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CASE REPORT

Mycobacterium shigaense sp. nov., a novel slowly growing scotochromogenic mycobacterium that produced nodules in an erythroderma patient with severe cellular immunodeficiency and a history of Hodgkin's disease

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ABSTRACT

A novel slow-growing scotochromogenic mycobacterium was isolated from skin biopsies from a patient with a history of Hodgkin's disease and severe cellular immunodeficiency as an opportunistic pathogen. Clinical characterization of these lesions revealed papules and nodules with pathological granuloma formation. Genotypic analysis using 16S rRNA misidentified this isolate as *Mycobacterium simiae*. However, multiple gene analysis using the internal transcribed spacer between the 16S and 23S rRNA genes, and the *rpoB* and *hsp65* genes revealed the presence of a novel mycobacterium. The antimicrobial susceptibility of this isolate was completely different from that of *M. simiae.* On the basis of these findings, we propose naming this new species *Mycobacterium shigaense* sp. nov., and conclude that multiple gene analysis is required for the appropriate diagnosis and treatment of non-tuberculous mycobacterial infections.

Key words: cellular immunodeficiency, *Mycobacterium shigaense* sp.nov., non-tuberculous mycobacteria, opportunistic infection.

INTRODUCTION

Non-tuberculous mycobacteria (NTM) have been well recognized as causative agents of human diseases. Recently, a number of new species have been added to the NTM. Some of these cause opportunistic infections in immunocompromised patients, not only those with AIDS, but also non-AIDS associated infections. Here, we report an additional new species of mycobacterium that caused cutaneous infection occurring as an opportunistic infection.

CASE REPORT

A 55-year-old Japanese male with a history of treatment of neck-oriented Hodgkin's disease in 2000, presented with erythema accompanied by generalized itching in 2005. The lesion persisted and worsened after treatment with a low dose of oral corticosteroids, resulting in erythroderma and scattered cutaneous nodules on the body trunk in 2007. On physical examination, he presented with itchy erythema over more than 90% of the total body surface (erythroderma) (Fig. 1a); scattered nodules on his chest, back and extremities (Fig. 1b); and multiple papules (Fig. 1c) on his back. He also presented with high-grade fever and slight lymphadenopathy of the neck and axilla. Cytomegalovirus (CMV) retinitis was diagnosed by ophthalmologists and several positive values of CMV antigen were detected at the end stage. His symptoms progressed, and after 2008 he was treated with ganciclovir or valganciclovir. Laboratory tests showed elevated levels of white blood cells ($10.2 \times 10^3/mm^3$; normal range [NR], 3.0–8.0×10^3) composed of 79.8% segmented neutrophils (NR, 40–74), 3.9% eosinophils (NR, 0–7), 11% lymphocytes (NR, 15–48) and 0% atypical lymphocytes, lactate dehydrogenase (295 IU/L; NR, 100–210), C-reactive protein (1.9 mg/dL; NR, <0.3), immunoglobulin E (188 498 IU/mL; NR, <400) and soluble interleukin-2 receptor (7470 U/mL; NR, 135–483). The platelet counts, liver and renal functions, serum immunoglobulin levels, complement values and angiotensin-converting enzyme were all within normal ranges. Antibodies against human T-lymphotropic virus-1 and HIV-1 were negative. Phenotypic analysis of peripheral lymphocytes revealed an increase in CD3 (95%; NR, 60–78%), T-cell receptors (TCR)-$\alpha\beta$ (94%) and -$\gamma\delta$ (1%), CD4 (93%; NR, 28–47) CD8 (3%; NR, 25–42) and CD19 (1%; NR, 6–16). Southern blot of peripheral lymphocytes revealed no monoclonal band. A tuberculin skin test for purified protein derivative was

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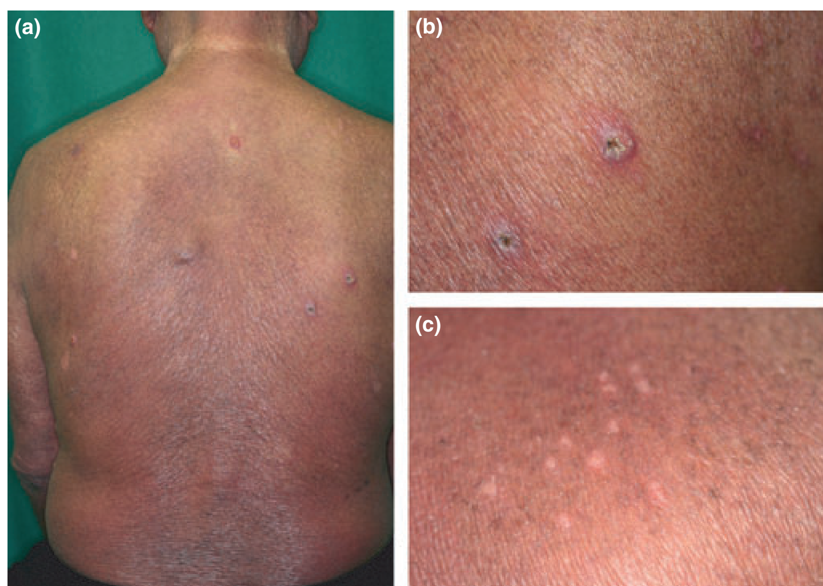

