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DIAGNOSTICS

Sub-speciation of *Mycobacterium tuberculosis* complex from tuberculosis patients in Japan

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SUMMARY

Mycobacterium tuberculosis is the major causative agent of tuberculosis in humans. It is well known that *Mycobacterium bovis* and other species in the *M. tuberculosis* complex (MTC) can cause respiratory diseases as zoonosis. We analyzed the MTC isolates collected from tuberculosis patients from Japan in 2002 using a multiplex PCR system that detected *cfp32*, RD9 and RD12. A total of 970 MTC isolates that were representative of the tuberculosis cases throughout Japan, were examined using this method. As a result, 966 (99.6%) *M. tuberculosis*, two *Mycobacterium africanum* and two *Mycobacterium canettii* were identified using a multiplex PCR system, while no *M. bovis* was detected. Two isolates that lacked RD9 were initially considered to be *M. canettii*, but further analysis of the *hsp65* sequence revealed them to be *M. tuberculosis*. Also two *M. africanum* were identified as *M. tuberculosis* using the −215 *narG* nucleotide polymorphism. Though PCR-linked methods have been used for a rapid differentiation of MTC and NTM, from our cases we suggest careful interpretation of RD based identification.

1. Introduction

Tuberculosis (TB) is a disease caused by the *Mycobacterium tuberculosis* complex (MTC), which includes *M. tuberculosis* sensu stricto, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium caprae*, *Mycobacterium canettii*, *Mycobacterium microti*, and *Mycobacterium pinnipedii*, as of 2012. *M. bovis* is a common zoonotic pathogen that can infect a wide range of hosts. *M. bovis* primarily infects cattle, but can be transmitted between humans, especially among HIV-infected patients.

On the other hand, Non-tuberculous mycobacteria (NTM) also known as mycobacteria other than tuberculosis (MOTT) are acid fast bacilli that do not belong to *M. tuberculosis* complex or *Mycobacterium leprae*. These mycobacteria are environmental organisms and are found in natural bodies of waters, biofilms, soil, water damaged walls, etc. NTM may also be found in drinking water supplies.

Recently it has been estimated that approximately 5–10% of the global TB burden may be due to *M. bovis* [1]. There are several reports regarding the infections caused by MTC other than *M. tuberculosis* sensu stricto, but only limited data is available on the total incidence or prevalence of mycobacterial disease caused by specific MTC members [2,3]. Japan is still a medium prevalence country for TB, with an incidence of 18.2/100,000 in 2010 [4]. Bacteriologically-identified TB cases in Japan are diagnosed based on the detection of MTC, in general, using commercial identification kit like Capilia TB (TOUNS, Japan). Although all mycobacterial isolates from patients in Japan are submitted for species identification, very few isolates will be subjected to further analysis to identify the MTC species. It is possible to differentiate MTC members by conventional laboratory culture and chemical testing methods; however, the procedures are time-consuming and not practical in clinical laboratories. The lack of surveillance for the specific

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causative agents of TB is mainly due to the absence of simple tests in diagnostic laboratories. Differentiation of MTC members is important for the accurate diagnosis of mycobacterial disease, public health surveillance and appropriate case management. Recently, polymerase chain reaction (PCR) based methods have been developed to differentiate TB from bacille Calmette-Guérin (BCG) disease in HIV-infected infants, where accurate diagnosis has implications for both clinical management and public health policy [5]. In this study, we utilized a simple multiplex-PCR diagnostic method to differentiate MTC members known to cause disease in humans. The method amplified one conserved MTC locus [6] (*cfp32*), and two chromosomal region-of-difference (RD) loci [7] (RD9, RD12). When necessary, additional loci [6] (MiD-3, RD4, RD7, *hsp65*, −215 *narG*) were characterized to allow rapid and conclusive differentiation of the relevant MTC species. Detailed information about the pathogenic MTC in Japan will provide a clear perspective for the management of patients diagnosed with TB and zoonotic situations.

