Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original research doi: 10.12980/jclm.4.2016J6-177

A detailed report on mycobacteriosis in *Pampus argenteus* along the coast of Bay of Bengal, West Bengal, India

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ARTICLE INFO

Article history:
Received 6 Sep 2016
Accepted 11 Sep 2016
Available online 18 Sep 2016

Keywords:
Mycobacteriosis
Acid fast bacilli
Granuloma
Nontuberculous mycobacteria
Ziehl-Neelsen

ABSTRACT

Objective: To investigate granuloma associated mycobacteriosis in *Pampus argenteus* (*P. argenteus*) (silver pomfrets) and its seasonal prevalence in marine and coastal aquaculture systems of West Bengal, India.

Methods: *P. argenteus* having granulomatous infection were sampled from coastal areas of East Midnapore District of West Bengal from January 2014 to December 2015, encompassing the various seasons. Tissue samples were collected for confirmation by biochemical studies and their prevalence and degree of infection and histopathology was conducted.

Results: A total of 197 fishes having haemorrhage like infections in skin and viscera were confirmed for mycobacteria by biochemical analysis, with prevalence of 22.6%. Post-winter season showed higher prevalence (47.97%) followed by winter (35.25%), pre-winter (27.81%), summer (12.5%) and rainy season (2.57%) respectively. Histopathological assessment showed necrotic granuloma in skin, gills and some internal tissues. Ziehl-Neelsen staining of infected tissue samples showed the deposition of acid fast organisms causing swelling and haemorrhages in the tissues. Severity of infection was evaluated by mean cumulative granuloma score (7.89) score. Majority of the acid fast bacilli were recovered from skin, gills and other organs. Biochemical assessment of the acid fast rods confirmed the presence of *Mycobacterium* spp.

Conclusions: The result suggests that the nontuberculous mycobacterial species are responsible for the granulomatous infection in *P. argenteus*. This infection seems to be influenced by environmental temperature, with the lower temperature favoring the pathogens.

1. Introduction

West Bengal is considered as the largest fish consuming state in India, enriched with extensive fish farming industries. Majority of the marine capture fisheries are integrated in the coastal regions of Bay of Bengal, especially throughout East Midnapore District of the state. Silver pomfrets [*Pampus argenteus* (*P. argenteus*)] have considerably high commercial value occupying about 82% of fishing grounds throughout the coastal regions of Bay of Bengal, West Bengal[1]. Extensive culture practices of *P. argenteus* have declined due to fish mortality caused by microbial infections and fishing hazards. A diverse range of bacteria can affect and colonize the epithelium, scales, fins and gills especially belonging to *Aeromonas* spp. and *Vibrio* spp.[2-6]. They can also be present as ubiquitous pathogens adhering to normal fishes without causing any infections.

Apart from these infections caused by common bacterial species, a disease called mycobacteriosis (or fish tuberculosis) has attributed to fish morbidity and mortality causing gross economic loss to fisheries sector. Mycobacteriosis is frequently caused by water-borne *Mycobacterium* spp., characterized by systemic granulomatous lesions with haemorrhagic ulceration throughout the skin, gills, parenchimal tissues, often penetrating through internal organs like intestine, liver and kidney of fishes[7-9]. Water-borne mycobacteria in general are aerobic, non-motile, Gram-positive, acid fast, pleomorphic rods belonging to the genus *Mycobacterium* and family Mycobacteriaceae. They are often referred to as nontuberculous mycobacteria *i.e.* other than *Mycobacterium tuberculosis* complex[10-12]. Outbreak of mycobacteriosis is closely related to the environmental temperature, stress and poor nutrition that weaken the immune and metabolic systems making the fishes more susceptible to infection by primary and secondary pathogens[13]. In our study, the sampling was done from a marine fish, *P. argenteus* having granulomatous ulcerations throughout the skin and different visceral organs collected from various fish markets and harbour of East Midnapore District. This study focuses on the isolation and characterization of granuloma

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The journal implements double-blind peer review practiced by specially invited international editorial board members.

associated mycobacteria and its seasonal predominance in *P. argenteus*, sampled from the Bay of Bengal coast in the state of West Bengal.

2. Materials and methods

2.1. Collection of samples

A total of 872 silver pomfrets (*P. argenteus*) identified with granulomatous ulcers and lesions were collected between January 2014 and December 2015, from East Midnapore (Figure 1). Fishes were examined for clinical signs of granulomatous infection (Figure 2) on skin, gills, peritoneal cavity and visceral organs like intestine, liver and kidney. Infected fishes were aseptically brought to laboratory for the isolation of the pathogens. Tissue samples were collected in sterile containers and transfer medium.

