EFFICACY AND COST OF MOLECULAR IDENTIFICATION OF CLINICAL MYCOBACTERIAL ISOLATES IN A RESOURCE LIMITED SETTING

Ratnatunga CN[1], Wickramasingha S[2], Thevanesam V[1], K. G .R. Athula Kumara[1]

[1] Department of Microbiology, Faculty of Medicine, University of Peradeniya, Sri Lanka
[2] Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka

ABSTRACT

Introduction: Many molecular methods of identification of mycobacteria are now available. With many molecular consumables now being available at low prices, routine identification in clinical laboratories is now possible, even in low/ middle income settings that have basic molecular facilities. This study was conducted to optimize a low cost PCR-RFLP based identification that can be used in such a laboratory.

Methodology: DNA was extracted from mycobacterial cultures using five methods. Three were heat extraction and two were kit extraction methods. Yield and purity of extracted DNA was evaluated and PCR-RFLP was done on extracts to ensure that the DNA could be used for molecular assays. The method giving the highest yield at low cost was selected and DNA was extracted from 105 mycobacterial cultures from patients diagnosed with pulmonary tuberculosis. *hsp65* PCR and restriction digestion with BstEII and HaeIII enzymes was done to identify mycobacteria, differentiate MTB complex from NTM and identify NTM species. *gyrB*PCR and restriction digestion with RsaI was done to identify MTB complex species. *hsp65*partial sequencing was done to confirm NTM species. Costs for molecular identification were calculated based on consumable cost.

Results: Heat extraction in water (80 ^{0}C for 1 hour) provided a mean DNA yield of 30.07ng/µland mean A260/280 ratio of 1.45. Heat extraction methods gave significantly higher DNA yield compared to the kit extraction methods (ANOVA, $p<0.05$). *hsp65* PCR-RFLP identified 102 isolates as MTB complex and 3 isolates as NTM. *gyrB*PCR-RFLP confirmed the 102 isolates as MTBC and showed that all isolates belonged to the MTB/ *M africanum/ M canettii*group. *Hsp65* partial sequencing identified the NTM as 2 isolates of *M avium*and 1 isolate that could not be identified. An algorithm for PCR-RFLP based identification was developed that allows identification of mycobacterial isolates at low cost (approximately USD 6.00 per sample). The NTM rate in this study population was 2.6%.

Conclusions: Using heat extraction in water, PCR-RFLP based identification of clinical mycobacterial isolates can be established at low cost in a laboratory that has basic molecular facilities.

Key words: Mycobacteria Identification; PCR-RFLP; *hsp65; gyrB*

INTRODUCTION

The methods used for identification of mycobacterial isolates depend on primarily on available laboratory facilities. Several different molecular methods are currently available and these have replaced the more cumbersome and time consuming conventional biochemical tests to a great extent.[1] Both *hsp65* and *gyrB*genePCR restriction digestion are robust methods for identification of Non Tuberculous Mycobacteria (NTM) and *Mycobacterium tuberculosis* complex (MTBC) respectively.[2,3] *Hsp65* restriction digestion with *Bst*EII and *Hae*III enzymes has been used extensively for NTM identification in multiple fields of study, while *gyrB* digestion with *Rsa*I and *Taq*Ihas been successfully used for *M. tuberculosis* (MTB) /*M.africanum* differentiation from *M.bovis/* BCG.[4-13]

Correspondence:
Dr. Champa Neelakanthi Ratnatunga
Department of Microbiology, Faculty of Medicine
University of Peradeniya, Sri Lanka
Email: champa26@gmail.com

In Sri Lanka, NTM were cultured in approximately 2-3% of all mycobacterial cultures done at the National Tuberculosis Reference Laboratory between 2005 and 2007[14,15] while a recent analysis of bronchoscopy cultures from patients in Kandy by Weeresekera et al. showed that -13-14 % were positive for NTM isolates, including *M.phocaicum*and *M. Smegmatis.*[16] Data on the true (overall) NTM infection rate among patients with pulmonary and extra pulmonary disease in Sri Lanka is not available as cultures are not routinely performed in suspected pulmonary tuberculosis. Even when cultured isolates are available, identification is not routinely done in most local laboratories.