Figure 1. (a) Itchy erythema covered more than 90% of the body surface. (b) Scattered cutaneous nodules on the trunk. (c) Multiple papules on the back.

negative. A systemic investigation using computed tomography, endoscopy and Gallium scintigraphy revealed no abnormalities or internal malignancies, including a recurrence of Hodgkin's disease.

Skin biopsies were taken from the erythema, nodules and papules. A biopsy specimen from the erythema showed only lymphocytic infiltration (primarily CD4 T cells) around superficial dermal vessels (Fig. 2a). The lymphocytes were histologically normal, and Southern blot analysis of the biopsy specimen revealed no monoclonal band. The papules were histologically diagnosed as molluscum contagiosum (MC), because numerous basophilic inclusion bodies were observed in keratinocytes which located in the upper dermis. The nodular lesions showed dense infiltration of histiocytes in the superficial dermis, which formed granulomatous lesions (Fig. 2b). Ziehl–Neelsen staining of repeated biopsy specimens from these nodules showed multiple copies of banded acid-fast bacilli (Fig. 2c).

From these findings, we initially diagnosed an opportunistic mycobacterium infection in a patient of cellular immunodeficiency and administrated 400 mg of oral clarithromycin and 400 mg of isoniazid daily. The nodules improved within a few weeks. Multiple biopsies and histological investigations with Ziehl–Neelsen staining failed to detect any bacilli. The medicines were administrated for 12 months. New lesions of MC sometimes occurred after cessation of drug therapy, but no nodules were found. Oral prednisolone was administrated for the erythroderma. The erythroderma often recurred after healing of the mycobacterial infection, but because none of the skin biopsies from the erythroderma and peripheral blood showed atypical cells, the origin of the erythroderma is unknown.

While oral corticosteroids were effective, a daily low dose was needed to control the erythroderma. In October 2009, he complained of abdominal pain. At the time, he was almost blind due to CMV retinitis. Computed tomography showed a mass in the small

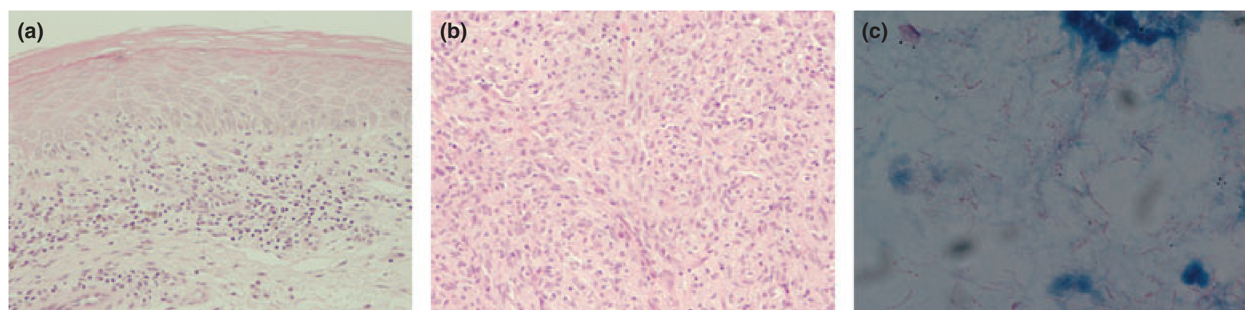

Figure 2. (a) Histological examination of erythema. Lymphocytic infiltration around superficial dermal vessels (hematoxylin–eosin [HE], original magnification ×100). (b) Histological examination of nodular lesions. Dense infiltrated histiocytes formed granulomatous lesions in the dermis (HE, ×200). (c) Ziehl–Neelsen staining of a skin biopsy from a nodule (oil immersion, ×1000).

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intestine and perforation of the gastrointestinal tract. Although a surgical resection of the mass in the small intestine was performed, he died of sepsis in November 2009. An autopsy was not performed. However, a histopathological examination of the mass revealed dense atypical lymphocytic infiltration without Hodgkin's cells, and Southern blot analysis showed a monoclonal band of TCR-$\alpha\beta$ cells. Therefore, we concluded that he died not of a recurrence of Hodgkin's disease, but of non-Hodgkin T-cell lymphoma (NHL) with severe immunodeficiency.