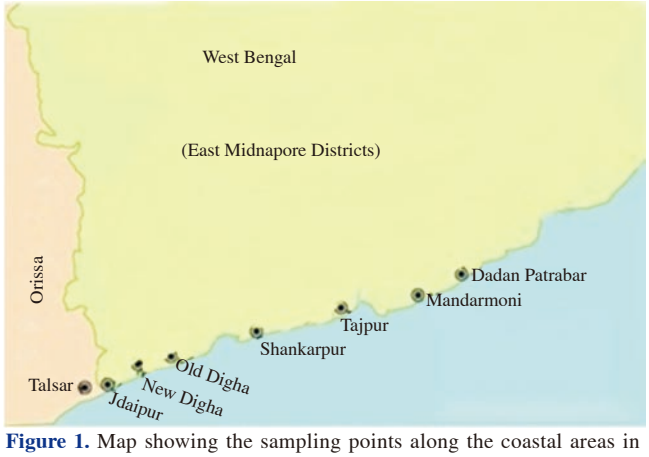

Figure 1. Map showing the sampling points along the coastal areas in East Midnapore District of West Bengal.

Figure 2. *P. argenteus* infected by *Mycobacterium* spp. showing hemorrhagic ulceration.

2.2. Infectious parameters

Prevalence of the granulomatous lesion in *P. argenteus* having seasonal variations with water temperatures were measured[14]. Degree of infection was categorized into mild, moderate and severe groups according to the different degrees of necrosis or ulceration[15]. Correlation between different water temperatures and mean prevalence of infection was measured and ranked according to their severity.

2.3. Recovery of mycobacterium spp. from infected organs

Exotic swab and tissue samples from the infected area of different organs were dehydrated in 70% alcohol and then microscopically examined after acid-fast staining (Ziehl-Neelsen method) and 2 g of tissue from positive samples were then vortexed with 2 mL of 0.85% physiological saline. One part of the tissue suspension was mixed with three parts of 4% sulphuric acid and centrifuged for 15 min at 9 000 r/min. Pellets were collected and inoculated on specific Lowenstein-Jensen medium slants and treated with cyclohexamide- penicillin antibiotic mixture and incubated at 35 ˚C. After 10–15 days of rough incubation, dry and whitish colonies were appeared. Gram stain and acid-fast stain of the Lowenstein-Jensen culture was done to ascertain the pathogenic species.

2.4. Biochemical confirmation of mycobacteria

Biochemical analysis of the isolated and suspected *Mycobacterium* spp. was done using nitrate reduction test, arylsulphatase activity, photoreactivity, catalase activity and NaCl tolerance. The bacterial stock culture was maintained in 20% glycerol and stored in –20 ˚C[16].

2.5. Histopathology

Tissues with granulomatous lesions isolated from infected gills, liver, intestine and kidneys were excised and preserved with 10% neutral buffer formalin, washed in running tap water, dehydrated with ethanol and embedded into paraffin wax. About 5 μm thin tissue sections were incised with rotary microtome. One set was stained with haematoxylin and eosin and another set was stained by Ziehl-Neelsen method. Slides were scored using the scale, with score 0 as normal, 1–2 minimal, 3–4 as mild, 5–6 moderate, 7–8 as severe and 9–10 as highly severe infections to estimate granuloma score (GS) of infection. Each fish showed a cumulative granuloma score *i.e.* sum of GS of different organs of one fish and mean cumulative granuloma score (MCGS) of a group[14,15,17].

3. Results

3.1. Description of granuloma

The granulomas as a form of mycobacterial infections were identified as red or pale yellow inflamed nodules of variable size found throughout the skin and internal organs of fishes with or without pus secretion and confirmed by acid-fast stain and biochemical test results. Haemorrhages often occurring in the nodular lesions were also sampled. The infections were seen to penetrate through the skin with single or multi-nodules which caused swelling or bulging of gills, liver, spleen and kidney parenchyma. Nodules were sometimes found to be embedded in the serosal surface of intestine.

3.2. Seasonal prevalence of infections

The seasonal prevalence of mycobacteriosis like infection (granuloma) in *P. argenteus* was presented in Table 1. A total of 872 diseased fishes were examined, of which 197 were found with granulomatic infection in skin and visceral organs with an overall prevalence of 22.6%. Higher prevalence was observed during post-winter months (47.97%) followed by winter (35.25%), pre-winter (27.81%), summer (12.5%) and rainy season (2.57%) respectively. The virulence of water-borne mycobacterial species was relatively high in low environmental temperature which indicated that the prevalence was significantly influenced ($P < 0.05$) by environmental temperature variations.

Table 1
Seasonal prevalence of mycobacteriosis like infection in *P. argenteus* varying with water temperature along with mean prevalence of granulomatic infections.

