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Original Article

Microbiology Section

Identification of a *Rothia mucilaginosa* Strain in a Clinical Specimen Based on PCR-Sequencing with *Mycobacterium hsp65* Primers

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ABSTRACT

Introduction: Tuberculosis (TB) is one of the top 10 causes of death worldwide. Xpert *Mycobacterium tuberculosis* (MTB)/Rifampicin (RIF) has been recommended by World Health Organisation (WHO) to diagnose TB, while *hsp65*-Polymerase Chain Reaction (PCR) has been used for mycobacteria identification.

Aim: To report a false positive result (*Rothia mucilaginosa*) using *hsp65*-PCR for detection of mycobacteria in a clinical specimen.

Materials and Methods: A sputum sample from a 58-year-old, male, suspected-TB patient was studied by Xpert MTB/RIF and *hsp65-PCR*. The amplified *hsp65*-PCR product was sequenced and analysed using bioinformatics for bacterial identification and primer specificity.

Results: Xpert MTB/RIF showed a negative result, while *hsp65*-PCR was positive, suggested the presence of Pulmonary Non-Tuberculous Mycobacterial (PNTM) Infection. The analysis of the sequencing result of the amplified *hsp65*-PCR fragment showed 98% similarity to *Rothia mucilaginosa*, a member of the normal flora of the human oropharynx and upper respiratory tract, which may cause pneumonia. Further analysis showed that the mycobacteria *hsp65* primers used have high percentage of similarity with *Rothia mucilaginos*a gene sequence, resulting in non-specific detection.

Conclusion: This study showed the possibility of false positive results in clinical specimens using PCR-*hsp65* primers considered specific for mycobacteria, therefore, this test should be used in clinical samples with caution and it is suggested the need of its further re-optimisation.

Keywords: Diagnosis, Mycobacteria, Polymerase chain reaction, Sequence, Specificity, Tuberculosis

INTRODUCTION

TB mainly caused by MTB in humans, is a global health concern as more than 10 million new cases and 1.5 million deaths were reported in year 2018 [1]. While in developed countries like Japan, United States and Australia, the incidence rates of PNTM is higher than TB [2]. Both MTB and Non-Tuberculous Mycobacteria (NTM) usually cause chronic lung disease and could not be distinguished via clinical symptoms [3]. Previous TB infection will also increase the risk for NTM infection [3].

In low-resource settings, direct acid-fast sputum microscopy is the most widely used method to diagnose TB, but it has low sensitivity and specificity [4]. Thus, several detection kits with high sensitivity and specificity have been developed and endorsed by WHO for TB diagnosis such as Xpert MTB/RIF [5], Loopamp™ MTB Complex (MTBC) Detection Kit (Eiken Chemical Co., Japan) [6], and GenoType MTBDRplus (Hain Lifescience GmbH, Germany) [7]. The diagnosis of NTM is difficult as more than 180 different NTM has been reported [8]. Several tests are used for rapid differentiation of MTBC and NTM such as NTM+MDRTB Detection Kit 2 (Nipro Co., Japan) [9], GenoType *Mycobacterium* Common Mycobacteria/Additional Species (CM/AS) (Hain Lifescience, Germany) [10], DR. TBDR/NTM IVD kit (DR. Chip Corporation, Taiwan) [11], and CapitalBio Mycobacteria Real-Time PCR Detection Kit (CapitalBio Corporation, China). Several target genes such as *hsp65, rpoB* and 16S rRNA have been used for mycobacteria identification [12-14].

Tb11 and Tb12 *hsp65* primers have been regarded as *Mycobacterium* genus-specific primers and used for PCR in the detection of mycobacteria [15,16]. *hsp65* PCR-Enzyme Analysis (PRA) has been reported as useful test for *Mycobacterium* species-level identification with higher discriminatory power compared to 16S rRNA and *rpoB* [12-14]. Molecular sequencing of the *hsp65* gene from clinical samples such as sputum, cerebrospinal fluid and biopsies has improved the diagnostic sensitivity, specificity and turnaround time [17].

Despite of these antecedents, here, authors have reported the possibility to have false positive results with *hsp65*-PCR due to the identification of *Rothia mucilaginosa* caused by the ambiguity of the Tb11 and Tb12 *hsp65* primers used.

