



Research Article

## Screening of Aeromonads as associated pathogens from Non-Tuberculous Mycobacterial infections in the aquaculture industry, West Bengal, India

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**Abstract:** The disease termed as 'Ulcerative disease' or 'Erythematous disease' is found in fishes and fish handlers and is caused by a group of waterborne *Mycobacterium* spp. called non-tuberculous mycobacteria (NTM). The wounds are frequently invaded by secondary pathogens like *Aeromonas* spp. which is ubiquitous in nature. NTMs are free-living bacterium inhabiting water bodies, causing skin lesions in fish and fish handlers. The secondary invaders are natural inhabitants and are found in excess due to anthropogenic pollution in aquatic environments affecting the fishes and human subjects as low-level latent infectants in wounds caused by NTM. This study highlights the various aspects mycobacteriosis followed by secondary infection and hemorrhagic septicemia caused by *Aeromonas* spp. in the state of West Bengal (WB), India. NTM and *Aeromonas* spp. samples were examined from different districts of WB. In the case of Fish handlers, NTM infection as well as *Aeromonas* spp. infected wounds were highly significant (correlation coefficient ( $\rho$ ) 0.859,  $p < 0.01$ ). Ulcerative fishes predominant with NTMs were significantly higher in the total samples studied (correlation coefficient 0.718,  $p < 0.01$ ) than the fishes infected with both *Aeromonas* spp. and NTM ( $\rho$  0.188,  $p < 0.5$ ). Systematic reporting of mycobacteriosis and associated pathogens studied here will help to improvise the drug regimes used in culture-based fisheries systems.

**Keywords:** *Mycobacterium marinum*, Mycobacteriosis, Aeromonads, Infection.

### 1. Introduction

Waterborne NTMs belonging to Actinomycetales and family Mycobacteriaceae are aerobic, gram-positive, pleomorphic rods that have been recognized as potential human pathogens (Christine *et al.*, 2003), and also affects aquatic organisms especially fishes of all kinds (Parikka *et al.*, 2012; Jacobs *et al.*, 2009). It causes skin infections in fish handlers, having open scars or cuts, especially from exposure to contaminated water (Collins *et al.*, 1984). The symptoms include skin lesions, nodules, necrosis and ulceration with or without pus formation. Isolation of this bacterial pathogen was first reported by Aronson (Aronson, 1926) from the liver, spleen, and kidney of tropical coral fishes kept in the Philadelphia Aquarium. About 20 species of NTM have been reported to cause granulomatous infection in fish as well as human (fish handlers), among which, *M. marinum*, *M. fortuitum*, *M. chelonae* are the most virulent ones (Decostere *et al.*,

2004). Norden and Linell (1951) and Ang *et al.*, (2000) demonstrated a case of granulomatous skin infection in human-caused by contaminated swimming pools where *M. marinum* was the causative agent.

*M. bohemicum*, *M. gastri*, *M. gordonae*, *M. smegmatis* have also been reported to infect the cultured fishes (Gauthier *et al.*, 2009). These infections were also reported to be zoonotic in nature to some extent (Jernigan & Farr, 2000). However, in the Indian context, the reports are substantially less.

In addition to the cases of mycobacteriosis, aquatic organisms are usually carriers of other bacterial species that are ubiquitous, causing primary as well as secondary infections, *Aeromonas* spp. being one of them (Phung *et al.*, 2013). *Aeromonas* spp. is naturally occurring waterborne pathogen, often found on the body surfaces of fishes without causing any signs of disease.

Poor water quality condition, stress, external aberrations or ulceration caused by facultative

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pathogens leads to disease outbreaks in fishes and shellfishes with this organism behaving as opportunistic pathogens, entering through either open scars or wounds caused by NTM infection or other microorganisms, causing secondary infections. *Aeromonas* spp. has been previously demonstrated as a pathogen affecting freshwater fishes in general, causing surface wounds and infections commonly known as hemorrhagic septicemia, Red sore disease etc. (Janda *et al.*, 2010).

Hemorrhagic skin ulceration can be the main symptom arising from *Aeromonas* spp. mostly occurring due to contaminated water systems (Hayes *et al.*, 2004). Several studies revealed that the most common cases of *Aeromonas* spp. is related to stress-mediated conditions, with poor nutrition and the presence of external wounds such as granulomas and ulcers caused by primary invaders *Mycobacterium* spp. which promotes the secondary invasion by *Aeromonas* spp. A case study reported *Aeromonas* spp. causing secondary infection on superficial surfaces of *C. catla* after an injury (Janda *et al.*, 2010). Thereby, both NTM and *Aeromonas* spp. can be released from diseased fishes and transmitted to other fishes and fish handlers having external skin wounds, cuts, scraps etc. as parallel transmission. Both men and women associated with fish rearing, handling and cleaning were reported with skin ulcers of unknown etiology (Hosseini Fard *et al.*, 2011). Confirmed results from clinical isolates of the severe infections showed the presence of acid-fast bacilli (AFB) followed by biochemical tests from samples confirming the presence of *Mycobacterium* spp. along with other opportunistic pathogens.

