition of bacterial growth.

Growth curve analysis/Time-kill assay: 27 nm size silver nanoparticles were found to be more effective in restricting growth of *E. tarda* in the broth as shown in Fig. 3.

Well and disk diffusion method: After 24 h of incubation on Mueller Hinton Agar using wells for Ag-NPs and Kirby-bauer disks for antibiotics, the diameter of zone of inhibition or clear zone exhibiting no bacterial growth around the disk/well was measured and is expressed in millimetres. Results of well and disk diffusion method are shown in the Fig. 4 and Table 4, respectively.

Minimum inhibitory concentration (MIC) and minimal bacterial concentration (MBC): The OD value of five bacterial culture pathogens viz. *Staphylococcus aureus*, *Edwarsiella tarda*, *Aeromonas hydrophila*, *Aeromonas cavaei* and *Vibrio parahaemolyticus* used in this test was recorded as 0.754, 0.574, 0.772, 0.974 and 0.510, respectively. Results of MIC and MBC for five fish pathogenic bacteria are shown in the Table 5 and Fig 5. Again, smaller sized Ag-NPs were found to be more effective and *V. parahaemolyticus* was found to be the most susceptible.

The presence of different sized silver nanoparticles at a concentration of 20 µg per plate inhibited the bacterial growth by 92–100%. The reduced size and peripheral

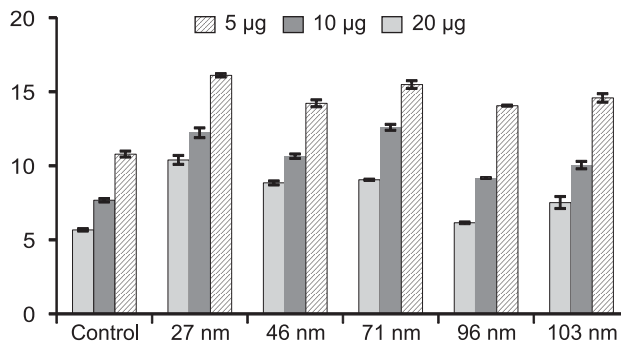


Fig. 4. Antibacterial effect of silver nanoparticles on *E. tarda* by well diffusion method.

Table 4. Diameter (mm) of zone of inhibition of silver nanoparticles (AgNPs) by disc diffusion method

| Treatment | Different concentration of silver nanoparticles | | |
|-----------|---|--------------------------|---------------------------|
| | 5 µg | 10 µg | 20 µg |
| Control | 6.2750 ^b ±0.08 | 8.265 ^a ±0.17 | 11.28 ^a ±0.08 |
| 27 nm | 8.1000 ^d ±0.2 | 13.10 ^c ±0.20 | 19.655 ^d ±0.25 |
| 46 nm | 6.6000 ^b ±0.1 | 11.15 ^d ±0.43 | 19.45 ^d ±0.15 |
| 71 nm | 7.25 ^c ±0.21 | 12.79 ^c ±0.01 | 19.83 ^d ±0.04 |
| 96 nm | 6.255 ^b ±0.05 | 9.71 ^c ±0.07 | 16.89 ^c ±0.11 |
| 103 nm | 5.375 ^a ±0.23 | 9.095 ^b ±0.23 | 15.295 ^b ±0.31 |

appearance of bacterial colonies on plates with 20 µg of nanoparticles is in accordance with findings of others (Sondi *et al.* 2004). The three *in-vitro* tests for effects of Ag-NPs, indicated greatest efficacy of nanoparticles in the smaller size range (27, 46.1 and 71nm) with intervariations amongst the tests. The 71 nm silver nanoparticles were found to be effective antibacterial agents in the TPC test and the possible reason behind this finding may be the co-occurrence of smaller sized Ag-NPs (1 nm to 10 nm) along with 71 nm Ag-NPs. NPs <10nm interact with bacteria and produce electronic effects which further enhances the reactivity of NPs (Morones *et al.* 2005). In tandem with the results, several references in literature also suggest that small sizes of silver nanoparticles have greater potential antibacterial activity as compared to larger Ag-NPs. Nanoparticles incubated along with *E. tarda* on BHI agar plates though showed the potential to completely inhibit bacterial growth, this inhibition depends not only on the concentration of Ag-NPs but also on the CFU of bacterium it was tested upon.

