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# APPLICATION OF COPPER NANOPARTICLES FOR THE CONTROL OF AEROMONAS HYDROPHILA INFECTION IN AQUACULTURE.

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**ABSTRACT** Aquaculture is prone to many challenges like climatic changes, environmental stress and bacterial infection. In the current study, the use of copper nanoparticles to control Aeromonas infection in Mahseer fish, a valued species for aquaculture has been explored.

Copper nanoparticles (CuNPs) were synthesized using a chemical method. Characterization of synthesized nanoparticles was carried out by UV visible spectrophotometry, atomic absorption and scanning electron microscopy. Antimicrobial test was done by the agar cup diffusion assay and minimum inhibitory concentration of CuNPs required to inhibit Aeromonas hydrophila determined by macrobroth dilution assay. Mahseer fry were kept in tanks containing dilute Aeromonas culture. Test fishes were given regular dips once a day in tanks containing copper nanoparticles and examined for signs of infection over a five day test period.

SEM studies revealed spherical nanoparticles approximately 85 nms in size. These CuNPs exhibited a maximum absorption peak at 520nm. Antibacterial activity by the agar cup diffusion assay indicated CuNPs to be a good bactericidal agent against Aeromonas hydrophila. MIC of copper nanoparticles against Aeromonas hydrophila was found to be 38 ppm. No evident sign of infection was seen in test fishes while control fishes died due to haemorrhage within two days. The current study hence demonstrates the potential use of copper nanoparticles in Mahseer aquaculture to prevent Aeromonas infection.

**KEYWORDS :** Mahseer, aquaculture, copper nanoparticles, Aeromonas hydrophila

## INTRODUCTION

Due to ever increasing human population coupled with the limited availability of space for land-based food production systems such as agriculture, a large number of people across the globe are affected by short or inconsistent supply of quality food. A plausible solution of the problem could be the utilization of water resources as more than 70% of the earth surface is covered with water. Amongst a variety of aquatic organisms present in the aquatic system, fishes are considered as the most important group of organisms suitable for human consumption.

As an important food product, fishes add value to rural livelihoods, creating employment, generating revenue and above all, ensuring global food security. This sector however is also prone to multiple challenges including overexploitation, pollution, climate change and above all, diseases resulting in retarded growth and substantial financial loss.

Among a sizable number of aquatic pathogens, the Gram negative organism Aeromonas hydrophila has been recovered from a wide variety of fishes and possesses the ability to grow in both aerobic and anaerobic conditions [1]. This pathogen is a representative species of the genus Aeromonas and is commonly involved in heavy mortalities in aquaculture organisms [2, 3]. It propels outbreak of various fish diseases like ulcers, fin-rot, tail-rot and hemorrhagic septicemia [1, 4]. In human beings, it reportedly causes various water-mediated gastrointestinal infections in children and immunocompromised persons [5]. Its infectivity enhances with environmental pollution, elevation of water temperature and addition of stressors in the aquatic medium.

Successful fish health management practices call for disease prevention rather than their cure. Sterilization techniques do not eliminate all the potential pathogens in the ecological niche of fish. Stressful conditions like suboptimal water quality, poor nutrition and immune suppression provides a conducive environment for opportunistic bacteria like A. *hydrophila* [6] and antimicrobials like antibiotics are generally used in order to prevent disease outbreaks. However, recent reports on A. *hydrophila* from various fish tissues reveal that the pathogen has developed resistance to many antibiotics like amoxicillin, ampicillin, lincomycin, novobiocin, oxacillin, penicillin, rifampicin and tetracycline [7] and thus research endeavors to find antibiotic alternatives like nanotechnology are gaining momentum [8].

The synthesis and use of metal nanoparticles have gained consideration due to their unique electrical, optical, catalytic and magnetic properties [9] which are different from bulk materials. In recent times, synthesis of inorganic nanoparticles has been demonstrated by many chemical and physical methods. But the importance of biological synthesis is being realized globally as chemical methods are toxic, costly and non-ecofriendly [10]. It is, therefore, important to develop synthetic strategies which are simple, cost-effective, environment friendly and easy to scale. Among transition metals, interest is being shown to copper nanoparticles because of its unique properties and potential applications as catalysts [11], electronic materials [12] and nanocomposite coatings [13]. Nanoparticles of silver, silicon, titanium, copper, gold and zinc have been extensively studied. Copper nanoparticles (CuNPs) have been considered as candidates for antimicrobial applications as biocides. These have been shown to hinder the growth of bacteria like *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa* and *Aeromonas hydrophila* [14].