A skin biopsy from the nodules confirmed multiple copies of acid-fast bacilli with Ziehl–Neelsen staining, although polymerase chain reaction (PCR) tests targeting *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium leprae* were all negative. The sequencing and genotypic analysis of DNA from the biopsy specimens using the first one-third of the16S rRNA gene showed the highest similarities to *Mycobacterium simiae* (99.54% identity with a 2-bp difference) and *Mycobacterium interjectum* (98.61% identity with a 6-bp difference) when compared with the Ribosomal Differentiation of Micro-organisms (RIDOM) database.[1]

Table 1. Phenotypic differentiation between isolate *Mycobacterium* sp. UN-152 and genotypically similar species of mycobacteria

Characteristics	Isolate UN152 of *Mycobacterium* sp.	*Mycobacterium simiae* ATCC 25275^T	*Mycobacterium interjectum* ATCC 51457^T
Growth† in 7 days	+	+	+
Growth† at:			
25°C	+	+	+
30°C	+	+	+
37°C	+	+	+
42°C	–	+	–
Colony morphology	Smooth	Smooth	Smooth
Colony pigmentation			
In the dark	+	–	+
Photoactivity	–	+	–
Growth† supplemented with:			
PNB (500 μg/mL)	+	+	+
NaCl (5%)	+	+	+
TCH (1 μg/mL)	+	+	+
TCH (10 μg/mL)	+	+	+
Iron uptake	–	–	–
Niacin	–	+	–
Tween-80 hydrolysis (5, 10 days)	–	–	–
Urease	+	+	+
Nitrate reduction	–	–	–
Semi-quantitative catalase	+	+	+
68°C catalase	+	+	+
Arylsulfatase (3 day)	+	–	–
Pyrazinamidase	+	+	+
MPB64 production	–	–	–

†Bacterial growth was examined on 2% Ogawa slants.

The mycobacterium was isolated from the skin biopsy using the BBL MGIT tube (Becton Dickinson, Franklin Lakes, NJ, USA) and designated *Mycobacterium* sp. UN-152. Phenotypic characteristics were analyzed after sub-culturing on 2% Ogawa egg slant medium (Table 1).[2] The strain was scotochromogenic with an intense yellow color in both light and dark conditions and had a banded appearance after Ziehl–Neelsen staining (Fig. 3), however, usual strains of *M. simiae* are photochromogenic. The strain was slow-growing, had a smooth colonial morphology, and was positive for 3-day arylsulfatase activity, 68°C and semi-quantitative catalase activity

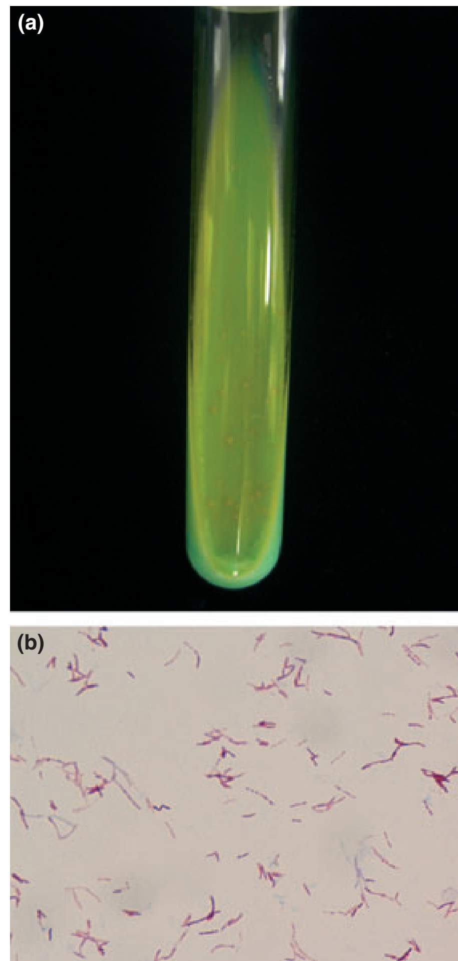

Figure 3. (a) Scotochromogenic colonies of *Mycobacterium* sp. UN-152 sub-cultured on 2% Ogawa egg medium. (b) Ziehl–Neelsen staining of *Mycobacterium* sp. UN-152 sub-cultured on 2% Ogawa egg medium (oil immersion, ×1000).

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and urease activity, but was negative for niacin activity, which suggested that this isolate was phenotypically different from *M. simiae*.