This study was designed to optimize selected molecular assays for basic identification of mycobacteria isolated from clinical samples. Development of identification algorithms that would be suitable for use in a clinical laboratory in the local setting and would provide the maximum species level differentiation in a minimum time and at low cost was targeted. Though many PCR targets have been evaluated for identification of mycobacteria, *hsp65* and *gyrB* were selected for this study as they have been successfully used in other studies and as *hsp65* sequencing provides accurate identification of many NTM species with online sequence databases and identification resources are available.

METHODOLOGY

Sputum samples from patients with suspected pulmonary tuberculosis were cultured on Lowenstein Jensen egg based media for 8-10 weeks. Positive cultures were then stained for acid fastness and molecular identification carried out according to the following protocol.

DNA extraction

DNA was extracted from a standard MTB H37Rv strain isolate and four clinical isolates (n=5) using five different extraction methods (A to E) of which three were heat extraction methods and two were kit extraction methods. The heat extraction methods were developed based on existing literature [17-19] and required a minimum amount of consumables, equipment and time. Kit extractions were done using the Invitrogen Pure Link R Spin Column DNA extraction kit. The first protocol followed was given by the manufacturer for DNA extraction from Gram positive organisms and the second protocol was a general extraction protocol.

Method A: Heat extraction (80 ^{0}C –heat inactivation)

Several colonies were suspended in sterile distilled water by brief vortexing and heated in a water bath to 80 ^{0}C for 1 hour. After centrifugation of the inactivated mycobacterial suspension, 400 µl of the supernatant was transferred into a new micro-centrifuge tube. (Extract A)

The cell pellet was then re-suspended in sterile distilled water by brief vortexing.5 drops of suspension were then cultured on Lowenstien Jensen (LJ) slopes to assess inactivation of cultures.500 µl each of suspension was then transferred into new microcentrifuge tubes (labelled B to E)for the next 4 extraction methods. For methods C, D and E the suspension was centrifuged again and the resulting cell pellet was used.

Method B: Heat extraction (95 ^{0}C)

B tubes were heated on a heat block at 95 ^{0}C for 30 mins. The tubes were then centrifuged at 5000 g for 10 mins and the resulting supernatant was transferred to new tubes. (Extract B)

Method C: Tris EDTA (TE) extraction

The cell pellet in C tubes was re-suspended in 500 µl of 1x TE (Tris EDTA) buffer with brief vortexing. The suspension was heated on a heat block at 95 ^{0}C for 30 mins. The tubes were then centrifuged at 5000 g for 10 mins and the resulting supernatant transferred to new tubes. (Extract C)

Method D: kit extraction – method for Gram positive organisms

DNA was extracted from the cell suspension in D tubes according to the protocol for Gram positive organisms provided by the manufacturer. (Extract D)

Method E: kit extraction – general protocol for non-specified samples

DNA was extracted from the cell suspension in E tubes according to the general protocol provided by the manufacturer. (Extract E)

Preparation of cell lysate: The cell pellet was suspended in 180 µlPureLinkR Genomic Digestion Buffer and 20 µlProtinase K and incubated at 55 °C for 1 hour. 20 µlRNase A (supplied with the kit) was added and incubated at room temperature for 2 mins. The lysate was centrifuged at 13,000g for 5 mins at room temperature to remove any particulate material. The supernatant was transferred to a fresh micro centrifuge tube and 200µLPureLinkR Genomic Binding Buffer supplied with the kit was added to the lysate. This was mixed well by vortexing to yield a homogenous solution. 200 µL 96–100% ethanol was added to the lysate and mixed well by vortexing for 5 seconds to yield a homogenous solution.The other steps for binding; washing and eluting DNA are as specified by the manufacturer.

The DNA content and purity of yield was measured using an automated DNA quantifier. 2µl of each extract was measured with respect to DNA content and Absorbance at 260 and 280nm using the NanoDrop 2000c/2000 UV-vis Spectrophotometer (Thermo Scientific). Mean DNA yield for each extraction method and mean A260/280 ratio were compared. Statistical analysis was performed with Minitab 14 statistical software.

