Journal of Advances in Microbiology

6(1): 1-7, 2017; Article no.JAMB.36783
ISSN: 2456-7116

RpoB Gene-Based Characterization of Non-Tuberculous Mycobacteria in Zimbabwe

Wadzanai Manjeese[1,2*], Boniface Muzividzi[3], Joshua Mbanga[1], Jonathan Mufandaedza[2] and Nyasha Chin'ombe[4]

[1]Department of Applied Biology and Biochemistry, National University of Science and Technology (NUST), P.O.Box AC 939, Ascot, Bulawayo, Zimbabwe.
[2]Department of Technical, National Biotechnology Authority, 21 Princess Drive, Newlands, Harare, Zimbabwe.
[3]National Microbiology Reference Laboratory, Harare Central Hospital, P.O.Box ST 14, Southerton, Harare, Zimbabwe.
[4]Molecular Microbiology Laboratory, Department of Medical Microbiology, University of Zimbabwe, P.O.Box A178, Avondale, Harare, Zimbabwe.

Authors' contributions

This work was carried out in collaboration between all authors. Author NC designed the study. Author BM isolated the bacteria and performed DNA extractions. Authors WM and NC performed RpoB gene amplification and the bioinformatics analysis of the results. Author WM wrote the protocol and the first draft of the manuscript. Authors Joshua Mbanga and Jonathan Mufandaedza managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2017/36783
Editor(s):
(1) Foluso O. Osunsanmi, Department of Biochemistry and Microbiology, University of Zululand, South Africa.
Reviewers:
(1) Mohan Khadka, Tribhuvan University Teaching Hospital, Nepal.
(2) Ketan Vagholkar, D. Y. Patil University School of Medicine, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/21444

Original Research Article

Received 15th September 2017
Accepted 12th October 2017
Published 17th October 2017

ABSTRACT

Aims: To characterize archived nontuberculous Mycobacteria (NTM) samples previously isolated from humans across Zimbabwe during the 2014 national tuberculosis survey. The *rpoB* gene of the isolates was targeted for characterization.
Study Design: The research was an observational study where NTM samples previously isolated from Zimbabwean population were analysed by *rpoB* gene sequencing and phylogeny to identify NTM species.

**Corresponding author: E-mail: wadzymanjeese@gmail.com;*

Place and Duration of Study: Department of Medical Microbiology, University of Zimbabwe between September 2015 and July 2016.
Methodology: We obtained 99 NTM DNA samples from 963 NTM at NMRL, which we characterised using *rpoB* gene analysis after PCR amplification. The amplicons were sequenced and bioinformatics tools were used for speciation. The *rpoB* gene of DNA extracts from the NTM was amplified and the sequences were analysed using bioinformatics tools to identify the NTM to species level.
Results: From the 99 NTM isolates, 40 were sequenced and analyzed. The NTM were identified as belonging to 13 species. The species were *M. palustre* (14.8%), *M. aroisense* (29.6%), *M. parascrofulaceum* (3.7%), *M. arupense* (7.4%), *M. asiaticum* (3.7%), *M. malmoense* (3.7%), *M. lacus* (3.7%), *M. avium* (7.4%), *M. nonchromogenicum* (3.7%), *M. gordonae* (3.7%), *M. aromaticivorans* (3.7%), *M. novocastrense* (3.7%), *M. bourgelatii* (3.7%). One sample (3.7%) belonged to *Mtb complex species* (3.7%) and another one (3.7%) was closely related to *S. oryzae*. The most common species belonged to *M. aroisense* and *M. palustre*. The species showed a high degree of *rpoB* sequence diversity. Sequence analysis of the rifampicin resistance determining region (RRDR) showed the existence of only silent mutations.
Conclusion: Species of NTM from Zimbabwe showed a high degree of *rpoB* gene sequence diversity. This characteristic feature can therefore be used in diagnosis and identification of NTM in clinical specimens.

Keywords: rpoB gene; nontuberculous mycobacteria; sequencing; alignments; RRDR; Mutations.