Seasons	Month	Water temperature (℃)	Number of fish sampled	Number of granulomatous fishes	Prevalence (%)	
					Month	Season
Winter	December	10–11	64	23	35.93	35.25
	January	8–10	75	26	34.66	
Post-winter	February	15–20	82	41	50.00	47.97
	March	20–25	66	30	45.45	
Summer	April	30–36	87	19	21.83	12.50
	May	38–40	80	9	11.25	
	June	36–40	73	2	2.73	
Rainy	July	36–38	56	0	0.00	2.57
	August	32–36	60	0	0.00	
	September	30–35	78	5	6.41	
Pre-winter	October	25–30	76	17	22.36	27.81
	November	18–22	75	25	33.33	

Correlation coefficient (R) between number of fish sampled and number of granulomatous fishes was 0.38 and level of significance was $P \leqslant 0.05$.

3.2. Biochemical identification of acid fast rods

A total of 260 bacterial cultures ($P < 0.01$) were positive for acid fast rods. Cultures were grown on the Lowenstein-Jensen medium slants that were selected for only *Mycobacterium* spp. and growth temperature ranging from 28 ℃ to 35 ℃. The biochemical test results highlighted in Table 2 confirmed the presence of *Mycobacterium* spp. A large number of samples showed nitrate reduction activity ($P < 0.01$) followed by arylsulfatase activity and tolerence to 5% NaCl. Maximum samples were shown to have very low growth rate at temperatures ranging between 25 ℃ and 40 ℃.

3.3. Histopathology

Histological assessment showed necrosis in skin, gills and in some internal tissues. The sections of liver tissues showed hepatomegaly characterized by diffused infiltration of hystiocytes, lymphocytes and multinucleated cells. Acid fast bacilli were deposited at the central necrotic core of granuloma. Ziehl-Neelsen staining of gill tissues highlighted the necrosis with presence of acid fast organisms (Figure 3). A moderate number of acid fast bacteria were found in kidney but necrosis was not observed. Figure 3 describes the intensity of penetration of infections throughout different layers of tissues. Degree of infection and granuloma score corresponding to the different organs were presented in Table 3. Figure 4 describes the percentage of variations in the infections throughout different tissues.

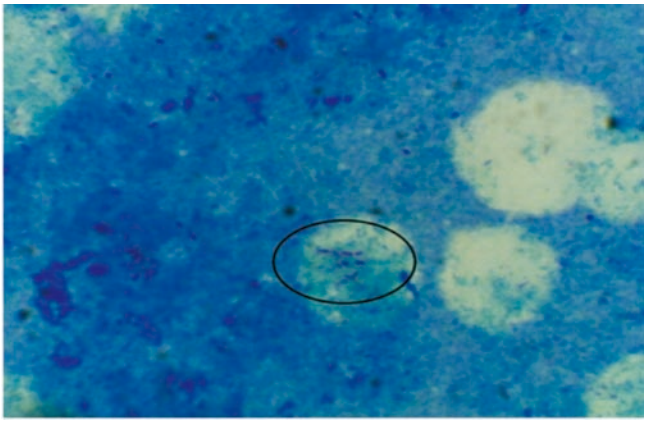

Figure 3. Ziehl-Neelsen staining of necrotic gill tissues of *P. argenteus* showing presence of acid fast bacilli.
Pink coloured rod shaped mycobacteria was visible within blue stained gill tissues.

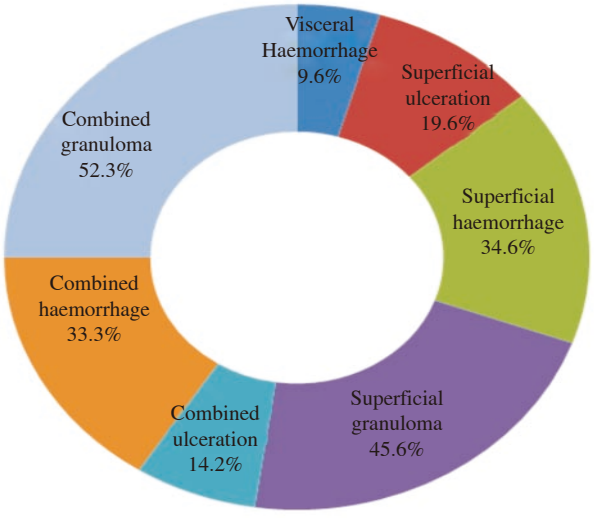

Figure 4. Percentage of different forms of infection in various fish tissues.

Table 2
Selective biochemical conformation for the number of sample isolated.

