

Pathogenicity Test of *Aeromonas* Isolated from Motile *Aeromonas* Septicemia (MAS) Infected Nile Tilapia on Some Freshwater Fish

¹Forhad Karim Saikot, ¹Rashed Zaman and ²M. Khalequzzaman

¹Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, 6205, Bangladesh

²Department of Zoology, University of Rajshahi, Rajshahi, 6205, Bangladesh

ABSTRACT

Background: Test of pathogenicity of fish pathogens usually done by injection or ingestion of the bacterial samples on the fish. Objective of the current research was to introduce a new method of testing pathogenicity of fish pathogens through an artificial infection system. The targeted method was to observe the pathogenicity in aquarium condition by direct infection of the system water with bacterial suspension. **Methods:** Pathogenic *Aeromonas* was isolated from naturally infected Nile Tilapia having symptoms similar to those reported for MAS. The isolate was identified by microscopic observation and biochemical assay. Liquid culture of the pathogenic isolate was taken to observe its pathogenic effect on four freshwater fish, e.g., *Labeo rohita*, *Cirrhinus cirrhosus*, *Oreochromis niloticus* and *Hypophthalmichthys molitrix* in aquarium condition. Water was directly infected with bacterial liquid culture instead of injecting the fish intra-muscularly. Antibiotic susceptibility was determined by agar disc diffusion assay. **Results:** *Aeromonas* was non-harmful for *L. rohita*, *C. cirrhosus* and *H. molitrix* while it remarkably infected *O. niloticus* even in lab condition with a lethal rate of $66.7 \pm 11.7\%$. *Aeromonas* was found to be susceptible to ciprofloxacin and resistant to ceftazidime. **Conclusion:** The research study showed that pathogens could harm the fish hosts who are susceptible in aquarium condition.

Key words: *Aeromonas*, pathogenicity test, freshwater fish, Nile tilapia, motile aeromonas septicemia, antibiotic susceptibility

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INTRODUCTION

Fish suffer from different diseases. They can be attacked by bacteria, fungi, virus and parasites or by physical ailments. However, bacterial diseases are most significant since their occurrence has the maximum value. Fish are infected by numerous bacterial diseases. A lot of pathogenic bacteria have been reported¹. Perhaps, *Aeromonas* is the most prominent pathogen that can infect fish. Many *Aeromonas* species are responsible for several fish diseases including *A. hydrophilla*, *A. formicans*, *A. liquefaciens* and *A. salmonicida*². The most reported infections caused by *Aeromonas* are bacterial hemorrhagic septicemia or Motile *Aeromonas* Septicemia (MAS), furunculosis, erythrodermatitis, cutaneous ulcer etc.³ Pathogenic bacteria cause an enormous economic loss to fisheries. They don't only cause mortality and cytotoxicity but also create uselessness of live fish as they are responsible for spots, lesions and scale loss on the infected ones. Losses incurred by fish farmers are heavily related to diseases, especially bacterial diseases. Estimates of dollar losses due to bacterial disease are conservative figures because fingerling fish have a significantly higher

value per unit of weight than food-sized fish. Only for an example, the catfish industry reported that 39% of the 100 million fish lost in the 1988 production season were killed by bacterial diseases⁴. The trout industry reported losses during 1988 of 20.7 million fishes, 50% of which were lost to diseases caused by pathogenic bacteria⁵. However, now days the loss has been minimized to some extent, but still it is massive. Among them, the contribution of *Aeromonas* is very high, though there is no clear report on the economic value of the loss caused by them. However, *Aeromonas* infection causes death of both cultured and wild fish species every year. *Aeromonas* is responsible for the pathogenesis of many fish infections⁶. Experimental pathogenicity test of them on lab or aquarium condition has been done for several times. But in almost every case, the experimental fish is infected by injection⁷. However, to very best of our knowledge, there is no work regarding the pathogenicity test of *Aeromonas* on the experimental freshwater fish species that were selected for our investigation, i.e., Rui (*Labeo rohita*), Mrigel (*Cirrhinus cirrhosus*), Niloticus or Nile Tilapia (*Oreochromis niloticus*) and Silver carp (*Hypophthalmichthys molitrix*) in lab condition.