Presently, copper sulphate is added to fish tanks as a disinfectant for prevention of infection in fishes. In the current study, instead of copper sulphate, copper nanoparticles were used as a novel alternative for prevention of opportunistic infection caused by *Aeromonas hydrophila* in Mahseer fish. Mahseer (Tor Khudree) is a valued food fish all over the world, especially in South-Asian countries. It is a candidate species for aquaculture and conservation of Mahseer is in high priority. Mahseer aquaculture is known to be affected by microbial pathogens like *Aeromonas hydrophila.*

In current study, the use of copper nanoparticles to control *Aeromonas* infection in Mahseer fish has been explored. It is hypothesized that since nanoparticles possess higher surface to volume ratio, they will be more effective than copper sulphate in their antimicrobial activity; also development of resistance towards *Aeromonas* will be slower compared to antimicrobials which are no longer effective today.

## MATERIALS AND METHODS

### Procurement of culture and fish model system

Culture of *Aero monas hydrophila* MTCC 1739 was obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh and young Mahseer fish fry procured from Mahseer Hatchery, Lonavala (Tata Power Company).

### Chemical synthesis of copper nanoparticles

Copper nanoparticles were synthesized using chemical reduction method involving the reduction of copper sulphate by sodium borohydride and the stabilizing agent being polyethylene glycol 6000. The four-step preparation scheme for copper nanoparticles was started by dissolving 0.1 M $CuSO_4 .5H_2O$, in distilled water to obtain a blue solution. Next, 0.04 M PEG 6000 was dissolved in water and added to the aqueous solution containing the copper salt while vigorously stirring. In this step, the solution changed from blue to white. In the third step, 0.04 M ascorbic acid and 1 M NaOH was dissolved in water and was added to the synthesis solution. Color change occured in the aqueous phase from white to yellow. Finally, a solution of 1 M $NaBH_4$ in distilled water was prepared and added to the solution under continuous rapid stirring. An instant color change occurred in the aqueous phase from yellow to blackish green indicating the presence of copper nanoparticles. The nanoparticles thus prepared the above method were stored in air tight tubes as they readily oxidize in ambient conditions.

### Characterization of CuNPs by UV–Vis Spectra Analysis

To confirm the presence of copper nanoparticles UV-visible Spectrophotometer (Systronics 117) was used to measure the peaks exhibited by the copper nanoparticles. Distilled water served as blank.

### Quantification by atomic absorption spectroscopy.

Quantification of the prepared copper nanoparticles was carried out by atomic absorption spectroscopy using air- acetylene flame. Prior to analysis, sample was digested using nitric acid on hot plate for 3hrs. The dry residue obtained was diluted with 5% nitric acid and filtered through 0.45μ filter and used for the analysis.

### Characterization by SEM

Morphology of the nanoparticles was determined by scanning electron microscope (Inspect F50). An aliquot was spread on a rigid groove, dried under vacuum and images taken.

### Testing the antibacterial activity of copper nanoparticles using agar cup diffusion assay

*Aeromonas hydrophila* was swabbed using sterile cotton swabs on nutrient agar plates. Wells were punched out and specified volume of the nanoparticles was used to fill the wells. Control wells contained an equivalent concentration of copper sulphate solution.The plates were then incubated at 370C/ 24hrs and zones of inhibition observed.

### Determination of minimum inhibitory concentration (MIC) of copper nanoparticles on Aeromonas hydrophila

Using stock solution of the prepared copper nanoparticles, various dilutions was prepared. 0.1 ml of the 24 hr old test culture Aeromonas hydrophila was added to all dilutions. Positive and negative controls were maintained. Tubes were incubated at 37°C for 24 hrs and the lowest concentration that did not show growth corresponded to the minimum inhibitory concentration (MIC).

### Animal studies

Fish model system comprising of Mahseer fry were tested in order to check for prevention of *Aeromonas* infection. Fishes in the test tank (five in number) were placed in tanks containing a dilute suspension of *Aeromonas*. These fishes were given regular dips everyday in tanks containing 0.2ppm of copper nanoparticles for 45 minutes. Fishes was examined for signs of infection which included red spots on the skin. Control tank (with five fishes) was maintained which had *Aeromonas* culture. These fishes were not subjected to dips in tanks with copper nanoparticles. The experiment was carried out over a period of five days.

## RESULTS AND DISCUSSION

Intensive and semi-intensive inland fish culture with the use of different species has become one of the important expanding practices in India. With the improved intensification of aquaculture, the problem of diseases caused by bacteria is also increasing. Bacterial diseases generally result in heavy mortality of fishes and consequently affect the economy of the fish farmers. The Indian major carps are highly nutritious and most prestigious of all fishes in India constituting nearly 90% of India's total production [15]. In India, majority of fishes are affected by *Aeromonas* group which include *Aeromonas hydrophila* [16].

Mahseer is a fresh water fish and a carp that inhabits both rivers and lakes; it is one of the 20 mega fishes of the world and can grow to about nine feet and weight up to 35-40 kgs. The Mahseer is commercially an important highly valued food fish [17].

