Chapter 5: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Sequencing of Heat shock protein 65 kD (hsp65) gene for Identification of Aquatic Mycobacterium spp.
5.1 Introduction

Mycobacteria are ubiquitous bacteria in the environment, particularly in aquatic reservoirs (Ortega et al., 2013). Mycobacteria are common fish pathogens and the diseases rank among the most prevalent chronic diseases in tropical and sub-tropical freshwater aquarium fish (Antonio et al., 2000; Gauthier and Rhodes, 2009). Mycobacteria are a heterogeneous group of bacteria in terms of their genotypic features and disease association (Nasr-Esfahani et al., 2006).

Generally, fish mycobacteriosis is considered a common systemic disease (Gauthier and Rhodes, 2009) in which the primary pathological lesion involves classic granulomatous inflammation in different organs or tissues (Roberts, 2012). Mycobacteriosis in different aquarium fish of Carassius sp.; Danio sp.; Colisa sp. and Trichogaster species were reported in previous studies (Marzouk et al., 2009, Peterson et al., 2013, Novotny et al., 2010) however there are only a few previous studies related to the incidence of mycobacteria in clinically healthy fish populations (Mrlik et al., 2012; Beran et al., 2006). The economically significant consequences of these infections include mortality, morbidity and effects of subclinical infection such as decreased feed efficiency, decreased growth rates and decreased marketability (Zanoni et al., 2008). The zoonotic nature of the organism, the massive economic losses which have occurred to the aquaculture industry due to this disease and the lack of effective treatment highlight the need for mycobacteria to be rapidly detected and identified to species level (Dai et al., 2011; Pourahmad et al., 2009).

Conventionally, mycobacterial species are identified by their growth characteristics (such as growth rate, colonial morphology, pigmentation and photo reactivity) and bio-chemical reactions. In addition, the procedures of these tests are time consuming, complicated and
sometimes even then fail to provide a precise identification (Ong et al., 2010). Therefore, several molecular based techniques have been developed for the rapid detection and identification of mycobacteria species. To date, methods such as the AccuProbe system, INNO-LiPA or Genotype *Mycobacterium* assays are commercially available. Even with desirable sensitivity and specificity, these assays are limited to a small number of mycobacteria. Molecular methods such as PCR-restriction enzyme analysis and sequence analysis offer a significant alternative for rapid selection and identification of these bacteria.

Restriction enzyme analysis of the amplified nucleic acids is relatively cheap, easy to perform and provides a rapid means of detecting interspecies polymorphism in different mycobacterial species (Telenti et al., 1993). Molecular methods linked to Polymerase Chain Reaction (PCR) - Restriction Enzyme Analysis and sequence analyses are more reliable and faster for identification of NTM (Non-Tuberculous Mycobacteria) (Pourahmad et al., 2009). In this regard, PCR of the 441 bp of heat shock protein 65kD gene (*hsp65*), followed by restriction fragment pattern analysis by *Bst*EII and *Hae*III enzymes proposed by Telenti et al., (1993), has provided a rapid method for identification of *Mycobacterium* and closely related species (Harmsen et al., 2003). The *hsp65* gene is present in all *Mycobacterium* species and can be used for identification of NTM to species level because of its interspecies variability compare to some other conserved genes such as *16S rRNA* (Harmsen et al., 2003). Therefore, it is a suitable target for identification of NTM to species level than other methods (Hafner et al., 2004).

The PCR-RFLP technique is relatively inexpensive and does not need specialized equipment; whereas, sequencing is more discriminative but requires highly sophisticated equipment. Unlike the extensive use of these methods in clinical medicine, there are limited
reports of using these techniques for detection and identification of fish mycobacteria. The aims of this chapter was to evaluate the potential use of PCR-RFLP and sequence analyses based on the 441 bp fragment of hsp65 gene in identification and differentiation of aquatic mycobacteria isolated from freshwater fish and pond water during this study.

5.2 Materials and Methods

Materials and methods for this chapter have been described in Chapter 2 (see Sections see Sections 2.2.4.; 2.2.5; 2.2.5.2; 2.2.5.4; 2.2.6; 2.2.7; 2.2.8).

5.3 Results

Mycobacterium spp. were isolated from 15 out of 60 fish examined, representing all eight species of fish samples. Three additional Mycobacterium strains related with two different spp. were isolated from three different pond water samples. In this chapter the molecular identification of all the isolates was performed by amplification, RFLP and sequencing of a 441 bp fragment from a highly conserved region of the mycobacterial hsp65 gene.

