Polymerase chain reaction – restriction fragment length polymorphism analysis for the differentiation of mycobacterial species in bronchial washings

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(Index words: *M. tuberculosis*, non-tuberculosis bacteria, PCR)

Abstract

Objectives To identify mycobacterial species in bronchoscopy specimens with a simple assay based on polymerase chain reaction and restriction enzyme digestion.

Methods Sputum smear negative, bronchoscopy specimens (n=202) were collected from patients attending the Central Chest Clinic and the Teaching Hospital Kandy, Sri Lanka. DNA, extracted from the mycobacterial cultures (n=43) were amplified using known mycobacterial specific Sp1 and Sp2 primers. Resulting products were digested with *Hae*III and *Cfo*I restriction enzymes and DNA sequencing was performed for the selected isolates.

Results Among the culture positive patients, PCR was able to distinguish 12 rapid growers (~280-320 bp), 15 slow (~200-220 bp) and 10 patients having both rapid and slow and one having two rapid growing mycobacteria. DNA Sequence analysis revealed the presence of *M. intracellulare* (n=3), *M. phocaicum* (n=7), *M. tuberculosis* complex (n=13), Nocardia (n=2), *M. smegmatis* (n=1) and *Mycobacterium* sp (n=12). The identified organisms got digested upon exposure to *Hae*III restriction enzyme whereas when exposed to *Cfo*I, only *M. phocaicum* yielded 80 bp and 230 bp DNA fragments while others remained undigested. Consequently, six patients were confirmed to have *M. tuberculosis* complex, seven had both *M. tuberculosis* and non-tuberculosis bacteria (NTM) in their bronchoscopy specimens while 21 had NTM.

Conclusions Optimised PCR-RFLP assay was able to differentiate *M. tuberculosis* complex bacteria from non-tuberculosis mycobacteria and *Nocardia*. Molecular analysis confirmed the presence of NTM in bronchoscopy specimens and according to the study a significant proportion of patients (13% to 14%) of the study population were found to have NTM in their bronchial washings.

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Introduction

The World Health Organization (WHO) reports that, tuberculosis (TB) remains a major public health problem as a first-line infectious disease [1]. Effective care for *Mycobacterium tuberculosis* (MTB) infected individuals requires a proper diagnosis [2]. The rising incidence of human infection by nontuberculous mycobacteria (NTM) is also a serious public-health concern [3].

Important aspects of the control of tuberculosis are rapid and correct diagnosis of cases and the optimisation of anti-tuberculosis treatment [4]. The conventional diagnosis of TB is based on Ziehl-Neelsen acid-fast bacilli (AFB) stain and culture of MTB [5,6]. AFB staining is a rapid and cheap method, but lacks sensitivity. Also, many TB patients have negative AFB smears with a subsequent positive culture. The culture requires time and viable microorganisms, are difficult to obtain, especially in treated patients. Several rapid methods for MTB diagnosis, such as DNA probes, have been developed [7]. The polymerase chain reaction (PCR) is a method that can amplify a small fragment of DNA, and has high specificity for the diagnosis of infectious diseases [8-12]. It is widely used in the diagnosis of various bacterial infections including TB and NTM [12]. This innovation enables an early diagnosis, and treatment could start immediately after diagnosis [12]. Recently, bronchoalveolar lavage (BAL) was recommended for the diagnosis of various lung diseases, including pulmonary infections [13]. In this study, conventional polymerase chain reaction – restricted fragment length polymorphism (PCR-RFLP) technique was used as a rapid and direct molecular method for the detection of mycobacteria in clinical specimens. For this the 16S rDNA intergenic spacer region (ISR) was targeted. It is a potential tool for the identification of mycobacteria because 16S ISR is highly conserved among mycobacterial species and present in all mycobacteria.

According to the WHO, estimated incidence of TB in Sri Lanka for the year 2010 was 66-per 100,000 population. In 2010, a total of 9103 TB patients were registered by the National Programme for Tuberculosis

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Control and Chest Diseases (NPTCCD). Of this, 6817 had pulmonary disease [14]. A total of 671 were registered in the Kandy District (which has the fourth highest population density) for 2010 out of which 489 had TB [14]. Similar to countries in Africa, microscopic examinations. are routinely done for the diagnosis of TB in Sri Lanka, the involvement of acid-fast NTM in tuberculosis like syndromes might result in the misdiagnosis of TB [15].

