Acta Medica et Biologica
Vol. 47, No.4, 169-173, 1999

Molecular Epidemiology of Intrafamilial *Mycobacterium tuberculosis* Infections in Niigata, Japan

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Received November 12 1999; accepted November 30 1999

Summary. **A case of intrafamilial infection of *Mycobacterium tuberculosis* in Niigata in 1998 was investigated by DNA hybridization assay with the insertion sequence IS*6110* probe. A male patient (the source of the *M. tuberculosis* transmission) and his daughter, but not his mother, were infected with the same *M. tuberculosis* strain, which showed at least 19 *Pvu*II- digested, IS*6110*-positive bands in the assay. His mother's strain and strains from five patients living in various parts of Niigata showed at least 13 to 19 bands in the assay, and were genetically divergent greatly from the family strains. The IS*6110*-probed hybridization assay was useful to analyze recent trends in the contagion of *M. tuberculosis* in Niigata.**

Key words—*Mycobacterium tuberculosis,* intrafamilial infection, DNA typing, IS*6110*.

INTRODUCTION

Tuberculosis remains a major public health problem in both developing and developed countries. In 1993, the World Health Organization (WHO) declared a state of emergency, recognizing that the tuberculosis endemic was out of control in many parts of the world, with 7.5 million cases and 2.5 million deaths worldwide in 1990[1]. In Japan, approximately 42,000 people are infected with *M. tuberculosis*, and some 2,700 of these people died in 1997. Faced with serious problems such as the growing incidence of group infections in school, medical facilities, and facilities for the elderly, the Ministry of Health and Welfare of Japan also declared a state of emergency concerning tuberculosis in 1999[2].

M. tuberculosis strains carry up to about 20 copies of the insertion sequence called IS*6110* (1,355 base pairs, bp, in length) on the chromosome[3-7]. Based on differences in the location and copy number of IS*6110*, each *M. tuberculosis* strain can usually be identified by DNA hybridization[8]. This IS*6110*-probed hybridization assay is specific to *M. tuberculosis*[9].

In this study, we investigated a case of intrafamilial transmission of *M. tuberculosis* in Niigata by the IS*6110*-based hybridization assay.

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MATERIALS AND METHODS

Patients and derived bacterial strains

A total of eight patients with pulmonary tuberculosis were studied. The patients lived in Niigata Prefecture, and their places of residence are marked on a map of the prefecture (Fig. 1). Three of them were members of the same family, in which a 44-year-old man living in Niigata City became ill in the fall of 1998 and was thought to have been the source of infection (Gaffky number observed with sputum was VII); this bacterial strain was designated KIJ1. His 12-year-old daughter living with him had no symptoms, but chest radiographs showed abnormalities in family contact examinations with were conducted in November 1998 (bacterial strain, KIJ2). His 68-year-old mother, living separately in Kashiwazaki City, met him for several hours in August 1998. While she had no symptoms and radiographs of her chest

