



Investigation of Mortality of Fishes in Backyard Fish Farming in Meghalaya by Culture and Molecular Methods

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ABSTRACT

Background: The present study investigated the mortality of fish reared in cemented tanks in backyard farming in Meghalaya.

Methods: Five dead fish and two water samples from two different tanks were brought for laboratory investigation to ascertain the cause of death. The samples (water and fish) were subjected to arrays of microbiological and biochemical tests to detect *Escherichia coli*, *Salmonella sp.*, *Aeromonas sp.*, *Vibrio sp.* and *Listeria sp.* by culture and molecular methods respectively.

Result: The present study showed that all the fish samples were found to be negative for *Vibrio sp.* but three samples were positive for *Aeromonas sp.* by PCR against the genes *ahh1* (130bp) and *aerA* (309bp) and one of the fish samples was also positive for *Salmonella sp.* The most Probable Count (MPN) of the water samples was not satisfactory i.e. >40 MPN/ 100 ml, the content of dissolved oxygen was also low and two of the water samples were also positive for *Aeromonas sp.* by PCR for the same genes. Thus, from the investigation results the death of fish was inferred due to multiple factors viz. to low dissolved oxygen, very high microbial load and detection of *Salmonella sp.* and *Aeromonas sp.* Further intervention was suggested regarding optimum holding time, pH, aeration, dissolved oxygen, stocking density, clean water and feeds to control the mortality and future prevention.

Key words: Fishes, Microbiological and Biochemical Tests, Mortality, PCR, Water quality.

INTRODUCTION

One of the foremost hindrances to aquaculture's expansion and successful enterprise is diseases, leading to great economic losses in many countries including India. Among the other causes, diseases caused by bacteria are one of the most common etiology, of which *Aeromonas* spp. poses a major threat. *Aeromonas hydrophila* is a ubiquitous organism reported to cause epizootic ulcerative syndrome diseases in fresh and marine fishes in various parts of the Southeast Asian countries like Malaysia, Japan, Sri Lanka, Bangladesh including India like Andaman, Assam, Meghalaya (Nahar *et al.*, 2016; Shome *et al.*, 2005; Janda and Abbott, 2010; Das and Mukherjee, 1997; Miyazaki *et al.*, 2001).

Aeromonas hydrophila has the potential to survive in diverse environmental conditions, however despite being common and widely spread, it is mostly able to cause diseases when the fishes are under stress (Grizzle and Kiryu, 1993). The virulent of *A. hydrophila* is determined by the production of various toxins (Yu *et al.*, 2005) and many genetic markers like *ahh-1*, *aerA*, *hlyA*, *etc.*, can be detected to ascertain the virulence nature of the *A. hydrophila* (Gonzalez-Serrano *et al.*, 2002; Shome *et al.*, 2005).

Moreover, physiochemical parameters of the water at the optimum level also play a significant role in controlling the fish's overall health conditions, which are positively correlated to the fish's immune response (Rameshkumar *et al.*, 2019). Various anthropogenic activities, release of harmful chemicals and effluents, algal blooms, overstocking of fish, *etc.*, cause mass fish kill in rivers (Khatri and Tyagi, 2015; Saleem Raja *et al.*, 2019). Similarly, fish kill in various lakes and reservoirs due to low dissolved oxygen in the water

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which was caused by the discharge of sewage and industrial effluents had been reported by several workers (Sheikh and Slathia, 2018; Verhoeven *et al.*, 2006; Benjamin *et al.*, 1996; Krishnamoorthy and Visweswara, 1963). On this contention, (Bhatnagar and Devi, 2013) highlighted the role of temperature in stabilizing the biochemical activity of an organism, otherwise, higher temperature affects the oxygen availability and solubility thereby causing an imbalance in ammonia concentration in water. Akongyuure and Alhassan (2021) pointed out that temperature and dissolved oxygen are the major determinant factors for maintaining a favorable environment for the living water body. At this juncture, for the overall development of backyard fish farming in the mid-hills condition of Meghalaya, assessment and understanding of fish mortality is highly essential and no such similar studies have been

reported so far from this region. Therefore, the present study was directed to investigate the plausible causes of fish mortality, so that a proper management strategy can be recommended for the prevention of such cases and to identify the other associated risk factors contributing to the infection and overall mortality in the fish farm.