2. Methods

2.1. Clinical isolates of the M. tuberculosis complex

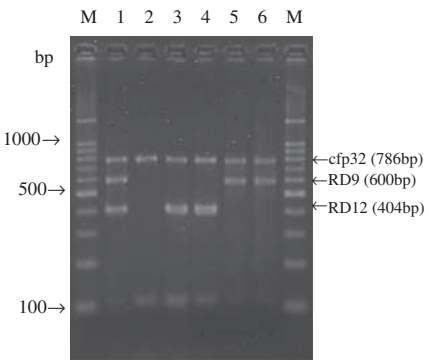
A total of 970 MTC isolates were randomly but systematically selected from the collection of the Ryoken anti-tuberculosis drug resistance survey in 2002 [8]. The Tuberculosis Research Committee (Ryoken) is a nationwide coalition of TB hospitals in Japan. The Committee has been conducting nationwide drug resistance surveys approximately every 5 years since 1957 with the participation of almost all the TB hospitals across the country and the surveys have resulted in high reliability in our country. In the survey in 2002, the MTC isolates numbered 3122 and a total of 99 major TB hospitals all over Japan participated and the entire country was divided into six geographical regions. All patients who had chemotherapy for culture-confirmed TB in the participating hospitals were eligible for the study and corresponded to approximately 10% of all MTC isolates in that year. Also in the drug resistance survey, children were indeed included and the patients consisted of 2211 males and 911 females, with a mean age of 61.1 ± 18.5 years (range 0–100) and 61.0 ± 22.4 years (0–100), respectively. In that survey a total of 2011 MTC isolates were recovered by using BACTEC MGIT 960 (Nippon Becton Dickinson, Japan) or BacT/ALERT 3D (Sysmex-Biomerieux, Tokyo, Japan), and the others were isolated using solid culture media. MTC isolates were identified by the lateral flow immunoprecipitation method (Capilia TB: TOUNS, Numazu, Japan), DNA–DNA hybridization method (DDH mycobacteria: Kyokuto Pharmaceuticals, Tokyo, Japan), and/or direct 16S rRNA sequencing [9]. The obtained 16S rRNA sequence data was subjected to Ribosomal Differentiation of Microorganisms (RIDOM) on the web site (http://www.ridom-rdna.de) for similarity searches. In our study a total of 970 MTC isolates were analyzed and the number of isolates was 1/3 of the drug resistance survey performed in 2002. This may have resulted in a bias in the selection of number, age and hospitals. However, including all the major TB institutions of the country, the adjustment for the regions made no substantial change in the estimates of the prevalence figures, which suggests that the results obtained in our study accurately represent the actual prevalence of MTC isolates differentiation in Japan. Also in age, in Japan in 2002, among children aged 0–4 years childhood tuberculosis notification rates was 1.4/100,000, aged 5–9 years was 0.6/100,000, aged 10–14 years was 0.7, on the other hand among total the rate was 25.8/100,000 [4]. These data indicates that Japan is a low-burden country in younger children tuberculosis, and then we can recognize that isolate selection in our study lesser influenced the our observed results in a younger age and our study result would not contradict the fact that no *M. bovis* was isolated in our study (Pages 5, lines 149–155). We couldn't analyze too much more isolates and if we had analyzed much more isolates, the result might have been slightly different from our study. But there have never been larger reports about MTC isolates differentiation in our country. After all at least our study result actually indicated the differentiation of 970 MTC isolates in 2002 in Japan and in this point we think that our study is of value to report the proper situation of MTC isolation in Japan.

2.2. Species identification of M. tuberculosis complex

DNA specimens were prepared using the ISOPLANT extraction kit (Nippon Gene, Japan). A multiplex PCR was performed to detect *cfp32*, RD9 and RD12, using a Type-it Microsatellite PCR Kit (QIAGEN, Japan) [6,10–13]. The expected sizes of the PCR products generated using each primer pair were 786, 600, and 404 bp, respectively. DNA templates from *M. tuberculosis* (H37Rv, ATCC 27294) were used as positive controls. The amplified products were analyzed on 2% agarose gels (Figure 1) and automated gel electrophoresis (QIAxcel system, QIAGEN, Japan). When MTC species other than *M. tuberculosis* sensu stricto were identified, further analyses were performed detecting RD4, RD7 and MiD3 [7,14–16]. The expected RD loci for each MTC species are summarized in Table 1.

2.3. Analysis of hsp65 for unidentified species

Any isolates showing discrepant or abnormal RD identification patterns that did not correspond to the typical morphotype were further analyzed by sequencing *hsp65* [17]. If the nucleotide at the position corresponding to 631 of the homologous *hsp65* of *M. tuberculosis* H37Rv was cytosine, the isolate was considered to be *M. tuberculosis* sensu stricto.



1. *M. tuberculosis* H37Rv ATCC 27294
2. *M. bovis* ATCC 19210
3. *M. africanum* ATCC 25420
4. *M. microti* ATCC 19422
5. strain A (this study)

Figure 1. Electrophoretic separation of multiplex PCR products.

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