Research Square

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Development of a cost-effective line probe assay for rapid detection and differentiation of Mycobacterium species

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Abstract

Background Line probe assay (LPA) is one of the most accurate diagnostic tools for detection of different Mycobacterium species. There are several commercial kits based on line probe assay for detection of Mycobacterium species. Because of their high cost, especially for underdeveloped and developing countries, and the discrepancy of NTM prevalence across geographic regions, it would be reasonable to consider the development of an in-house LPA. The aim of this study was to develop a LPA to detect and differentiate mycobacterial species, and also to evaluate the usefulness of PCR-LPA for direct application on clinical samples. Method One pair of biotinylated primers and fifteen designed DNA oligonucleotide probes were used based on multiple aligned ITS sequences. Specific binding of the PCR amplified products to the probes immobilized on a strip of nitrocellulose membrane was evaluated by hybridization method. Experiments were performed three times on separate days to evaluate the repeatability of the assay. Further, evaluation of the PCR-LPA was carried out directly on 9 clinical samples and cultivated isolates. Results All the fifteen probes used in this study were hybridized specifically to ITS sequences of the corresponding standard species. Results were reproducible for all of the strains in different days. Mycobacterium species of nine clinical specimens and their relevant cultivated isolates were correctly identified by PCR-LPA and confirmed by sequencing. Conclusions In this study, we described a PCR-LPA which is readily applicable in clinical laboratory. This assay is a fast, cost-effective and highly specific method which requires no radioactive materials.

Introduction

Mycobacterium tuberculosis, the causative agent of 'tuberculosis', is an ancient human pathogen, which has re-emerged as a major health problem worldwide. According to the WHO reports, about one-third of world's population has been infected with *M. tuberculosis*. In 2017, 1.3 million people died from tuberculosis (TB) and about 54 million deaths recorded during 2000-2017 [1]. This disease is a life-threatening infection and can easily be transmitted through respiratory droplets. Therefore, rapid and accurate diagnosis of TB can result in the appropriate therapy and preventing its spread within the community.

In recent years, the prevalence of *non-tuberculous mycobacteria* (NTM) infections has increased significantly. Some infections of NTM, especially *M. chelonae*, *M. avium* complex (MAC) and *M. fortuitum*, are rapidly increasing worldwide [2]. Unlike *M. tuberculosis*, NTMs are organisms that are often isolated from natural resources such as water and soil. Therefore, contamination with these organisms is almost inevitable especially in elderly people or immunodeficiency population [3]. Although, many of NTM cause the same pulmonary disease as TB, however the type of drugs, their doses and duration of treatment are different from those of TB [4]. For example, the presence of *erm* genes (*erm 38*, *erm 39*, and *erm 41*) in *M. abscessus* group, *M. fortuitum* and *M. smegmatis* induces resistance to macrolides and may lead to treatment failure [5-7].

Therefore, rapid identification and differentiation of *Mycobacterium* species are very necessary to ensure effective therapy of the disease. Traditional strategies based on culture methods and phenotypic characteristics like colonial morphology, growth rate, producing pigment and biochemical characteristics are very time-consuming, difficult to interpret and unreliable [8]. Several molecular techniques like PCR (polymerase chain reaction), RFLP (restriction fragment length polymorphism) and real-time PCR methods have also been developed as rapid tests for detection of *Mycobacterium* species. However, none of these techniques can simultaneously differentiate several species of *Mycobacterium* in the clinical laboratory. Therefore, a high discriminatory method is still needed for laboratory diagnosis and epidemiological studies. Line probe assay (LPA) is a diagnostic tool based on the detection of a

specific RNA or DNA sequence by hybridizing the complementary strand of a nucleotide probe to a part of the target sequence [9]. This method has considerably improved the accuracy and rapidity of *Mycobacterium* spp. *differentiation* [10].

There are several target genes or sequences which are used to differentiate *Mycobacterium* species including: *16S rRNA* [11], *16S-23S rRNA* or internal transcribed spacer (*ITS*) [12], *23S rRNA*[13], *recA*[14], *rpoB* gene[14], *dnaJ*[15], *secA1*[16], *dnaA*[17], *gyrB* [18], *sod*[14] and *hsp65* genes [19]. Among these, *ITS* target has been found to have an outstanding potential for this purpose, because of its variations in size and sequence from species to species and containing both variable and highly conserved regions. Previous studies have also indicated that *ITS* has a good reproducibility and high level of variation in mycobacteria [12, 20].

There are several commercial kits based on line probe assay for detection of *Mycobacterium* species. Because of their high cost, especially for underdeveloped and developing countries, and the discrepancy of NTM prevalence across geographic regions, it would be reasonable to consider the development of an in-house LPA for rapid identification and differentiation of *Mycobacterium* species.

Therefore, the main aim of the present study was to design an in-house and cost-effective LPA-based method targeting *ITS* to identify prevalent NTM species. It was additionally evaluated for direct application to DNA from clinical specimens compared to that of extracting from bacterial colonies of the same specimen.