ABBREVIATIONS

MAC: Mycobacterium avium-intracellulare complex; NTM: nontuberculous mycobacteria; Mtb: Mycobacterium tuberculosis; NMRL: National Microbiology Reference Laboratory; Mtb: Mycobacterium tuberculosis; RRDR: rifampicin resistance determining region.

1. INTRODUCTION

Nontuberculous mycobacteria (NTM) are diverse and ubiquitous acid-fast microorganisms that are found in several environments [1]. In recent years, several species of NTM have been found to be opportunistic human pathogens in both immunocompromised and immunocompetent individuals [2]. The diagnosis of infection by these NTM and identification of individual species are still challenging. In most low-resource countries such as Zimbabwe, microscopy is used for diagnosis of infection by mycobacteria including *Mycobacterium tuberculosis*. However, most phenotypic methods such as microscopy cannot distinguish the different NTM species. Molecular methods such as gene sequencing are becoming useful in diagnosis and identification of microorganisms to species level [3]. The 16S rRNA gene sequencing has been popularly used in the identification of several NTM species [4]. Other genes such as *hsp*65, *sod*A, *rec*A and *rpo*B have also recently been explored in characterization and identification of NTM [5]. The sequencing of the *rpoB gene,* which encodes the β subunit of RNA polymerase, has been very useful for the phylogenetic characterization of NTM [6]. No previous studies have investigated the clinical utility of using *rpoB* gene sequencing in characterizing and identifying NTM species in Zimbabwe. In this pilot study, we therefore set out to characterize NTM isolates in Zimbabwe by *rpoB* gene amplification and sequencing.

2. MATERIALS AND METHODS

2.1 Archived NTM Isolates

The archived NTM isolates used in this study were retrieved at the National Microbiology Reference Laboratory, Harare. They were isolated throughout Zimbabwe during the national tuberculosis survey of 2014 [7]. The bacterial samples were retrieved, cultured and DNA extracted as previously described [7].

2.2 Amplification of *rpo*B gene, Sequencing and Bioinformatics

The *rpoB* gene of NTM from the extracted DNA samples was amplified by polymerase chain reaction using *Mycobacterium* specific forward

primer, 5´-GCTGATCCARAACCAGATCCG-3´ and reverse primer 5´-CTCGATGAACCCGAACGGGT-3´. Amplification was performed according to the following cycling profile: initial incubation at 94°C for 5 minutes, 35 cycles of 30 seconds denaturation at 94°C, 1 minute annealing at 55°C, 30 seconds elongation at 72°C and a final extension of 5 minutes at 72°C. The NTM *rpoB* gene amplicons (10 µl) were analyzed by gel electrophoresis. Samples with strong gel bands of approximately 392 bp were selected and sent for DNA sequencing at Inqaba Biotechnical Industries, South Africa.

2.3 Analysis of DNA Sequencing Data

DNA sequences were analyzed using common bioinformatics softwares such as Geneious Basic program (Biomatters, USA), Chromas Lite, BioEdit, MAFFT and BLAST programs. Different NTM species were identified by comparison of the alignment with sequences in the Genbank. Phylogenetic analysis of the DNA sequences of *rpoB* gene was performed using the Geneious Basic program.

3. RESULTS

During the national tuberculosis prevalence survey conducted throughout in Zimbabwe in 2014, 963 NTM samples were isolated and kept at the National Microbiology Reference Laboratory [7]. In this study, we extracted DNA from 99 of the bacterial isolates and performed amplification of the *rpoB* gene. Only 59 (59.6%) of the samples showed positive PCR results with an expected band of approximately 392 bp (Fig. 1). Nineteen samples (19.2%) also showed positive results but with faint bands of the expected 392 bp on agarose gel. Negative results were shown by 21 samples (21.2%) that did not amplify and there were no bands produced on agarose gel. In the amplification, samples designated W1, W2, W3, W4 and W5 were included and these were isolates of *Mycobacterium tuberculosis* (Mtb). The five samples (W1-W5) were used as positive PCR controls and showed amplification of *rpoB* gene on gel electrophoresis (results not shown). Forty (40) amplicons from the 59 positives were sequenced.