Corresponding Author: M. Khalequzzaman, Department of Zoology, University of Rajshahi, Rajshahi, 6205, Bangladesh Tel: +8801711302249 Fax: +880721750064

In the present study, an assessment was done to understand the pattern of pathogenicity of *Aeromonas* isolated from naturally MAS affected Nile Tilapia on the four experimental fish species. Concentration was also implied on infecting the fish by means of direct contamination of aquarium water. It was done to observe the possibility of infection through the presence of bacteria in water system. The present study, therefore, would help significantly to formulate preventive recommendations and therapeutic guidance for the disease.

MATERIALS AND METHODS

Collection of fish samples: The diseased fish sample of Nile Tilapia was collected from a fish farm of Meherchondi, Rajshahi, Bangladesh. Initially three MAS infected fishes were found. For the experiment, live and disease free fingerlings of the four experimental fishes [Nile Tilapia (*Oreochromis niloticus*), Rui (*Labeo rohita*), Mrigel (*Cirrhinus cirrhosus*), Silver carp (*Hypophthalmichthys molitrix*)] were required. These were collected from a pond maintained by a fingerling dealer near Meherchondi, Rajshahi, Bangladesh. Fifteen fingerlings of each sample were collected for the entire experimentation. Size of the fingerlings ranged within 14-23 cm (measured using a measuring tape) and weighted within 20-50 gram (measured using a kitchen scale).

Isolation and identification of the bacterial strain:

Isolation of pathogen was done according to standard protocol⁸.

Naturally infected fish were taken under laminar airflow and their infected parts (gill, scale and abdomen) were cut off with sterilized tools and washed them in 0.1% saline water for 2 to 3 minutes in a 100 mL beaker under laminar airflow and were washed thoroughly with autoclaved distilled water to remove the excess sodium chloride. The infected parts were then homogenized in mortar-pestle and the homogenate was poured into a 250 mL conical flask containing Luria-Bertoni (LB) broth medium through an autoclaved filter paper. Standard procedure followed to isolate single bacterium. Orange to yellow, irregular, raised, undulate colonies were targeted. Subculture was done at every week for successive duplication of the culture. The bacterium was later identified by microscopic analysis and biochemical characterization⁹.

Infection of the fingerlings with the isolated

***Aeromonas*:** As said earlier, in this experiment, instead of injecting the fish with bacterial culture, a target was set to infect the water directly. For this purpose, a number of

80 L aquariums were cleaned and were filled with 70 L clean water. Water was left for 24 h to become settled and to gain room temperature. Aquariums were kept in a place with plenty of light and air but away from direct sunlight. Four fingerlings were put in each of the pre-settled aquarium water. One species was kept in a single aquarium. The fingerlings were supplemented with high protein packet food and also with oxygen by the help of aerators. The top of the aquariums were covered with net to prevent dust and insects. The fingerlings were left to be adapted at least for 2-3 days. To infect the fingerlings, overnight grown 100 mL liquid bacterial culture was taken as inoculum seed and simply poured into aquarium. One aquarium for each fish species was left un-infected as control. Observation was done at every 6 h for 7 days to find any abnormality or symptoms on the fingerlings. Data collection was done accordingly.

Antibiotic sensitivity: Antibiotic sensitivity test on *Aeromonas* was done by standard agar disc diffusion assay¹⁰.

RESULTS

Clinical examination of the diseased sample:

Naturally infected fish samples of Nile Tilapia were subjected to clinical examination for the confirmation of the presence of the disease. Disease symptoms were found on them including reddening of the skin, gill and tail, scale loss, paleness, skin loss and exposure of the muscle below. All these are well described symptoms of MAS.

Identification of the isolated bacterium:

Isolated bacterium on LB agar plate having orange to yellow, irregular, raised, undulate colony was subjected to microscopic examination and biochemical test sets for identification. The bacterium was gram negative rod, catalase positive, urease negative, H₂S (Hydrogen Sulphide) negative and VP negative. Thus the sample was confirmed to be *Aeromonas*.