Materials And Methods

Strains and clinical specimens: In this study, 25 bacterial species were used including 15 *Mycobacterium* and 10 non-mycobacterium species (Table 1). Moreover, nine clinical specimens and their relevant isolates from culture medium were collected from Pasteur Institute of Iran, to evaluate the results of our developed LPA in a pilot study. The clinical samples were positive for acid-fast bacilli and the cultivated isolates were negative for niacin test, and (i.e., the patients were suspected of NTM infection). These samples were evaluated in parallel by PCR-LPA and sequencing.

Probe and primer design: The 55 sequences of *ITS* gene of mycobacteria were obtained from NCBI GenBank database (Table 2). Multiple alignment analysis of available sequences were performed using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA) and species-specific probes were selected from the polymorphic and conserved regions of mycobacterial sequences to ensure probe specificity. Afterwards, Gene Runner software (Hastings Software, Colorado, USA) was used to compute the probes parameters. Then, selected oligonucleotide probes were analyzed with BLAST search (Basic Local Alignment Search Tool) at nucleotide collection database to determine the specificity of the selected regions. A set of primers *Sp1* (5'-ACC TCC TTT CTA AGG AGCACC-3') and *Sp2* (5'-GAT GCT CGC AAC CAC TAT YCA-3'), targeting *ITS* region of *Mycobacterium* were used from the previous study [12]. Primer *Sp2* was labeled with biotin at the 5' end and a poly-(T) tail was added at the 3' end of oligonucleotide probes.

DNA extraction and PCR amplification: The extraction of DNA from samples was carried out with boiling method [21]. Briefly, after two times of washing with TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA), 50 µl TE buffer was added to the samples and then boiled for 30 min in a water bath. Afterward, the microtubes were centrifuged at 10000 rpm for 10 minutes. The supernatant solution was used as template for the PCR reaction. PCR was performed in a total volume of 25 µl containing 1 µl each of forward and reverse primers (10 pmol), 12.5 µl PCR Master Mix (Parstous biotechnology, Iran), and 1 or 10 µl DNA samples (1µl for cultivated samples and 10 µl for

clinical samples). PCR was performed by a Mastercycler Gradient (ASTEC, Japan). The reaction scheme was as follows: an initial denaturation step at 95°C for 5 min, a total of 40 cycles with the following steps: denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 10 min. Analysis of the PCR products was performed by electrophoresis on 2% agarose gel stained with Green viewer™. The predicted sizes of the amplicons (varied from 200-300 bp) were visualized by UV-transilluminator. For each PCR run, sterile water and 1 ng of *M. tuberculosis* H37Rv genomic DNA were used instead of template as negative and positive control, respectively.

Line probe assay: the LPA was modified from a previous publication [22]. Briefly, 10 µl of 20 pmol/µl species-specific probes in TE buffer were immobilized onto a nitrocellulose membrane (pore size 0.45 µm; Amersham Pharmacia Biotech) as parallel lines (line 2-16) by Bio-Dot SF microfiltration apparatus (Bio-Rad, USA), with the first line as positive-conjugated control containing a biotinylated DNA (PCR product).The strips were incubated for 1 hour at 80°C (Oven) to fix the probes on the strips. Equal volumes (20 µl) of biotinylated PCR product (50 ng/µl) and denaturation solution (400 mM NaOH + 10 mM EDTA) were mixed in a microtube and incubated at 37°C for 15 min. At the same time, 2 ml of hybridization buffer 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS) and 200 µl BSA 10% (bovine serum albumin) was added onto the each strip at room temperature. Afterwards, 2 ml of prewarmed (60°C) hybridization buffer mixed with denatured PCR product was added to the strip and incubated at 59°C for 30 min in a shaking ProBlot 12 hybridization oven (Labnet International, Woodbridge, NJ). Then, the strips were washed once in 2 ml of 2X hybridization solution for 1 min at room temperature and once in 0.1 X SSC solution for 5 min at 62°C in hybridization oven. Then, the strips were incubated with 2 ml of a 1:1000 diluted alkaline phosphatase-labelled streptavidin (Sigma) in Tris buffered saline at pH 8 on a rotating platform at room temperature for 30 min. The strips were washed in 1 X SSC solution for 10 min at room temperature. Finally, 2 ml of 5-bromo-4-chloro-3-indolylphosphate and nitroblue-tetrazolium (BCIP/NBT, Sigma) were added to the strips and incubated in a shaking plate at room temperature for 10 min in a dark condition. The color reactions were occurred and the strip results were visually interpreted by probe alignment guide which shows the exact position of the probes in each line. In order to evaluate the repeatability or intra-assay precision, PCR-LPA was performed three times on separate days.

Nucleotide sequence analysis: In order to confirm the LPA results, PCR products of cultivated samples were sequenced (Macrogen, Seoul, South Korea) in one direction using the *SP1*as primer. The nucleotide sequences of *ITS* obtained were compared by the BLAST search of NCBI GenBank in nucleotide collection database.