DNA–DNA hybridization to identify the species (DDH Mycobacteria Kyokuto Pharmaceutical Industrial, Tokyo, Japan) produced no matches with any of the 18 mycobacteria species included in the panel with *M. simiae.*[3] Further genotypic analysis was performed in an attempt to identify this isolate. Sequence analysis targeting fragments of the 16S rRNA gene, the internal transcribed spacer between the 16S and 23S rRNA genes (ITS region), and the *rpoB* and *hsp65* genes was performed (Table 2). Amplified PCR products were sequenced using an ABI Prism 310 PCR Genetic Analyzer (Applied Biosystems, Foster City, CA).[8] The sequences of isolate UN-152 were compared to those from the *M. simiae* (ATCC25275^{T}) type strain and the *M. simiae* clinical isolate 51808 from Japan.[9] We also performed a similarity search using BLAST to find identical and/or closely-related species of mycobacteria.[10] Phylogenetic analyses were performed using the neighbor joining method with Kimura's two-parameter distance correction model with 1000 bootstrap replications in the MEGA version 4.0.2 (Build#: 4028) software package.[11]

The sequence of the first one-third of the 16S rRNA gene from a sub-culture was identical with that from the previously examined skin biopsy. There were only four sites of a point difference between the sequence of UN-152 and that of *M. simiae* (99.7%

Table 2. Primers used in this study

Primer	Sequence	Target (amplified fragment size)	Reference
8F16S	5′-AGAGTTTGATCCTGGCTCAG-3′	16S rRNA gene (~1500 bp)	4
1047R16S	5′-TGCACACAGGCCACAAGGGA-3′		
830F16S	5′-GTGTGGGTTTCCTTCCTTGG-3′		
1542R16S	5′-AAGGAGGTGATCCAGCCGCA-3′		
ITSF	5′-TTGTACACACCGCCCGTC-3′	16S-23S ITS region (~340 bp)	5
ITSR	5′-TCTCGATGCCAAGGCATCCACC-3′		
MF	5′-CGACCACTTCGGCAACCG-3′	*rpoB* gene (351 bp)	6
MR	5′-TCGATCGGGCACATCCGG-3′		
TB11	5′-ACCAACGATGGTGTGTCCAT-3′	*hsp65* gene (441 bp)	7
TB12	5′-CTTGTCGAACCGCATACCCT-3′		

Table 3. DNA sequence similarities between isolate *Mycobacterium* sp. UN-152 and highly similar species of mycobacteria

	% identity			
Species†	16S rRNA (1471 bp)	ITS (280 bp)	*rpoB* (315 bp)	*hsp65* (401 bp)
Mycobacterium sp. UN-152	100	100	100	100
Mycobacterium simiae ATCC 25275^{T}	99.7	88.4	90.2	94.0
M. simiae 051808	99.7	88.4	90.8	94.0
Mycobacterium sherrisii ATCC BAA-832^{T}	99.5	ND	ND‡	93.0
Mycobacterium triplex ATCC 700071^{T}	99.1	85.7	ND	94.3
Mycobacterium cookii CIP 105396^{T}	ND	ND	95.9	93.3

†Sequence data (accession number in parenthesis) of three species were taken from database: *M. sherrisii* (AY353699, AY365190), *M. triplex* (U57632, GQ153291, AF334028) and *M. cookii* (AF547824, AY544904). ‡Not determined.

Table 4. Antibiotic susceptibility tests

	Minimal inhibitory concentration (μg/mL)		
Antibiotics	Isolate UN152 of *Mycobacterium* sp.	*Mycobacterium simiae* isolate	*M. simiae* (ATCC 25275T)
Streptomycin (SM)	8	16	4
Ethambutol (EB)	>128	32	44
Kanamycin (KM)	4	8	4
Isoniazid (INH)	>32	32	4
Rifampicin (REF)	0.03	>32	>32
Levofloxacin (LVFX)	0.5	2	1
Clarithromycin (CAM)	1	8	2
Ethionamide (TH)	>16	4	4
Amikacin (AMK)	4	8	4