In the growth curve analysis study, AG-NPs reduced the growth of bacteria in decreasing order of efficacy (27 nm >71 nm>46.1 nm). The possible reason to this finding may again be attributed to the small size range of the Ag-NPs. It is notable that Ag-NPs, even at high concentration in liquid media caused only a delay in growth of *E. tarda* as also previously reported in a study on *E.coli* (Sondi *et al.* 2004).The bactericidal effect in the well-diffusion assay (27 nm> 71 nm > 46.1 nm) in decreasing order of efficacy may again be attributed to greater efficiency of smaller NPs

Table 5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of different sized silver nanoparticles against five bacteria

| Size of AgNPs (nm) | <i>Edwardsiella tarda</i> | | <i>Vibrio parahaemolyticus</i> | | <i>Aeromonas hydrophila</i> | | <i>Aeromonas cavaei</i> | | <i>Staphylococcus aureus</i> | |
|--------------------|---------------------------|---------|--------------------------------|---------|-----------------------------|---------|-------------------------|---------|------------------------------|---------|
| | MIC(µg) | MBC(µg) | MIC(µg) | MBC(µg) | MIC(µg) | MBC(µg) | MIC(µg) | MBC(µg) | MIC(µg) | MBC(µg) |
| 27 | 20 | 10 | 1.25 | 1.25 | 20 | 10 | 5 | 5 | 20 | 10 |
| 46.1 | 10 | 10 | 2.5 | 2.5 | 20 | 10 | 10 | 10 | 5 | 5 |
| 71 | 10 | 10 | 1.25 | 1.25 | 20 | 10 | 20 | 20 | 2.5 | 2.5 |
| 96 | 10 | 10 | 2.5 | 2.5 | 10 | 10 | 20 | 20 | 5 | 5 |
| 103 | 10 | 10 | 2.5 | 2.5 | 10 | 10 | 20 | 10 | 5 | 5 |

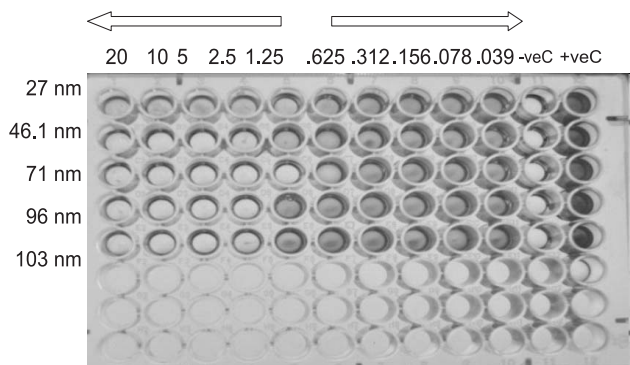


Fig. 5. MIC of various sized silver nanoparticles against gram negative *V. parahaemolyticus*.

(Sarkar *et al.* 2008, Morones *et al.* 2005, Kim *et al.* 2007). The smaller the size of the nanoparticles, the larger the surface area it possess in order to come in contact with the bacterial cells and hence, will have a higher percentage of interaction than bigger particles (Morones *et al.* 2005, Kreibig and Vollmer 1995, Mulvancy 1996, Pal *et al.* 2007).

The finding of antibacterial effect by using disk-diffusion method was similar to that of well diffusion method where among the five different sizes silver nanoparticles, 27 nm >71 nm >46.1 nm Ag-NPs were found to possess higher bactericidal effect compared to others at different concentration. Between the two methods, well and disk diffusion, the larger zone of diffusion in disk diffusion test may be due to faster diffusion rate of silver nanoparticles through the surface of the agar plate. In well diffusion method, the thicker agar surrounding the Ag-NPs may have lowered the diffusion rate of Ag-NPs.

In the MIC study, among five fish pathogen that has been used in MIC test, it was found that *V. parahaemolyticus* was the most susceptible to the small sizes (27 nm and 71 nm) than the large sizes (96 nm and 103 nm) of silver nanoparticles. This could probably be due to the reason that *Vibrio* species are all gram-negative bacteria. Previous reports have also indicated greater sensitivity of gram negative bacteria to Ag-NPs as compared to gram positive (Yoon *et al.* 2007, Kim *et al.* 2007). In case of *E. tarda*, all the five silver nanoparticles at 20 µg concentration showed antibacterial property. Hence, it is clear that Ag-NPs exhibit excellent bacteriostatic and bactericidal effect towards all pathogenic bacteria tested regardless of their drug-resistant

mechanisms albeit mild in gram-positive bacteria (Ansari *et al.* 2011). In this context, it is noteworthy that there are fewer studies on the *in-vivo* effect of Ag-NPs on pathogenic bacteria causing disease in commercially important aquatic organisms.

In-vivo antibacterial effect: After 21 days, it was found that the cumulative survival percentage in Ag-NP treated fishes significantly varied as compared to the control and was highest (64.62%) in the injection route of administration followed by immersion (48.07%) and feed (38%). The results are shown in the Fig. 6.

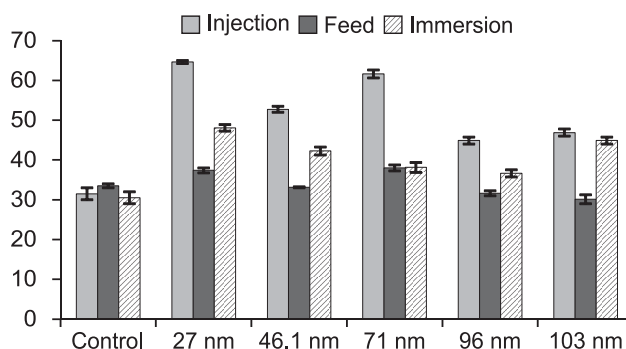


Fig. 6. Cumulative survival percentage of *E. tarda* infected *Labeo rohita*.

