Gene Reports 10 (2018) 26–32

Contents lists available at ScienceDirect

Gene Reports

journal homepage: www.elsevier.com/locate/genrep

Species identification of aquatic mycobacterium isolates by sequencing and PCR-RFLP of the *16S–23S* rDNA internal transcribed spacer (*ITS*) region

Shubhra Shukla[a,*], Sanjeev Kumar Shukla[b], Rolee Sharma[a]

[a] Department of Biosciences, Integral University, Lucknow, Uttar Pradesh 226026, India
[b] Department of Biotechnology, Bundelkhand University, Jhansi, Uttar Pradesh 284128, India

ARTICLE INFO

Keywords:
Mycobacterium
Aquarium fish
ITS
PCR-RFLP

ABSTRACT

Fast and reliable identification of clinically significant Mycobacterium species strains attempted to identify on the basis of amplification, RFLP and sequencing of the internal transcribed spacer 1 (*ITS1*) *16S–23S* Mycobacteria in Apparently Healthy Freshwater Aquarium Fish in Uttar Pradesh, India. Sixty apparently healthy freshwater fish aquariums belonging to eight different species have been harvested from six outlets in several aquarium cities and processed for the isolation of Mycobacterium species. Using the initial decontamination protocol tissue homogenates (with 1 N HCl and NaOH 2 N) and incubating at 30 °C for two months, Mycobacterium species was isolated of 25% of them. These isolates have been identified by standard biochemical tests. All the fragments of genes of the *ITS* region of the *23S rRNA* were amplified by *16S* fragment performance of different dimensions between 230 bp and 350 bp digested by two fast digest restriction enzymes; *Hae*III and *Sau*96I. The digested products were analyzed using gel electrophoresis with agarose. There has also been sequencing amplified fragments of the *ITS* gene. The isolates were identified as five strains of *M. abscessus*, *M. gordonae* as three, two *M. fortuitum*, *M. Parascrofulaceum*, *M. arupense*, one isolate as *M. senegalense*, *M. aubagnense* and *M. conceptionense*. The incidence of mycobacteria in freshwater fish, seemingly healthy is terrible and the study is relevant due to the variety of Mycobacterium related to aquarium fish and zoonotic importance. All Mycobacterium species isolated during this study are well known pathogens both to fish and humans.

1. Introduction

Mycobacterial species reported in the aquatic environment has increased significantly, especially water reservoirs (Herbst et al., 2001; Rhodes et al., 2003). Therefore, we have developed various molecular methods based on the rapid detection and identification of mycobacterial species. PCR followed by restriction fragment length polymorphism (RFLP) and sequence analysis were developed in response to the need to identify further species. Analysis of amplified nucleic acid restriction enzymes is relatively inexpensive, easy to perform and provides a rapid method for detecting polymorphism among the various species of mycobacteria (Telenti et al., 1993; Roth et al., 2000). The presence and distribution of mycobacterial species in clinically healthy freshwater aquarium fish had not been adequately explored. Phenotypic methods for identifying mycobacteria, such as acid-fast staining, are not useful for identifying species of mycobacteria and biochemical tests may be delayed and even then, cannot distinguish between the different mycobacterial species under examination. Molecular methods try to solve these problems (Rhodes et al., 2005). Mycobacteria are common pathogenic fish diseases and diseases are among the most common chronic diseases in the aquarium tropical and subtropical freshwater fish (Gauthier and Rhodes, 2009). Conventionally, mycobacterial species are identified by their growth characteristics (such as growth rate, colony morphology, pigmentation and photo reactivity) and biochemical reactions. In addition, these test procedures are time consuming, complicated and sometimes do not even provide accurate identification and isolates from different aquatic animal sources (Nasr-Esfahani et al., 2006; Zanoni et al., 2008; Nguye et al., 2017; Puk et al., 2017). For many years, PCR has been clinically used for the rapid identification of Mycobacterium species. Given the number of species closely related to genus Mycobacterium, there is often a different species of PCR cross reactivity, an additional method is needed to identify species specific mycobacteria. Polymerase Chain (PCR) Linked Molecular Methods, Enzyme Restriction Analysis (ERA) and sequence analysis are more reliable and faster to identify NTM (non-tuberculous mycobacteria) (Pourahmad and Richards, 2013; Emmerich and Fabri,