5.3.1 Restriction enzyme analysis of the amplified products

The results obtained from the RFLP analysis of eighteen mycobacterial isolates are concised in Table. 5.1. For easy elucidation of the RFLP profiles produced by each isolate, a 50 bp size ladder was used as a reference. HaeIII and BstEII digestion of the 441 bp hsp65 PCR amplicon produced fragments ranging from 50 to 325 bp. Restriction fragments smaller than 50 bp were omitted in order to reduce confusion with primer-dimer bands, and restriction fragment band sizes were rounded to the nearest 5 bp, as recommended by Telenti et al., (1993). Some species, such as M. gordonae are known to have several subtypes and in this study, subtypes of
M. gordonae generated distinctive restriction profiles (Fig. 5.2 and Fig. 5.3 lanes 2, 5, 9). Consequently, the results clearly indicated that this method can differentiate mycobacteria at the species and even the subspecies level. All M. fortuitum and M. conceptionense (Fig. 5.3 lanes 6, 7, 8, 15) displayed similar patterns on BstEII digestion (240, 120, 85); however, these species could be discriminated from the HaeIII digestion. The strains of M. fortuitum were indistinguishable from M. senegalense strains due to the same digestion pattern for HaeIII and BstEII similar pattern (Fig. 5.2 and Fig. 5.3 lanes 3, 6, 7, 15).

5.3.2 Sequence analysis of hsp65

All rapidly and slowly growing mycobacteria had best matches with 99% to 100% similarity with their corresponding species in the databanks. The phylogenetic relationships among these eighteen isolates representing six species of Mycobacterium are presented in Fig. 5.4. All species screened were easily discriminated from each other. M. fortuitum, M. senegalense and M. conceptionense the three species with the highest degree of similarity for the RFLP pattern of hsp65 gene had readily differentiated from sequences analysis. The advantage of sequence analysis over RFLP was most obvious for the species having same digestion pattern and species with new RFLP patterns which could only be specified via sequencing (Table. 5.1).

5.3.3 Phylogenetic results of hsp65

The sequences of all the isolates found in this study were compared with those of several closely related isolates available in the National Centre for Biotechnology Information’s GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The best matches of our sequences with their corresponding species in the databanks studies by Basic Local Alignment Search Toll
(nBLAST) and obtained hsp65 gene sequences were downloaded and constructed phylogenetic tree.

The phylogenetic analysis of hsp65 gene was performed to characterize the phylogenetic relationships of these mycobacteria in a constructed phylogenetic tree (Fig. 5.4). The sequences of hsp65 gene of Mycobacterium isolates (MF1, MF2, MF3, MF4, MF5, MF6, MF7, MF8, MF9, MF10, MF11, MF12, MF13, MF14, MF15, MF16, MF17 and MF18) were highly matched with the most closely related species in GenBank database.

When their sequences were compared against databanks, they were closely related to M. abscessus (99% similarity), M. gordonae (99% similarity), M. senegalense (100% similarity), M. parascrofulaceum (99% similarity), M. fortuitum (99% similarity), M. conceptionense (100% similarity), M. aubagnense (99% similarity) and M. arupense (100% similarity). After RFLP and sequencing of hsp65; fifteen isolates from fish were confirmed as: five isolates of M. abscessus, three as M. gordonae, two as M. fortuitum, two as M. conceptionense, two as M. parascrofulaceum and one isolate as M. senegalense while the three isolates from pond water samples were identified as two strains of M. arupense and one of M. aubagnense.
Fig. 5.1: The PCR products obtained from amplification of hsp65 gene of isolated aquatic *Mycobacterium* spp.

M = molecular size marker 50bp Ladder

Lane 1) [MabCaLu]  Lane 7) [MfHtDl]  Lane 13) [MabCaaGp]
Lane 2) [MgHtLu]  Lane 8) [McHtGy]  Lane 14) [MpfEfDd]
Lane 3) [MsCaaBmp]  Lane 9) [MgCaaGau]  Lane 15) [McTcMU]
Lane 4) [MpaClBly]  Lane 10) [MabCaaStm]  Lane 16) [MarPwBhm]
Lane 5) [MgCaAl]  Lane 11) [MbBmKnp]  Lane 17) [MarPwBr]
Lane 6) [MfHtDl]  Lane 12) [MabBmStm]  Lane 18) [MauPwAg]
Fig. 5.2: RFLP pattern of hsp65 PCR products using HaeIII restriction enzyme.