Increasing recovery of NTM from environmental and clinical sources has prompted the development of laboratory methods to determine their taxonomic affiliation and identify them precisely [16]. Therefore this study proposes to optimise a simple assay based on PCR and restriction enzyme digestion (PCR-RFLP) for the identification of mycobacterial species in bronchoscopy specimens.

Methods

Sputum smear negative bronchoscopy specimens were collected from patients (n=202) attending the General Hospital, Kandy, Sri Lanka from April 2009 to January 2011 who had pulmonary symptoms, nodular or cavitatory opacities on chest radiograph, or an HRCT scan that showed multifocal bronchiectasis with multiple small nodules. Approval was obtained from the Ethic Review Committee, General Hospital, Kandy, Sri Lanka and informed consent was obtained from the patients.

Decontamination of specimens was done using standard NaOH/Na citrate technique [17]. Decontaminated specimens were inoculated onto Lowenstein-Jensen (LJ) media and Middle brook 7H-10 and cultures were observed for 8 weeks at 28°C and 37°C in light and dark conditions until the appearance of colonies. Genomic DNA was extracted from AFB positive cultures with standard cetyltrimethylammonium bromide (CTAB/NaCl) method [18]. The PCR was performed as described using the, primerssp1 (5'-ACCTCCTTTCTAAGGAGCACC-3') and sp2 (5'-GATGCTCGCAACCACTATCCA-3') targeting the 16S-23S ISR region [19]. DNA amplification was carried out with an initial incubation of 94°C for 10 minutes then 40 cycles of 94°C for 1 minute, 59°C for 1 minute, 72°C for 1 minute and 4°C indefinitely. The sensitivity of the PCR method was tested, with serial dilutions containing 10 ng, 1 ng and 100 pg of each standard $H_{37}Rv$, *M. bovis* and *M. phocaicum* DNA per vial. DNA sequencing was performed on amplified PCR products using same primers. Twenty microlitres of purified PCR products were used for custom DNA sequencing in Macrogen Inc., South Korea using ABI 3730XL sequencer. Sequencing data obtained were analyzed using the software programmes BioEdit 7.0.9 and Chromas 2.33. The results were compared with the sequences from the Genbank database.

RFLP was carried out for the PCR products obtained from the 16S-23S rDNA ISR. Initial electrophoresis was carried out to determine the nature of mycobacteria present by determining the presence and size of the PCR product (200-220 bp for slow growing and 280-320 bp for rapidly growing mycobacteria) [20]. The enzymes used for the digestion were *Hae*III and *Cfo*I (Promega, USA). Eight µl of PCR product was digested with 10 units of each enzyme at an optimal temperature of 37°C according to manufacturer's instructions. Both the digested and undigested PCR products were electrophoresed on 2% agarose in TBE buffer at 120 V for 1 hour. After electrophoresis, the gel was visualized using Gel Documentation System (Syngene, UK) and the digestions were analysed with gene tool software (Syngene, UK).

Results

Of the 202 specimens, 81 had a growth within eight weeks of incubation. The remaining 121 did not grow. Of the 81, 75 grew within seven days of incubation. Using AFB staining, bacteria (slender, curved rods of pink colour) were observed only in 43 patients. In DNA amplification for the positive cultures, 12 had the unique ~280-320 bp fragment confirming the presence of rapidly growing mycobacteria. Fifteen yielded the unique ~200-220 bp amplification indicating the presence of a slow growing mycobacterium. Eleven patients had mixed cultures and 10 patients had both bands indicating the presence of both rapid and slow growing mycobacteria in specimens. However, in one patient though the culture was mixed two rapid growers were present. No DNA fragments were present in six cultures.