Bioinformatics analysis using Chromas Lite and the Geneious 9.0.5 program showed that only 27 sequences (36.5%) (designated R1, R2, R3, R6, R7, R9, R10, R11, R12, R14, R15, R16, R18, R19, R21, R25, R26, R27, R28, R29, R30, R31, R34, R35, R36, R37 and R40) of the total 40 sequences could be analysed and used for phylogenetic characterization. The other 13 sequences (32.5%) were not eligible for further analysis because they showed DNA sequences that were either too short for analysis or the chromatograms had “noisy” sequence peaks with low quality scores. The 27 sequences were aligned with sequences in the Genbank using the BLAST tool in Geneious program and identified (Table 1, Fig. 2). The identified species were *M. aroisense*, *M. parascrofulaceum*, *M. palustre*, *M. arupense*, *M. asiaticum*, *M. malmoense*,

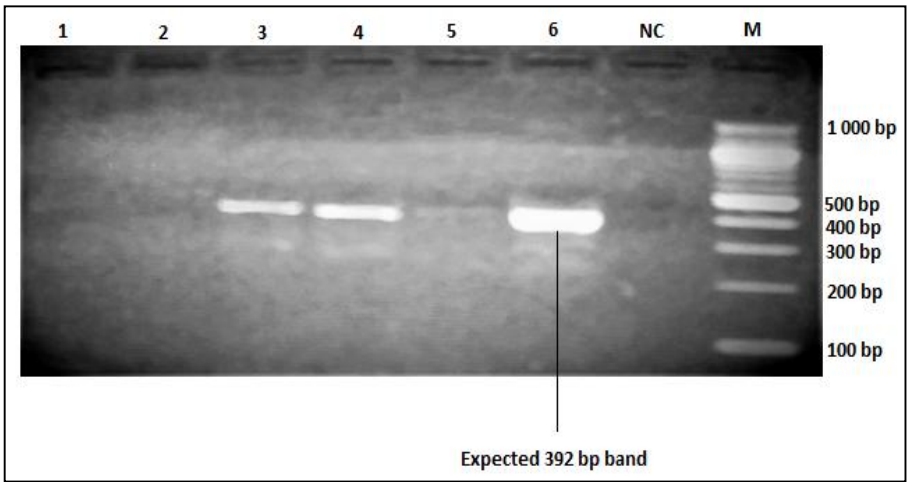

Fig. 1. A representative gel of *rpoB* gene amplification from NTM isolates. M is the molecular weight marker (Promega™, Wisconsin); lanes 3, 4 and 6 show positive NTM *rpoB* gene amplicons, lanes 1 and 2; no amplification. Lane 5; feint bands. Lane NC; negative PCR control. The expected PCR band for *rpoB* gene was approximately 392 bp.

M. lacus, *M. avium*, *M. nonchromogenicum*, *M. aromaticivorans*, *M. novocastrense*, *M. gordonae* and *M. bourgelatii* (Table 1). Sample R30 could not be identified using Blast N because it aligned 97.8% with *M. gordonae* and *M. bourgelatii* strains from Genbank. Using Blast X tool, the protein coded for by R30 aligned 100% with *M. gordonae*. All sequences needed a further analysis since they had an alignment of less than 100% with sequences in Genbank (using BlastN). A non-mycobacteria species was identified in sample R11 as belonging to *Streptacidiphilus oryzae*.

To investigate the genetic relatedness of the NTM isolates, a phylogenetic tree was constructed (Fig. 2). The tree was rooted using the Mtb control sequences (W1-W5, H37Rv). The NTM sequences were clustering into about 5 groups based on their genetic relatedness (Fig. 2). R29 sequences were almost the same as those of the positive controls and could be one of the *Mtb* complex members such as *Mycobacterium bovis*, *M. Tuberculosis*, *M. Africanum, M. Orygis, M. Microti, M. Canetti, M. Caprae, M. Pinnipedii, M. suricattae and M. mungi.* The sequences of all the isolates showed great diversity even within the same species (Figs. 2 and 3).