Pathogenicity test results:

Pathogenicity test results were recorded as per the number of fingerlings infected inside the aquarium after inoculating water. *Aeromonas* was detected non-hazardous for *L. rohita*, *C. cirrhosus* and *H. molitrix*. But it showed pathogenic effect against *O. niloticus*. On first trial, symptom was observed on a fish after 60 h. The infected fish died after 72 h. A second fish was infected after 78 h and died at 96 h. Another fish was infected on the fifth day and died right after 12 h of infection. Thus, *Aeromonas* caused death to 3 fishes while a single fish remained healthy even after 7 days. After three replications, 2.7 ± 0.4 fingerlings die out of 4 due to

the infection of *Aeromonas*. It showed a lethal rate of $66.7 \pm 11.7\%$. However, no abnormality or death occurred in the control (Table 1).

Antibiotic sensitivity test results on the pathogenic *Aeromonas*:

Antibiotic sensitivity of *Aeromonas* was determined by using antibiotic discs. Antibiotic discs taken for the experiment were Ciprofloxacin (CIP 10), Ampicillin (AMP 10), Vancomycin (VA 30) and Ceftazidime (CAZ 30) (Fig. 1). A 22 mm zone of inhibition was measured for the CIP 10 disc which proved the bacteria sample was susceptible for ciprofloxacin (Fig. 1a).

A very brisk zone of only 7 mm was obtained in case of AMP 10 disc (Fig. 1b). It expressed that the sample was moderately resistant to the antibiotic. VA 30 discs resulted in a 13 mm zone (Fig. 1c). Thus the bacterium was intermediate resistant to the antibiotic. No zone was exhibited in case of CAZ 30 (Fig. 1c), which proved the complete resistance of *Aeromonas* against ceftazidime (Table 2).

DISCUSSION

Typically *Aeromonas* was isolated successfully from infected samples of Nile Tilapia. Described knowledge regarding phenotypic identification schemes often fails

Table 1: Pathogenicity test results of *Aeromonas* on four experimental freshwater fish fingerlings

Fish	Control			Treatment		
	Fingerlings taken	Fingerlings died (M±SE)	Lethal rate (%) (M±SE)	Fingerlings taken	Fingerlings died (M±SE)	Lethal rate (%) (M±SE)
<i>L. rohita</i>	4	0.0±0.0	0.0±0.0	4	0.0±0.0	0.0±0.00
<i>C. cirrhatus</i>	4	0.0±0.0	0.0±0.0	4	0.0±0.0	0.0±0.00
<i>O. niloticus</i>	4	0.0±0.0	0.0±0.0	4	2.7±0.4	66.7±11.7
<i>H. molitrix</i>	4	0.0±0.0	0.0±0.0	4	0.0±0.0	0.0±0.00

Experiment was done in three replicates and mean data has been presented as result, M: Mean, SE: Standard error