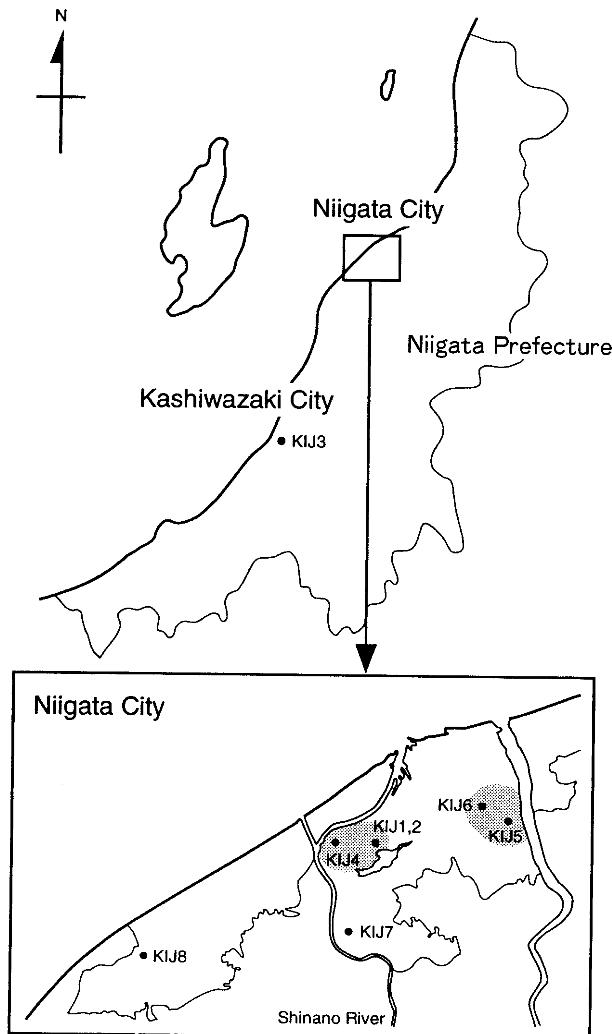

Fig. 1. Maps of Niigata Prefecture and Niigata City. Dots in the map indicate the location of isolation of each *M. tuberculosis* strain. Closely-related strain areas are shaded.

showed no abnormalities in ordinary health examinations for the elderly conducted in October 1998, abnormalities did appear in family contact examinations conducted in November of 1998 (bacterial strain, KIJ3). Five other patients who had no contact with the family were independently infected with *M. tuberculosis* between 1998 and 1999 (bacterial strains, KIJ4 to KIJ8).

Bacterial isolation and culture

Bacterial strains were isolated from the sputum or gastric juice of patients between 1998 and 1999. They were cultured on the Ogawa egg medium (Kyokuto Pharmaceutical Co., Tokyo). Prior to DNA extraction, bacterial strains were grown on the Ogawa egg medium for 3 to 4 weeks at 37℃.

Preparation of bacterial DNA

Bacterial colonies on the Ogawa egg medium were suspended in a lysozyme solution (100 mg/ml), and then lyzed using the isoplant kit (Nippon Gene, Tokyo). DNA in the aqueous solution was precipitated with 0.6 volumes of isopropanol. DNA was then rinsed with 70% ethanol and redissolved in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

Digoxigenin-labeled IS*6110* probe

The IS*6110* probe was prepared by using the DIG DNA labeling mix (Boehringer Mannheim Biochemicals, Indianapolis, USA) essentially according to the manufacturer's instructions. Briefly, the 245-bp region within IS*6110* present on the chromosome DNA of *M. bovis* BCG was amplified by PCR with a primer set INS-1 (5'-CGTGAGGGCATCGAGGTGG-C) and INS-2 (5'-GCGTAGGCGTCGGTGACAAA)[10]. The 245-bp amplicon was simultaneously labeled with digoxigenin (DIG)-11-dUTP. The DIG-labeled probe was purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany).

IS*6110*-probed hybridization assay

Bacterial DNA was digested with *PvuII* and the digests were electrophoresed in a 0.8% agarose gel with 1 kb DNA ladder (Life Technologies, Gaithersburg, MD) used as molecular size standards. Southern hybridization was performed as described previously[11] using a nylon membrane (Amersham International, Amersham, UK). DNA hybrids on the membrane were treated with anti-DIG-alkaline phosphatase, Fab fragment (Boehringer Mannheim Biochemicals), and then visualized by color development by adding nitroblue tetrazolium chloride and 5-brome-4-chloro-3-indolyl-phosphate, 4-toluidine salt (substrate) (Boehringer Mannheim Biochemicals).

Computer analysis

The computer-assisted analysis of the IS*6110*-positive fragment patterns was performed with a program called Molecular Analyst Fingerprinting Plus (Bio-Rad, Tokyo), according to the UPGMA clustering algorithm[12,13]. In this analysis, the Dice coefficient (S_D) = $2n_{AB}/(n_A + n_B)$ (where n_A is number of bands in lane A, n_B is number of bands in lane B,

