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DRUG RESISTANCE IN *MYCOBACTERIUM FORTUITUM* ISOLATED FROM GOLD FISH, *CARASSIUS AURATUS*

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Abstract: Mycobacteriosis is a chronic disease of freshwater and marine fish. In the present study, *Mycobacterium fortuitum* from a diseased gold fish, *Carassius auratus* was isolated and identified by biochemical and molecular (PCR) method. The isolate was found to be resistant to rifampicin, isoniazid, pyrazinamide and ethambutol and myoconda® a nanodrug combination of rifampicin, clarithromycin and clofazamine by broth microdilution method (BMM). Presence of such drug resistant *Mycobacterium fortuitum* in ornamental fishes is of great concern due to its ability to cause disease in fishes and immunodeficient fish handlers.

Keywords: Mycobacteriosis, *Carassius auratus*, Gold fish, Drug resistance.

Introduction

Ornamental fish keeping is a popular hobby worldwide. The growing interest in aquarium fishes has resulted in increasing demand for aquarium fishes and trading globally. The value of ornamental fish trading is increasing at an average growth rate of approximately 14 % per year (Rani et al. 2014). Hence, the practice of ornamental fish culture in intensive production systems like raceways and recirculating aquaculture systems are also increasing. Improper management of such intensive fish production systems often leads to high organic load, poor water quality resulting in disease outbreaks. Mycobacteriosis is a chronic or acute, systemic, granulomatous bacterial disease that occurs in ornamental fish which is also referred to as nontuberculosis mycobacteriosis (Decostere, 2009). The disease has been reported in a broad range of fish species from fresh water, marine and brackish water environment (Kaattari et al., 2006). The symptoms of the disease include loss of scales, granulation and skin ulcers. Infected fish act as a source of vertical and horizontal transmission, which significantly increase the risk of infection in the breeding stock (Floyd, 2011). Further, mycobacterial diseases can be zoonotic which when handled by immunocompromised individuals may

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contract the infection (CFSPH, 2006). Recent reports indicate an increase in the resistance to antimycobacterial first line of drugs which could become a serious public health issue (Horan and Cangelosi, 2009). The present study was undertaken with an objective to assess the drug resistance in a mycobacterial isolate from infected gold fish.

Materials and Methods

Isolation of Mycobacterium sp.

Mycobacterium sp (SDDLUM-2/12) was isolated from a diseased gold fish, *Carassius auratus* with the ulcerated granuloma in the dorsal tail which was collected from a commercial aquarium outlet at Chennai, Tamilnadu, India following the method described by Beran et al.(2006) with slight modification. Briefly, the tip of the swabs from the samples were cut and placed in a microcentrifuge tube containing 500ul of PBS. The tubes were vortexed for 1 min and centrifuged at 8,000× *g* for 15 min at room temperature. The swabs were removed and 100µl of 0.7% hexa decyl pyridium chloride was added and incubated at room temperature for 1 h. After incubation, the suspensions were centrifuged at 8,000× *g* for 15 min at room temperature. The pellet was washed twice with sterile PBS solution by centrifuging at 8,000× *g* for 5 min and the sample was resuspended in 200µl of PBS. Lowenstein Jensen slants and Herrold's egg yolk medium (HEYM) were prepared and each tube was inoculated with 100µl of sample and incubated at 37^0C. The culture tubes were examined periodically for the presence of characteristic colonies for a period of 10 days. The conventional characterization methods *viz.,* microscopy after Ziehl-Neelsen staining, evaluation of growth on Lowenstein-Jensen slants and the selection of NTM colonies were carried out based on the description of colonial morphologies by Glover et al.(1994).

Identification and characterization Mycobacterium sp

The isolate was identified based on biochemical characterization and molecular method (PCR). Pure culture of Mycobacterium sp (SDDLUM-2/12) was obtained by sub culturing and subjected to biochemical characterization *viz*. growth at 45ºC, 37ºC and 24°C, growth rate, growth in MacConkey agar, NaCl tolerance, pigment production, nitrate reduction and Aryl sulfatase test. For PCR amplification of 16sRDNA and sequencing, DNA was extracted from the isolate (SDDLUM-2/12) grown in HEYM using a commercial genomic DNA extraction Kit (Qiagene, Germany) following the manufacturer's protocol. The DNA was checked for the purity and quantified (Nanodrop spectrophotometer, Thermo scientific). Specific amplification of mycobacterial 16s rRNA fragment was carried out using the primers, 285-F (5' GAG AGT TTG ATC CTG GCT CAG 3') and 264-R (5'TGCACA CAG

GCC ACA AGG GA 3') which allows amplification of hyper variable region (Ronald and Kavitharani, 2013). Mycobacterial DNA (50 ηg) was amplified in a 25µl reaction mixture with an initial denaturation at 94^{o}C for 3 minutes, 35 cycles of 94^{o}C for 1 minute, 55^{o}C for 1 minute, and 72^{o}C for 1 minute and a final extension at 72^{o}C for 7 minutes. The PCR products were resolved on 1% agarose gel, purified using PCR product purification kit (Qiagene, Germany) and sequenced to determine the species- specific nucleic acid sequence of hyper variable region of 16s rRNA. The sequence information was submitted to the Genbank, NCBI .