MATERIALS AND METHODS

Background and sample collections

There was a report of fish mortality in a backyard fish farm in Meghalaya and after that, a field investigation was carried out in 2021. It was observed that fishes were reared in cemented tanks of size 0.05 ha with a water level of 2 meters depth; mix cultured of fishes was reared *Gonius (Labeo gonius)* and Common carp (*Cyprinus carpio*) according to the standard ratio. The disease was reported during the December month of the winter season and mortality was observed in all the fish types. The gross pathological lesions recorded were ulceration of skins, eyes were enlarged, fins were necrosed and hemorrhages were noted on the ventral side. On postmortem, most of the organs like the liver, kidney, spleen and intestines were enlarged and hemorrhagic. To ascertain the plausible causes of death, a total of 5 dead fishes and 2 water samples from different tanks were collected aseptically and transported at 4°C for further laboratory investigation.

Bacterial isolation and identification

All the samples, namely 5 dead fishes and 2 water samples, were subjected to bacterial isolation and identification targeting the following bacterial spp. *Aeromonas* spp., *Listeria* spp., *Salmonella* spp. and *Vibrio* spp. Accordingly, the dead fishes were opened aseptically and liver, skin, intestine and other tissue samples were collected for inoculations. The study was conducted at the Division of Animal and Fisheries Science laboratory, ICAR RC NEHR, Umiam.

Accordingly, for *Aeromonas* spp., the samples (tissues and water) were first enriched in Alkaline peptone water (APW, HiMedia) overnight to 18 hrs at 37°C. A loopful of cultures was inoculated on Ampicillin Dextrose agar plates (ADA, HiMedia) and incubated at 37°C for 24 hours. Smooth and opaque yellow colonies were further subjected to various biochemical tests after visualizing Gram's negative coccobacillary morphology. Subjected biochemical tests were oxidase test, haemolysis test, Lecithinase test, esculin test, TSI, Citrate, Urease, Lysine, Arginine, Ornithine, Nitrate, Voges-Proskauer, Methyl red and Carbohydrate tests (Mannitol, Sucrose, Inositol, Cellobiose, Arabinose, salicin and Lactose) following already described protocols (West and Colwell, 1984; Shome *et al.*, 2005).

Targeting for *Vibrio* spp., water and ground tissue samples were pre-enriched in alkaline peptone water containing 3% NaCl at pH 8.5 and incubated at 37°C for 18 hrs. Further, a loopful of overnight culture were streaked on thiosulphate citrate bile salts sucrose agar (TCBS, HiMedia)

and incubated at 37°C for 24 hrs. However, there was no growth of any typical colonies specific to *Vibrio* spp. (green colonies with blue center), it was not investigated further.

To isolate *Listeria* spp., water and minced tissue samples were inoculated in University of Vermont (UVM)-1 (HiMedia) broth enriched with acriflavin and Nalidixic acid (inhibitor of gram-positive and negative organisms) at 30°C for 24 hrs and further on UVM-II. However, further growth was not detected, which arbitrarily ruled out the presence of *Listeria* spp.

Lastly for *Salmonella* spp. the samples were inoculated in test tubes containing 5 ml of Rappaport-Vassiliades broth as an enrichment medium. The tubes were incubated aerobically at 42°C for 24 to 48 hours. A loopful of broth culture was then streaked onto MacConkey Lactose agar. After that, the non-lactose fermented colonies were streaked on Brilliant Green Agar (BGA) and incubated for 24 hours at 37°C. *Salmonella* suspected pink or red colonies were smeared and stained by Gram's staining method and examined microscopically. Characterization and preliminary identification of suspected *Salmonella* cultures were made based on morphology, colony characteristics and biochemical tests (Quinn *et al.*, 2002).

Samples were also inoculated on MacConkey Lactose agar, discerning lactose fermenter and non-lactose fermenter, lactose fermenter typical of *E. coli* were streaked on Eosin methylene blue agar for confirming its metallic sheen nature.

Molecular confirmation

Based on the cultural, morphological and biochemical tests the isolates were designated as *Aeromonas hydrophila* and *Salmonella* spp. The pure colonies of these isolates were subjected to bacterial DNA isolation using a kit (Qiagen). Thereafter, a Polymerase chain reaction was conducted in a thermal cycler (Biorad) for detecting the virulence-determining genes of *Aeromonas hydrophila* viz *ahh-1* (*haemolysin gene*) and *aer-A* (*aerolysin gene*) following the described PCR protocols of Wang *et al.*, 2003. The primer sets corresponding to *ahh1* and *aerA* will yield an amplified product of 309bp and 130bp respectively. The PCR was set up for the final volume of 25 µl reaction mixture comprising 12.5 µl of 2x PCR master mix (Fermentas, Life Sciences, Germany), 10 pm of each forward and reverse primers, nuclease-free water and 1 µl of respective template DNA. The cycling parameters were an initial denaturation for 5 min at 95°C, followed by 35 cycles of 50 sec at 95°C, annealing at 59°C for 50 sec and extension at 72°C for 50 sec, followed by a final extension at 72°C for 10 min and hold at 4°C.