Results

Designed specific probes: The *ITS* sequences of 55 mycobacteria were evaluated to select the best conserved region of each species. It was essential to design more than one probe for some of the species like *M. fortuitum* and *M. kansasii*. Some other species like *M. ulceranse* and *M. marinum* was not differentiable by *ITS* target. So, one genus-specific and fifteen species-specific probes for 12 *Mycobacterium* species were designed after consideration of length and melting temperature. According to table 3, the lengths of designing probes were19-23 bp, which was suitable for hybridization procedure. The optimal melting temperatures (Tm) were in range 60.6-62.5 °C (Tm difference ≤ 1.9 °C) and the optimal free energy values (ΔG) for hairpin and self-dimer were lower than -2.2 and -5.3 kcal/mol, respectively (Table 3). Then, the selected probes were checked with the nucleotide collection database and it was confirmed that there was no homology among the designed probes and non-related bacterial sequences.

PCR-LPA: The *ITS* fragments were amplified successfully for all *Mycobacterium* species by PCR with the *SP1* and *SP2*-biotinylated primers. The PCR reaction was performed at annealing temperature of 59°C and produced a single band ranging in length approximately from 220 to 300 bp for different *Mycobacterium* species (Figure1). In this study, the rapidly growing *Mycobacterium* species like *M. fortuitum* show larger amplicons (300 bp) than slowly growing species. The genus-specific primers (*Sp1* and *Sp2*) were shown to be specific for mycobacteria as were unable to amplify any DNA fragment from bacteria other than *mycobacterium* species. All immobilized oligonucleotide probes on the nitrocellulose membrane strips were successfully hybridized with their relevant biotinylated PCR products from *Mycobacterium* species. The specificity of LPA assay was 100% using DNA extracted from mycobacterial and non-mycobacterial reference strains (Table 1 & Figure 2). LPA results were reproducible for all of the strains in different days. However, there are several considerations about our PCR-LPA assay: 1) to detect different types of *M. kansasii* and *M. fortuitum* respectively two and three different kinds of probes are required, 2) *M. marinum* and *M. ulcerans* are not differentiable due to 100% sequence homology between their *ITS* region, 3) The members of *M. tuberculosis* complex cannot be differentiated from each other. 4) Both *M. avium* and *M. intracellulare* are detected by INT/AV probe and can be differentiated shed from each other using AV probe which only detects *M. avium*. The results of hybridization on LPA strips are presented in Figure 2.

PCR-LPA assay on clinical specimens and mycobacterial isolates: Totally, nine clinical specimens and their relevant isolates from culture medium were blindly examined by PCR-LPA assay in a pilot study. Then, our findings were compared with the sequencing results. Two clinical specimens were shown to each contain two different NTM species by gel electrophoresis (specimens No. 7 and No. 9).The presence of two different types of *M. fortuitum* was approved by PCR-LPA in one of these specimens (No.7). However, co-infection with *M. simiae* and *M. fortuitum* was proved in the other specimen (No. 9) using PCR-LPA. NTM species of the rest of clinical specimens were identified as *M. fortuitum*. The results of PCR-LPA performed on clinical specimens and their relevant clinical isolates were comparable. The results of PCR-LPA and sequence analysis were also comparable except for the two mixed infection specimens in which the sequencing results were not interpretable. For this reason, these two specimens were amplified again, and then PCR products were purified using a gel extraction kit (GeNet Bio, Korea) and the purified PCR products were sent for re-sequencing. The results showed that the two species extracted from sample 7 belong to different types of *M. fortuitum* and sample 9 contained *M. fortuitum* and *M. simiae*, which was similar to PCR-LPA result.

Discussion

Due to increasing incidence of *Mycobacterium* infection, rapid diagnosis and differentiation of the *Mycobacterium* species is crucial for successful treatment. Traditional methods usually take about 4-10 weeks (or more) to give the results. Therefore, molecular methods can be an appropriate alternative for rapid diagnosis and differentiation of *Mycobacterium* species. Although, *Mycobacterium* genome is more than 4000 bp in length, a few genes can be the candidate target for molecular diagnosis of these bacteria. Some of the most prevalent NTM species lack a unique sequence within the candidate genome region to be differentiated from each other, due to the high genomic similarity among the *Mycobacterium* species [23].

Most of the PCR assays which are used for detecting mycobacteria, identify a single or limited number of *Mycobacterium*s species [24]. Therefore, these methods seem to be inefficient for identifying *Mycobacteria* to the species level. Restriction fragment length polymorphism (RFLP) is known as a reliable method for differentiation of mycobacteria[25], but interpretation of its results is actually quite difficult, because of low distance between band sizes in some species and the emergence of new patterns that have not been reported before [26]. Furthermore,

RFLP method is unreliable in detection of mixed cultures and is not applicable to clinical samples. Real time PCR technique is also too costly and requires considerable expertise. Although, sequencing is known as the gold standard method in detection of mycobacteria, it is unable to identify mixed culture samples or co-infection with more than one NTM species in a patient as it requires a high quality and pure PCR product. Generally, the advantages and disadvantages of different molecular techniques are summarized in table 4.

Due to disadvantages of these diagnostic techniques, use of a molecular method based on reverse hybridization can be useful in detecting and differentiating of *Mycobacterium* species. The PCR-LPA is one of the reverse hybridization techniques which is able to differentiate a large number of *Mycobacterium* species from each other. LPA is faster than traditional methods, more accurate than chromatographic tests, and less expensive than sequencing for diagnosis of mycobacteria [12]. However, LPA assay is a PCR-based method. The low amount of DNA and high amount of inhibitors in the clinical specimen can lead to false-negative results, especially in smear-negative specimens [27].