PCR and restriction digestion –*hsp65*and*gyrB*PCR-RFLP

*gyrB*PCR was then performed on all extracts to ensure that extracted DNA could be successfully amplified. Based on the results obtained from above tests, method 'A' was selected for DNA extraction, and extraction was performed on 105 clinical isolates obtained from sputum of patients with pulmonary tuberculosis.

hsp65 PCR was done using the following protocol. GoTaqFlexi DNA polymerase (Promega. USA) (5U/µl) -0.25 µl; primers 10pmol conc. (Tb11: 5'-ACCAACGATGGTGTGTCCAT-3` Tb12: 5' - CTTGTCGAACCGCATACCCT- 3`)- 1.5 µl each; 2.5 mMdNTP -2 µl; 25 mmol $MgCl_2$- 3 µl; template DNA - 5 µl; total reaction volume 25 µl.The 441bp product was visualized on 2% agarose gel. Restriction digestion was done with *Bst*EII (Promega. USA. Ref. R6641) and *Hae*III (Promega. USA. Ref. R6171) enzymes according to established protocols[1]. Digested fragments were visualized on 4% agarose gel with 25bp DNA ladder. RFLP-gel images were analyzed using GelAnalyzer 2010 (Lazar software. Available from http://www.gelanalyzer.com/).*Hsp65 R*estriction digestion patterns were then analyzed using the PRASITE database (available at http://app.chuv.ch/prasite/index.html).

gyrB PCR was performed on all isolates using established protocols[1].Primers Mtubf-5`-TCGGACGCGTATGCGATATC-3`Mtubr-5`-ACATACAGTTCGGACTTGCG-3`. PCR product (1020bp) was visualized on 1% agarose gel with 100bp DNA ladder. *GyrB*positive isolates were confirmed as MTBC and negative isolates were confirmed as NTM. Restriction digestion was done with*Rsa*I (Promega. USA. Ref. R6371) restriction enzyme and digested fragments were visualized on 2% agarose gel with 50bp DNA ladder.

Identification of NTM isolates (*hsp65* PCR positive, *gyrB*PCR negative isolates) was then confirmed by partial sequencing of the *hsp65* product (Macrogen (Seoul, Korea)), manual checking and annotation using BioEdit software (version 7.2.5; Tom Hall, Carlsbad, CA [http:// www.mbio.ncsu.edu/bioedit/bioedit.html]) and aligned using ApE software (v2.0.47; M WayneDavis.[http://biologylabs.utah.edu/jorgensen/wayned/ape/]). The edited sequences were then aligned with sequences available in GenBank using a BLASTN (Basic Local Alignment Search Tool) search (available from http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi.)

Cost per sample was calculated based on the cost of consumables required for identification assuming that an optimal number of samples will be processed at a given time.. Equipment and labour costs were not included in the calculations.

RESULTS

DNA extraction

The minimum, maximum and mean yield of DNA from the five extraction methods used as well as the purity of extracts based on A260/280 ratio are shown in Table 1. Analysis of variance (ANOVA) showed a significant effect of extraction method on the DNA yield. (p=0.001), with an R^2(adjusted) value of 48.79% indicating that approximately 49% of the variability seen in the DNA yield was due to the effect of the extraction method. 2 way-

ANOVA including sample as a factor increased the R^2(adjusted)to 60%, though as a factor it did not have a significant effect on the yield (p=0.093). Extract A had a significantly higher yield than extract D and E (kit extraction). There is no difference in the yield between methods A, B and C.Amplification of a 1020 bp segment of the *gyrB* gene was successfully done from extracted DNA. Figure 1 shows *gyrB* PCR products after successful PCR amplification of DNA extracted by all five methods. All samples were *gyrB* positive and are therefore MTBC. LJ cultures from all 25 extracts gave no growth after 10 weeks incubation. Inactivation of organisms with initial heating to 80 ^{0}C was confirmed.