The suspected *Salmonella* isolates were confirmed as *Salmonella* by detecting the *Salmonella*-specific gene by simplex PCR using a specific primer reported by Elder *et al.* (1997) corresponding to the *invA* gene. The PCR reaction mixture was set up for a reaction volume of 25 µl and the reaction mixture composition was similar as above except for respective primers. The cycle parameters were

1 cycle of 5 min at 94°C; 30 cycles of 45 sec at 94°C, 40 sec at 60°C, 1 minute at 72°C; 1 cycle of 5 min at 72°C and hold at 4°C. The positive reaction will yield an amplified product of 457 bp.

DNA extraction for the suspected *E. coli* colonies was done using the snap-chilled method. The crude DNA extracted was subjected to detection of the *stx1* and *stx2* genes using already reported primers and protocols. (All primers used in the study are detailed in Table 1).

The amplified products of all the genes were analyzed by electrophoresis in 1.5% agarose gel in Tris-borate buffer containing 2 µl ethidium bromide (10 mg/ml), visualized with a UV transilluminator and photographed by gel documentation system.

MPN determination

To assess the microbial load of the water from the fish tank, the most probable count (MPN) of coliform was done per standard microbiological methods (Quinn *et al.*, 2002). The aseptically collected water samples were processed within the same day of collection per the standard protocol using an inverted Durham's tube in 9 Mac Conkey broth test tubes (HiMedia). The tubes were arranged in three rows of 3 tubes per row. The first row contains 10 ml of double-strength broth plus 10 ml of water samples. Subsequent rows contain 10 ml single-strength broth inoculated with 1 ml and 0.1 ml water respectively giving up to 1:1000 dilutions. All the tubes for separate samples were incubated at 37°C for 24 to 48 hr. The MPN index determined the MPN.

Water analysis- Temperature, pH, dissolved oxygen, alkalinity, chloride, nitrite, conductivity

The temperatures of the fish tanks were recorded and the pH of the water was also determined. Limnological water analysis was performed in the laboratory following standard techniques (APHA, 2005).

RESULTS AND DISCUSSION

Bacterial isolation and identifications

24 bacterial isolates were recovered from the 5 dead fishes and two water samples. Out of 24 isolates, 7 isolates were *Aeromonas hydrophila* and one isolate was of *Salmonella* spp., 10 isolates were *E. coli* and others were common

enterobacteria. There was no recovery of *Vibrio* spp. or *Listeria* spp. In our study, we have noted that gross and postmortem lesions like ulceration of the skin, eyes were enlarged, fins were necrosed and enlarged (dropsy) and hemorrhages of most of the organs viz. liver, kidney, spleen and intestines. This corresponds to the lesions noted by many researchers during *Aeromonas* infection (Shome *et al.*, 1999, 2005; Nahar *et al.*, 2016, Srinath and Uma, 2024). Therefore, it is evident that *A. hydrophila* may be attributed as the major etiological agent for the mortality of the fishes along with other contributing environmental factors, evading the immune status of the fishes which is in corroboration with the findings of various researchers (Shome *et al.*, 2005; Erdem *et al.*, 2011; Sarkar *et al.*, 2012; Nahar *et al.*, 2016, Laitlanmawia *et al.*, 2023, Mamun *et al.*, 2019).