Assessment of drug resistance by broth microdilution method (BMM)

The *Mycobacterium* sp (SDDLUM-2/12) isolate was resuspended in sterile distilled water to match the turbidity of 0.5 MacFarland standard. The working stock solutions for the drugs were prepared as described in RNTCP training manual (2009) and BMM was carried out following the method described by Liete et al. (2000). Briefly, Middlebrook 7H9 medium (100µl) was dispensed to all the 12 wells from A – F in a 96 well microtitre V bottom plate. Row A:1-6 served as positive control and 7-12 as negative control. Row B was kept blank to avoid accidental spilling. The first well of Row C,D,E and F were added with 100 µl of working solution of rifampicin, isoniazid, pyrazinamide and ethambutol. Serial two fold dilutions was done and 100µl from the 12th well was discarded. The drug concentrations were 100µg, 50 µg,25 µg,12.5 µg,6.25 µg upto 0.048 µg in the descending order. About 5 µl of culture suspension was uniformly added to all the wells. The isolate was also compared with reference bacterial strain *M.tuberculosis* H_{37} R_v for drug resistance. The plates were sealed and kept in sterilized autoclave bags and incubated at 37^{0}C. The plates were observed for bacterial growth (turbidity/button formation) after 3-5 days for the minimum inhibitory concentration. Similarly, drug resistance was also assessed for myoconda® a nanodrug combination of rifampicin, clarithromycin and clofazamine. MIC was done in duplicate for the isolate.

Results

The Mycobacterial isolate was identified as *M.fortuitum* based on the biochemical characterization as presented in Table 1. PCR amplification of 16srDNA gene of mycobacterium resulted in specific amplification of 1487 bp product which was then confirmed as *M.fortuitum* by DNA sequencing and analysis (Fig.1). The Genbank (NCBI) accession no. for the sequence information of *M.fortuitum* is KC832402. The results of broth microdilution method for the isolates against Pyrazinamide, rifampin, isoniazid, ethambutol

and Myoconda® showed that the isolate was resistant to Pyrazinamide, rifampin, isoniazid , ethambutol and Myoconda® (Fig.2)

Discussion

Mycobacterial infection is the most common chronic disease in the fishes of temperate and tropical regions (Noga, 1995, Kiesch, 2000). Mycobacterium is of great concern to aquaculture industries, especially in intensive culture units with recirculating systems as it is characterized by conditions such as warm water temperatures, low dissolved oxygen levels, acidic pH, high soluble zinc, high fulvic acid, and high humic acid that favours the growth of mycobacteria (Floyd, 2011).

In the present study, Mycobacteria isolated from the gold fish was identified following conventional and molecular techniques as Phenotypic and molecular methods have been followed for identification of nontuberculous mycobacteria from various sources (Sharareh, 2012). Beran et al., (2006) isolated *M. fortuitum, M. peregrinum* and *M. chelonae* in fishes without macroscopic lesions typical of mycobacteriosis since they are common saprophytes in the aquatic environment. Their presence appears to be strongly related to poor managemental practices resulting in abnormal stress to fish and a reduction in their normal resistance. Zanoni et al. (2008) reported that macroscopic lesions suggestive of mycobacteriosis in only 10.8% of the fish samples screened and only in the fishes which were positive on microscopic examination. A new isolate of mycobacterium similar to *M. marinum* and *M. ulcerans* that causes disease in wild striped bass (*Morone saxatilis*) in the Chesapeake Bay has been reported (Rhodes et al. 2001). As mycobacterial infections of fish are zoonotic, they are likely to cause disease in persons involved in fish handling (Street et al., 1991; Lehane and Rawlin, 2000). Investigations on the mycobacteria in ornamental fish in India are scanty. A report from India (Seema, 2014) states that, 70.6% of the aquarium fish samples collected in a study showed culture positivity for mycobacteria from parenchymatous organs with granuloma.

There are very few reports on the drug resistance in mycobacterial isolates from fish. Nicole et al., (2011) have reported resistance to rifampicin in a *M.marinum* strain. The mycobacteria sp. isolated in the present study showed drug resistance to all the primary antimycobacterial drugs.