The present study, as a pilot survey was carried out in a few districts of West Bengal to study the distribution of Mycobacteriosis like disease associated with secondary invasion by *Aeromonas* spp. in human and fish samples collected from varying climatic and physicochemical conditions from within the state of WB.

## 2. Materials and Method

Swab and pus samples were taken from wounds of people involved in fish handling, fish packaging and fish trading business with significant signs and symptoms of mycobacteriosis like infection represented by nodular or hemorrhagic lesions in extremities. Marine and freshwater fishes having skin ulceration were also sampled. Swabs were also taken from an equal number of normal and healthy fishes that were devoid of any infection. Fresh and marine fishes were brought from different geographic locations along with human samples surveyed for NTM infection. The Districts covered were Cooch Behar, Malda, North 24 Parganas and Kolkata in the state of WB. Pus and swab samples of the human subjects were collected from nodular skin lesions and ulcers found at the upper arm, fingers and palm areas of fish handlers, fishermen and

fish traders of the localized region. Sterile cotton swabs were used to collect the mucous from the necrotic parts mainly at branchial and abdominal region, at base of the fin and pectoral, pelvic and tail fin margin of the infected fishes and were also sampled from the same regions of healthy fishes and both swab and pustules were stored in sterile leak-proof containers in a sealed plastic bag aseptically for transport.

For screening of the pathogen, inoculation was done on nutrient agar, Trypticase soy agar, blood agar and MacConkey's agar for *Aeromonas* spp. and incubated at 32°C. Small, white colony appeared after 24 to 48 hours of incubation from both healthy and infected fish samples and to be very lesser extent to human samples. Presence of *Aeromonas* spp. was confirmed by Gram staining as gram-negative bacilli and biochemical characterization such as oxidase test, glucose fermentation test, H<sub>2</sub>S production, starch hydrolysis etc. as positive (Karunasagar *et al.*, 1986) with PCR amplification of 16S rDNA gene fragment by using primers forward 8F 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse 1492R 5'-ACG GCT ACC TTG TTA CGA CTT-3'.

For detection of different *Mycobacterium* spp. samples were decontaminated by washing with 5% NaOH solution referred to as Petroff's concentration method (Chauhan *et al.*, 1999) and then inoculated on 7H10 agar media supplemented by 10% OADC (Braunstein *et al.*, 2002) and two sets of Lowenstein-Jensen medium slants (Beli *et al.*, 2006) i.e. one set containing 2% glycerol and another 2% pyruvate supplement and incubated at different temperatures ranging from 30°C to 42°C. Slants were observed on a weekly basis and growth appeared after 14 days up to 5 weeks. Colonies were then identified according to their morphological characteristics like coloration and shape of the colony (Tobin *et al.*, 2001) followed by gram staining and acid-fast staining (Joseph *et al.*, 2008) (Williams & Riordan, 1973). The strongly gram-positive and acid-fast positive bacilli (AFB) were screened and confirmed as NTM by further biochemical tests such as Niacin production, nitrate reduction test (Ostland *et al.*, 2008), arylsulphatase activity, catalase activity, tween 80 hydrolysis, NaCl tolerance, presence of urease etc. against reference strains. The bacterial stock culture was made in 20% glycerol and stored in -80°C freezer (Ostland *et al.*, 2008).

## 3. Results

All our studies presented in this manuscript are documented after analysis of a vast and diverse pool of NTM and *Aeromonas* spp. related infections in both human and fish populations. This data is obtained from fish handlers located in various districts both in saline and freshwater environments. Table 1 shows the number of infected people and fishes from different districts.