In our study, *Aeromonas hydrophila* was presumptively confirmed based on cultural, morphological and arrays of biochemical tests. Acquiring and identifying *A. hydrophila* were done using cultural, morphological and biochemical tests. The APW pre-enriched broth when streaked on ADA yielded many colonies of which the clear, yellow, smooth and opaque colonies were suspected to be *A. hydrophila*. After sub-culturing on nutrient agar, these pure colonies were subjected to Gram's staining and various biochemical and sugar fermentation tests. The isolates were negative for Gram stain and coccobacillary morphology. The isolates induced haemolysis, exhibited lecithinase, urease activity, reduced nitrate, produced gas and acid, no H₂S production, hydrolyzed arginine, esculin, utilized citrate, no ornithine and lysine activity, positive for oxidase, catalase, methyl red, indole, VP, esculin tests. All the isolates fermented mannitol, sucrose, arabinose, salicin and lactose and do not ferment inositol and cellobiose (Table 2). *A. hydrophila* is an opportunist pathogen and is described as one of the major sources of losses in fish aquaculture. Many other workers have reported similar biochemical test results for *A. hydrophila* (Shome *et al.*, 2005; Erdem *et al.*, 2011; Sarkar *et al.*, 2012; Nahar *et al.*, 2016; Samal *et al.*, 2014) results have shown Lysine positive isolates, unlike our results. Our results show negative results for lysine and ornithine activity for the *A. hydrophilla* isolates and positive for most other biochemical tests i.e. positive for oxidase, catalase, nitrate, citrate, methyl red, indole, VP, esculin, arginine, lecithinase,

Table 1: Primer Sequence for detection and virulence genes determinants.

Primer	Primer sequence (5' - 3')	Amplicon size	Reference
<i>A. hydrophila</i>	Forward-GCCGAGCGCCCGAGAAGGTGAGTT	130 bp	Wang <i>et al.</i> , 2003
<i>Ahh1</i>	Reverse-GAGCGGCTGGATGCGGTTGT		
<i>A. hydrophila</i>	Forward-CAAGAACAAGTTCAAGTGGCCA	309 bp	
<i>aerA</i>	Reverse-ACGAAGGTGTGGTTCCAGT		
<i>Salmonellae</i>	Forward-TGC CTA CAA GCA TGA AAT GG	457 bp	Elder <i>et al.</i> , 1997
<i>(invA)</i>	Reverse-AAA CTG GAC CAC GGT GAC AA		
<i>E. coli</i>	Forward-ATAAATCGCCATTTCGTTGACTAC	180 bp	Paton and Paton 1998
<i>Stx1</i>	Reverse- AGAACGCCCACTGAGATCATC		
<i>E. coli</i>	Forward- GGCAGTGTCTGAACTGCTCC	255 bp	
<i>Stx2</i>	Reverse- TCGCCAGTTATCTGACATTCTG		

urease and haemolysis tests. Our results have shown that all the isolates fermented mannitol, sucrose, arabinose, salicin and lactose and do not ferment inositol and cellobiose, consistent with Nahar *et al.* (2016).

Molecular confirmation

Screening the isolates of *A. hydrophila* for the presence of virulence gene corresponding to haemolysin and aerolysin all the isolates (n=7) gave positive results for Ahh-1 gene with amplified product of 130 bp and only 5 isolates were positive for aerA gene (309 bp product size). Besides, one fish sample was found positive for the *Salmonella* inva gene. None of the *E. coli* isolates gave positive results for

stx1 or stx2 genes (Table 3 and Fig 1 and 2). The virulence gene of *A. hydrophila* was determined by molecular methods *i.e.* Polymerase chain reaction based on the haemolysin (ahh-1) and aerolysin (aerA) genes. Consistent with the present finding, many researchers have reported detecting ahh-1 and aerA genes as virulence factors (Shome *et al.*, 2005, Khalil and Mansour 1997). In our study all the *A. hydrophila* isolates (n=7) carried ahh1 genes in accord with the results of Shome *et al.* (2005), some possessed aerA gene (n=3) and notably, the isolates positive for ahh-1 were positive for aerA as well but not vice versa (Wang *et al.*, 2003; Heuzenroeder, 1999).

Table 2: Biochemical profile of *A. hydrophila* isolates.

Sample code	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Water 1	Water 2
	F1	F2	F3	F4	F5	W1	W2
Gram's stain	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
H ₂ S production	-	-	-	-	-	-	-
TSI	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+
Lysine	-	-	-	-	-	-	-
Arginine	+	+	+	+	+	+	+
Ornithine	-	-	-	-	-	-	-
Nitrate	+	+	+	+	+	+	+
Voges proskeur	+	+	+	+	+	+	+
Methyl Red	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+
Haemolysin	+	+	+	+	+	+	+
Lecithinase	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-
Arabinose	+	+	+	+	+	+	+
salicin	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+

Table 3: Molecular characterization of Virulence genes determinants of *A. hydrophila* (AH), *Salmonella* spp (S) and *E. coli* (EC).