Therefore, cultivation of bacteria from clinical specimens and testing on colonies is the most appropriate way for NTM identification. However, a major drawback with mycobacterial culture is the overgrowth of one NTM species that may cause to miss the growth of another one in the cases of co-infection with more than one NTM species.

In the present study, *ITS* showed a strong potential for discriminating closely related *Mycobacterium* species. The sequence analysis of *ITS* in different *Mycobacterium* species revealed that the 16S-23S spacer sizes of slowly growing *Mycobacteria* are shorter than those of rapidly growing species (Figure1). Moreover, the differences in the *ITS* length of various *Mycobacterium* species have made it easy to detect the mixed culture or co-infections by electrophoresis of PCR products. We found the presence of more than one species in 2 samples according to the electrophoresis and sequencing results. The rest of the samples (1,2,3,4,5,6,8) were showed only one species (Figure3). Therefore, this advantage of *ITS* target can be very useful for diagnostic purposes.

To date, several lung infections have been reported with more than one species of NTM or more than one genotype of a specific species. However, information on such infections is very limited. The highest incidence of simultaneous infection with two NTM species is related to *M. avium* complex and *M. abscessus* co-infection [28], but in this study, we found a co-infection with *M. simiae* and *M. fortuitum*, and a mixed infection with different *M. fortuitum* genotypes.

However, PCR-LPA analysis indicated that the discrimination between *M. marinum* and *M. ulcerans* which have a high degree of sequence homology is impossible. Interestingly, PCR-LPA was able to discriminate between *M. chelonae* and *M. abscessus* with two nucleotide differences between their *ITS* sequences, and between *M. avium* and *M. intracellulare* which cannot be distinguished by chromatographic methods [12].

In our pilot study on nine clinical samples and their cultured isolates, M*. fortuitum* was the most common mycobacterial species in the sample collection region (Tehran, Iran). In consistent with our results, Irandoost and colleagues reported the same species as the most prevalent one in the same region [29]. Our finding showed that this method is capable of detecting mycobacterium species in clinical samples as accurate as culture or sequencing methods (Figure 4). Although, the results of LPA performed on clinical samples showed band intensity weaker than those of cultured isolates, we had no problem for interpretation of the results.

In the present study, we described a kind of PCR-LPA which is readily applicable for respiratory specimens in clinical laboratories. This is the first study which compared the results of PCR-LPA in clinical and cultivated samples from

the same patients. Our PCR-LPA based on the *ITS* target can differentiate 12 mycobacterial species and genotype by the use of 15 probes. The speed of the assay is similar to commercial kits with high specificity and required no radioactive material. Moreover, the cost of this test is very low compared to the similar available kits. Each test only has a cost of 6-7€ (Table 4). We routinely performed the amplification by PCR in less than 3 h, followed by a line probe assay in 4 h.

Conclusions

PCR-LPA identified mixed cultures or co-infections in clinical samples which cannot be recognized by PCR, and interpreted by nucleotide sequence analysis. Therefore, it is recommended to integrate PCR-LPA into the routine microbiological laboratory practice for diagnosis of mycobacterial infection, especially by its direct performing on clinical samples.

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

EA, MS, RKK, ZM contributed in the study design and target finding. MS designed the probes. RKK, EA collected and evaluated the clinical specimens. RKK, MS wrote the manuscript. All authors approved the final version of the paper.

Ethics approval and consent to participate

The ethical approval for performing this study was obtained from the Ethics Committee of the Mashhad University of Medical Sciences (Ethics code of IR.mums.fm.rec.1396.195) and written informed consent was obtained from participants.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

LPA: Line probe assay; MTBC: Mycobacterium tuberculosis complex; NCBI: National Center for Biotechnology Information; NTM: Nontuberculous Mycobacteria; PCR: Polymerase Chain Reaction;

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Tables

Table 1 The bacteria and reference strains used in this study

Bacterial species	Reference strains*
Mycobacterial species	
M. abscessus	DSMZ 44196T
M. avium	ATCC 25291
*M. avium*subsp.*silvaticum*	DSMZ 44175
M. bovis	Clinical isolate
M. chelonae	Environmental isolate
M. fortuitum	TMC 1530
*M. fortuitum*subsp.*acetamidolyticum*	ATCC 35391
M. gordonae	Clinical isolate
M. intracellulare	ATCC 13950
M. kansasii	TMC 1204
M. marinum	Environmental isolate
M. scrofulaceum	ATCC 35785
M. simiae	Clinical isolate
M. tuberculosis	H37Rv
M. ulcerans	ATCC 35840
Non-mycobacterial species	
Acinetobacter baumannii	NCTC 13304
Corynebacterium diphtheriae	Clinical isolate
Escherichia coli	ATCC25922
Enterococcus faecalis	ATCC29212
Klebsiella pneumonia subsp. *pneumonia*	ATCC700603
Listeria monocytogenes	ATCC7644
Pseudomonas aeruginosa	ATCC27853
Salmonella enteritidis	RITCC1624
Salmonella enteric serovar Typhimurium	ATCC14028
Staphylococcus aureus	ATCC25923

*ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von MikroorganismenundZellkulturen GmbH; TMC, Trudeau Mycobacterial Culture; NCTC, The National Collection of Type Cultures; RITCC, Razi Institute Type Culture Collection. .