**Table 1. Distribution of *M. marinum* related mycobacteriosis in the different districts of West Bengal.**

District	No. of market sampled	Total no. of fishermen sampled	No. of fisherman With ulcer	No. of NTM infected people	No. of infected people with NTM and <i>Aeromonas</i> spp.	Total no. of Fish sampled	No. of fishes with ulcers	No. of NTM infected Fish	No. of fishes with NTM and <i>Aeromonas</i> spp.
Cooch Behar	10	427	242	144	99	647	374	245	162
Malda	10	375	218	119	61	522	337	177	127
Kolkata	10	396	243	124	72	650	374	256	157
24 Parganas (N)	10	367	239	112	53	617	400	240	142
<b>Total</b>	<b>40</b>	<b>1565</b>	<b>942</b>	<b>499</b>	<b>285</b>	<b>2436</b>	<b>1485</b>	<b>918</b>	<b>588</b>

Cooch Behar and Malda fall in the comparatively low-temperature zone (10°C to 34°C) and 24 Parganas and Kolkata in the relatively higher temperature zone (18°C to 38°C). The northern regions and southern region of West Bengal namely Cooch Behar, Malda, and North 24 Parganas, Kolkata show a huge number of NTM infection in both fish and fish handlers (Cooch Behar: fisherman-144; fish-245. Malda: fisherman-119; fish-177. North 24 Parganas: fisherman-112; fish-240 and Kolkata: fisherman-124; fish-256 respectively). But a comparatively lesser number of NTM infection cases followed by *Aeromonas* spp. invasion was found from these regions (Cooch Behar: fisherman-99; fish- 162. Malda: fisherman-61; fish-127. North 24 Parganas: fisherman-53; fish-142 and Kolkata: fisherman-72; fish-157).

Correlation coefficient between fish handlers and ulceration due to NTM infection is 0.718 and is significant at 0.001 level of significance. NTM infected fishes is significantly ( $p < 0.001$ ) correlated with total no. of the infected fishes (0.859) in all four districts. Fishes infected with both *Aeromonas* spp. and *Mycobacterium* spp. isolated from a pool of the infected fishes sampled from Cooch Behar showed higher significant value (0.188) and were correlated showing that NTM is responsible for infections in the aquatic environment. The results suggest that in fishes, aeromonads occur simultaneously with NTM in many of the sampled regions and the rate of infection followed by secondary pathogens in the sampled population is noticeable (Shayo *et al.*, 2012). Similar results was not found in fish handlers, suggesting that aquatic organisms are more prone to infection by secondary pathogens and suitable treatment regime should be followed, keeping this in mind.

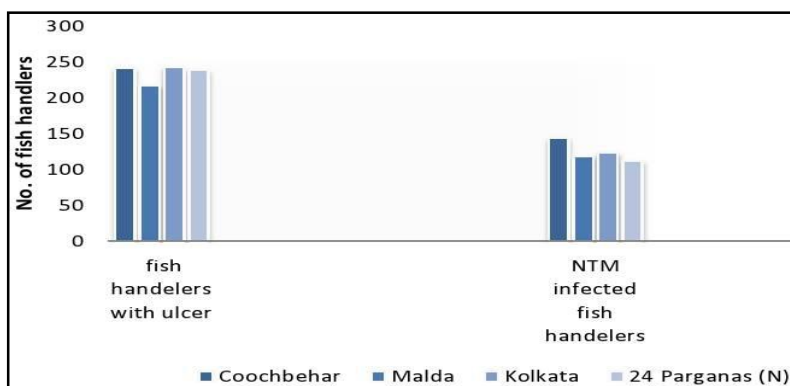
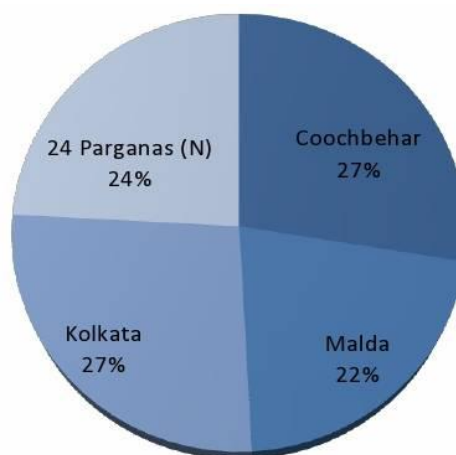
**Fig. 2. Comparative study of total number of infection and NTM infection in fish handlers.**

Fig. 1 highlights the percentage of fish samples infected with both *Aeromonas* and NTMs throughout the districts. Infections were predominantly observed in the District Kolkata (27%) and North 24 Parganas (24%) whereas Malda (22%) and Cooch Behar (27%) showed relatively lesser cases of infected fishes.

A comparative study of the total number of infected population in relation to NTM infection is shown in Fig. 2. The figure suggests that the NTM infection is lesser in number than the total infection by other pathogens in all four districts.

Fig. 3 shows the 1.5 kb amplified gene fragment of 16s gene isolated from *Aeromonas* spp. isolated from skin ulcer by 16s rDNA amplification. This states that *Aeromonas* spp. acts as secondary invaders in wounds made by mycobacterial spp. (Popovic *et al.*, 2000).