Source	Sourcecode	Isolates code	Ahh1	aerA	invA	Stx1	Stx2
Fish 1	F1	AH1	+	+	*NA	All screened EC (1-10) were negative for both the genes	
Fish 2	F2	AH2	+	-	NA		
Fish 3	F3	AH3	+	-	NA		
Fish 4	F4	AH4	+	-	NA		
Fish 5	F5	AH5	+	+	NA		
Water 1	W1	AH6	+	-	NA		
Water 2	W2	AH7	+	+	NA		
Fish 2	F2	S	NA	NA	+		

*NA: Not applicable.

MPN determination

Coliform determination in the two water samples was done using MPN tube dilution techniques. The water samples gave an MPN value of >40MPN/100ml per the MPN index. One water sample gave 40 MPN/100ml and the other showed 43 MPN/100ml. The coliform MPN count was above 40, which is relatively high rendering the water not fit for drinking and

Table 4: Physico-chemical parameters of the water sample.

Parameters	Units
Temperature (°C)	18
pH	6.5
D.O (mg/l)	4.6
Alkanity (mg/l)	25
Chlorides (mg/l)	40
Phosphate (mg/l)	0.1
Nitrite (mg/l)	0.1
Conductivity (mS/cm)	35

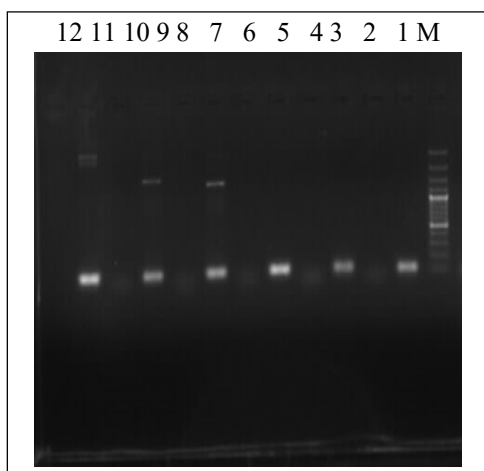


Fig 1: Ahh1 gene positive samples (M, 1 positive control, 3, 5, 7, 9, 11 positive samples, 2, 4, 6, 8 negative samples, 10- NTC) 130bp.

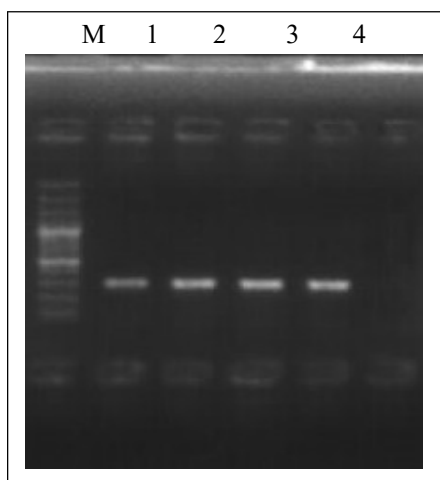


Fig 2: aerA gene positive samples (M, 1 positive control, 2-4 positive samples, 5-NTC) 309 bp.

capable of inducing stress to the fish within its microenvironment. *A. hydrophila* is ubiquitous in nature and has been assigned as a major pathogen of fishes under stress. Other factors contributing to a stressful environment viz., pH, temperature, dissolved oxygen, alkanity, chloride, phosphate, nitrite, etc. The low value of DO below the favorable range may be one of the reasons that have caused stress to the fish leading to less productivity resulting in low photosynthetic activity of the water conditions in the cemented tank which has a similar finding of Vasisht and Sekhar (1979). The positive correlation existing between DO and fish mortality has been supported by various studies conducted in India and abroad (Powers, 1938; Banerjee *et al.*, 1956; Bhagat *et al.*, 1979; Ruparelia *et al.*, 1986; Small *et al.* 2014). All the above factors examined have contributed to the mortality of fishes in the present study. However, there may be other contributing risk factors, which we have not measured like stocking density, organic load- ammonia, ammonium, phosphate, nitrate, etc. which might have resulted in the increased susceptibility of the fishes to the widely distributed opportunistic *A. hydrophila*.

CONCLUSION

Therefore, it can be concluded that various stress factors like temperature, pH, dissolved oxygen, organic matter, stocking density and others have led to stressful conditions for the fishes to keep a stable immune balance and overall general health. This environmental condition has increased the susceptibility of the fishes to *Aeromonas hydrophila* and other secondary bacterial pathogens leading to its mortality. Thus, it will be highly imperative to keep the ideal condition of the water bodies to keep the various stressors in check and prevent future mortality.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from using this content.

Conflict of interest

All authors declare that they have no conflict of interest.

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