Abbrevations: ITS, Internal transcribed spacer; M, Mycobacterium; RFLP, Restriction fragment length polymorphism; PCR, Polymerase Chain Reaction; REA, Restriction Enzymatic Analysis

* Corresponding author at: Faculty of Applied Science, Department of Bioscience, Integral University, Lucknow, Uttar Pradesh 226026, India.
E-mail addresses: shubhra85@gmail.com (S. Shukla), sanjeevcloning@gmail.com (S.K. Shukla).

http://dx.doi.org/10.1016/j.genrep.2017.10.013
Received 29 August 2017; Received in revised form 27 September 2017; Accepted 31 October 2017

2017). Therefore, it is an appropriate target for identifying the level of NTM species with respect to other methods (Hafner et al., 2004). In general, mycobacterium fish is considered a common systemic disease (Gauthier and Rhodes, 2009), where primary pathological lesions involve classical granulomatous inflammation in various organs or tissues, Ocular localization of mycobacterial lesions (Roberts, 2012; Phillips et al., 2017; Vanhooteghem and Theate, 2017). The incidence of mycobacteria in clinically healthy fish populations (Mrlik et al., 2012; Beran et al., 2006). In this study so many researchers are try to make a successful tuberculosis vaccine, Myllymaki and colleague identification of new candidate antigens for an adult zebrafish (*Danio rerio*) tuberculosis vaccine (Myllymaki et al., 2017).

Affected fish might also lose their appetite, show up debilitated and emaciated, have impaired growth and come to be extra inclined to contamination by using opportunistic bacteria. Skin lesions can also now not be present. If present, the severity of the lesions can differ from small blisters to shallow ulcerations (Heckert et al., 2001). Some fish may additionally have fin damage, skin ulcerations where lesions lying in the muscle without delay beneath the pores and skin rupture to the outside. Others can also have pigment alterations, being brighter in color or duller (Austin and Austin, 2007). Some affected fish can also show issues in their copy device (e.g., releasing eggs). However, severity of contamination varies dramatically, from simply nuisance infections in which a few fish die from continual infections to severe outbreaks with high mortality (Whipps et al., 2003).

Several regions were studied in the genome of Mycobacterium and were compared among the species and include the *16S* gene *rRNA* (Kirschner et al., 1993), the internal transcribed spacer 1 (*ITS1*) region sequence located between *16S rRNA* and *23S rRNA* genes (Roth et al., 1998), the ß subunit of RNA polymerase gene (*rpoB*) (Kim et al., 1999). The sequential analysis of *16S rRNA* has been extensively used. Due to the sequence variability of the spacer region *16S–23S*, several *ITS1* based tests were developed as an alternative method to the identification of mycobacteria (Mohamed et al., 2005; Gurtler et al., 2006).

Variation in *ITS* sequences (which only needs two primers for getting a clear sequence, compared to *16S rRNA* gene which is conserved and has a longer sequence) among Mycobacterium species, the direct sequencing of this genetic element has been shown to be useful in the identification of new aquatic mycobacteria species. PCR amplification can be used to differentiate slowly growing and rapidly growing species within this genus. In the present study, analysis by *ITS*-PCR and ITS restriction fragment length polymorphism (RFLP) was found to be a useful, rapid method compared to current molecular and phenotypic techniques and routinely amplified to its small size associated to the availability of highly conserved flanking sequences (Katoch et al., 2007).

The aim of this study was to identify, by applied culture techniques, the analysis of the gene sequence of PCR-RFLP and *ITS* of various aquatic species Mycobacterium in apparently healthy freshwater aquarium fish in India.