MATERIALS AND METHODS

Sample

In the context of a prospective study, a sputum sample from a 58-year-old, male with TB-like symptoms (fever and chronic cough) from a local community in Kota Kinabalu, Malaysia was collected. The study was approved by the Ethical Committee (Human) of Universiti Malaysia Sabah (UMS), in accordance with Declaration of Helsinki {JKEtika 2/2016(6)}. The ethical clearance for the TB screening program was obtained in June 2016, approved for three years of study. Informed consent was obtained from the patient.

Xpert MTB/RIF assay

Sputum (1 mL) was processed according to the manufacturer's protocol by mixing with sample reagent (2 mL) in a 15 mL falcon tube and incubated at room temperature for 15 minutes. Then, the inactivated material (2 mL) was transferred to a test cartridge, loaded into Xpert MTB/RIF machine (Cepheid, Sunnyvale, CA) and the presence of MTB was determined by real-time PCR assay [18].

Sputum Processing and DNA Extraction

Sputum (1 mL) was decontaminated and digested with BBL® MycoPrep™ (Becton Dickinson, USA) (1 mL) for 15 minutes in a 50 mL falcon tube. The tube was top-up with Phosphate Buffered Saline (PBS) and the bacteria was pelleted by centrifugation at 3,000 g for 20 minutes. Bacterial DNA was extracted using Kaneka Easy DNA Extraction Kit version 2 (Kaneka Co., Hyogo, Japan), according to the manufacturer's protocol. Solution A (100 µL) was added to the bacterial pellet and was incubated at 98°C for 8 minutes. Then,

solution B (14 µL) was added to stop the chemical lysis. The DNA was stored at -20°C [19].

High-Fidelity *hsp65*-PCR and Sequencing

The *hsp65*-PCR assay was carried out in a total reaction volume of 50 µL with 5X PrimeSTAR GXL Buffer, 0.2 mM Deoxyribonucleoside Triphosphates (dNTPs), 200 nM Tb11 forward primer, 200 nM Tb12 reverse primer, and 1.25 U PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Japan) [20]. The DNA oligonucleotides, Tb11 (5'-ACCAACGATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT) primers [16], were synthesised by Integrated DNA Technologies (IDT), Pvt., Ltd., Singapore. The extracted DNA (20 µL) was added to the master mix and amplified using the following thermal-cycling parameters: 1 cycle (95°C/2 min); followed by 45 cycles (98°C/10 sec, 60°C/15 sec and 68°C/1 min); and a final extension (68°C/5 min). DNA (1 µg/µL) from MTB H37Rv, *Mycobacterium bovis* BCG Pasteur strain TMC 1011, *Mycobacterium avium* subsp. *paratuberculosis* strain K-10, *Mycobacterium avium* subsp. *avium* strain 2285, *Mycobacterium intracellulare* strain 1956, *Mycobacterium kansasii* strain 824, *Mycobacterium simiae* strain MO-323 and *Mycobacterium abscessus* strain MA 1948, obtained from ATCC and BEI Resources, USA, were used as positive controls.

The amplified PCR products were analysed on a 2% agarose gel containing FloroSafe DNA stain (1st BASE, Singapore) and visualised using Molecular Imager® Gel Doc™ (Bio-Rad, USA).

The amplified PCR product from the patient was excised from the gel and purified using FavorPrep GEL/PCR Purification Kit (FAVORGEN Biotech Corp, Taiwan) according to the manufacturer instructions and sent for sequencing with Tb11 and Tb12 primers (Apical Scientific Sdn. Bhd., Malaysia).

Gene Alignment and Bioinformatic Analysis

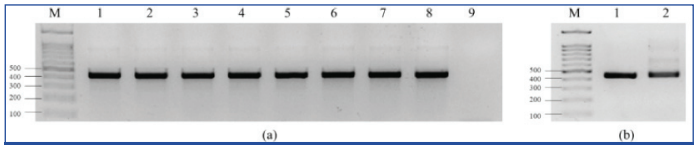
The forward and reverse gene sequences obtained from sequencing were aligned using BioEdit software [21] and the National Center for Biotechnology (NCBI) Basic Local Alignment Search Tool (BLAST) software under blastn program [22] was used for bacterial identification. The sequence similarity between Tb11 and Tb12 primers with the identified bacteria was studied to determine the specificity of the primers using Primer-BLAST [23].