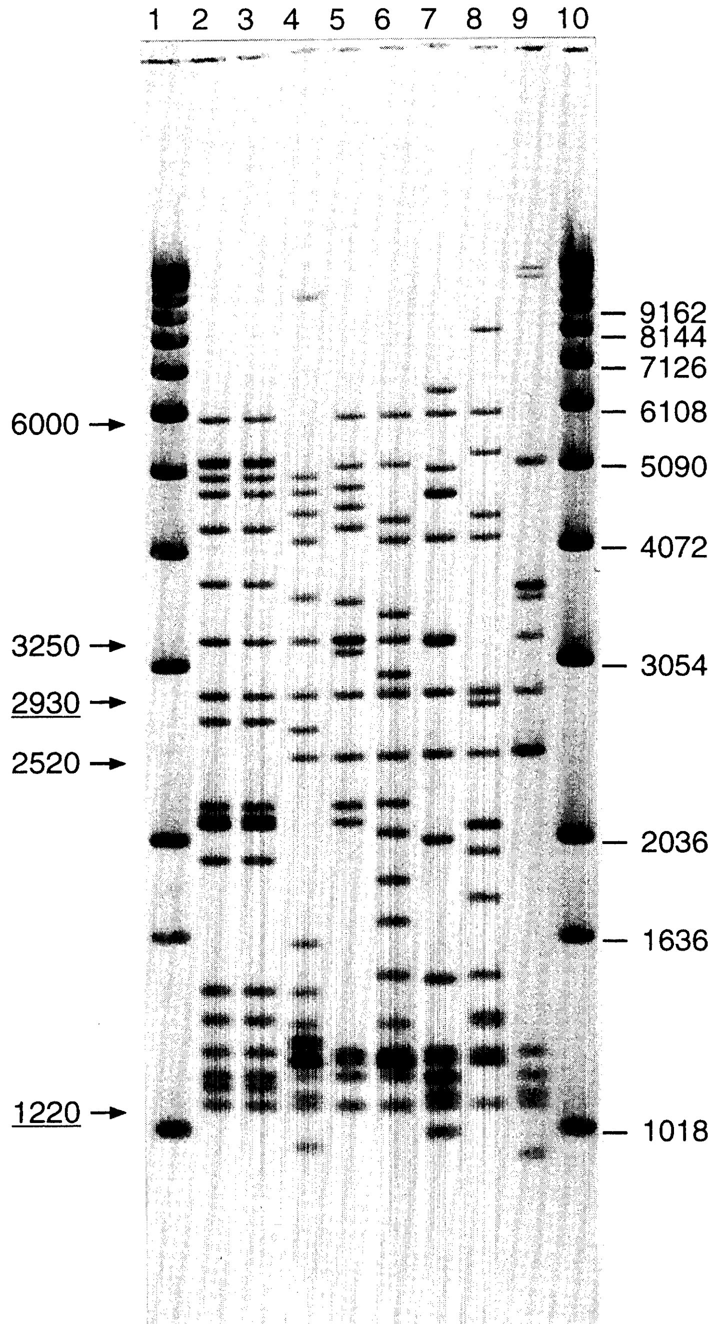

Fig. 2. Southern hybridization analysis of *Pvu*II-digested fragments of *M. tuberculosis* DNA with the IS*6110* probe. Samples: lanes 1 and 10, 1 kb DNA ladder; lane 2, strain KIJ1 (44-year-old male patient's strain); lane 3, strain KIJ2 (his daughter's strain); lane 4, strain KIJ3 (his mother's strain); lane 5, strain KIJ4; lane 6, strain KIJ5; lane 7, strain KIJ6; lane 8, strain KIJ7; lane 9, strain KIJ8. Numbers on the right indicate sizes in bp of the molecular weight markers. On the left, numbers with and without underlining indicate sizes of the common fragment and frequently-found fragment, respectively.

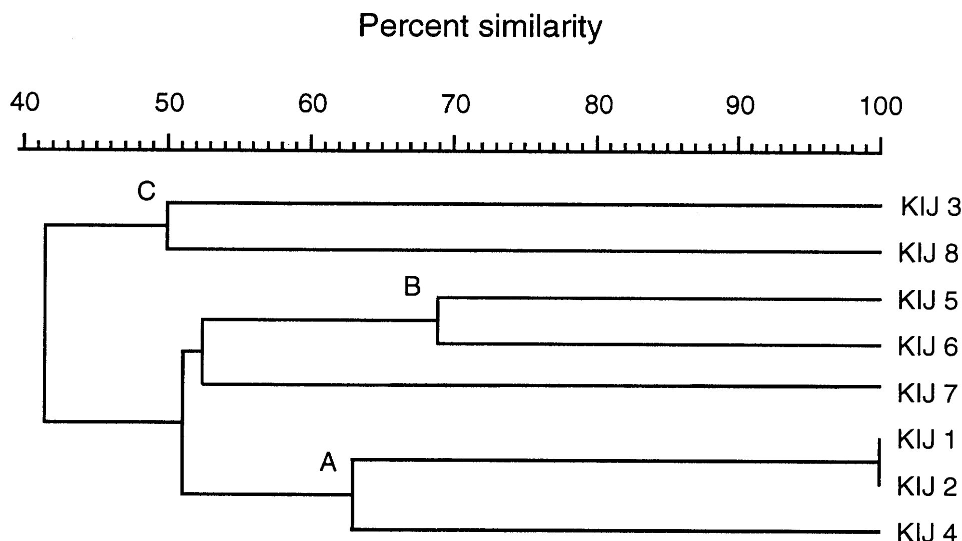

Fig. 3. Dendrogram constructed by computer-assisted comparison of the IS*6110*-probed hybridization analysis data. The data were taken from Fig. 2.

and n_{AB} is number of bands found in both lanes A and B) and the matching tolerance of 0.8% were employed.