4. DISCUSSION

NTM species have been linked to diseases in humans such as pulmonary disease, soft tissue infections and skin abscesses [8]. Some of the species of NTM associated with human disease are *M. avium*, *M. kansasii*, *M. intracellulare, M. paratuberculosis*, *M. scrofulaceum*, *M. simiae*, *M. interjectum*, *M. xenopi*, *M. szulgai*, *M. fortuitum*, *M. immunogenum*, *M .chelonae*, *M. marinum*, *M. malmoense*, *M. ulcerans*, *M. smegmatis*, *M. goodii*, *M. neoaurum*, *M. palustre*, *M. elephantis*, and *M. septicum* and *M. nonchromogenicum* [9]. *RpoB* gene sequencing has been previously used for the molecular identification of different bacterial genera, including *Mycobacterium* [5,6]. In this study, we identified 27 isolates as belonging to 13 known *Mycobacterium* species (Table 1). Some of the species identified have previously been associated with diseases in humans. The most commonly isolated NTM species in our study was *M. aroisense* (29.6%) followed by *M. palustre* (14.8%). Other NTM species identified in this study included, *M. arupense* (7.4%), *M. aromaticivorans* (3.7%), *M. bourgelatii* (3.7%), *M. novocastrense* (3.7%), *M. gordonae* (R30) and *M. asiaticum* (3.7%) (Table 1). *M. avium* and *M. aroisense* (MAC members) have been associated with most pulmonary NTM infections especially in immunocompromized patients [10]. *M. palustre* is generally isolated from environmental sources such as water bodies and veterinary samples, and is associated with paediatric lymphadenitis [11]. The *M. lacus* species has been associated with bone and joint infections [12]. *M. arupense* has been associated with tenosynovitis and is now regarded as an emerging pathogen [13]. *M. asiaticum* has been

Table 1. Identification of NTM species using *rpoB* gene sequencing

Mycobacterium species	# of strains identified	Sample sequence code (% identity)
M. palustre	4 (14.8%)	R1 (97.3%), R2 (97.0%), R16 (99.5%), R27 (99%)
M. aroisense	8 (29.6%)	R3 (97.8%), R6 (98.7%), R9 (98.1), R10 (97.5%), R25 (98.7%), R28 (99.1%), R34 (98.8%), R36 (99.3%)
M. parascrofulaceum	1 (3.7%)	R7 (99.2%)
M. arupense	2 (7.4%)	R12 (98.6%), R26 (98.7%)
M. asiaticum	1 (3.7%)	R14 (98.3%)
M. malmoense	1 (3.7%)	R15 (98.2%)
M. lacus	1 (3.7%)	R18 (98.6%)
M. avium	2 (7.4%)	R19 (99.6%), R31 (99.5%)
M. nonchromogenicum	1 (3.7%)	R21 (98.2%)
Mtb complex (eg *M. bovis*)	1 (3.7%)	R29 (99.8%) *
M. gordonae	1 (3.7%)	R30 (97.1%) **
M. aromaticivorans	1 (3.7%)	R35 (99.6%)
M. novocastrense	1 (3.7%)	R37 (92.5%)
M. bourgelatii	1 (3.7%)	R40 (99.1%)
S. oryzae	1 (3.7%)	R11 (94.3%)*

**Non-mycobacterium species, ** sequence aligned with more than 1 Genbank sequence using Blast N tool*

found to be associated with keratitis, lyphadenitis, bursitis and wound infections [14]. *M. parascrofulaceum*, *M. malmoense*, *M. nonchromogenicum*, *M. gordonae and* M. novocastrense are known to cause pulmonary infections [15-19]. *M. aromaticivorans* is found in the soil and has not previously been associated with human disease [20]. *M. bourgelatii* was previously isolated only in cattle and its clinical relevance in humans is uncertain [21]. We also identified R29 to be a Mtb complex member and not an NTM member (Table 1), this shows that the sample was previously wrongly characterised during culturing, Ziehl Neelsen's (ZN) smear microscopy and biochemical identification of isolates were carried out at NMRL prior to commencement of this study. We identified R11 as *S. oryzae* through *rpoB* gene sequencing. However, the *rpoB* sequence similarity was only 94.3% and hence it was not likely to be *S. oryzae*. Since the sequence clustered with other samples, it could just be an unidentified NTM species with no sequence homologies in Genbank.

