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ORIGINAL ARTICLE

Molecular typing of *Mycobacterium tuberculosis* isolated from adult patients with tubercular spondylitis

Ching-Yun Weng [a], Cheng-Mao Ho [b,c,d], Horng-Yunn Dou [e], Mao-Wang Ho [b], Hsiu-Shan Lin [c], Hui-Lan Chang [c], Jing-Yi Li [c], Tsai-Hsiu Lin [c], Ni Tien [c], Jang-Jih Lu [c,d,f,*]

[a] *Section of Infectious Diseases, Department of Internal Medicine, Lin-Shin Hospital, Taichung, Taiwan*
[b] *Section of Infectious Diseases, Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan*
[c] *Department of Laboratory Medicine, China Medical University Hospital, Taichung, Taiwan*
[d] *Graduate institute of Clinical Medical Science, China Medical University, Taichung, Taiwan*
[e] *Division of Clinical Research, National Health Research Institutes, Zhunan, Taiwan*
[f] *Department of Laboratory Medicine, Linkou Chang-Gung Memorial Hospital, Taoyuan, Taiwan*

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Background/Purpose: Tuberculosis (TB) is endemic in Taiwan and usually affects the lung, spinal TB accounting for 1—3% of all TB infections. The manifestations of spinal TB are different from those of pulmonary TB. The purpose of this study was to define the epidemiological molecular types of mycobacterial strains causing spinal TB.
Methods: We retrospectively reviewed the medical charts of adult patients diagnosed with spinal TB from January 1998 to December 2007. Patients with positive culture results and/or pathological findings characteristic of TB were enrolled in this study. Spoligotyping was performed to type the *Mycobacterium tuberculosis* isolates.
Results: A total of 38 patients with spinal TB were identified. Their mean age was 68 years, and their median duration of symptoms was 60 days (range 3—720 days). The lumbar and thoracic spine accounted for 76% of the sites involved. Thirteen specimens (from seven male and six female patients) were available for typing. Spoligotyping of these 13 specimens revealed three Beijing (23%) and 10 non-Beijing types (77%). The non-Beijing types included two EAI2 Manilla (15%), two H3 (15%), two unclassified (15%), and one each of BOVIS1, U, T2, and orphan type. No significant predominant strain was found in this study, and no drug-resistant Beijing strains were identified.

* Corresponding author. Department of Laboratory Medicine, China Medical University Hospital, 2 Yuh-Der Road, Taichung City, Taiwan.
E-mail addresses: janglu45@gmail.com, d17405@mail.cmuh.org.tw (J.-J. Lu).

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Conclusion: TB spondylitis was found to occur in older patients. Spoligotyping results showed that most of the TB spondylitis cases were caused by non-Beijing type *Mycobacterium tuberculosis*.

Introduction

Taiwan implemented its National Tuberculosis Program more than 50 years ago, and the prevalence and mortality rate of tuberculosis (TB) has gradually declined since then. However, TB remains a leading notified infectious disease in Taiwan. In 2007, the TB notification and mortality rates were 63.2 and 3.4 per 100,000 people, respectively.[1] Most cases of TB were pulmonary, extrapulmonary cases accounting for 6% of the total notified cases of TB. Bone and joint TB was rare, accounting for only 1% of the cases notified. The diagnosis of extrapulmonary TB is more difficult than that of pulmonary TB because it mainly depends on tissue culture and pathology.

TB spondylitis is an indolent and progressive neurological disease.[2] Unlike pulmonary TB, spinal TB rarely exhibits constitutional symptoms such as fever, body weight loss, or night sweating.[3,4] This atypical presentation of TB spondylitis raises the question of whether the causative TB strains are different from those of pulmonary TB.

The development of various TB typing methods has enabled epidemiological studies of TB. Using spoligotyping, Jou et al. found that 44.4% of *Mycobacterium tuberculosis* (MTB) isolates from different regions in Taiwan were of Beijing family genotypes. The proportion of the Beijing genotype isolates was the highest in patients under the age of 24 years (61.5%).[5] However, there have been no studies using spoligotyping to investigate TB spondylitis.

Spoligotyping is a polymerase chain reaction (PCR)-based method used to analyze the strain polymorphisms in the MTB short direct repeat chromosomal region. This region consists of 36 identical 36 bp direct repeats interspersed by 35—41 bp nonrepetitive spacer sequences.[6] The purpose of this study was to use spoligotyping to determine the epidemiological molecular types of MTB isolates causing TB spondylitis.

Materials and methods

Patients

The basic data and clinical presentation of all patients diagnosed with spinal TB at the China Medical University Hospital, Taichung, Taiwan from January 1998 to December 2007 were collected by reviewing medical charts. Spinal TB was diagnosed based on the following criteria: (1) the presence of osteomyelitis in one or more vertebral segments, (2) MTB isolated from an abscess or tissue biopsy specimen, and (3) histology of a tissue specimen demonstrating caseating granuloma or granulomatous inflammation with a positive acid-fast bacillus smear.[3] Patients who fulfilled criterion (1) plus criterion (2) or (3) were included in this study. Probable cases, not confirmed microbiologically or pathologically but responding to anti-TB therapy, were excluded. All patients enrolled in this study were adult ($\geq$18 years old). The study was approved by the Institutional Review Board of China Medical University Hospital, Taichung, Taiwan (DMR100-IRB-016).