RESULTS

IS*6110*-positive restriction fragment patterns

M. tuberculosis strains KIJ1 and KIJ2 (strains from the 44-year-old male patient and his daughter) produced at least 19 *Pvu*II-digestion bands and shared the same fragment pattern in the IS*6110*-probed hybridization assay (Fig. 2, lanes 2 and 3). *M. tuberculosis* strain KIJ3 (the strain from his mother) also showed at least 19 *Pvu*II-digestion bands; however, of the 19 bands, only 7 were identical to those from the family strains KIJ1 and KIJ2, and the remaining 12 were divergent (Fig. 2, lane 4).

M. tuberculosis strains KIJ4 to KIJ8 produced at least 13 to 19 *Pvu*II-digestion bands and showed markedly different fragment patterns (Fig. 2, lanes 5 to 9). Of those, the pattern of strain KIJ4 was more similar to the pattern of the family strains (KIJ1 and KIJ2); 9 of 16 bands were identical (Fig. 2, lane 5).

Dendrogram of strains

M. tuberculosis strains were classified based on the number and patterns of the *Pvu*II-digestion fragments, and a dendrogram showing the similarity between any two strains or group of strains was constructed by computer (Fig. 3). The family strains KIJ1 and KIJ2 and strain KIJ4 constructed a 63% branch similarity (branch A). To a similar extent of similarity, strains KIJ5 and KIJ6 formed another branch (B). Strains KIJ3 and KIJ8 (composing branch C) were located far from the other branches.

DISCUSSION

The copy number of IS*6110* in each *M. tuberculosis* strain is determined from the number of restriction bands that are hybridized with the IS*6110* probe. When M. tuberculosis strains carry multiple copies of IS*6110*, each *M. tuberculosis* strain is identified by the IS*6110*-probed hybridization assay[8]. This study demonstrated that the *M. tuberculosis* strains in Niigata have at least 13 to 19 copies of IS*6110* on the chromosome, although our experience has shown that a precise estimation of the number of IS*6110* per genome may be considerably difficult depending on the conditions of agarose gel electrophoresis employed. The Niigata strains were genetically heterogeneous to a high extent and shared only two *Pvu*II digestion fragments of 1.2 and 2.9 kbp in size (Fig. 2).

Intrafamilial transmission of *M. tuberculosis* in Niigata has unambiguously been demonstrated in this study. Initially, three strains from a family (KIJ1 from a man, KIJ2 from his daughter and KIJ3 from

his moter) were supposed to be the same based on epidemiological information. Indeed, strains KIJ1 and KIJ2 isolated from the family members who were living together were the same in the IS*6110*-probed hybridization assay. However, one strain (KIJ3) from a family member who was living separately from the other two in a distinctly different area in Niigata (although she came into contact with the other two infected family members) was markedly divergent from the intrafamilially-transmitted strains KIJ1 and KIJ2. It is likely that she had become infected with a different strain from a different source.

When the IS*6110*-probed hybridization patterns were analyzed by computer, strains KIJ1, KIJ2, and KIJ4 constructed a branch (A) with a close similarity. One other patient infected with strain KIJ4 lived near the infected family (Fig. 1). Patients infected with strains KIJ5 and KIJ6 (which constructed a branch [B] with a close similarity) also lived near each other (Fig. 1). The location of isolation of strains KIJ3 and KIJ8 which constituted a branch (C) (Fig. 3) was the southeast side of the Shinano River in Niigata City (Fig. 1). These data indicate geographic differences among the strains, although the number of *M. tuberculosis* strains analyzed in this study was small. Further genetic analysis with greater numbers of *M. tuberculosis* strains of various geographic origin will be necessary to know the precise status infection in Niigata.

Acknowledgments. We wish to thank Kazuhiro Kishida and Yasuo Mizuguchi for helpful suggestions for the *M. tuberculosis* hybridization experiments.

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