M = molecular size marker 50bp Ladder

Lane 1) [MabCaLu]    Lane 7) [MfHtDl]       Lane 13) [MabCaaGp]
Lane 2) [MgHtLu]     Lane 8) [McHtGy]       Lane 14) [MpfEfDd]
Lane 3) [MsCaaBmp]   Lane 9) [MgCaaGau]     Lane 15) [McTcMU]
Lane 4) [MpaClBly]   Lane10) [MabCaaStm]    Lane 16) [MarPwBhm]
Lane 5) [MgCaAl]    Lane 11) [MbBmKnp]      Lane 17) [MarPwBr]
Lane 6) [MfHtDl]    Lane 12) [MabBmStm]    Lane 18) [MauPwAg]
Fig. 5.3: RFLP pattern of hsp65 PCR products using BstEII restriction enzyme.

M = molecular size marker 50bp Ladder

Lane 1) [MabCaLu] Lane 7) [MfHtDl] Lane 13) [MabCaaGp]
Lane 2) [MgHtLu] Lane 8) [McHtGy] Lane 14) [MpfEfDd]
Lane 3) [MsCaaBmp] Lane 9) [MgCaaGau] Lane 15) [McTcMU]
Lane 4) [MpaClBly] Lane 10) [MabCaaStm] Lane 16) [MarPwBhm]
Lane 5) [MgCaAl] Lane 11) [MbBmKnp] Lane 17) [MarPwBr]
Lane 6) [MfHtDl] Lane 12) [MabBmStm] Lane 18) [MauPwAg]
### Table 5.1 Mycobacterium species identified by sequencing of the *hsp65* PCR products and patterns obtained by RFLP of *hsp65* gene by *Hae*III and *Bst*EII restriction enzymes.

<table>
<thead>
<tr>
<th>Isolate Code</th>
<th>Source</th>
<th>Sequencing Result (% of best match)</th>
<th>RFLP pattern (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF1 [MabCaLu]</td>
<td><em>Carassius auratus</em></td>
<td><em>M. abscessus</em> (99)</td>
<td>200, 60, 55, 235, 205</td>
</tr>
<tr>
<td>MF2 [MgHtLu]</td>
<td><em>Helostoma temminckii</em></td>
<td><em>M. gordonae</em> (99)</td>
<td>130, 110, 240, 210</td>
</tr>
<tr>
<td>MF3 [MsCaaBmp]</td>
<td><em>Carassius auratus auratus</em></td>
<td><em>M. senegalense</em> (100)</td>
<td>145, 120, 50, 240, 120, 105</td>
</tr>
<tr>
<td>MF4 [MauPwAg]</td>
<td>Pond water</td>
<td><em>M. aubagnense</em> (99)</td>
<td>150, 80, 65, 200, 140, 70</td>
</tr>
<tr>
<td>MF5 [MpaCIBly]</td>
<td><em>Colisa lolia</em></td>
<td><em>M. parascrofulaceum</em> (99)</td>
<td>130, 95, 80, 235, 210</td>
</tr>
<tr>
<td>MF6 [MarPwBhm]</td>
<td>Pond water</td>
<td><em>M. arupense</em> (100)</td>
<td>145, 130, 330, 115</td>
</tr>
<tr>
<td>MF7 [MgCaAl]</td>
<td><em>Carassius auratus</em></td>
<td><em>M. gordonae</em> (99)</td>
<td>200, 60, 325, 130</td>
</tr>
<tr>
<td>MF8 [MfHtDi]</td>
<td><em>Helostoma temminckii</em></td>
<td><em>M. fortuitum</em> (99)</td>
<td>145, 120, 50, 240, 120, 105</td>
</tr>
<tr>
<td>MF9 [MfDrLkm]</td>
<td><em>Danio rerio</em></td>
<td><em>M. fortuitum</em> (99)</td>
<td>145, 120, 50, 240, 120, 105</td>
</tr>
<tr>
<td>MF10 [McHiGy]</td>
<td><em>Helostoma temminckii</em></td>
<td><em>M. conceptionense</em> (100)</td>
<td>140, 125, 60, 55, 240, 120, 105</td>
</tr>
<tr>
<td>MF11 [MgCaaGau]</td>
<td><em>Carassius auratus auratus</em></td>
<td><em>M. gordonae</em> (99)</td>
<td>200, 60, 325, 130</td>
</tr>
<tr>
<td>MF12 [MabCaaStm]</td>
<td><em>Carassius auratus auratus</em></td>
<td><em>M. abscessus</em> (99)</td>
<td>200, 60, 55, 235, 205</td>
</tr>
<tr>
<td>MF13 [MbBmKnp]</td>
<td><em>Balantiocheilus melanopterus</em></td>
<td><em>M. abscessus</em> (99)</td>
<td>200, 60, 55, 235, 205</td>
</tr>
<tr>
<td>MF14 [MabBmStm]</td>
<td><em>Balantiocheilus melanopterus</em></td>
<td><em>M. abscessus</em> (99)</td>
<td>200, 60, 55, 235, 205</td>
</tr>
<tr>
<td>MF15 [MabCaaGp]</td>
<td><em>Carassius auratus auratus</em></td>
<td><em>M. abscessus</em> (99)</td>
<td>200, 60, 55, 235, 205</td>
</tr>
<tr>
<td>MF16 [MpfEfDd]</td>
<td><em>Epalzeorhynchos frenatus</em></td>
<td><em>M. parascrofulaceum</em> (97)</td>
<td>145, 140, 75, 235, 205</td>
</tr>
<tr>
<td>MF17 [McTcMU]</td>
<td><em>Trichogaster chuna</em></td>
<td><em>M. conceptionense</em> (100)</td>
<td>140, 125, 60, 55, 240, 120, 105</td>
</tr>
<tr>
<td>MF18 [MarPwBr]</td>
<td>Pond water</td>
<td><em>M. arupense</em> (100)</td>
<td>145, 130, 330, 115</td>
</tr>
</tbody>
</table>
Chapter 5: PCR-RFLP and Sequencing of hsp65 gene for identification of aquatic mycobacteria