The sensitivity of the optimized PCR assay was ~ 1 ng /µl of genomic DNA. DNA sequence analysis revealed the presence of *M. intracellulare* (n=3), *M. phocaicum* (n=7), *M. smegmatis* (n=1), *M. tuberculosis* complex (n=13) and *Mycobacterium* sp (n=12). In addition there were *Nocardia farcinica* (n=2), an organism similar to *Rhodococcus sp* and in one patient, organisms somewhat similar to *Corynebacterium pseudotuberculosis*/ *M.sp* and *Corynebacterium ulcerans*/ *M.sp* were identified. RFLP profiles produced by digestion with enzyme *Hae*III for 220 bp PCR fragments of $H_{37}Rv$ and *M. bovis* were of 110 and 50 bp while the same enzyme *Hae*III cleaved 220 bp PCR fragment of *M. intracellulare* yielding a ~ 100bp fragment (Table 1).

The enzyme *Cfo*I did not cleave the 220 bp PCR fragments of $H_{37}Rv$, *M. bovis* or *M. intracellulare*. Both enzymes had the cleaving sites for 320 bp PCR product of *M. phocaicum* (Table 1).

According to Gene tool analysis the PCR fragment size of *M. tuberculosis* complex was in the range of 210-220 bp and the *Hae*III digested two fragments were in the ranges of 103-110 and 47-50 bp. Comparatively PCR fragment size of *M. intracellulare* complex was at 230-235 bp and the *Hae*III digested fragments were in the range of 110-120 bp. Therefore, *Hae*III alone was adequate to differentiate *M. tuberculosis* complex isolates from *M. intracellulare*. Of 43 culture positive patients, six were confirmed as having *M. tuberculosis* complex, seven had both *M. tuberculosis* complex and NTM while 21 had NTM. Therefore according to the analysis, 13% to 14% of the study population had NTM in their bronchial washings.

Table 1. PCR-RFLP Analysis of standard and known *Mycobacterium* cultures

Identified Organism	*Fragment size (bp)*	*Restriction fragment length size (bp) – Direct visualization*		*Fragment size (bp) – Gene tools*	*Restriction fragment length size size (bp) – Gene tools*	
		*Hae*III	*Cfo*I		*Hae*III	*Cfo*I
$H_{37}Rv$	220	50, 110	ND	211.47	103.72, 47.40	ND
M. bovis	220	50, 110	ND	217.46	107.57, 50	ND
M. phocaicum	320	120, 200	80, 230	320.49	103.75, 205.02	223.81, 95.48
Nocardia	320	150, 160	ND	320.49	239.09,181.71, 165.10, 75.79, 50	ND
M. sp.	220	120,50	ND	200	118.41, 53.59	ND
M. intracellulare	220	100	ND	232.84	110.45	ND

ND - no digestion

Discussion

In recent years, infections caused by mycobacteria other than *Mycobacterium tuberculosis* have been increasingly reported [21]. In a variety of clinical settings disease caused by rapidly growing mycobacteria usually follows accidental trauma or surgery [22,23].

In this study, a detailed study of non-tuberculosis mycobacteria in patients with lung problems in the district of Kandy was carried out by combining microbiological and molecular methods. PCR-based sequencing has become the gold standard for identification of mycobacterial species [24]. It also allows for direct detection of mycobacterial species that cannot be grown on conventional culture media [25,26]. Early mycobacterial identification to the species level is important because it would help the initiation of early and appropriate treatment. However, identification of mycobacteria by conventional methods is time-consuming and mostly inconclusive [27]. Therefore this assay could be used to augment conventional methods of diagnosis of mycobacterial diseases and thus help clinicians to differentiate between *M. tuberculosis* and NTM. It also could help to select appropriate chemotherapeutic agents early, which could considerably reduce the morbidity due to mycobacterial diseases [28].

PCR-RFLP is simple to perform, easy to read, reproducible and rapid, which are features that make it highly attractive for use in routine clinical laboratories [29]. In addition it can differentiate numerous mycobacteria within a single experiment [28].