Table 2 GenBank sequence identification numbers (accession number) for Mycobacterium *species*

Gene	Mycobacterial Species	Accession Number
ITS (internal transcribed spacer)	*M. tuberculosis complex*	L15623, DQ133991, AJ315568, L26330, AJ315570, AJ315571, L26328, AJ315569, L26329
	M. chelonea	AJ291583, AJ291584, AJ314873, AJ314874, AF144327
	M. abscessus	AJ314869, AJ291580, AJ314871, AJ314870
	M. gordonae	AJ315574, AJ315575
	M. ulcerans	X99217
	M. marinum	Y14185, AJ315572, AJ315573, GU827996
	M. simiae	Y14187, Y14186, Y14188, Z46426
	M. intracellularae	X74057, Z46425, Z46423, AJ306711, AJ314865, AJ314864
	M. avium	AJ314863, L15620, AJ314862, Z46422, Z46421, X74054
	M. scrofluaceum	L15622, AJ314884
	M. kansasii	L42262, L42263, L42264, X97632
	M. fortuitum	AJ291593, AJ291592, AJ291587, AJ291588, AJ291589, AJ291590, AJ291591, AF144326

Table 3 The sequences and the parameters of oligonucleotide probes targeting mycobacterium*ITS*. *

Species	Sequence	Length (mer)/Position	Tm (∘C)	Self-dimer ΔG (kcal/mol)	Hairpin ΔG (kcal/mol)
M. tuberculosis complex	ACTTGTTCCAGGTGTTGTCCCAC	23/134-157	61.5	1.8	1.1
M. chelonea	CAGCCGAATGAGCTTGGGAA	20/16-36	62.0	-1.3	-0.6
M. abscessus	TCCCAGTCGAATGAACTAGGGAA	23/13-36	61.8	-2.8	-2.2
M. gordonae	CGTGAGGGGTCATCGTCTGTAG	22/42-64	60.6	1.5	2.4
M. ulcerans/marinum	ACATCTCTGTTGGTTTCGGGATG	23/124-147	61.8	0.0	0.6
M. simiae	CCGACTTCGGTTGAAGTGGTGT	22/134-156	62.5	-3.3	-0.8
M. intracellularae	GTCGATCCGTGTGGAGTCCCT	21/133-154	61.9	-1.8	1.2
M. intracellularae/avium	CCGTGTGGAGTCCCTCCATCT	21/139-160	61.9	-3.2	-2.2
M. scrofluaceum	CAACACTCGGCTCGTTCTGAGT	22/124-146	60.6	0.5	-0.2
M. kansasii (KAN1)	GGACTTGTCTGGGTCGTTGTCC	22/133-155	61.7	-1.0	-0.4
M. kansasii (KAN2)	TCGGGCTCTGTTCGAGAGTTGT	22/129-151	62.5	-1.8	-1.0
M. fortuitum (F1)	CCACGAAAAGGGTTGAGACACTG	23/8-31	62.5	NA	NA
M. fortuitum (F2)	ACGTTGAGCCGCAAGGGAT	19/92-111	61.2	-1.2	0.6
M. fortuitum (F3)	CGTAAATCGCGGGATCAGCT	20/29-49	61.5	-5.3	2.8

* The parameters were determined at 330 mM salt concentration. NA, not applicable; ΔG, Gibbs free energy.

Table 4 Comparison of several molecular methods for detection and differentiation of mycobacterium species.

Method	Time	Cost	Clinical or Cultivated samples	Difficulty	Detection of multiple-species	Results
PCR	3 h	Low	Both	Requires specific target for each species	+ (Limited)	Electrophoresis
RFLP	up to 2 days	Low	Cultivated	Difficulty in interpretation	-	Electrophoresis
Real time PCR	3 h	High	Both	Requires considerable expertise	-	Computer analysis
LPA (In this study)	7 h	6-7 €	Both	Requires considerable expertise	+ (12 species)	visually
Commercial LPA	7 h	High	Both	Requires expertise and extra instrument	+ (the number of species)	visually
sequencing analysis	Up to 1 weeks	High	Cultivated	Requires high quality and pure product	-	BLAST search

PCR: Polymerase chain reaction, RFLP: Restriction fragment length polymorphism, LPA: Line probe assay