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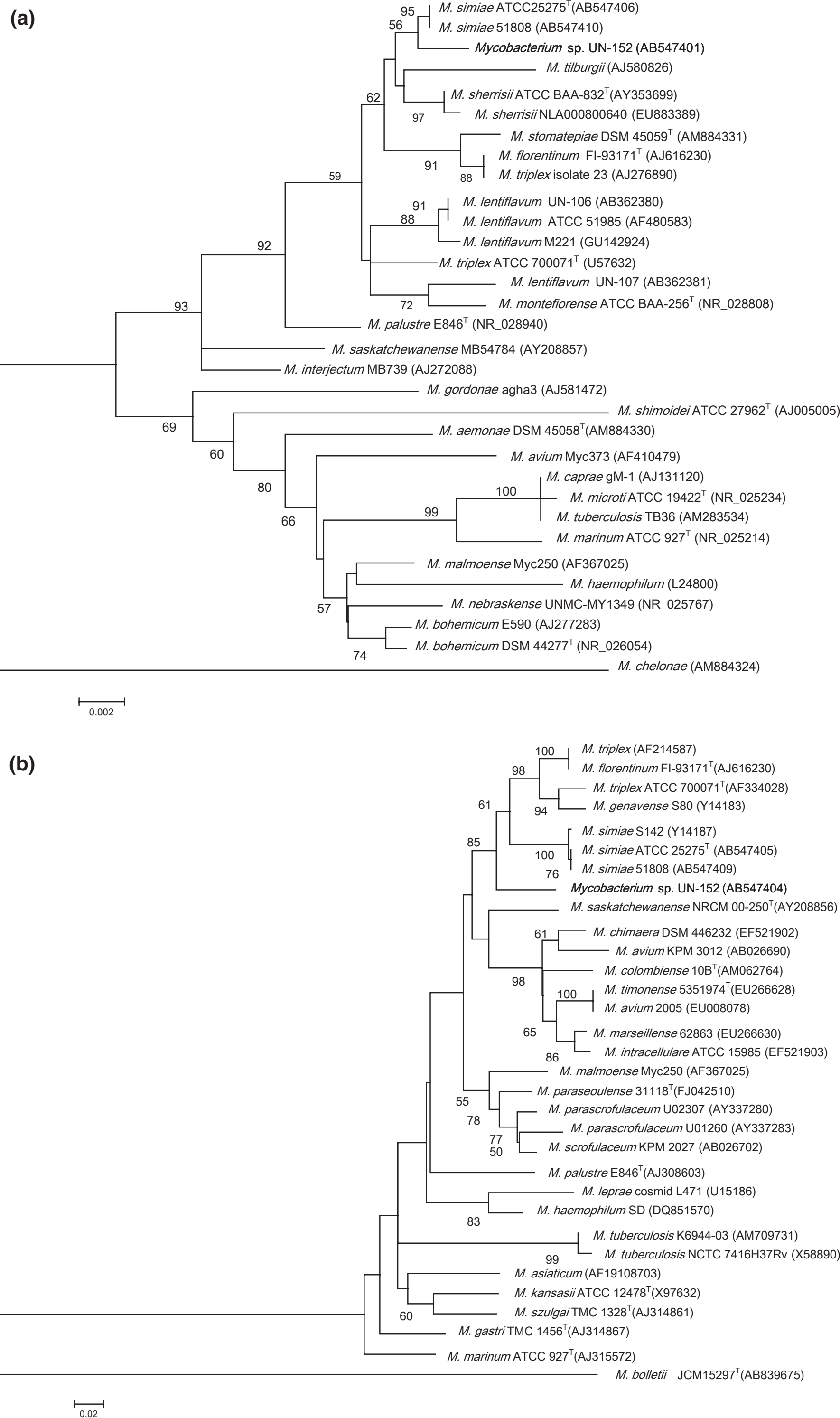
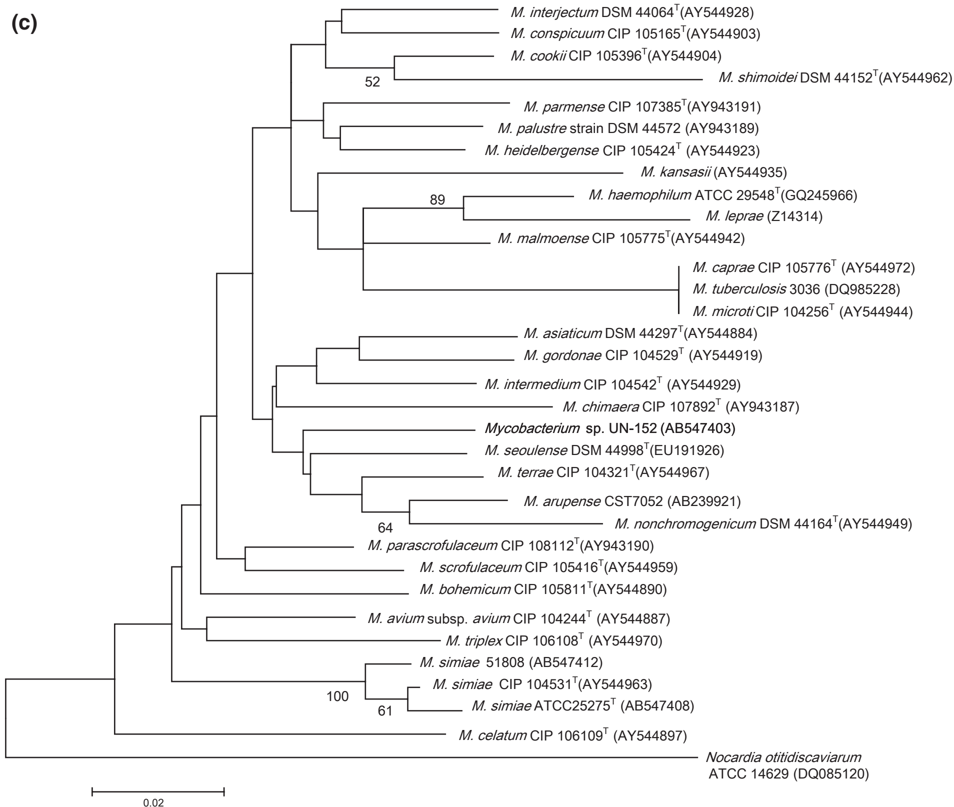