5.4 Discussion

Cultures of ornamental fish are usually exposed to several potential diseases causing organisms such as *Mycobacterium* spp. Conventional detection / identification of fish mycobacteria are based on culture and biochemical characteristics. However, these methods have their own limitations. All the isolates in the present study could not be identified by biochemical tests. It is essential to do the molecular identification of mycobacteria for routine speciation of these organisms. At present, methods such as the AccuProbe system, INNO-LiPA, high performance liquid chromatography (HPLC), restriction fragment length polymorphism (RFLP) using various target regions are available (Nasr-Esfahani et al., 2012, Kim et al., 2005). Among these different methods, PCR-restriction fragment length polymorphism is preferred since it offers an easy, rapid and inexpensive means of identifying mycobacteria species (Kim et al., 2005). Furthermore, this PCR-RFLP technique has been applied to several genes, for example *16S rRNA* (Turenne et al., 2001) *hsp65* gene (hsp65), *ITS* and *rpoB* (Koning et al., 2005, Nasr-Esfahani et al., 2006, Harmsen et al., 2003). Of these, a relatively simple method providing promising results is PCR-RFLP of *hsp65* gene. Despite being widely used for differentiation of mammalian mycobacteria, this method has only been used in the identification of a small number of aquatic mycobacteria (Pourahmad et al., 2009). PCR-RFLP schemes targeting *hsp65* have been most widely used, since this molecule is conserved in all mycobacteria and related strains, and because it shows sufficient sequence variation to allow mycobacteria differentiation at the species or strain level (Devallois et al., 1997; Telenti et al., 1993).
Mycobacterial isolates were subjected to PCR-restriction fragment length polymorphism of the *hsp65* gene. All the isolates had been correctly identified using the PCR-RFLP and sequencing method. Both isolates of *M. fortuitum* included in the study had patterns utmost like to *M. conceptionense* when matched to patterns obtained by agarose gel. PCR-RFLP patterns with a more accurate size were obtained using a 10% polyacrylamide gel (Brunello et al., 2001). However, in practice polyacrylamide gels are more difficult to use than agarose gels (Devallois et al., 1997). The use of PCR-RFLP for subtyping mycobacteria may improve our epidemiological understanding of these organisms in the aquatic environment (Pourahmad et al., 2009). However, because of the number of fragment patterns not yet reported, this technique could not be used solely as a tool for identification of rare or novel species of mycobacteria and (Pourahmad et al., 2009). In this study, we reported two subtypes of *M. gordonae* species: *M. gordonae* type IV (pattern of 200, 60 for *Hae*III digestion and 325, 130 for *Bst*EII digestion) and *M. gordonae* V (pattern of 130,110 for *Hae*III digestion and 240, 210 for *Bst*EII digestion).