Table 1. Yield and purity of DNA extracted by 5 methods

Method	No. of sample	DNA yieldng/µl			Standard deviation	A 260/280 (Nano drop 200)
		Min	Max	Mean		
A (80^{0}C heat in water)	5	5.10	31.70	18.40*	10.220	1.540
B (95^{0}C heat in water)	5	2.40	10.60	6.34*	3.270	1.692
C (95^{0}C heat in TE buffer)	5	4.20	23.30	12.80*	8.090	1.880
D (kit-gram+ve protocol)	5	1.30	4.40	2.86	1.155	1.902
E (kit-general protocol)	5	0.50	3.30	1.86	1.234	0.988
*ANOVA – p<0.05						

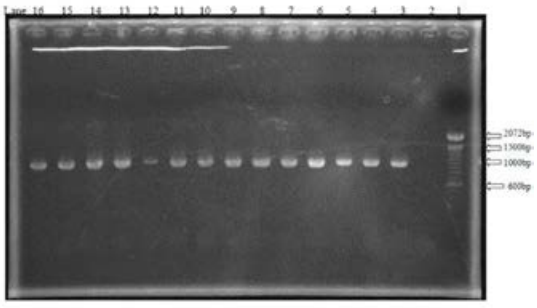
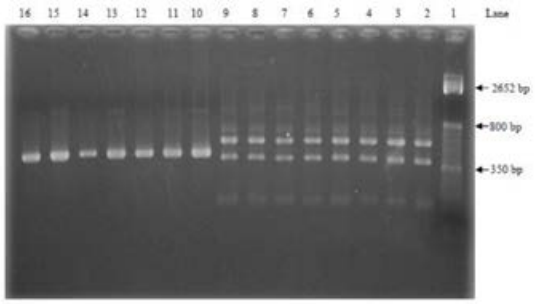


Figure 1. Gel electrophoresis of PCR products (1020 bp) of GyrB gene. Lane 1- 100 bp DNA ladder. Lane 2 - negative control. Lane 3- amplified product from standard TB strain H37Rv, which was used as positive control in all subsequent amplifications. Lanes 8-16 show products from amplification of clinical isolate extracts using methods A to E.

Hsp65 and*gyrB*PCR –RFLP

PCR amplification of the *hsp65* target 441 bp segment was successfully done. All 105 samples tested were positive, indicating that all samples were mycobacteria. (see figure 2.)



(***Figure 2.****GyrBRsaldigestion andhsp65 PCR. Lane 1 – 100 bp DNA ladder.Lane 2 to 9- gyrB- RsaI digestion with 3 bands (100, 385, 560 bp). Lane 10-16 hsp65PCR 441bpamplicon. Lane 10 - positive control –H37Rv. Lanes 11,12 and 13 - PCR products from the isolates that were gyrB negative. Lane 11- M aviumcomplex, lane 12- Mycobacteriumspp, lane 13-Mycobacterium aviumcomplex.)*

Restriction digestion of *hsp65* PCR product from 102 isolates showed the same digestion pattern as the standard H37Rv strain as seen in figure 3. These isolates were therefore identified as belonging to the MBTC. Mixed infection with NTM was unlikely. However the possibility of mixed infection with NTM that had the same RFLP pattern as MTBC organisms could not be excluded. Digestion pattern was different in PCR product from 3 isolates identifying them as NTM.

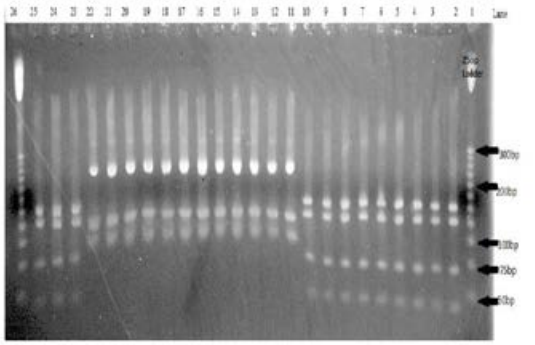


Figure 3. BstEII and HaeIII digestion patterns of hsp65 PCR product from MTBC isolates. Lane 1 and 26 - 25bp DNA ladder. Lane 2 to 10 – HaeIII digestion (4 fragments).Lane 11 to 22-BstEII digestion (3 fragments).Lane 23 to 25-HaeIII digestion.)