NTM infections are treated by drug therapy. The treatment regimens vary according to the species causing the infection. Patients with pulmonary MAC are usually treated with multidrug regimens including rifampicin. In Mtb, almost all of the Rifampicin resistant clinical isolates carry a mutation in the *rpoB* gene. Nucleotide changes within this region are very rare, hence changes denote resistance to Rifampicin. In our findings, all mutations causing rifampicin drug resistance were found to be silent because they were mostly in the last nucleotide of a codon hence amino acid sequences were highly conserved. This suggested that the NTM species have not yet developed rifampicin drug resistance because they have not yet been exposed to the drug for a long time.

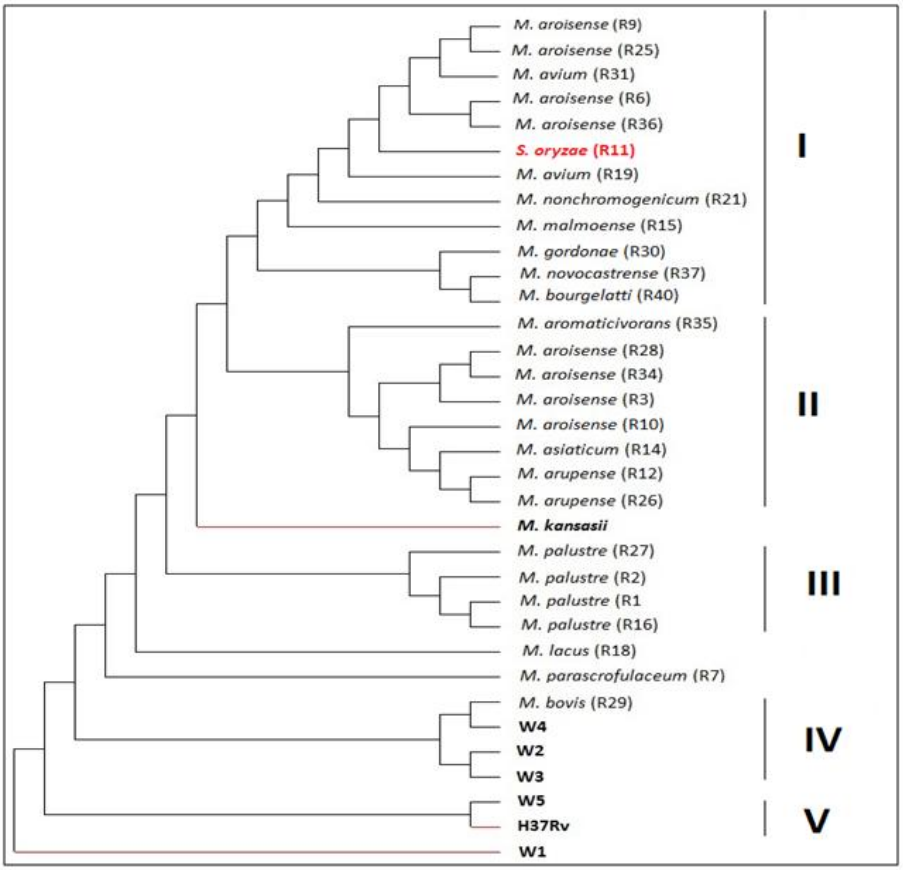

Fig. 2. Phylogenetic tree of NTM species. Tree was rooted with *Mtb* Complex species (W1) which was one of the control samples in the study. The sequence of *Mycobacterium tuberculosis*, H37Rv was also used as a positive control in the analysis.