Nevertheless, intraspecies polymorphism in the 441 bp target region observed in individual *M. parascrofulaceum* species, such as *M. parascrofulaceum*, was shown to produce different restriction patterns (for *Hae*III digestion) for distinct isolates of the same species, thereby increasing the uncertainty of the method (Wilson et al., 2001). Previous studies showed that differences between calculated sizes of restriction fragments and published patterns because of running conditions, type of agarose used and the computer programme used, as well as confusion in interpretation of patterns due to lack of standardization are major drawbacks of PCR-RFLP (Devallois et al., 1997; Hafner et al., 2004).
Application of at least two restriction endonuclease enzymes is required in PCR-RFLP methods used for the identification of mycobacteria to species level. In this regard, *Hae*III in combination with *Bst*EII has been traditionally employed in the PCR-RFLP of 441 bp hsp65 gene. However, in this study the use of the *Bst*EII enzyme resulted in the production of identical fragment patterns for different species of mycobacteria, e.g. *M. abscessus* and *M. parascrofulaceum*. Thus, replacement of this restriction enzyme by a more discriminative restriction enzyme seems necessary.

PCR followed by restriction fragment length polymorphism (RFLP) and sequence analysis have been developed in response to the need for complementary species identification (Pourahmad et al., 2013). The patterns in Table 5.1 obtained by PCR-RFLP were confirmed by sequence analysis. The sequencing results were valuable for the accurate identification of *M. fortuitum* and *M. conceptionense*. Both isolate of *M. fortuitum* having an identical hsp65 sequence were determined by sequencing.

The isolate MbBmKnp which was identified as *M. bolletii* by 16S rRNA sequencing, was confirmed as *M. abscessus* after RFLP and sequencing of hsp65. It is proposed that *M. bolletii* should be considered as a sub species of *M. abscessus* (Leao et al., 2011). Similarly, the isolate MpfEfDd which was identified as *M. paraffinicum* by 16S rRNA sequencing, was confirmed as *M. parascrofulaceum* after RFLP and sequencing of hsp65. hsp65 analysis provided a resolving power higher than 16S rRNA gene analysis for differentiation among species (Kim et al., 2005b). Furthermore, separation between closely related species such as between *M. abscessus*, *M. bolletii* and *M. chelona* is poorly achieved by 16S rRNA gene analysis due to the small amount of sequence variation (Kirschner et al., 1993), but these species can be clearly separated by
hsp65 analysis (Kim et al., 2005b). Secondly, in the multiple alignments of hsp65 sequences of fifteen mycobacterial isolates, no gaps or additions were found. This means that all the sequence information can be deliberated for phylogenetic analysis without deletion of gap sequences. In fact, when sequencing of 441 bp hsp65 fragments have done to fifteen aquatic isolates, no ambiguous results were observed. Sequence analysis of the hsp65 gene has proven a useful method to integrate into species identification to specify both slowly and rapidly-growing species of aquatic mycobacteria. The constructed phylogenetic tree (Fig. 5.4) was useful to show the relatedness of the isolates to a known species or group of mycobacteria. Furthermore, when an isolate could not be matched as a certain species, the phylogenetic tree showed the relatedness of that isolate to a known species or group of mycobacteria.

*M. fortuitum, M. abscessus* and *M. gordonae* isolated from fish in this study are well known pathogens for fish and cause fish tuberculosis (Beran et al., 2006) and these were also classified as PPM which can cause infections to human (Elko et al., 2004). *M. conceptionense* isolated from two fishes in present study is one of the most common cause of fish mycobacteriosis (Pourahmad et al., 2009) and may cause various pulmonary, skin, or soft-tissue infections in humans (Adekambi et al., 2006). *M. parascrofulaceum* is a known pathogen for fish and also related with pulmonary tuberculosis in human (Tortoli et al., 2005). *M. senegalense* isolated during this study was previously reported to cause tissue infection in a child after fish tank exposure (Talavlikar et al., 2011). *Mycobacterium* strain MF6 and MF18 were 100% match with *M. arupense*, a new species of mycobacteria that has recently been described (Cloud et al., 2006). More recently, Masaki et al. (2006) have reported two human isolates of this species, in Japan.
In conclusion, PCR-RFLP of *hsp65* is simple, rapid and inexpensive method for the identification of mycobacteria and useful alternative for phenotypic identification in diagnostic laboratories. In addition, sequence analysis of the *hsp65* gene has proven useful as a method to species identification to indicate both slowly- and rapidly-growing species of aquatic mycobacteria. As sequencing facilities are becoming more accessible, sequencing of this 441 bp fragment may increasingly be used with restriction enzyme analyses as a means of molecular identification of *Mycobacterium* species.
Fig. 5.4: Phylogenetic tree of the hsp65 gene of isolated mycobacteria prepared by using the neighbour-joining method and Kimura’s two-parameter distance correction model. The support of each branch, as determined from 1000 bootstrap samples, is indicated by the value at each node (as a percentage). The scale bar indicates a 0.5 % difference in nucleotide sequences.
Chapter 5: PCR-RFLP and Sequencing of hsp65 gene for identification of aquatic mycobacteria

References