GyrB PCR was then performed on all extracts. Of the 105 sample extracts that were amplified, *gyrB* product was positive in 102 samples, confirming

that they were of the MTBC. PCR was negative in 3 samples which qA confirmed by repeat PCR on these extracts and these isolates were therefore identified as NTM. *Rsa*I restriction digestion patterns of the positive isolates are shown in figure 4. The samples showed three bands at 100bp, 380bp and 560bp which are seen in MTB, *M.africanum* and *M. canettii* isolates. These isolates were were therefore identified as belonging to one of these three species, with MTB being the most likely.

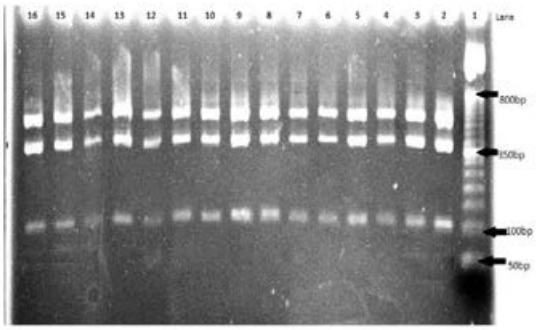


Figure 4. RsaI digestion pattern of GyrB PCR products. Lane 1- 100 bp DNA ladder. Lane 2- digested fragments from MTB H37Rv. Lane 3-16- digestion of products from clinical isolates.

Hsp65 PCR restriction analysis (PRA) patterns obtained from the 3 NTM isolates were entered into PRASITE database and tentatively identified. Two isolates were identified as *M. terrae* and one as *Mavium complex* (MAC) or *M.columbiense* (a member of the MAC described in 2006 [20]MAC-X. All of the seven novel isolates gave a positive result with the MAC-specific AccuProbe (Gen-Probe). However specific identification was not possible as there were a large number of possible species given in the output with very similar digestion patterns.

Based on partial sequences of *hsp65* product and BLASTN search results, these 3 isolates were subsequently identified as two isolates of *M avium* complex (misidentified as *M terrae* in PRA pattern) , and one isolate that could not be specifically identified as the closest matches had only 96% identity (identified as MAC in PRA). These sequences are available in the GENBANK data base. (Accession numbers: *M avium*– GenBank:KJ820768, *M avium* – GenBank:KJ820770, *Mycobacterium species*- GenBank:KJ820769).

In this study, the NTM infection rate in patients diagnosed as having PTB was 2.58%.

Cost

PCR –RFLP based identification of MTBC isolates could be done at approximately LKR 850.00 per sample for consumables (USD 6.00) if optimal numbers of samples are processed simultaneously.

Based on the above results the optimum identification algorithm for routine use in this clinical laboratory for identification of clinical isolates of mycobacterium is shown in figure 5. Using this algorithm would reduce costs further as *hsp65* amplification would only be done if *gyrB*PCR was shown to be negative.

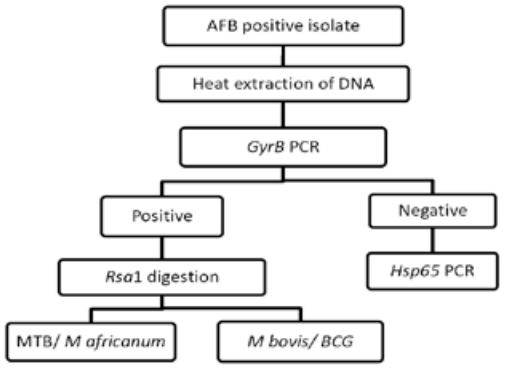


Figure 5. Optimized algorithm for molecular identification of clinical mycobacterial isolates

DISCUSSION

Molecular methods of identification have advantage not only in speed, accuracy and reproducibility, but also in safety as only DNA is required. Cultures can be inactivated safely prior to DNA extraction making molecular methods attractive even in low income settings where safety equipment, protocols and laboratory safety training is minimal. The obstacles for routine use are first, the cost of equipment and consumables, and secondly the availability of technical expertise required for molecular testing.