Non tuberculous mycobacteria (NTM) are reported to be relatively resistant to many of the first- and second-line drugs and their mechanisms to intrinsic drug resistance vary among mycobacterial species which is mediated by a single chromosomal mutation or accumulation

of mutations that occur due to direct exposure of the organism to a particular drug. Recent environmental surveys of both freshwater- and salt water-borne bacteria have documented unexplained antibiotic resistance to various antimicrobial drugs, including rifampin (Smith et al., 2013). Sharareh et al. (2012) reported a resistance of more than 57% in non tuberculosis mycobacterium (NTM) species to rifampin, isoniazid and ethambutol. Drug resistance in NTM isolates may also contribute to the development of drug resistance in human beings involved in handling of fishes in aquariums and fish farms.

Conclusion

M.fortuitum isolated from the goldfish, *C.auratus* samples with ulcers was identified by biochemical characterization and 16sRNA sequencing. The results of the study shows that Mycobacteriosis is prevalent in the gold fish, *C.auratus*. Further MIC revealed that the isolates are resistant to Pyrazinamide, rifampin, isoniazid, ethambutol and Myoconda® which need to be addressed seriously with regard to public health aspect.

Table 1: Biochemical Identification of the *Mycobacterium fortuitum* isolate SDDLUM-2/12

Tests	**Result**
Rate of growth	2 days
Growth at 25°C	+
Growth at 37°C	+
Growth at 45°C	+
Aryl sulfatase test , 3 days	+
Pigmentation	No pigmentation
Nitrate reduction	+
Growth on Mac Conkey	+
Tolerance to 5% NaCl	+

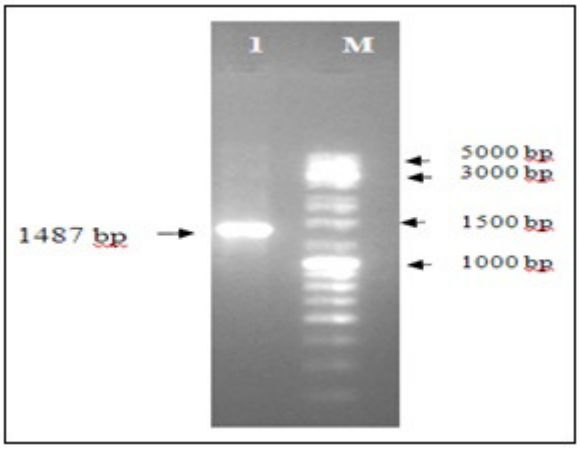
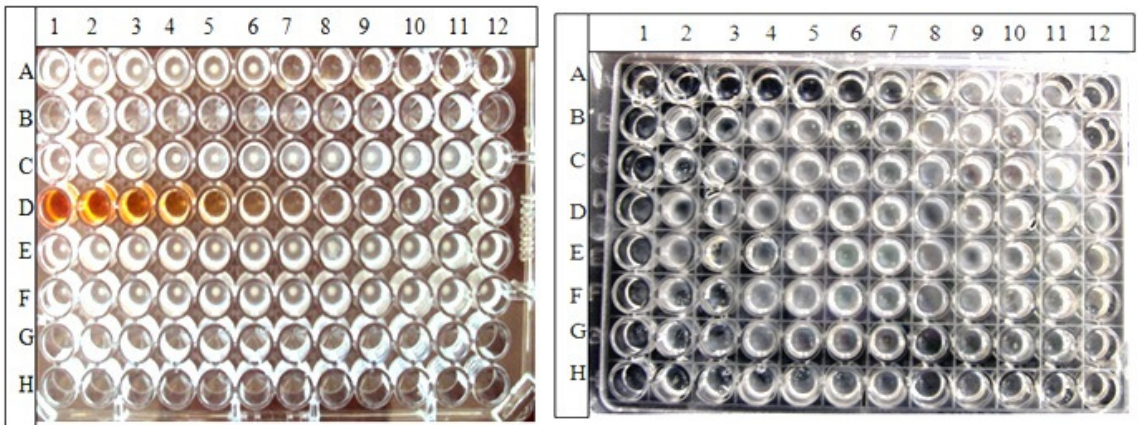


Fig. 1.16sRDNA amplification of Mycobacterial isolate SDDLUM-2/12 isolated from goldfish

Lane: 1- PCR amplicon of 16S rDNA gene; M- Molecular weight marker.



Row A: 1 – 6 well – Negative control;
7 –12 wells – Positive control
Row B - H: Myoconda® drug dilution

Rows A: 1 – 6 well – positive control
B :7 – 12 wells – Negative control
C: Pyrazinamide drug dilution;
D: Rifampicin drug dilution
E: Ethambutol drug dilution
F: Isoniazid drug dilution

Fig. 2. Broth Microdilution method to assess sensitivity to antimycobacterial drugs and Myoconda® for the mycobacterial isolate SDDLUM-2/12

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