RESULTS

Sputum Analysis with Xpert MTB/RIF and *hsp65*-PCR

The study of the patient's sputum with Xpert MTB/RIF assay showed a negative result, suggesting non-Mycobacterium Tuberculosis Complex (MTBC) infection.

Tb11 and Tb12 primers specifically amplified the reference MTB and Non-Tuberculous Mycobacterial (NTM) DNA with the detection of a 441 bp *hsp65* gene fragment [Table/Fig-1a]. Analysis of the DNA extracted from patient's sputum also showed a positive amplification with similar characteristic [Table/Fig-1b], suggesting NTM infection or an Xpert MTB/RIF false negative result.



[Table/Fig-1]: a) Amplification of DNA from *Mycobacterium* reference strains with Tb11 and Tb12 primers showed positive amplification with band size of 441 bp. M=DNA markers, 1=MTB H37Rv, 2=*Mycobacterium bovis* BCG Pasteur strain TMC 1011, 3=*Mycobacterium avium* subsp. *paratuberculosis* strain K-10, 4=*Mycobacterium avium* subsp. avium strain 2285, 5=*Mycobacterium intracellulare* strain 1956, 6=*Mycobacterium kansasii* strain 824, 7=*Mycobacterium simiae* strain MO-323, 8=*Mycobacterium abscessus* strain MA 1948, 9=Negative control; b) Amplification of DNA extracted from patient's sputum with Tb11 and Tb12 primers showed positive amplification with same band size with MTB H37Rv. M=DNA markers, 1=MTB H37Rv, 2=Patient's sputum.

Bioinformatic Analysis of the Amplified PCR Product from Patient

The DNA sequence alignment of the amplified PCR product from patient with BioEdit showed that the amplified gene fragment had 438 bp.

The bioinformatic analysis with blastn showed that the amplified product was closely related to *Rothia mucilaginosa* DY-18, with 100% query match and 98.17% of identity with eight nucleotide mismatches and E-value (expected value) of 0.0 [Table/Fig-2].