Smaller sized Ag-NPs showed better antibacterial activity *in-vivo* also, and improved the survival percentage of fishes infected with *E. tarda*. *In-vivo* antibacterial activity was found to be most effective in injection followed by immersion and feed possibly due to higher bioavailability and direct uptake in bloodstream of AgNPs via injection as compared to immersion and feed, where the nanoparticles are subject to digestive absorption and filtering action by the gills respectively. Since, the treated fishes are food fishes of commercial importance, it becomes important to investigate the possibilities of trophic transfer. It is unlikely that treatment at concentration of 3.2mg/kg body weight (via injection) is a cause for concern, but it does rule out the possibility of increasing the amount of dissolved silver in the aquatic environment in which the fish is cultured. However, the acute lethal dose of AgNO₃ to humans is ~10g as prescribed by the World Health Organisation. Hence, the associated risks with silver nanoparticle treatment are

minimal but need to be further ascertained by studying the residence time of silver nanoparticles in treated fishes.

This study showed that silver nanoparticles have excellent antibacterial activity. Specific findings are that (i) smaller size particles showed higher stability, (ii) larger sizes (103 nm) silver nanoparticles are more toxic to *L. rohita* as compared with the smaller sizes (27 nm) of silver nanoparticles and the toxicity was found to be size, route, dose and exposure time dependent. The 27 nm silver nanoparticles showed more effective antibacterial properties. Larger zone of inhibition against *E. tarda* and AgNPs treated fish provide resistance against *E. tarda* infection and reduces mortality in *L. rohita*. The present studies, integration of nanotechnology and bacteriology can help in the development of new drug against pathogenic bacteria. However, future studies on antimicrobial activity of silver nanoparticles are required soon in commercialization of silver nanoparticles as an effective drug in the field level of aquaculture and fisheries and to find out the best size of silver nanoparticles which gives the better antimicrobial activity against particular pathogen.

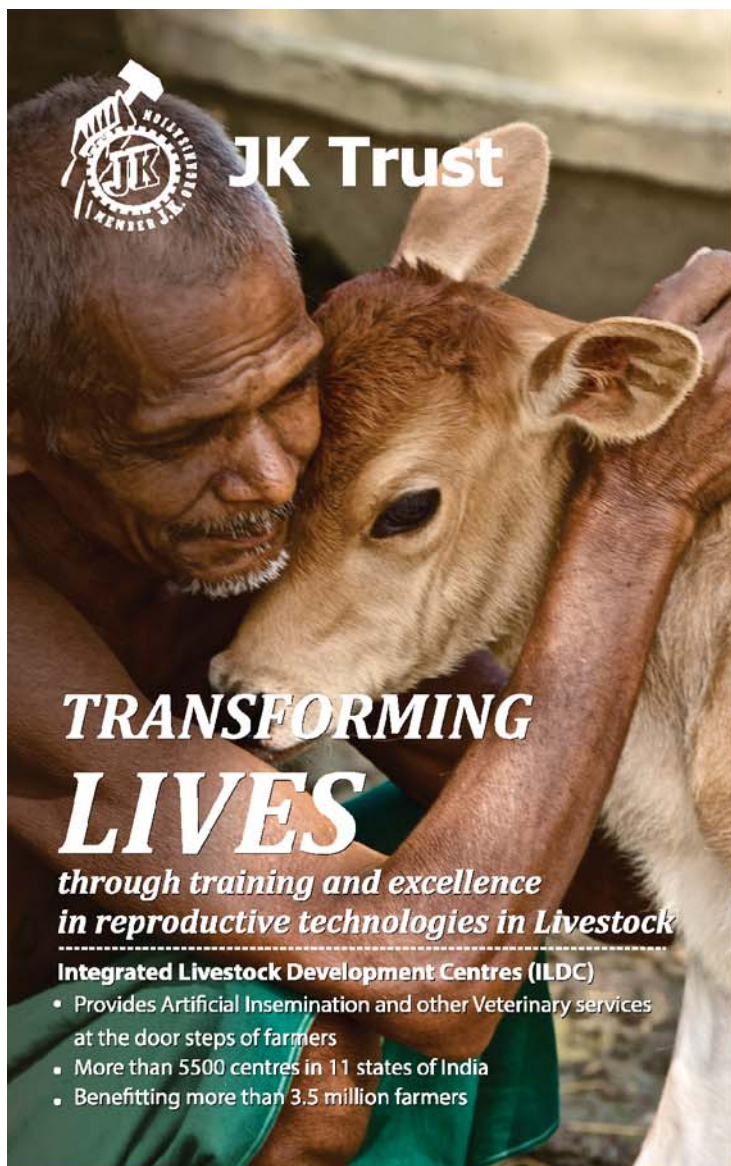
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