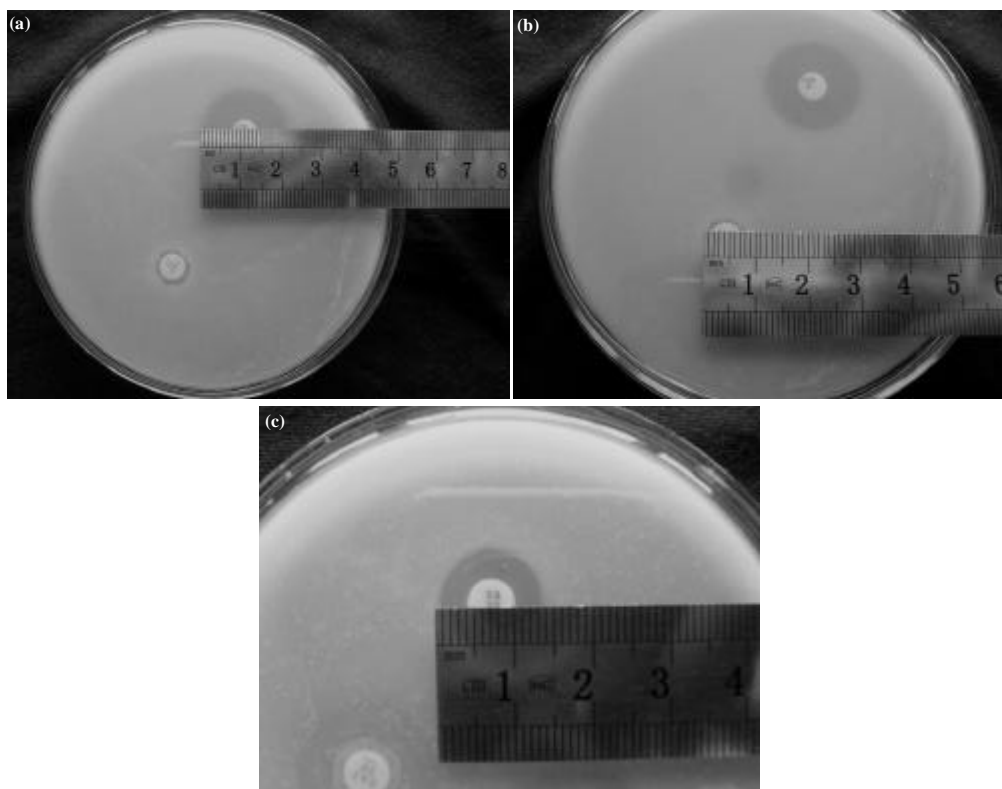


Fig. 1(a-c): Antibiotic sensitivity test results on the pathogenic *Aeromonas*, (a) Zone inhibition by ciprofloxacin, (b) Zone inhibition by ampicillin and (c) Zone inhibition by vancomycin and ceftazidime

Table 2: Antibiotic sensitivity test results of four selected antibiotics against *Aeromonas*

Antibiotic	Zone inhibition (mm)	Sensitivity
Ciprofloxacin (CIP 10)	22	Susceptible
Ampicillin (AMP 10)	7	Highly resistant
Vancomycin (VA 30)	13	Intermediate resistant
Ceftazidime (CAZ 30)	0	Resistant

due to overlapping behavior among the flora of bacteria. Advanced knowledge based on biochemical identification raised a compact identification tool for the isolate.

Pathogenicity test of the isolated *Aeromonas* was done. There was a vision to confirm the pathogenicity of the isolate as well as testing its' effect on some other freshwater fish. The approach of infection was completely different from many other scientists who injected the suspension of pathogenic culture to the challenged fish^{7, 11, 12, 13, 14, 15}. But instead of injecting, an enthusiasm was working to infect water directly with the bacterial culture. *Aeromonas* was not infectious for *L. rohita*, *C. cirrhosus* and *H. molitrix*. However, the pathogenic *Aeromonas* only infected Nile Tilapia (*O. niloticus*) with a mortality rate of 66.7%. Very lesser works are defined in such decorum; in fact, there is no work with *Aeromonas*. Al-Sunaiher¹⁵ reported 83.3% mortality when infected with *Vibrio*. Such approach is quite common these days as many scientists are adopting them^{8, 16, 17}. This study confers knowledge regarding the maintenance of the experimental fish species. It is likely to say, the pond or water system containing *Aeromonas* is not that risky for the culture of *L. rohita*, *C. cirrhosus* and *H. molitrix*. However, that system is not suitable for the culture of Nile Tilapia (*O. niloticus*). Assessment on antibiotic sensitivity of *Aeromonas* was performed due to two noteworthy reasons. First of all its' pathogenic nature was encouraging enough to find out suitable antibiotic sensitivity for future remediation. Secondly, literature regarding the antibiotic sensitivity of *Aeromonas* was not sufficient. Result enumerated that *Aeromonas* was totally susceptible to Ciprofloxacin (CIP 10) and resistant to ceftazidime (CAZ 30). Kirkan *et al.*¹⁴ found similar results with Ciprofloxacin on *Aeromonas*. Similarity is also described on *Vibrio* species¹⁵. Hopefully, the information provided through this research would be helpful for the fish farmers. At the same time it would provide knowledge for the future workers.

CONCLUSION

Isolated *Aeromonas* was taken to examine whether it has any pathogenic effect on the four investigating fish. It was found to be affectless against *L. rohita*, *C. cirrhosus* and *H. molitrix*. However, *Aeromonas* was confirmed as pathogenic as they infected *O. niloticus* with a lethal rate of

66.7±11.7%. *Aeromonas* is susceptible to ciprofloxacin providing a zone of 22 mm, while it is resistant to ceftazidime.