Figure 4. (a) Phylogenetic position of *Mycobacterium* sp. UN-152 (bold font) based on 1471 bp of the 16S rRNA gene. The percentages at the nodes represent bootstrap levels (values of <50% are not shown). The scale bar represents differences in nucleotide sequences. (b) Phylogenetic position of *Mycobacterium* sp. UN-152 (bold font) based on 280 bp of the 16S-23S rRNA ITS region. (c) Phylogenetic position of *Mycobacterium* sp. UN-152 (bold font) based on 315 bp of the *rpoB* gene. (d) Phylogenetic position of *Mycobacterium* sp. UN-152 (bold font) based on 401 bp of the *hsp65* gene.



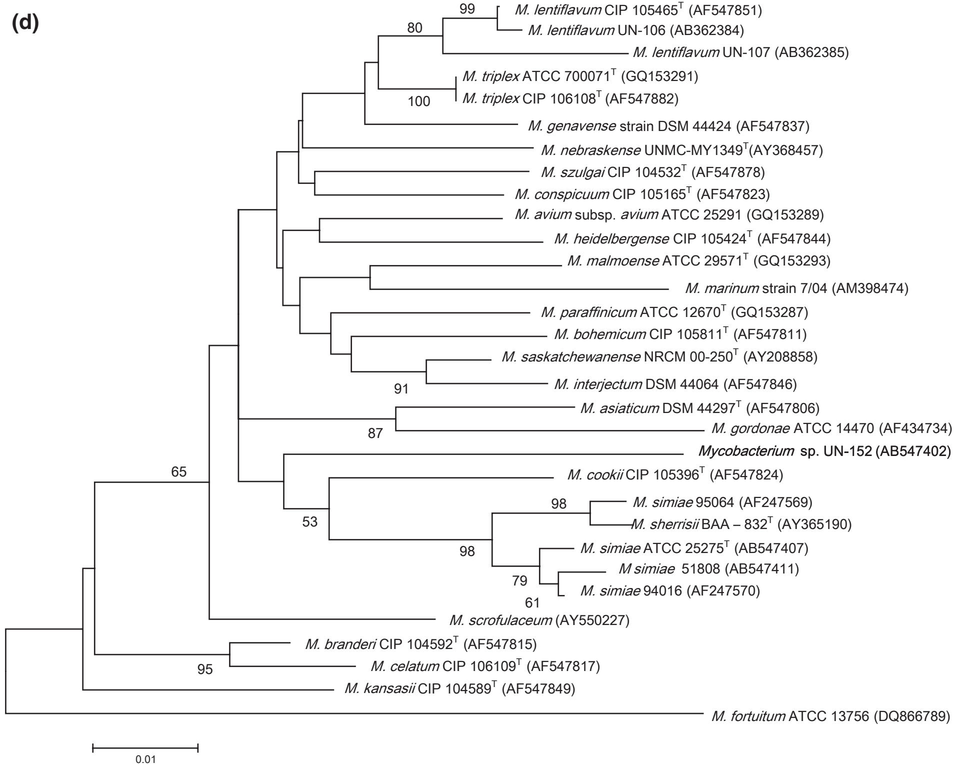


Figure 4. (*Continued*).