Figures

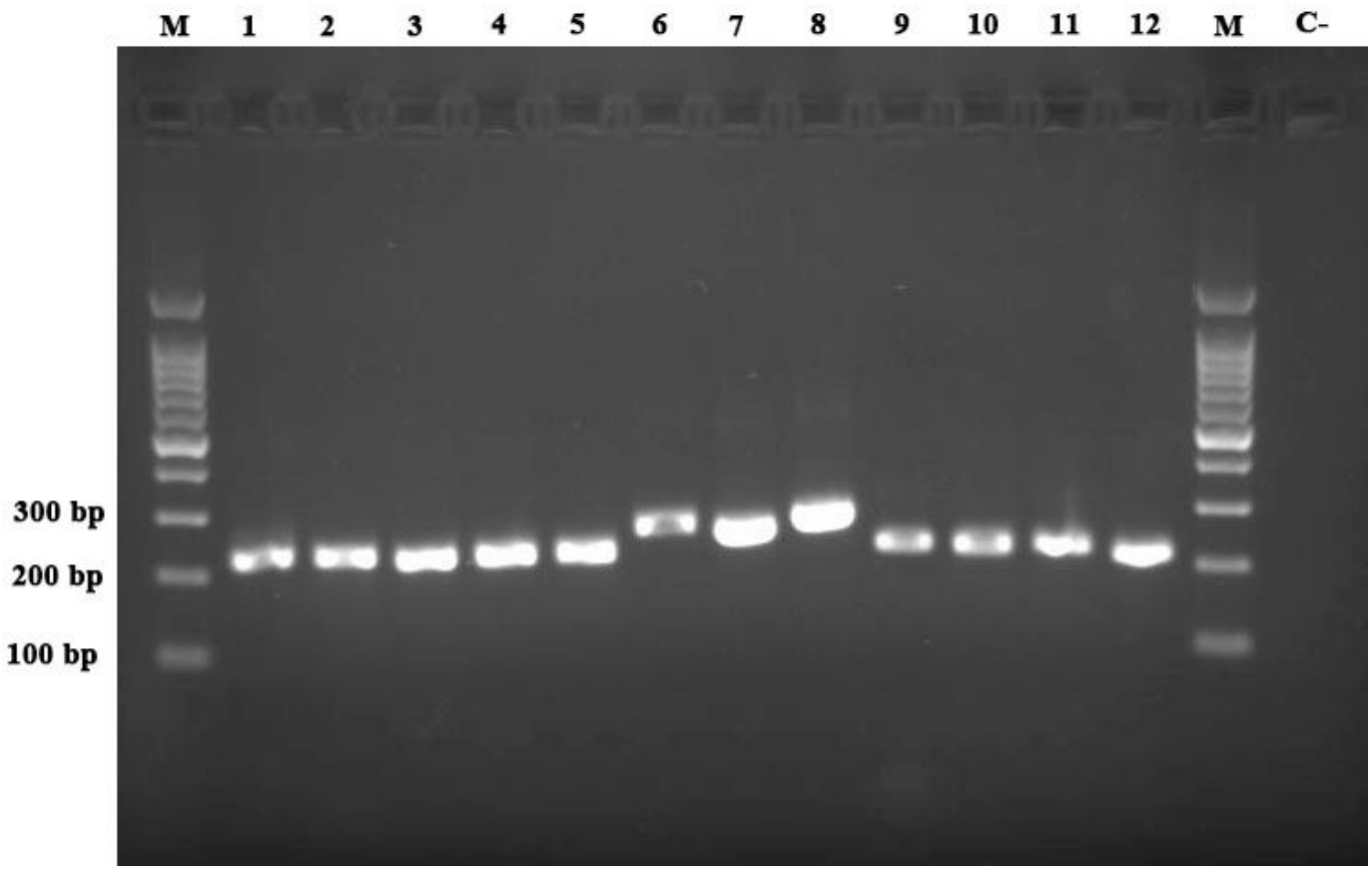

Figure 1

The results of ITS-PCR for standard mycobacterium species. Lane 1: M. tuberculosis H37Rv, Lane 2: M. simiae (clinical isolate), Lane 3: M. intracellulare ATCC 13950, Lane 4: M. avium ATCC 25291, Lane 5: M. kansasii TMC 1204, Lane 6: M. chelonae (environmental isolate), Lane 7: M. absessuss DSMZ 44196T, Lane 8: M. fortuitum TMC 1530, Lane 9: M. scrofulaceum ATCC 35785, Lane10: M. ulcerans ATCC 35840, Lane 11: M. marinum (environmental isolate), Lane 12: M. gordonae (clinical isolate). M, 100 bp DNA marker; C, negative control.