In this study, we applied the PCR-RFLP method on DNA of culture positive Mycobacterial isolates. The long-term aim is to use PCR-RFLP to identify and differentiate *M. tuberculosis* from NTM using a technique that would not only be rapid but also simple enough to be used in a non-referral laboratory so that it could be used to augment conventional techniques in a mycobacteria diagnostic laboratory. The rate of growth is still useful for preliminary broad classification of a NTM [30]. However, some of the mycobacterial isolates in our study formed colonies in less than seven days (considered as rapid), but in PCR analysis those yielded a fragment size of 220 bp which categorised them as slow growing mycobacteria.

M. avium complex or MAC (also referred to as MAI) includes two mycobacterial species, *M. avium* and *M. intracellulare*. These species cannot be differentiated by traditional physical and biochemical tests [30]. *M. avium* is the more important pathogen in disseminated disease, whereas *M. intracellulare* is the commoner respiratory pathogen [30]. Four specimens yielded organisms somewhat similar to *Rhodococcus, Corynebacterium* and *Nocardia* species, which are phylogenetically close to mycobacteria which produced 280-320 bp DNA amplicons and identified by DNA sequencing and by restriction digestion. *N. farcinica* is a Gram-positive branching filamentous bacillus causing many localized and disseminated infections in humans, including pulmonary and wound infections, brain abscesses, and bacteremia [31]. The complete genome sequence of a clinical isolate (*N. farcinica* IFM 10152) revealed the presence of many candidate genes for virulence and antibiotic resistance. By conducting a genomic analysis the researchers suggest that this bacterium can survive not only in the soil environment but also in animal tissues, resulting in human disease [32].

Lung disease due to NTM occurs commonly in structural lung disease, such as chronic obstructive pulmonary disease (COPD), bronchiectasis, cystic fibrosis (CF), pneumoconiosis, prior tuberculosis, pulmonary alveolar proteinosis, and esophageal motility disorders [33,34]. Abnormal CF genotypes and 1-antitrypsin (AAT) phenotypes may predispose some to NTM infection [35]. Bronchiectasis and NTM infection, usually MAC, often

coexist, making causality difficult to determine [30]. Clinical studies have established the validity of bronchial washings as a culture source for *M. tuberculosis* [36]. Limited data suggest that bronchial lavage may also be useful for diagnosing NTM (MAC) lung disease [37]. There is expert consensus that bronchial washings are more sensitive than routine expectorated sputum testing and less likely to be affected by environmental contamination if the bronchoscopy specimens are protected from tap water [30]. The routine use of bronchoscopy for diagnosis and follow-up of patients with NTM lung disease is not established [30]. Because NTM can be isolated due to environmental contamination, in general, more than one culture-positive specimen for NTM is necessary for diagnostic purposes [30]. The exception is the patient with classic symptoms and radiographic findings for nodular/ bronchiectatic NTM disease who is unable to produce sputum for AFB analysis [30]. For these patients, the isolation of NTM, especially MAC, from one bronchoscopy specimen is considered adequate for the diagnosis of NTM disease [30]. Therefore the present study amply demonstrates the usefulness of bronchial washings as the preferred specimen in diagnosis of NTM lung disease by culture as well as from molecular techniques. Given the incidence of NTM and other nontuberculous organisms which have been identified in a significant proportion of patients in this study, the appearance of NTM and TB, in a ZN smear and that standard anti TB treatment (ATT) is not effective in many NTM spp. It is appropriate to question whether all patients with smear positivity should be treated as TB. Given the resources needed for rapid species identification and the current treatment approach manages to clear sputum (removal of AFB) in a majority of smear positive patients justifies treating all smear positive patients with ATT unless proven otherwise. As this study was done in patients who were smear negative at the onset we actually cannot extrapolate that a similar proportion of smear positive patients will have NTM in their sputum.

If the country has already developed sufficient laboratory capacity, culture and DST should be performed at the start of treatment and, if smears are positive, at any of these points in time [38].

In conclusion optimized PCR-RFLP assay was able to differentiate *M. tuberculosis* complex from NTM and *Norcardia*. Molecular analysis confirmed the presence of NTM in bronchoscopy specimens. According to the study 13% to 14% of the study population had NTM in their bronchial washings.

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