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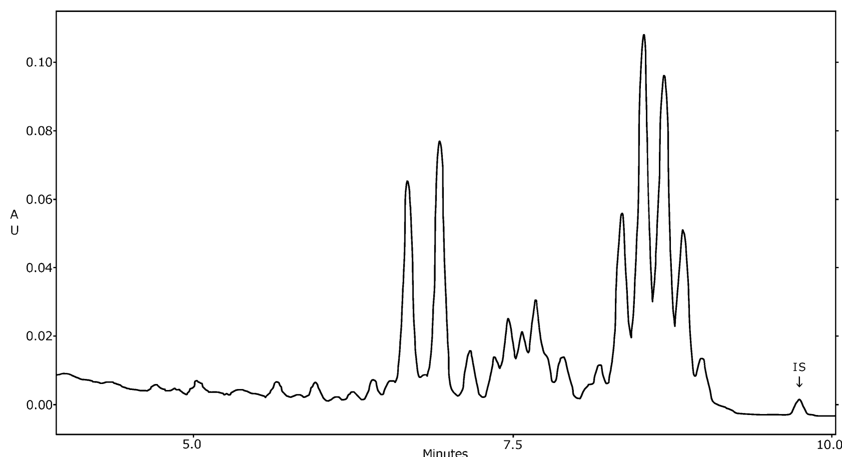

Figure 5. Mycolic acid analysis in *Mycobacterium* sp. UN-152 using high-performance liquid chromatography. IS, high molecular mass internal standard.

identity) when compared to almost all portions of the 16S rRNA gene (1471 bp). In contrast, the sequence identity of the ITS region was only 88. 4% between UN-152 and the *M. simiae*. *Mycobacterium cookii* revealed the highest similarity (95.9% identity) in the *rpoB* gene while *M. simiae* showed only 90.2–90.8% identities. *Mycobacterium triplex* exhibited the highest similarity in the *hsp65* gene (94.3% identity) while *M. simiae* was 94.0% similar (Table 3). However, there was no single mycobacterium species that showed the highest similarity across these four gene fragments, which suggested that this clinical isolate, *Mycobacterium* sp. UN-152, was a novel mycobacterium (Fig. 4).

Table 4 shows the results of antimicrobial susceptibility tests against *Mycobacterium* sp. UN-152, and the two strains of *M. simiae* (BrothMIC NTM; Kyokuto Pharmaceutical Industrial). *Mycobacterium* sp. UN-152 was highly susceptible to rifampicin and exhibited good susceptibility to clarithromycin and levofloxacin. Conversely, the minimal inhibitory concentration of rifampicin to *M. simiae* reference and clinical strains was more than 32 μg/mL, demonstrating that the antimicrobial susceptibility profile of the unknown isolate was different from that of *M. simiae*. The sequences obtained from the multiple gene analysis of the unknown clinical isolate (*Mycobacterium* sp. UN-152) and the *M. simiae* reference strains (ATCC25275^T and 51808) were deposited into the International Nucleotide Sequence Databases (INSD) through the DNA Databank of Japan (DDBJ)[12] under the accession numbers AB547401 to AB547412.

Finally, high-performance liquid chromatography of mycolic acid methyl esters was performed according to the CDC guidelines.[13] The pattern of bromophenacyl esters of mycolic acids can be used as an alternative method of discrimination of mycobacteria. The chromatographs revealed a representative profile characterized by three late clusters of peaks grossly resembling *M. simiae* or *Mycobacterium lentiflavum* (Fig. 5).[14]

DISCUSSION

Based on colony morphology, this clinical isolate may belong to NTM Runyon II. Because a slow-growing mycobacterium has a long culture time, the genotypic analysis of clinical samples would enable rapid diagnosis and treatment.[8,15] Genotypic analysis using the 16S rRNA gene is now contributing to diagnostics as an identification methodology for novel NTM.[16] In this case, the result of 16S rRNA gene sequences strongly suggested that *M. simiae* was the etiological strain; however, the identification was not supported by the scotochromogenic colony morphology and negative niacin accumulation. Additional sequence analysis targeting the ITS region and the *rpoB* and *hsp65* genes suggested the different species of mycobacteria, leading to the discovery of a novel NTM. We propose naming this new species *Mycobacterium shigaense* sp. nov.

Some species of recently registered mycobacteria may be of dubious clinical significance.[16] This isolate was determined to be clinically significant and not an environmental contaminant because: (i) it was isolated from multiple nodules, not from the erythema; (ii) the nodules improved after treatment with clarithromycin and isoniazid; and (iii) the isolate was no longer detectable once the nodules improved, a result which was repeatedly confirmed histologically. Unfortunately we could not know the source of this mycobacterium infection from medical interview.

In this case, MC infection was frequently seen in the trunk, CMV retinitis was diagnosed and treated for several months, and finally NHL was diagnosed. These findings were indicative of a significant cellular immunity deficiency because MC, CMV and NHL are frequently found in advanced AIDS patients with significant cellular immunodeficiency.[17–19] We propose that the decrease of peripheral CD8 T cells and the history of Hodgkin's disease resulted in severe immunodeficiency and that the cutaneous *M. shigaense* infection occurred as an opportunistic infection.