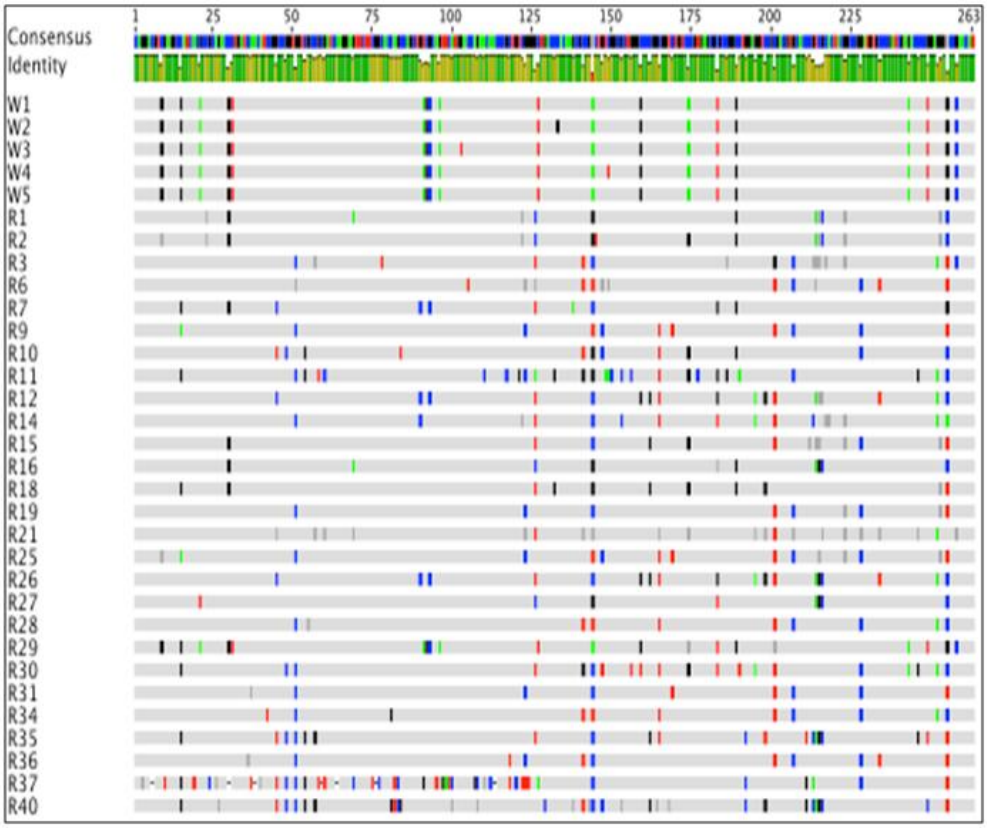

Fig. 3. Genetic diversity of NTM isolates from Zimbabwe. All the NTM sequences were aligned and the hypervariable and conserved sections of the rpoB gene were shown. The grey areas represent the conserved regions, the hypervariable regions are represented by blue (Cytosine), green (Adenine), red (Thiamine) and black (Guanine) regions in the rpoB gene

The main limitation of this study was that only a small sample size of NTM isolates was used. Although there were more than 900 NTM isolates archived at the NMRL, limited resources only forced us to analyze few samples.

5. CONCLUSION

This study revealed the different types of NTM species found in clinical samples in Zimbabwe. NTM species were characterised as belonging to 13 different *Mycobacterium* species. The species of the NTM showed a high degree of *rpoB* gene sequence diversity and this characteristic feature can therefore be used in future in diagnosis and identification of NTM in clinical specimens.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Falkinham JO. 3rd Current Epidemiologic Trends of the nontuberculous mycobacteria (NTM). Curr Environ Health Rep. 2016;3(2):161-7.
2. Ryu YJ, Koh WJ, Daley CL. Diagnosis and treatment of nontuberculous mycobacterial lung disease: Clinicians' Perspectives. Tuberc Respir Dis. 2016;79(2):74-84.
3. Trotha R, Hanck T, König W, König B. Rapid ribosequencing-an effective diagnostic tool for detecting microbial infection. Infection. 2001;29(1):12-6.
4. Slany M, Pavlik I. Molecular detection of nontuberculous mycobacteria: Advantages and limits of a broad-range sequencing approach. J Mol Microbiol Biotechnol. 2012;22(4):268-76.
5. Ade´kambi T, Drancourt M. Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by

16s rRNA, *hsp*65, *sod*A, *rec*A and *rpo*B gene sequencing. International Journal of Systematic and Evolutionary Microbiology. 2004;54:2095-2105.