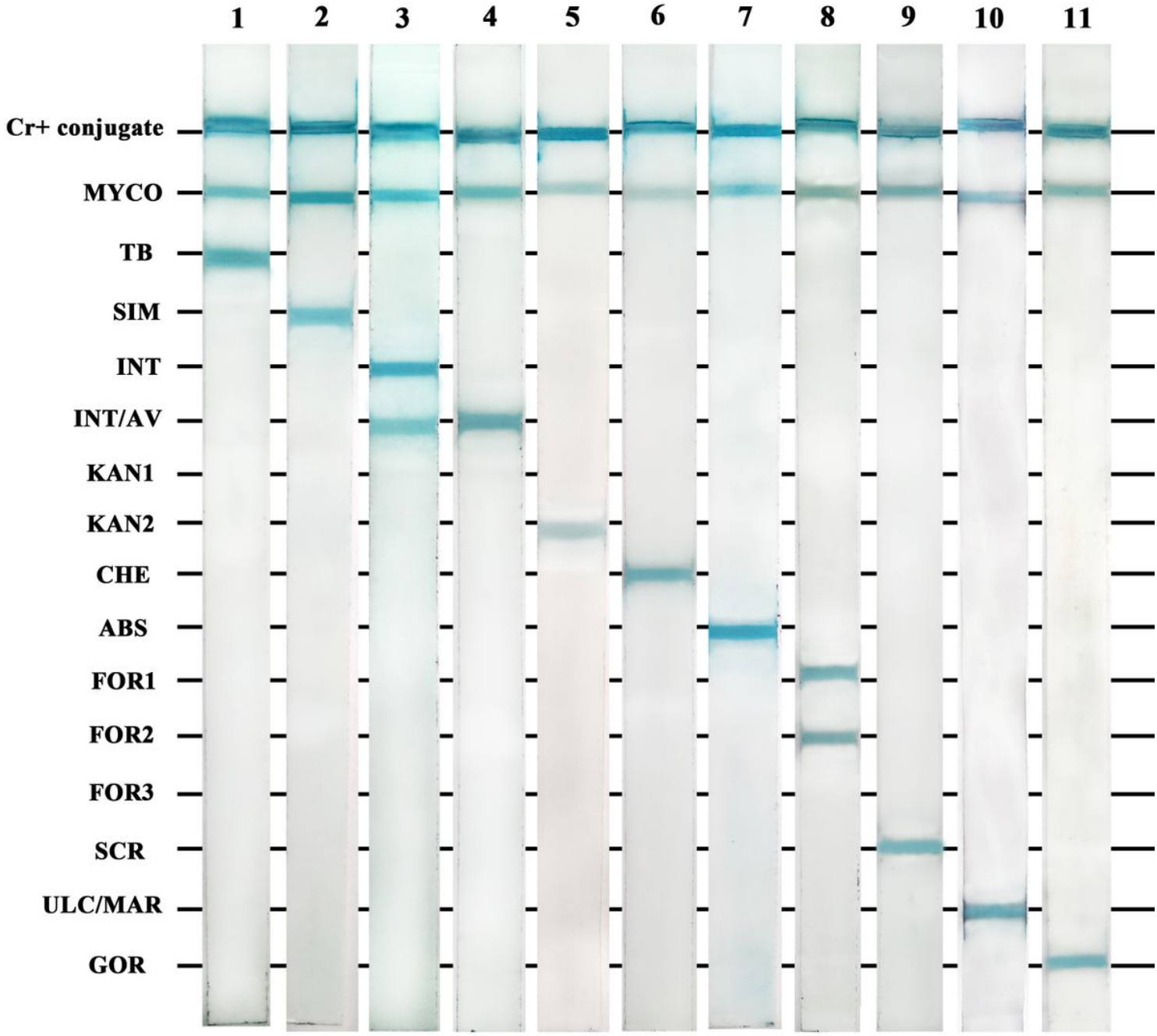

Figure 2

LPA for standard mycobacterium species. strip 1: M. tuberculosis H37Rv, strip 2: M. simiae (clinical isolate), strip 3: M. intracellulare ATCC 13950, strip 4: M.avium ATCC 25291, strip 5: M. kansasii TMC 1204, strip 6: M. chelonae

(environmental isolate), strip 7: M. absessuss DSMZ 44196T, strip 8: M. fortuitum TMC 1530, strip 9: M. scrofulaceum ATCC 35785, strip 10: M.ulcerans ATCC 35840 and M. marinum (environmental isolate), strip 11: M. gordonae (clinical isolate). M, 100 bp DNA marker; C, negative control.