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REFERENCES

1. Austin, B., 2006. The bacterial microflora of fish, revised. *Scient. World J.*, 6: 931-945.
2. Aberoum, A. and H. Jooyandeh, 2010. A review on occurrence and characterization of the *Aeromonas* species from marine fishes. *World J. Fish Mar. Sci.*, 2: 519-523.
3. Roberts, R.J., 1989. *Fish Pathology*. 2nd Edn., Bailliere Tindall, London, Pages: 466.
4. Meyer, F.P., 1991. *Aquaculture disease and health management*. *J. Anim. Sci.*, 69: 4201-4208.
5. USDA, 1989. Annual report on trout production. USDA, NASS, Washington, DC., USA. <http://usda01.library.cornell.edu/usda/nass/TrouProd/1980s/1989/TrouProd-09-29-1989.pdf>.
6. Rahman, M., P. Colque-Navarro, I. Kuhn, G. Huys, J. Swings and R. Mollby, 2002. Identification and characterization of pathogenic *Aeromonas veronii* Biovar Sobria associated with epizootic ulcerative syndrome in fish in Bangladesh. *Applied Environ. Microbiol.*, 68: 650-655.
7. Yardimci, B. and Y. Aydin, 2011. Pathological findings of experimental *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*). *Ankara Universitesi Veteriner Fakultesi Dergisi*, 58: 47-54.
8. Patil, R.C., M.V. Upadhye, V.D. Kolatkar, V.I. Katchi, G.J. Kulkarni and Y.S. Shouche, 2011. Isolation and identification of two new strains of *Aeromonas* spp. from diseased gourami fish and aquarium water. *Int. J. Life Sci.*, 5: 25-31.
9. Holt, J.G., 1986. *Bergey's Manual of Systematic Bacteriology*. Vol. 2, Williams and Wilkins, Baltimore, USA., ISBN-13: 9780683078930, Pages: 2648.
10. Cheesbrough, M., 2000. *District Laboratory Practice in Tropical Countries*, Part 2. 2nd Edn., Cambridge University Press, UK., ISBN-13: 9780521665452, Pages: 434.
11. Iqbal, M.M., K. Tajima and Y. Ezura, 1999. Pathogenicity of motile *Aeromonas* species isolated from fishes with Epizootic Ulcerative Syndrome (EUS) in Southeast Asian countries. *Bull. Fac. Fish. Hokkaido Univ.*, 50: 93-100.

12. Sarkar, M.J.A. and M.M. Rashid, 2012. Pathogenicity of the bacterial isolate *Aeromonas hydrophila* to catfishes, carps and perch. J. Bangladesh Agric. Univ., 10: 157-161.
13. Hossain, M., 2008. Isolation of pathogenic bacteria from the skin ulcerous symptomatic gourami (*Colisa lalia*) through 16S rDNA analysis. Univ. J. Zool. Rajshahi Univ., 27: 21-24.
14. Kirkan, S., E.O. Goksoy, O. Kaya and S. Tekbiyik, 2006. *In-vitro* antimicrobial susceptibility of pathogenic bacteria in rainbow trout (*Oncorhynchus mykiss*, Walbaum). Turk. J. Vet. Anim. Sci., 30: 337-341.
15. Al-Sunaiher, A.E., A.S.S. Ibrahim and A. Al-Salamah, 2010. Association of *Vibrio* species with disease incidence in some cultured fishes in the Kingdom of Saudi Arabia. World Applied Sci. J., 8: 653-660.
16. Shayo, S.D., C. J. Mwita and K. Hosea, 2012. Ulcerative *Aeromonas* infections in *Tilapia* (*Cichlidae: Tilapia*) from Mtera Hydropower Dam, Tanzania. Open Access Sci. Rep., Vol. 1, No. 1. 10.4172/scientificreports.115
17. Nielsen, M.E., L. Hoi, A.S. Schmidt, D. Qian, T. Shimada, J.Y. Shen and J.L. Larsen, 2001. Is *Aeromonas hydrophila* the dominant motile *Aeromonas* species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China? Dis. Aquat. Organisms, 46: 23-29.