Rothia mucilaginosa DY-18 DNA, complete genome

Sequence ID: AP011540.1 Length: 2264603 Number of Matches: 2

Range 1: 552975 to 553412 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
755 bits(836)	0.0	430/438(98%)	0/438(0%)	Plus/Plus

```
Query  1       ACCATCGATGGTGTGTCCATTGCCCGCGAGATTGAACTGGACGACCCGTACGAGAACCTG  60
               |||| ||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  552975  ACCAACGACGGCGTGTCCATTGCCCGCGAGATTGAACTGGACGACCCGTACGAGAACCTG  553034

Query  61      GGTGCCCAGCTGGCGAAGGAAGTCGCCACCAAGACCAATGACGTTGCCGGTGACGGCACC  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553035  GGTGCCCAGCTGGCGAAGGAAGTCGCCACCAAGACCAATGACGTTGCCGGTGACGGCACC  553094

Query  121     ACCACCGCAACCGTGCTCGCGCAGGCGCTGGTGAACGAGGGCCTGCGCAACGTGACCAGC  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553095  ACCACCGCAACCGTGCTCGCGCAGGCGCTGGTGAACGAGGGCCTGCGCAACGTGACCAGC  553154

Query  181     GGTGCGGCTCCCGCCGACGTCAAGCGCGGCATCGAGGCGGCCGTGGAGGCTGTCTCCGCA  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553155  GGTGCGGCTCCCGCCGACGTCAAGCGCGGCATCGAGGCGGCCGTGGAGGCTGTCTCCGCA  553214

Query  241     CGCCTGCTCGAGAACGCACGTCCCGTGCAGGGTCCCGAGGTGGCGCACGTCGCCGCTATT  300
               |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  553215  CGCCTGCTCGAGAACGCACGTCCCGTGCAGGGCCCCGAGGTGGCGCACGTCGCCGCTATC  553274

Query  301     TCCGCGCAGTCCGAGCAGATTGGTGAGCTGCTGGCTCGCGCCTTCGAGAAGGTCGGCCAG  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553275  TCCGCGCAGTCCGAGCAGATTGGTGAGCTGCTGGCTCGCGCCTTCGAGAAGGTCGGCCAG  553334

Query  361     GACGGCGTTATCACCATCGAGGACGGTTCCTCCACCGAGATTGAGCTGGAGATCACCGAG  420
               |||||||| |||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  553335  GACGGCGTCATCACCATCGAGGACGGTTCCTCCACTGAGATTGAGCTGGAGATCACCGAG  553394

Query  421     GGTATGCGGTTCGACAAG  438
               ||||||| ||||||||||
Sbjct  553395  GGTATGCAGTTCGACAAG  553412
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[Table/Fig-2]: Analysis of the 438 bp gene fragment showing 100% query match and 98% similarity with *Rothia mucilaginosa* DY-18 whole genome (Image extracted from NCBI BLAST).

The top 20 sequences producing significant alignments with the *hsp65*-PCR amplified sequence in this study has been shown in [Table/Fig-3].

The analysis of the primers' specificities with *Rothia mucilaginosa* DY-18 showed that the 20 bp TB11 primer has 18 similar nucleotides and two mismatches, while the reverse Tb12 primer has 19 similar nucleotides with one mismatch, based on the alignment of the Tb11 (forward primer) and Tb12 (reverse primer) with the *Rothia mucilaginosa* DY-18 whole genome DNA sequence (template) [Table/Fig-4]. According to the results of molecular study, the strain identified was *Rothia mucilaginosa*. Since this case was regarded as non-mycobacterial infections, the patient was not treated at specialised TB clinic and was referred to a nearby general health clinic for further management.

DISCUSSION