With many molecular consumables becoming available at lower prices and new economical methods being investigated, a re-evaluation of costs is necessary. In addition, many molecular methods used today are more robust than earlier methods. Conventional PCR and RFLP are easy to perform if optimized protocols are available. This study was designed to optimize an identification algorithm for clinical mycobacterial isolates that can be performed at minimal cost with minimal

expertise in a laboratory in a low-income setting. The research student and technical officer who carried out the work in this study had no previous experience with molecular techniques and learned all required methods and developed the extraction protocols within a short period of time.

GyrB gene PCR was selected as MTBC is the most common infecting organism and this can be detected with a single PCR with no need for additional restriction digestion. Further analysis with RFLP or *Hsp65* PCR can be done only if required. Other gene candidates for differentiation of mycobacterium species are also available including 16S-23S rDNA ISR[16] which were not assessed in this study. The results show that simple heat extraction at 80^0C for 1hour is the adequate as a method of DNA extraction from mycobacterial cultures. The yield is high and of good purity. The efficacy of inactivation of organisms at 80 ^{0}C was also compatible with published literature.[21] As heating for 20 mins has been shown to have variable results, with some studies showing complete inactivation and others showing some positive cultures[19] the safer option of 60 mins was used. Mycobacteria were inactivated by this procedure which rendered the suspensions safe for use. This extraction procedure requires minimum technical ability, equipment or other reagents and can easily be done in any laboratory with basic facilities. However the yield was relatively constant across all samples, whereas heat extraction gave varying results.

It is possible that the kit method will give a constant yield whatever the amount of DNA present in the original sample and therefore may be useful in low DNA samples. However for extraction of DNA from culture, this method shows no advantage. Better yield can probably be obtained using the spin column method by increasing incubation time with lysis digestion buffer and protienase K However a kit method would always be more expensive and require more equipment than the heat extraction method described. As the cell suspension used for method A extraction/ inactivation had a greater volume and higher number of organisms than the other methods B to E (for which equal volumes of the suspension were use) the yield shown here for this method is an overestimate when compared to the other methods. Repeat testing with equal numbers of organisms was not done and is one of the limitations of this study. To ensure that the extracts were all suitable for PCR-RFLP, both *GyrB* PCR and *Rsa*1 digestion were carried out on all 25 extracts. All 25 extracts gave positive PCR bands and digestion bands showing that even the water extracts were of adequate purity.

GyrB PCR has proved an excellent method of identifying MTBC organisms. It is a simple and easy to perform procedure and does not have the disadvantage of gene negative strains that are seen with the IS6110 PCR, which is one of the more widely used PCR methods for TB diagnosis. However, RFLP is necessary to differentiate species and even then it can only differentiate between 4 species of the complex (MTB, *M. bovis, M. africanum* and *M. microti*) when two restriction enzymes are used. It does not have the genotyping power of IS6110 RFLP. This method, therefore, is a practical method for routine use in a clinical laboratory where identification to group level is adequate. Use of a single enzyme that made the important differentiation between MTB and *M bovis/ BCG* was evaluated so costs would be minimal.

*Rsa*Idigestion was successfully performed and based on the band patterns, all 102 samples tested belong to the MTB, *M. africanum, M. canettii* group. The absence of *M. bovis/ BCG* and *M.microti* isolates is not surprising as these strains are rare causes of disease in Sri Lanka.[15] Of the 3 possible species, *M canettii* is unlikely as it is a rare cause of disease in humans. Between MTB and *M.africanum*, the most likely species is MTB.

Hsp65 PCR-RFLP was successfully performed and this accurately differentiated NTM from MTBC. If the digested band pattern is the same as that of the positive control, they are relatively easy to identify and estimate band sizes. However when differing band patterns are seen, which is the case with most NTM, then precise estimation of the band molecular weight is very difficult even with a 4% agarose gel. Using image analyzer software is a useful way of interpreting gel images and with free software available this is now possible at no extra cost.