2. Materials and methods

2.1. Collection of samples (fish)

For this study, we have chosen six private aquarium shops, two shops in each city Allahabad, Bareilly and Lucknow (Uttar Pradesh, India) as sampling sites. The selection of fish species was based on the availability of species in all selected samples. A total of sixty ornamental fish without apparent behavioral changes and macroscopic lesions of eight species namely *Carassius auratus auratus*, *Carassius auratus*, *Helostoma temminckii*, *Colisa lolia*, *Danio rerio*, *Balantiocheilus melanopterus*, *Epalzeorhynchos frenatu* and *Trichogaster chuna* were informally collected from aquarium shops. From each aquarium, we collected a total of ten fish, three fish of *C. auratus auratus* and a fish of each species remaining.

2.2. Homogenization and decontamination of tissue

Live fish was euthanized with an overdose of tricain methanol (MS-222, Sigma-Aldrich, USA) and immediately examined. In the case of small fish (< 5 g of weight), whole fish was used, while in larger fish the samples of dermal and internal tissues were aseptically harvested, assembled and homogenized in sterile 0.85% NSS (Normal Saline Solution). About two gm of tissue samples in two ml of NSS were used to prepare homogeneous. Tissue samples were de-contaminated according to the Shitaye method (Shitaye et al., 2009).

2.3. Isolation of bacterial culture

The decontaminated homogenates (0.05 ml) were inoculated on the Lowenstein-Jensen (LJ) slants with supplement Gruft (Hi-media, India). Inoculated cultures were incubated at 30 °C and examined for two months. All morphologically distinct colonies were streaked to Middlebrook 7H11 agar (MDA, Hi-media, India) plates supplemented with 10% (*v*/v) oleic acid-albumin dextrose catalase (OADC) and 0.5% (v/v) glycerol to obtain pure cultures. The culture media were prepared according to the manufacturer's instructions.

2.4. Phenotypic bacterial identification

Healthy Orange or yellow colonies cultivated within three to sixty days were subjected to rapid Ziehl Neelsen (*Z*-N) staining Acid Fast coloration (Hi-media stains, India) and examined under the microscope. Characterization of the isolated bacilli acid was performed according to morphological characteristics, growth rate, pigmentation and growth at various temperatures (25 °C, 30 °C and 37 °C). The isolates were characterized by purification using the following biochemical characteristics: LJ growth with 5% NaCl, pigment production, nitrate reduction, Tween-80 catalase hydrolysis resistance, decreased tellurite, arylsulfatase activity, pyrazinamidase production and TCH resistance (Thiophene-2-hydroxyl acid hydrazide). All tests were performed at 30 °C and diagnostic clues (Roth et al., 2000) were used to assign an isolated species when applicable.

2.5. DNA isolation from mycobacterial isolates

Mycobacterial cells grown in Middlebrook 7H9 broth (MDB, Hi-media, India) containing ADC (albumin dextrose catalase) and 0.05% glycerol were pelleted to 5000 g for five minutes and used for DNA extraction. Purified DNA was extracted from mycobacterial isolates using the CTAB method using lysozyme and proteinase K (Sambrook et al., 1989). DNA quality was evaluated with 0.8% electrophoresis with agarose and ethidium bromide (EtBr).

2.6. Identification by molecular methods polymerase chain reaction (PCR)

PCR amplifications were always performed in a clean PCR room (amplified without DNA). Each 50 µl of PCR reaction mixture contained in 0.2 ml flat cap PCR tubes (Axygen Scientific, USA) 25 µl of Master Mix 2× PCR (Thermo Scientific, USA), ultra-pure (Millique) water, 1 µM of each primer and 1 µl of extracted bacterial DNA. The amplification was performed in an automated thermocycler (Bio-Rad Laboratory, USA). Each PCR analysis contained a negative control (1 µl of TE buffer or 1 µl of ultra-pure water instead of template DNA) and a positive control (1 µl of amplifiable DNA known in place of the DNA template). 5 µl of each PCR product was analyzed by gel electrophoresis with agarose.

2.7. Study of 16S–23S spacer region

The spacer region between *16S rRNA* and *23S rRNA* genes in mycobacteria was amplified using the forward primer Sp1 (5′-ACC TCC