Diagnosis of TB via conventional sputum smear microscopy has low sensitivity, compared to culture and molecular methods [24]. Molecular detection of mycobacteria from direct clinical specimens has significantly reduced the time of detection [24]. Tb11 and Tb12 primers have been commonly used for direct *hsp65* gene amplification in clinical samples and pure culture for the detection of mycobacteria [17,25,26]. However, in this study, *Rothia mucilaginosa* was identified using *hsp65*-PCR diagnostic method, showing a false positive identification, which could wrongly be interpreted as an NTM infection or an Xpert MTB/RIF false negative result.

The analysis of the top 20 sequences producing significant alignments with the *hsp65*-PCR amplified sequence in present study showed that *Rothia mucilaginosa* DY-18 had the highest identity and lowest E-value, which supported the identification of this strain with high degree of confidence. The E value obtained for *Rothia mucilaginosa* DY-18 (0.0) means that zero sequences can be expected to match better [27].

Rothia mucilaginosa, is part of the normal flora residing in the human oropharynx and upper respiratory tract [28]. It is recognised

#	Description	Max score	Total score	Query cover (%)	E-value	Per. ident (%)	Accession
1	*Rothia mucilaginosa* DY-18 DNA, complete genome	755	951	100	0.0	98.17	AP011540.1
2	*Rothia mucilaginosa* strain FDAARGOS_369 chromosome, complete genome	628	802	100	6e-176	91.78	CP023510.1
3	*Rothia mucilaginosa* DNA, complete genome, strain: NUM-Rm6536	628	780	100	6e-176	91.78	AP014938.1
4	*Kocuria* sp. BT304 chromosome, complete genome	444	661	100	5e-120	82.42	CP030039.1
5	*Kocuria rhizophila* strain NCTC8340 genome assembly, chromosome: 1	439	666	100	6e-119	82.19	LR134409.1
6	*Kocuria rhizophila* strain FDAARGOS_302 chromosome, complete genome	439	666	100	6e-119	82.19	CP022039.2
7	*Kocuria rhizophila* DC2201 DNA, complete genome	439	666	100	6e-119	82.19	AP009152.1
8	*Kocuria indica* strain CE7 chromosome, complete genome	421	643	100	2e-113	81.28	CP035504.1
9	*Kocuria* sp. KD4 chromosome, complete genome	416	625	100	7e-112	81.05	CP050449.1
10	*Arthrobacter* sp. 11W110_air genome assembly PRJEB5507_assembly_1, scaffold CONTIG000002	416	416	100	7e-112	81.05	LN483071.1
11	*Sinomonas atrocyanea* strain KCTC 3377, complete genome	412	631	100	8e-111	81.06	CP014518.1
12	*Kocuria rosea* strain ATCC 186 chromosome, complete genome	411	613	100	3e-110	81.09	CP035103.1
13	*Kocuria rosea* strain NCTC7512 genome assembly, chromosome: 1	411	613	100	3e-110	81.09	LR134487.1
14	*Kocuria rosea* strain NCTC7514 genome assembly, chromosome: 1	411	613	100	3e-110	81.09	LR134391.1
15	*Rothia dentocariosa* strain NCTC10918 genome assembly, chromosome: 1	410	573	100	3e-110	81.02	LR134521.1
16	*Rothia aeria* DNA, complete genome	407	575	100	3e-109	80.83	AP017895.1
17	*Rothia dentocariosa* strain NCTC10207 genome assembly, chromosome: 1	406	579	100	1e-108	80.79	LR134479.1
18	*Kocuria flava* strain HO-9041, complete genome	397	597	100	6e-106	80.68	CP013254.1
19	*Rothia dentocariosa* ATCC 17931, complete genome	397	560	100	6e-106	80.32	CP002280.1
20	*Arthrobacter* sp. QXT-31, complete genome	385	600	100	1e-102	79.45	CP019304.1

[Table/Fig-3]: Top 20 sequences producing significant alignments with the *hsp65*-PCR amplified sequence in this study (438bp) (Data extracted from NCBI BLAST).
Per: ident-percentage identification

>AP011540.1 Rothia mucilaginosa DY-18 DNA, complete genome