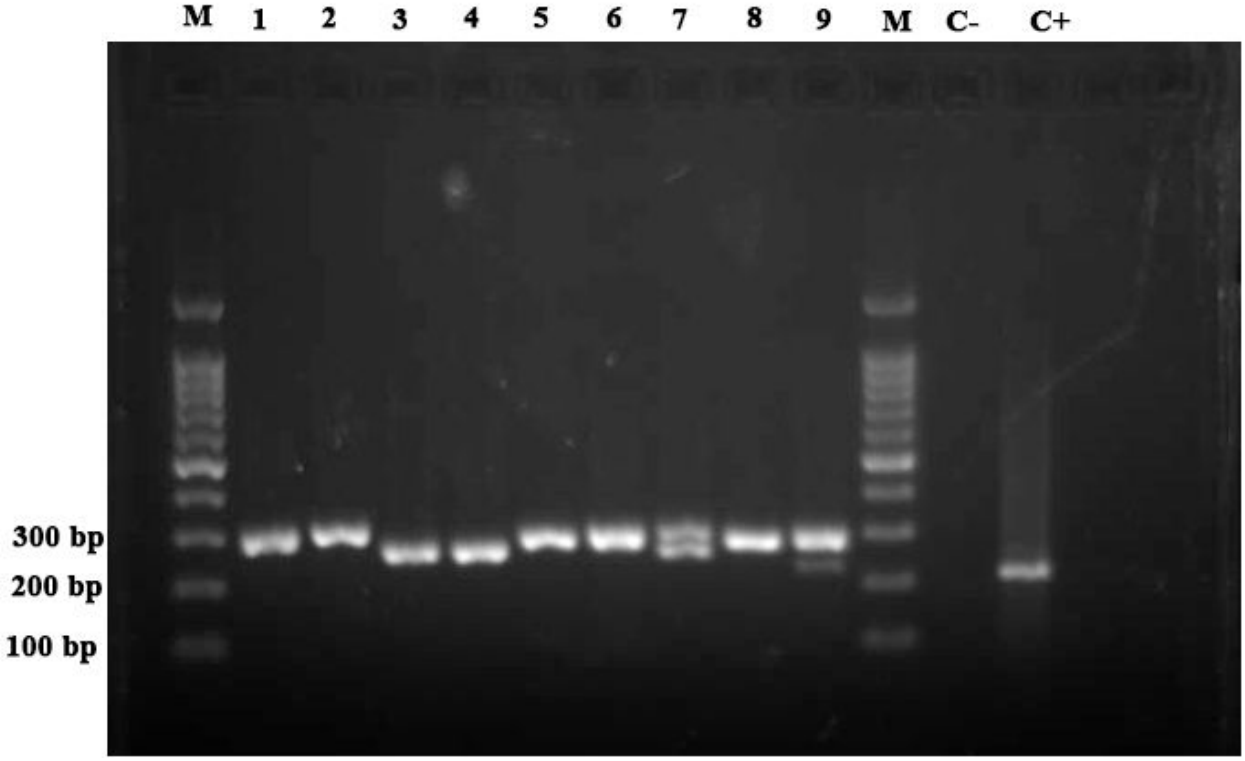

Figure 3

The results of ITS-PCR for 9 cultivated samples.1: M. fortuitum, 2: M. fortuitum, 3: M. fortuitum, 4: M. fortuitum, 5: M. fortuitum, 6: M. fortuitum, 7: M. fortuitum, 8: M. fortuitum, 9: M. fortuitum+ M. simiae, M, 100 bp DNA marker; C-, negative control; C+, positive control.

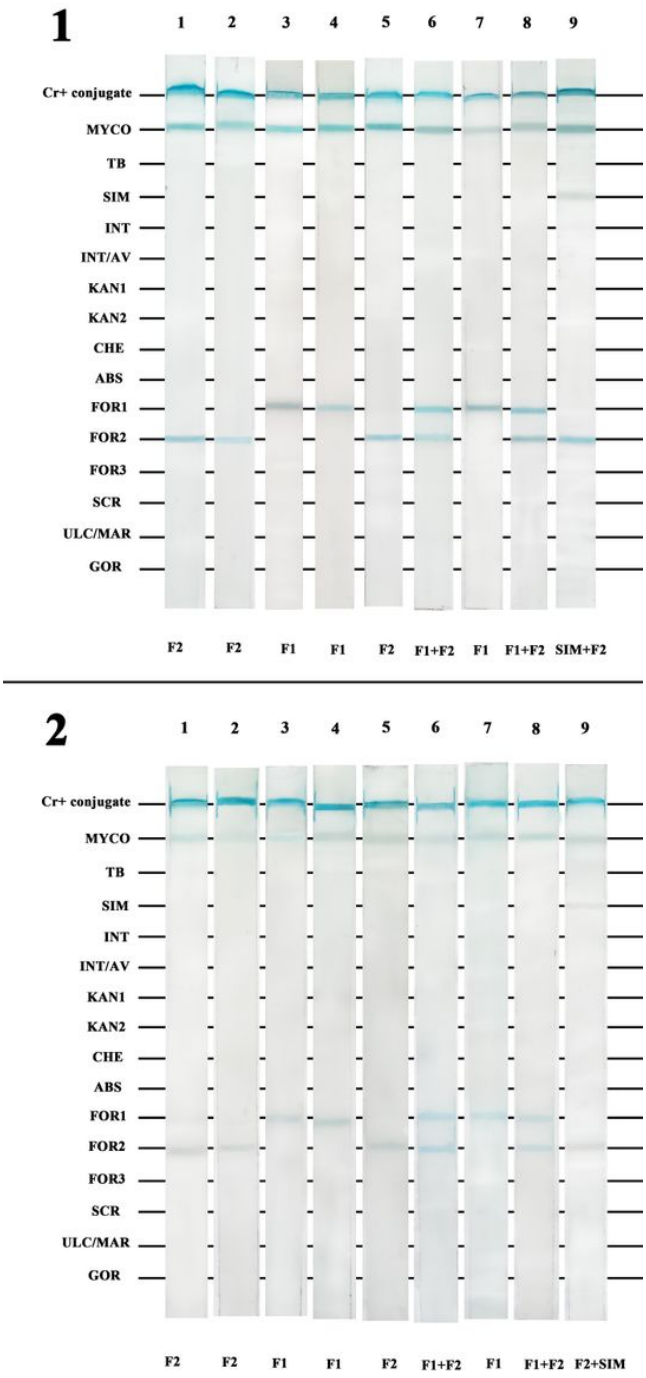

Figure 4

The results of LPA fornine cultivated samples. 2) The results of LPA fornine clinical samples. 1-8: different types of M. fortuitum; 9: M. simiae+M. fortuitum.