No. samples	Biochemical Tests									
	Arylsulfatase activity	5% NaCl tolerance	Scotochromogenic	Non-chromogenic	Photochromogenic	Nitrate reduction	SQ catalase	Catalase	Growth at 25 ℃	Growth at 45 ℃
47	+	+	+	-	-	-	-	-	-	-
43	+	+	-	+	-	+	-	-	-	-
42	-	-	-	-	+	+	+	-	-	-
37	-	+	-	+	-	+	-	+	-	-
29	-	-	+	-	-	+	-	-	-	+
25	-	-	-	+	-	+	-	+	+	-
19	-	-	-	+	-	+	-	+	-	-
11	-	-	-	+	-	-	-	+	+	-
7	-	-	-	-	-	-	-	-	-	-

SQ: Semiquantitive.

Table 3
Microbiological and histopathological observation of infected fish tissues explaining the MCGS of respective tissues and recovery of the acid-fast bacilli from the infected tissue samples.

Site of infections	Number of infected fishes	Mean of degree of infections	MCGS	Recovery of acid-fast bacilli (No. of bacterial culture grown/No. of tissue sample examined)
Skin, eye, gill cover, gill	17	Moderate	5.58	37/53
Skin, peritoneal cavity, intestine	21	Moderate	5.42	44/58
Intestine, liver	10	Mild	3.30	9/20
Skin, inner muscles	21	Severe	7.90	26/43
Base of tail, tail fin	18	Severe	7.61	17/28
Oesophagus, intestine	3	Mild	3.28	3/8
Base of fins, gill cavity	8	Moderate	5.00	9/17
Skin, base of fins	32	Severe	7.71	41/65
Gill cover, gills, gill cavity, nostrils, skin	19	Severe	7.89	22/40
Intestine, liver, kidney	5	Moderate	5.80	8/19
Gill cavity	9	Mild	3.44	5/8
Gill cover, gill	21	Moderate	5.04	18/35
Gill, gill cover, skin	13	Moderate	6.00	21/28

4. Discussions

The present study was conducted to assess the infectivity and histopathological changes caused by mycobacteriosis like infections and their seasonal prevalence in *P. argentes*, visible signs being granulomatous inflammations. This was the first survey on granulomatous infection of such marine fishes from the coastal aquaculture sector of West Bengal. Fish mortality due to pathogenic microorganisms belonging to various groups and taxa has been reported by various researchers[17-19]. Granulomatous infection is commonly characterized by formation of systemic granuloma with inflammation or swelling of infected tissues and organs[8,9,18,20]. Haemorrhages and secretion of pus seem to be common with inflammations and similar results were observed here. Mycobacteria are ubiquitous pathogens that can survive and accumulate in the environment for prolonged periods[19,20]. Fishes usually acquire these infections through contact with contaminated water sources. Isolations of this species from our samples corroborates with this results. Haemorrhagic ulceration is often misdiagnosed as other diseases, thereby hampering the treatment regimen.

Recovery of rod shaped acid fast bacteria from excised tissue samples was the first step in detection of the species suggesting that the infection was caused by the bacteria studied here. The individual characteristic of genus *Mycobacterium* is the acid fast sensitivity due to presence of thick layers of cell wall lipids which makes them distinct from others[21]. Table 3 describes the recovery of the acid-fast rods from the infected skin, gills and gill covers with high MCGS (7.89). This particular region may be accountable for the infestation of the pathogens leading to infection which can penetrate through the internal organs of the fishes[19,20,22]. Gradually, this infection spreads through the visceral organs like digestive tract, liver and kidneys[8,9,17,20]. Histopathological observations showed the highest GS in the skin, inner muscles and gill regions in comparison to others organs, as these are the most exposed and prone to be in contacted with the infected organisms and environment (Table 1). The site of the inflammation related to the different organs does not directly influence the degree of infection and relative granuloma score. The study exhibits that multiple organs can be affected but the location and consistency of granuloma can be diverse[23,24].

Initial confirmation was done by biochemical profiling shown in the Table 2[16,17]. The Lowenstein-Jensen medium enriched with malachite green and cycloheximide-penicillin antibiotic mixture was used for normal growth and biochemical test. Malachite green inhibits fungal growth and antibiotic supplement resists the growth of other contaminants.

Seasonal prevalence of mycobacteriosis related to different water temperature was viewed in our study where higher rate of infection was found during winter to post-winter season ($P \leqslant 0.05$) in comparison to summer. Very low intensity of infection was found through rainy season which may be due

to the availability of fresh water in the monsoon. These results suggest that the environmental temperature may play a major role in pathogenicity and virulence of *Mycobacterium* spp. Salinity and pH of culture water of all regions were almost similar during the period of survey indicating that is not an immediate contributor to this disease especially for marine sector where a relatively uniform salinity was observed[25]. Mycobacteria associated infections are at raise in the coastal areas and also in the aquaculture systems. Precise identification of these pathogens should be considered a priority for understanding the diversity of pathogenic microflora of fish in the state of West Bengal and documentation of the same.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We are thankful to Department of Biotechnology, Government of West Bengal and Department of Science and Technology, Government of India.

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