```
product length = 438
Forward primer  1       ACCAACGATGGTGTGTCCAT  20
Template        552975  ........C..C........  552994

Reverse primer  1       CTTGTCGAACCGCATACCCT  20
Template        553412  ..........T.........  553393
```

[Table/Fig-4]: Tb11 and Tb12 primer sequence similarity with *Rothia mucilaginosa* DY-18 complete genome (Image extracted from Primer-BLAST).

as opportunistic pathogen in immuno-compromised individuals, resulting in pneumonia [28].

The non-specific amplification obtained with the *hsp65*-PCR assay used can be explained by high similarity of the primers used with the *Rothia mucilaginosa* gene sequence, with few mismatches to the middle of the primers. It is considered that a target can be amplified even if there are few mismatches in the middle or towards the 5' end of the primers sequence [23]. According to the general rules in specific primer design, two base mismatches at the 3' end are generally required to prevent non-specific amplification [23].

A study by Randima GDD et al., to detect mycobacteria in elephant nasal secretions collected from trunk wash samples, using the same set of primers of present study, showed poor sensitivity in mycobacteria detection and DNA sequencing of the amplified products showed the best match with *Rothia dentocariosa* [29].

Busatto C et al., used *hsp65* as a marker (primer sequences not disclosed) for detection of *Mycobacterium avium* in suspected-TB patients' DNA samples that were IS6110-PCR negative. The sequencing results of positive *hsp65* amplifications showed the presence of not only mycobacteria (MTBC, *Mycobacterium avium* and *Mycobacterium monacense*), but also other organisms such as *Rhodococcus* spp., *Rothia mucilaginosa*, *Gordonia* spp., *Cryobacterium* spp., *Streptomyces* spp., *Nocardia* spp., and *Corynebacterium* spp., suggesting the low accuracy of the diagnostic technique [30].

Sarti E et al., using the same primers, reported the amplification of *Bifidobacterium crudilactis, Kocuria rhizophila* and *Kocuria palustris* in cheese samples [31]. The presence of *Kocuria* spp., had been reported in the skin and mucosa of animals and human [32], and this could be another source of false positive result when *hsp65*-PCR is used for identification of mycobacteria.

Kim BJ et al., using different primers, designed a *hsp65* nested PCR-direct sequencing method to increase the sensitivity for species identification from sputum, which according to the primers design avoid *Rothia* spp. amplification [33]. The use of this method could be an alternative to eliminate the possibility of *Rothia* spp. amplification.

Limitation(s)

The culture method (gold standard) for detection of mycobacteria either on Löwenstein-Jensen medium or in Mycobacteria Growth Indicator Tube (MGIT) was not used in this study due to limited sample availability.

CONCLUSION(S)

The present study showed the possibility of false positive results in clinical specimens using PCR-*hsp65* primers considered specific for mycobacteria, therefore, this test should be used in clinical samples with caution and it is suggested the need of its further re-optimisation.

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REFERENCES

[1] WHO. Fact sheets: Tuberculosis. Geneva: World Health Organisation, Geneva. 2018. Available from: http://www.who.int/en/news-room/fact-sheets/detail/tuberculosis.
[2] Brode SK, Daley CL, Marras TK. The epidemiologic relationship between tuberculosis and non-tuberculous mycobacterial disease: A systematic review. Int J Tuberc Lung Dis. 2014;18(11):1370-77.
[3] Hu C, Huang L, Cai M, Wang W, Shi X, Chen W. Characterization of non-tuberculous mycobacterial pulmonary disease in Nanjing district of China. BMC Infect Dis. 2019;19(1):764.
[4] Denkinger CM, Kik SV, Pai M. Robust, reliable and resilient: Designing molecular tuberculosis tests for microscopy centers in developing countries. Expert Rev Mol Diagn. 2013;13(8):763-67.

[5] WHO. Policy update: Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF system for the diagnosis of pulmonary and extrapulmonary TB in adults and children. Geneva: World Health Organisation. 2013. Available from: http://apps.who.int/iris/handle/10665/112472.
[6] WHO. The use of loop-mediated isothermal amplification (TB-LAMP) for the diagnosis of pulmonary tuberculosis: policy guidance. Geneva: World Health Organisation. 2016. Available from: https://www.who.int/tb/publications/lamp-diagnosis-molecular/en/.
[7] Ling DI, Zwerling AA, Pai M. GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: A meta-analysis. Eur Respir J. 2008;32(5):1165-74.
[8] Adjemian J, Daniel-Wayman S, Ricotta E, Prevots DR. Epidemiology of nontuberculous mycobacteriosis. Semin Respir Crit Care Med. 2018;39(3):325-35.