The genotypes obtained from a skin biopsy and subculture were identical, suggesting that the bacterium is responsible for the multiple nodules. In addition to the clinical significance, this case shows the insufficiency of single 16S rRNA gene analysis. Multiple gene analysis would be required to identify the species of mycobacteria.[20] This species has been never reported, but may have been previously misidentified as *M. simiae*. Because *M. shigaense* and *M. simiae* differ significantly in their susceptibility to rifampicin,

clinicians must differentiate the two isolates in terms of the treatment. Therefore, multiple gene analysis that includes the ITS region and *rpoB* and *hsp65* genes is required for the appropriate treatment and diagnosis of NTM.

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REFERENCES

1 Turenne CY, Tschetter L, Wolfe J *et al.* Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. *J Clin Microbiol* 2001; **39**: 3637–3648.
2 Della-Latta P, Weitzman I. Mycobacteriology. In: Isenberg HD, ed. *Essential Procedures for Clinical Microbiology*, 1st edn. Washington, DC: ASM Press, 1998; 169–203.
3 Kusunoki S, Ezaki M, Tamesada Y *et al.* Application of colorimetric microdilution plate hybridization for rapid genetic identification of 22 *Mycobacterium* species. *J Clin Microbiol* 1991; **29**: 1596–1603.
4 Springer B, Wu WK, Bodmer T *et al.* Isolation and characterization of a unique group of slowly growing mycobacteria: description of *Mycobacterium lentiflavum* sp. nov. *J Clin Microbiol* 1996; **34**: 1100–1107.
5 Roth A, Fischer M, Hamid ME *et al.* Differentiation of phylogenetically related slowly growing mycobacteria based on 16S–23S rRNA gene internal transcribed spacer sequences. *J Clin Microbiol* 1998; **36**: 139–147.
6 Kim B-J, Lee S-H, Lyu M-A *et al.* Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). *J Clin Microbiol* 1999; **37**: 1714–1720.
7 Telenti A, Marchesi F, Balz M *et al.* Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 1993; **31**: 175–178.
8 Nakanaga K, Ishii N, Suzuki K *et al.* ''*Mycobacterium ulcerans* subsp. *shinshuense*'' isolated from a skin ulcer lesion: identification based on 16S rRNA gene sequencing. *J Clin Microbiol* 2007; **45**: 3840–3843.
9 Yoshimura K, Imao M, Goto H *et al.* A case of pulmonary infection due to *Mycobacterium simiae*. *Nihon Kokyuki Gakkai Zasshi* 2005; **43**: 32–36.
10 Altschul SF, Madden TL, Schaffer AA *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; **25**: 3389–3402.
11 Tamura K, Dudley J, Nei M *et al.* MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; **24**: 1596–1599.
12 Kaminuma E, Mashima J, Kodama Y, *et al.* DDBJ launches a new archive database with analytical tools for next-generation sequence data. *Nucleic Acids Res* 2010; **38**: Database issue D33–D38.
13 Butler WR, Margaret MS, Floyd M, *et al.* Standardized method for HPLC identification of mycobacteria. 1996 [WWW document]. [cited 20 Apr, 2011] Available from URL: www.cdc.gov/ncidod/publications/hplc.pdf.
14 Tortoli E, Bartoloni A. High-performance liquid chromatography and identification of mycobacteria. *Rev Med Microbiol* 1996; **7**: 207–219.
15 Ishiwada N, Hishiki H, Watanabe M *et al.* Usefulness of PCR in rapidly diagnosing subcutaneous abscess and costal osteomyelitis caused by *Mycobacterium bovis* BCG. *Kansenshogaku Zasshi* 2008; **82**: 30–33.
16 Griffith DE, Aksamit T, Brown-Elliott BA *et al.* An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* 2007; **175**: 367–416.
17 Hoshino Y, Nagata Y, Gatanaga H *et al.* Cytomegalovirus (CMV) retinitis and CMV antigenemia as a clue to impaired adrenocortical function in patients with AIDS. *AIDS* 1997; **11**: 1719–1724.
18 Hoshino Y, Nagata Y, Taguchi H *et al.* Role of the cytomegalovirus (CMV)-antigenemia assay as a predictive and follow-up detection tool for CMV disease in AIDS patients. *Microbiol Immunol* 1999; **43**: 959–965.
19 Jung AC, Paauw DS. Diagnosing HIV-related disease: using the CD4 count as a guide. *J Gen Intern Med* 1998; **13**: 131–136.
20 Devulder G, Pérouse de Montclos M, Flandrois JP. A multigene approach to phylogenetic analysis using the genus Mycobacterium as a model. *Int J Syst Evol Microbiol* 2005; **55**: 293–302.

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