6. de Zwaan R, van Ingen J, van Soolingen D. Utility of rpoB gene sequencing for identification of nontuberculous mycobacteria in the Netherlands. J Clin Microbiol. 2014;52(7):2544-51.
7. Chin'ombe N, Muzividzi B, Munemo E, Nziramasanga P. Molecular Identification of nontuberculous mycobacteria in Humans in Zimbabwe Using 16S Ribosequencing. Open Microbiol J. 2016; 10:113-23.
8. Katoch V. Infections due to non-tuberculous mycobacteria (ntm). Indian Journal of Medical Research. 2004;4:290.
9. Tortoli E. Microbiological features and clinical relevance of new species of the genus mycobacterium. Clinical microbiology reviews. 2014;27(4):727-752.
10. Henkle E, Winthrop KL. Nontuberculous mycobacteria infections in immunosuppressed hosts. Clin Chest Med. 2015;36(1):91-9.
11. Torkko P, Suomalainen S, Iivanainen E, Tortoli E, Suutari M, Seppänen J, Paulin L, Katila ML. Mycobacterium palustre sp. nov., a potentially pathogenic, slowly growing mycobacterium isolated from clinical and veterinary specimens and from Finnish stream waters. Int J Syst Evol Microbiol. 2002;52:1519-25.
12. Turenne C, Chedore P, Wolfe J, Jamieson F, Broukhanski G, May K, Kabani A. *Mycobacterium lacus* sp. nov., a novel slowly growing, non-chromogenic clinical isolate. Int J Syst Evol Microbiol. 2002;52(Pt 6):2135-40.
13. Lopez FK, Miley M, Taiwo B. *Mycobacterium arupense* as an emerging cause of tenosynovitis. Emerg Infect Dis. 2016;22(3):559-61.
14. Croce O, Robert C, Raoult D, Drancourt M. Draft genome sequence of *Mycobacterium asiaticum* strain DSM 44297. Genome Announc. 2014;2(2):00320-14.
15. Kim KB, Park SG, Park JS, Lee W, Ha JS, Ryoo NH, Jeon DS, Kim JR. First Case of Pulmonary *Mycobacterium parascrofulaceum* infection in a patient with bronchiectasis in Korea. Ann Lab Med. 2015;35(3):379-81.
16. Lapierre SG, Fellag M, Magan C, Drancourt M. *Mycobacterium malmoense* pulmonary infection in France: A case report. BMC Res Notes. 2017;10(1):436.
17. Sawai T, Inoue Y, Doi S, Izumikawa K, Ohno H, Yanagihara K, Higashiyama Y, Miyazaki Y, Hirakata Y, Tashiro T, Kohno S. A case of *Mycobacterium nonchromogenicum* pulmonary infection showing multiple nodular shadows in an immunocompetent patient. Diagn Microbiol Infect Dis. 2006;54(4):311-4.
18. Porvaznik I, Solovič I, Mokrý J Non-tuberculous mycobacteria: Classification, diagnostics, and therapy. Adv Exp Med Biol. 2017;944:19-25.
19. Shojaei H, Hashemi A, Heidarieh P, Naser AD. *Mycobacterium novocastrense*-associated pulmonary and wound infections. Emerg Infect Dis. 2011; 17(3):550-1.
20. Kwak Y, Park GS, Lee SE, Li QX, Shin JH. Genome sequence of Mycobacterium aromaticivorans JS19b1(T), a novel isolate from Hawaiian soil. J Biotechnol. 2014; 186:137-8.
21. Guérin-Faublée V1, Flandrois JP, Pichat C, Boschiroli ML, Lamy B. *Mycobacterium bourgelatii* sp. nov., a rapidly growing, non-chromogenic species isolated from the lymph nodes of cattle. Int J Syst Evol Microbiol. 2013;63(Pt 12):4669-74.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/21444