Mycobacterial strains and genomic DNA

Various MTB culture and identification systems were used during the study period. The first was the BACTEC® MGIT™ 960 system (Becton Dickinson, Sparks, Maryland, USA). Clinical specimens were processed, and the centrifuged sediment was inoculated onto Löwenstein—Jensen medium (BBL; Becton Dickinson, Sparks, MD, USA) and Middlebrook 7H9 broth (BBL; Becton Dickinson). The cultures were incubated at 35°C in 5% carbon dioxide for up to 8 weeks. Identification of MTB was based on colony morphology and biochemistry reactions (nitrate reduction and niacin test). In 2004, the traditional biochemistry methods for MTB identification and drug susceptibility were replaced by the Protec ET system (Becton Dickinson).[7] MTB genomic DNA was extracted as previously described.[8]

Multiplex PCR for typing MTB

All MTB were stored bacterial specimens from positive MTB cultures, which is a standard method for the clinical diagnosis of TB. It has been determined that the region spanning genes *Rv2816* to *Rv2819* and part of *Rv2820* is deleted in the genome of all Beijing strains of MTB.[9] Based on this information, three sets of PCR primers were used to distinguish between Beijing and non-Beijing strains.[10]

The first set of primers, BjF (CTCGGCAGCTTCCTCGAT) and BjR (CGAACTCGAGGCTGCCTACTAC), produced a 129 bp PCR product containing the 3′ end of *IS6110* and the 5′ end of the *Rv2820* gene from the Beijing strains of MTB.[11] The second set of PCR primers, nBjF(AAGCATTCCCTTGACAGTCGAA) 1 and nBjR (GGCGCATGACTCGAAAG AAG), produced a 95 bp PCR product from the *Rv2819* gene of non-Beijing strains of MTB. The third primer set, IS59 (GCGCCAGGCGCAGGTCGATGC) and IS60 (GATCAGCGATCGTGGTCCTGC), amplified a 523 bp PCR product from *IS6110* that is present in all MTB isolates; this reaction served as the internal PCR control.

Multiplex PCRs with these three sets of primers were performed for all specimens. A Beijing strain of MTB was expected to produce two amplicons: the 523 bp internal control product and the 129 bp product. A non-Beijing strain was expected to produce the 523 bp and the 95 bp products.

Spoligotyping and spoligotype analysis

Spoligotyping was performed according to the manufacturer's instructions (Isogen Bioscience B.V., Maarssen, The Netherlands). The positive or negative hybridization result was represented by binary codes n and o, respectively, and analyzed using the Microsoft® Office Excel® 2007 (Microsoft Taiwan Corporation, Taipei, Taiwan) to group and order the hybridization patterns. Spoligotypes common to more than one strain were designated as shared types (ST) and assigned a shared international type number according to the updated version of the international spoligotype database SpolDB4.

Statistical analysis

Statistical Package for the Social Science (version 13.0; SPSS, Chicago, IL, USA) was used for statistical analysis. Data were presented as mean ± standard deviation.

Results

During the 10-year period of the study, 3240 cases of pulmonary TB and 404 of extrapulmonary TB were diagnosed. Among the extrapulmonary cases, 38 were spinal TB. The median age of these 38 patients with spinal TB was 72 years (interquartile range 18 years). The majority of patients (65.8%) were 70 years or older, and three were younger than 50 years old.

The median duration of symptoms before diagnosis was 60 days (range 3—720 days). At the time of diagnosis, nine of the 38 patients (24%) had constitutional symptoms. Back pain was the most common clinical complaint (38 patients, 100%), followed by weakness (20 patients, 53%) and numbness (10 patients, 26%).

Thirty-four of the 38 patients were followed up after completion of anti-TB treatment. Most patients recovered without any motor or sensory deficit in their daily activities, and no relapse was reported during the period of follow-up (mean duration 10.6 months). One patient was lost to follow-up, and three patients died of their comorbidities during the treatment period.

Thirteen MTB isolates from 13 of the 38 cases of spinal TB were available for further study. The results of drug susceptibility assays (Table 1) showed that nine (69.2%) of these isolates were sensitive to all four of the first-line anti-TB drugs (rifampin, isoniazid, ethambutol, and streptomycin), and four (30.7%) were resistant to at least one drug. No multidrug-resistant strain was detected. The median treatment duration was 11 months (range 6—32 months).