The PRASITE database generates the closest matches to the given band pattern. However, accurate species level identification with this method

is difficult for two reasons. Firstly, many species differ from each other with only a few base pair differences in fragment length. As image analyzing software is not completely accurate and molecular weights of bands are approximate calculations, identification is an approximation at best. Also, as seen in reference texts, several species can share the same band pattern. Therefore, even if the band molecular weight were accurate, species level identification would not always be possible. However, with this method, the most likely group of organisms can be determined. A drawback of this method is that it compares the band sizes with that of known species. Therefore a new strain would not be identified, but rather each query is put into the closest fitting group. This problem was seen in this study where all three NTM isolates were misidentified using the PRA method.

Hsp65 sequencing is used for accurate species level identification of many bacterial species. Studies have shown that the level of sequence similarity between isolates in a given species is >98.2% with some species like MTB showing 100% similarity in all isolates.[12] Phylogenetic analysis using this sequence has shown great similarity to the phylogenetic tree generated from 16S rRNA analysis.[12] *Hsp65* sequencing has been shown to have a greater resolving ability than 16S rRNA sequencing as well. GenBank contains an extensive database of *hsp65* sequences from different mycobacteria that can be accessed for comparison. Based on the GenBank database and other sources, a web accessible data base of h*sp65* sequences for mycobacterium species identification has also been published.[13] The problems associated with PRA method can be avoided by sequencing of the *hsp65* product, which will give a more accurate species identification. Sequencing costs are approximately the same as PCR-RFLP making this a viable option for the few NTM isolates that are cultured in this population. The NTM isolation rate of 2.58% is compatible with the 3% rate previously described by Elvitigala et al in Sri Lanka (2008).

BLAST analysis showed that two of the isolates were *M.avium*, which is compatible with the figures seen worldwide. *M. avium* is the most common NTM isolated from pulmonary specimens.[22]

An added advantage of using molecular methods is that the DNA extracts can be stored for future extensive study if required. Cost calculations for the optimal algorithm show that molecular identification can be established in a routine laboratory at reasonable cost to the provider/ patient.

This study highlights the following. First, that NTM disease rates (pulmonary) are between 2-3% in this population. Second, that a molecular identification algorithm can be established in a routine clinical laboratory at low cost. This protocol requires only basic equipment such as a thermocycler, electrophoresis apparatus, heat block / water bath, centrifuge and gel documentation system. As there are only a few laboratories (both state and private sector) that provide mycobacterial culture facility, most of these would already be equipped to perform this protocol. Consumables costs etc would be reasonable enough that most patients would be able to afford the test, or if necessary, state sponsoring of identification tests would be feasible. Currently, identification is not done routinely in state laboratories that preform mycobacterial culture. If at all, basic biochemical tests are used for differentiation of MTBC and NTM isolates and though cheap, these tests are time consuming and are not available for the first few months of clinical management of patients. Isolated studies like those mentioned in the introduction provide a glimpse into the NTM disease burden in Sri Lanka, but robust, large scale studies have not been done due to lack of identification data. The importance of having such data and routine identification of isolates is highlighted in studies that show the changing landscape of mycobacterial infections in other countries.[23]including hsp65, rpoB, and 16S-23S rRNA internal transcribed spacer (ITS Utilizing this protocol as a routine in culture laboratories would go a long way to fill this gap. As there are only 3-4 culture laboratories in the TB control programme of Sri Lanka, this method can be used to identify most of the mycobacterial isolates from here.

As we progress towards better TB control measures, improvement of diagnostics and laboratory infrastructure is a must. These improvements need to be targeted towards improving services that are needed in the country, based on local needs, not on foreign models. Improvement does not need to involve large investments. Making maximum use of available resources and implementation of optimised methods that give maximum results is the best way forward.

Limitations

Limitations of this study include the lack of optimization of the kit extraction methods which could potentially yieldhigher amounts of DNA. This method is for use on cultured mycobacteria and not for clinical samples. A further step of optimizing this method for direct DNA extraction from clinical samples was not performed.

CONCLUSIONS

The NTM disease rate in patients diagnosed with pulmonary TB in this cohort was 2.6%. A simple heat extraction in water provides a good DNA yield of adequate purity from mycobacterial cultures that can be used for molecular studies. The simple algorithm developed using PCR-RFLP based identification provides a low cost method for identification of clinical mycobacterial isolates that provides useful information for patient management.

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