[9] Nathavitharana RR, Hillemann D, Schumacher SG, Schlueter B, Ismail N, Omar SV, et al. Multicenter noninferiority evaluation of Hain GenoType MTBDRplus Version 2 and Nipro NTM+MDRTB line probe assays for detection of rifampin and isoniazid resistance. J Clin Microbiol. 2016;54(6):1624-30.
[10] Singh AK, Maurya AK, Umrao J, Kant S, Kushwaha RA, Nag VL, et al. Role of GenoType((R)) *Mycobacterium* Common Mycobacteria/Additional Species Assay for rapid differentiation between *Mycobacterium tuberculosis* complex and different species of non-tuberculous mycobacteria. J Lab Physicians. 2013;5(2):83-89.
[11] Lee MR, Cheng A, Huang YT, Liu CY, Chung KP, Wang HC, et al. Performance assessment of the DR. TBDR/NTM IVD kit for direct detection of *Mycobacterium tuberculosis* isolates, including rifampin-resistant isolates, and nontuberculous Mycobacteria. J Clin Microbiol. 2012;50(10):3398-401.
[12] Kim SH, Shin JH. Identification of nontuberculous mycobacteria using multilocous sequence analysis of 16S rRNA, *hsp65*, and *rpoB*. J Clin Lab Anal. 2018;32(1):e22184.
[13] Joao I, Cristovao P, Antunes L, Nunes B, Jordao L. Identification of nontuberculous mycobacteria by partial gene sequencing and public databases. Int J Mycobacteriol. 2014;3(2):144-51.
[14] Ong CS, Ngeow YF, Yap SF, Tay ST. Evaluation of PCR-RFLP analysis targeting *hsp65* and *rpoB* genes for the typing of mycobacterial isolates in Malaysia. J Med Microbiol. 2010;59(Pt 11):1311-16.
[15] Kumar P, Benny P, Jain M, Singh S. Comparison of an in-house multiplex PCR with two commercial immuno-chromatographic tests for rapid identification and differentiation of MTB from NTM isolates. Int J Mycobacteriol. 2014;3(1):50-56.
[16] Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol. 1993;31(2):175-78.
[17] Zakham F, Bazoui H, Akrim M, Lemrabet S, Lahlou O, Elmzibri M, et al. Evaluation of conventional molecular diagnosis of *Mycobacterium tuberculosis* in clinical specimens from Morocco. J Infect Dev Ctries. 2012;6(1):40-45.
[18] Blakemore R, Story E, Helb D, Kop J, Banada P, Owens MR, et al. Evaluation of the analytical performance of the Xpert MTB/RIF assay. J Clin Microbiol. 2010;48(7):2495-501.
[19] Kaneka Corporation. Kaneka Easy DNA Extraction kit version 2. 2019. Available from: https://fnkprddatablobcorewindowsnet/export/data/datasheet/KNK/KN-T110005_epdf.
[20] Takara Bio USA, Inc. PrimeSTAR GXL DNA Polymerase: Product manual. 2019. Available from: https://wwwtakaracokr/file/manual/pdf/R050A_ev1906Dapdf.
[21] Hall T. BioEdit: An important software for molecular biology. GERF Bulletin of Biosciences. 2011;2(1):60-61.
[22] Wheeler D, Bhagwat M. BLAST QuickStart: Example-driven web-based BLAST tutorial. Methods Mol Biol. 2007;395:149-76.
[23] Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 2012;13:134.
[24] Afsar I, Gunes M, Er H, Gamze Sener A. Comparison of culture, microscopic smear and molecular methods in diagnosis of tuberculosis. Rev Esp Quimioter. 2018;31(5):435-38.
[25] Gopinath K, Singh S. Multiplex PCR assay for simultaneous detection and differentiation of *Mycobacterium tuberculosis*, *Mycobacterium avium* complexes and other Mycobacterial species directly from clinical specimens. J Appl Microbiol. 2009;107(2):425-35.
[26] Liu H, Lian L, Jiang Y, Huang M, Tan Y, Zhao X, et al. Identification of species of nontuberculous mycobacteria clinical isolates from 8 provinces of China. Biomed Res Int. 2016;2016:2153910.
[27] Foox J. What is e-value? 2015. Available from: https://jonfooxwordpresscom/2015/10/07/what-is-e-value/.
[28] Maraki S, Papadakis IS. *Rothia mucilaginosa* pneumonia: A literature review. Infect Dis (Lond). 2015;47(3):125-29.
[29] Randima GDD, Abeysinghe KGDDA, Vandercone RPG, Bamunuarachchige TC. Screening of Asian elephants (Elephas maximus) in captivity at elephant orphanage, Pinnawala and ETH, Udawalawe for *Mycobacterium tuberculosis* and elephant endotheliotropic herpes virus type1 (EEHV 1) using direct amplification of pathogen DNA from trunk washes. Advances in Bio-Informatics, Bio-Technology and Environmental Engineering- ABBE 2016. 2016:27-32.
[30] Busatto C, Silva ABS, Groll AV, Ramos D, da Silva PEA, Ramis IB. Identification of *Mycobacterium avium* among suspected tuberculosis.2016. Available from: https://online.unisc.br/acadnet/anais/index.php/CBIPS/article/view/15975.
[31] Sarti E, Rodrigues R, Duarte L, Bacanelli G, Lilenbaum W, Araújo F. Detection of potentially pathogenic non-tuberculous mycobacteria in artisanal Coalho cheese from the state of Paraiba, Northeast Brazil. Mycobact Dis. 2018;8(3):1000266.
[32] Kandi V, Palange P, Vaish R, Bhatti AB, Kale V, Kandi MR, et al. Emerging bacterial infection: Identification and clinical significance of Kocuria species. Cureus. 2016;8(8):e731.
[33] Kim BJ, Park JH, Lee SA, Kim H, Cha CY, Kook YH, et al. Differentiation of mycobacteria in sputa by duplex polymerase chain reaction for mycobacterial *hsp65* gene. Diagn Microbiol Infect Dis. 2008;62(2):193-98.

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