Table 2 shows the spoligotypes of the 13 MTB isolates. Of these, three (23%) were of Beijing type. The others included two (15%) EAI2 Manilla strains, two (15%) H3, two (15%) unclassified strains, and one each of BOVIS1, U, T2, and orphan type. The multiplex PCR results showed three Beijing and 10 non-Beijing types, consistent with those of the spoligotyping.

Table 1 Genotypes and drug resistance patterns of *Mycobacterium tuberculosis* isolates from patients with culture-confirmed tuberculosis (TB)

Genotype family	Number of isolates	Resistant to any first-line anti-TB drug	Sensitive to all four first-line anti-TB drugs
Beijing	3	0	3
Non-Beijing	10		
EAI2	2	0	2
H3	2	2	0
Unknown	2	0	2
BOVIS1	1	1	0
U	1	0	1
T2	1	0	1
Orphan	1	1	0
Total	13	4	9

First-line anti-TB drugs were rifampin, isoniazid, ethambutol, and streptomycin.

Discussion

The Beijing genotype family of MTB isolates is well known to have a distinct and highly conserved genetic signature.[12] It is present worldwide and is more prevalent in certain areas, such as Asia[13,14] and Russia.[15] Drug-resistant Beijing family genotype strains and their transmissions have been documented.[16—18] Many MTB genotyping studies have been reported.[12,19—21] Most of these examined isolates from all infected sites as a whole, while some focused on pulmonary isolates. In this study, we investigated and genotyped isolates from patients with TB spondylitis in a medical center located in central Taiwan.

Spinal TB was first described in 1782 by Sir Percival Pott.[22] It accounts for 50% of skeletal TB,[22,23] and 1—3% of all TB cases.[24—26] In our institution, spinal TB represents 1% (38/3644) of all TB cases. Unlike those in the other reports,[27,28] the population of our subjects were older, with 65.8% being aged 70 years or older, probably due to the longer life expectancy in Taiwan.[28]

Similar to other studies,[3,28] back pain was most common for spinal TB, followed by weakness and numbness. Unlike pulmonary TB, spinal TB seldom exhibits constitutional symptoms such as fever, body weight loss or night sweating,[3] and this atypical feature was also observed in our study. The inability of MTB to grow in a low-oxygen environment such as the spine may be the reason for the atypical presentation of spinal TB. Considerable variation in the duration of symptoms was also observed in the present study, and this may be due to the insidious clinical course and nonspecific manifestations of spinal TB.[3,29]

The genetic heterogeneity of MTB isolates may influence their transmissibility and virulence.[30—32] The Beijing strain is rapidly increasing in prevalence.[33] It is more readily aerosolized and more infectious, and the infection progresses more quickly. Since spinal TB progresses slowly with more subtle clinical symptoms, it is possible that the causative strains are different from those of pulmonary TB, in which the Beijing strain predominates, even though only 13 MTB isolates underwent genotyping in this study.

In this study, one non-Beijing MTB isolate belonged to genotype OBVIS1. Because the diagnosis of MTB in our microbiology laboratory was ascertained using BD Protect, we were unable to differentiate *M. bovis* from

Table 2 Spoligotypes of the 13 *Mycobacterium tuberculosis* isolates

Sample number	Hybridization results of spoligotyping	Type
1	■■□■■■■■■■■■■■■■■■■□□■■■■■■■□□□□□■□■■■■■■■■■	(ST19,EAI2_MANILLA)
2	■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■■■■■■■■■■■	(Unknown)
3	■■□■■■■■■■■■■■■■■■■□□■■■■■■■□□□□□■□■■■■■■□	(ST849,H3)
4	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	Beijing (ST1)
5	■■■■■■■■■■■■■■■■■■■■□■■■■■■■□■□□□□□■■■□■■■	(Unknown)
6	■■	(ST523,U)
7	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□□■■■■■■	(ST50,H3)
8	■■□■■■■■■■■■■■■■■■■□□■■■■■■■□□□□□■□■■■■■■■■■	(ST19,EAI2_MANILLA)
9	■■□■■□■■□■■■■■■□■■■■■□■■■■■■■■■■■■■■■■■■□□□□□	(ST684,BOVIS1)
10	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□■■■□■■■	(ST52,T2)
11	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	Beijing (ST1)
12	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■■■■■■□□■■	(Orphan)
13	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	Beijing (ST1)

M. tuberculosis complex. However, the spoligotyping profiles of this isolate were typical of *M. bovis* with the absence of spacers 3, 6,9, 16, 21, and 39—43, which has also been found with human TB in Taiwan.[34] *Mycobacterium bovis* was highly suspected in this case of a 74—year-old Aboriginal female with degenerative scoliosis of the lumbar spine and bilateral lower limb weakness for about 2 weeks before admission. There was no history of drinking unpasteurized milk, eating raw meat, or close contact with infected animals.

In summary, we found that the MTB isolates from spinal TB were different from those from pulmonary TB. Non-Beijing strains were found to be predominant in this patient group. Since the sample size of this study was relatively small, additional studies with more enrollments are needed to confirm our results.

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