*Aeromonas hydrophila* is known to cause dermal ulceration, fin haemorrhages, fin rots, red sores, haemorrhages and necrosis of visceral organs [18] in Mahseer fishes [19]. Its infectivity enhances with environmental pollution, elevation of water temperature and addition of stressors. Development of resistance to antimicrobials is common. Recently, the in vitro antibacterial efficacy of copper nanoparticles against the Gram-negative bacteria *Aeromonas hydrophila* has been demonstrated [20].

In the current study, chemical reduction method was employed for the fabrication of copper nanoparticles. This method is economical, simple, fast and a better size distribution of nanoparticles can be obtained by controlling the experimental parameters. On addition of sodium borohydride, a greenish black coloured solution was obtained which indicated the formation of copper nanoparticles (Figure1).

**Fig 1: Grenish- black coloured copper nanoparticles.**

Primary characterization of the nanoparticles by UV- visible spectroscopy has proven to be a very useful technique. The absorption band of copper nanoparticles has been previously reported in the range of 500-600 nm [21, 22]. In the current study, copper nanoparticles were analyzed by UV-visible spectroscopy and absorption spectrum peak was observed at 520nm. Depending upon the method used for synthesis, the morphology of the particles differs. In this study, the morphophology demonstrated by the chemically synthesized copper nanoparticles was spherical and the diameter approximately 85 nm (Figure2).. Zhang Qiu-li et.al [23] prepared copper nanoparticles by a different chemical reduction method and reported 100nm diameter nanoparticles. Although similar copper powders with size of 35 nm can be prepared by the use of hydrazine method [24], this method has the disadvantage of toxic resource materials and is unsuitable for large-scale application [25].

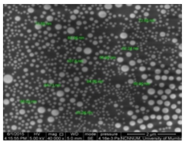

**Fig 2: SEM image of CCuNP's**

Atomic absorption spectrophotometry revealed the concentration of copper in the solution to be 38 ppm. Similar studies have been done using different methods and the concentration of copper was found out to be 5.670 ppm, by Katelyn A. Cohen et.al [26] which is very less compared to our findings. Results of the antibacterial activity of copper nanoparticles using agar cup diffusion assay has been shown in Table 1 and Figure 3.


**Fig. 3: Agar cup diffusion assay.**

As evident from Table 1, chemically synthesized copper nanoparticles exhibited significant bactericidal activity, much higher than an equivalent concentration (38ppm) of copper sulphate solution which was used as control. The MIC of copper nanoparticles for Aeromonas hydrophila was found out to be 38 ppm after 24hrs of incubation at 370C which is far lower than that of CuSO4 which has been previously reported as 82.3ppm [27]. Hence CuNPs are almost more than twice as effective in controlling Aeromonas as compared to copper sulphate.

**Table 1: Zones of inhibition against *Aeromonas hydrophila* MTCC1739**

| Copper nanoparticles | Zone of inhibition(mm |
|---|---|
| CuNPs (38 ppm) | 23±3 |
| CuSO4 (38 ppm) | 18±1 |

To check the antimicrobial effect of copper nanoparticles, experiments were performed using 20-25 days old Mahseer fry models. Fishes were transferred to indoor tanks in order to acclimatize them prior to the assay. During the acclimatization period, fishes were fed once daily with commercial feed. Two fish tanks was maintained each containing five fishes. In the control tank containing dilute Aeromonas culture, fishes died within two days and hemorrhage was observed near the gills (Figure 4), while fishes in the test tank showed no evident signs of infection up to five days (Figure 5); after this the experiment was discontinued. A concentration of 0.2 ppm of copper nanoparticles was selected for dips, since it is recommended that the concentration of copper sulphate dips must be within the range of 0.02-8 ppm in order to avoid copper toxicity. Since varied time periods are employed by different fish breeders (15 ) minutes to 1 hour), an exposure time of 45 minutes was selected for the current study [28].

**Fig. 4: Note: Haemorrhage near gills when infected in absence of copper nanoparticles.**

**Fig. 5: No evident sign of infection in fishes given copper nanoparticle dips.**

## CONCLUSION

Results of animal studies reveal that copper nanoparticles can certainly prove to be useful in controlling Aeromonas hydrophila infection in Mahseer fish. Although copper is an essential micronutrient in biological systems and is commonly employed to control diseases in aquaculture facilities, it is also known to possess certain toxicity at higher concentrations [29, 30]. Hence the optimum concentration of copper nanoparticles that can be used to give dips to Mahseer fry must be determined. It is also known that the toxicity of copper nanoparticles is dependent on the species of the fish [31] and that the liver of fishes is the central compartment of copper metabolism [32], hence it is advised that further histological studies be done in order to check for damages or changes in the liver and other internal organs of Mahseer fish.

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