**Fig. 1. District-wise distribution of fish sample infected with NTM and *Aeromonas* spp.**

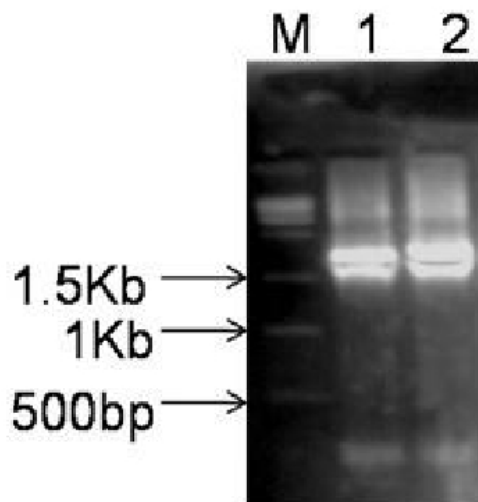


Fig. 3. Amplified DNA by gradient PCR of *Aeromonas* spp.

#### 4. Discussion

Since statistical data relating the NTM infection with *Aeromonas* spp. is not available, we have made an attempt here to find the etiology of ulcers (Plumb, 1994). The most common way to get infected by *Aeromonas* spp. is through open scars, either due to contaminated culture water or during handling infected fishes having necrotic ulceration (Eissa *et al.*, 2008). Various authors demonstrated that *Aeromonas* spp. acts as secondary pathogens in infections found in a variety of hosts (Doukas *et al.*, 1998). This bacteria can often cause secondary invasion in open wounds such as erythematous ulcer, cellulitis etc. which is usually caused by another pathogenic bacteria such as *Mycobacterium* spp., *Vibrio* spp. etc. (Oliver *et al.*, 2005). Our present study also corroborates the result. *Aeromonads* were found to be significantly higher in Mycobacterial infections, both in fishes and in human population as previously reported from other parts of the world (Jernigan & Farr, 2000). Fig. 1 shows that mycobacteria were found in various water systems collected from different regions.

Significant level (0.188) of association of NTMs with infection is reported here. Similar observations have also been reported by Parashar *et al.*, 2004.

In this study, a significant number of fish handlers were surveyed, having ulceration on the upper limbs and fingers of which maximum were found to have NTM invasion. A similar result was also shown by (Shukla *et al.*, 2013). A further study was done to identify the secondary pathogens.

Secondary pathogens such as *Aeromonas* spp., *Vibrio* spp. has been reported earlier from normal and NTM wounds by (Oliver, 2005). The result from fish samples were grouped for infection with only NTM's and NTM's with *Aeromonas* spp. The result showed

significantly ( $p < 0.1$ ) more numbers in the first case while it was lower in the second case. This states that mycobacteriosis is prevalent in the aquatic environment of West Bengal, and possibly throughout the country (Parashar *et al.*, 2004). The skin wounds caused by the pathogenic strain are liable to get infected by other ubiquitous microorganisms found in the water bodies or which may enter due to anthropogenic pollution. Austin (2007) also stated that the infective lesions caused by mycobacterial spp. weaken the immune system of the aquatic organisms making them prone to infection by other pathogens. A similar study with the human host working in the fish farming industry shows equal significance ( $p < 0.01$ ) for both NTM and secondary pathogen related infection. NTMs are known to be zoonotic in nature and cause infections and lesions in human beings who are in contact with them (Joseph *et al.*, 2008). NTM affected human samples were also reported in our study (33.72%). These results suggest that fishes were found to have number of granulomatous lesions showing NTM as an emerging pathogen. Granulomatous ulceration is signified with penetration of microorganism to the deeper tissues of the skin, scales, gills of fishes. In our study, mycobacteria were isolated from the deeper tissue samples (Astrofsky *et al.*, 2000), but *Aeromonas* spp. could be found only in extremities. *Aeromonads* have only been isolated from pus and swab on skin segment at the site of infection in both human and fish samples. They were also isolated from the mucous layer on the skin of normal, healthy fishes with no sign of any kind of ulceration. This suggests that they are normal flora of the skin, but their presence in greater numbers, can be of concern as a secondary or opportunistic invader to compromised hosts. Within a wide spectrum of waterborne bacteria, our study confirms that NTM and *Aeromonas* spp. cause ulceration in both aquatic organism and fish handlers.

This study will thus be an important step towards the selection of antibiotics and other medication for both the study group. Broad-spectrum antibiotics will be of greater help to the infected population (Conte, 2004). Probiotics can also be used to enhance the immune system of the targeted host so that they are less liable to be infected by secondary pathogens. This study will help to ensure a proper drug regime for the present aquaculture environment.

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