Genetic Characterization of Multidrug-Resistant Mycobacterium bovis Strains from a Hospital Outbreak Involving Human Immunodeficiency Virus-Positive Patients

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Nineteen multidrug-resistant (MDR) Mycobacterium complex strains isolated in a nosocomial outbreak were characterized at the molecular level. The strains were microbiologically characterized as Mycobacterium bovis. The mpt40 sequence was not present in chromosomal DNA from these strains, supporting the fact that they were M. bovis. All of the isolates were resistant to isoniazid, rifampin, pyrazinamide, ethambutol, streptomycin, para-aminosalicylic acid, clarithromycin, cycloserine, ethionamide, ofloxacin, capreomycin, and amikacin. By performing the standardized IS6110 fingerprinting by restriction fragment length polymorphism (RFLP) analysis, we were able to differentiate two groups (groups A and B) containing two (16 isolates) and three (3 isolates) IS6110 copies, respectively. These strains were typed by spoligotyping, developed to distinguish M. bovis strains and also to distinguish them from M. tuberculosis strains (J. Kamerbeek et al., J. Clin. Microbiol. 35:907–914, 1997). All the strains were confirmed to be M. bovis. In addition, spoligotyping showed a difference in only 1 of 43 spacers between RFLP groups A and B. The rpoB region of several strains representative of each identified group was cloned and sequenced, and identical mutations (Ser-531 to Leu) responsible for the rifampin resistance phenotype were found. To our knowledge, this is the first characterization at the molecular level of an MDR M. bovis strain responsible for a nosocomial outbreak.

The line of progressive decrease in the cases of human tuberculosis in developed countries unexpectedly stopped and then moved upward. During the last few years we are witnessing an impressive increase in the number of cases of human tuberculosis, particularly among human immunodeficiency virus (HIV)-positive individuals (14). The situation may be dramatic in those countries such as Spain, where high rates of HIV infection are superimposed on a high prevalence of tuberculosis. As expected, the resulting high number cases of tuberculosis in HIV-positive patients frequently occurs in a disadvantaged population in which the compliance with the scheduled standard antituberculosis treatments is very low. The consequence is the progressive selection of multiresistant Mycobacterium strains by uncontrolled treatments. Worth consideration is whether multiresistant strains produce a lower rate of infection compared with that produced by wild-type (not multiresistant) Mycobacterium tuberculosis strains when they are acquired by immunocompetent people. Nevertheless, organisms that acquire high transmissibility may evolve to higher pathogenicity. Studies on the epidemiology of tuberculosis are therefore crucial in a social landscape where a high number of immunocompromised patients may come into contact with a high number of potentially susceptible hosts.

Epidemiological studies of tuberculosis are greatly facilitated by the application of strain-specific markers (13). In order to type M. tuberculosis complex strains, most molecular methods are based on the detection of repetitive elements. The repetitive elements described so far in M. tuberculosis are the insertion sequences IS6110 and IS1081, the major polymorphic tandem repeats, the polymorphic GC-rich repetitive sequences, and the direct repeat sequence (9, 18). At present the most extensively used method for differentiating M. tuberculosis is the internationally standardized Southern blotting-based technique consisting of restriction fragment length polymorphism (RFLP) analysis with insertion sequence IS6110 as the probe (17). Several epidemics caused by multidrug-resistant (MDR) M. tuberculosis strains have recently been detected; however, only some epidemics due to MDR strains have been properly documented from a genetic point of view (1).

Mycobacterium bovis is the main organism responsible for tuberculosis in cattle, but its involvement as a causative agent of tuberculosis in humans was recognized a long time ago. Nevertheless, primary human disease due to M. bovis is very rare in developed countries, although a reactivated form of the disease is still encountered. Immunological factors preventing progression of infection caused by M. bovis, whether of animal or human origin, to overt human disease could well be suppressed by coinfection with HIV. Cases of HIV-related human tuberculosis due to M. bovis have been reported in England, France, and the United States (21). Human-to-human transmission of disease due to M. bovis in HIV-positive persons has recently been confirmed. A strain of M. bovis resistant to many antituberculosis drugs was the source of infection in five patients in a Paris hospital (2), but this nosocomial outbreak of MDR M. bovis was not characterized at the molecular level.

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Techniques other than RFLP for differentiating *M. bovis* strains with low IS6110 copy numbers have been based on polymorphic GC-rich repetitive sequence or direct repeat polymorphism (6). Recently, we have communicated the occurrence in our hospital of a presumptive *M. tuberculosis* complex outbreak. From December 1993 through February 1995, 19 cases of primary MDR tuberculosis in HIV-infected patients (16 males and 3 females; mean age, 31 ± 6.5 years; age range, 24 to 54 years) were detected. In all patients the strains were resistant to all the drugs tested (5). The index patient and two other patients had previously attended another hospital in Madrid that had an *M. tuberculosis* MDR epidemic. The other 16 patients were exposed in our infectious diseases ward to one of these three patients. All patients died, despite treatment with multiple first- and second-line antituberculosis drugs. Standard microbiological identification techniques demonstrated that all strains were *M. bovis*. The epidemic was controlled after implementation of a control policy.

In this report we present data resulting from the application of different molecular biology-based methods for characterization of the strains isolated in the cited outbreak. Strains were typed by the standardized method for *M. tuberculosis* strains, RFLP analysis with IS6110, and by spoligotyping, a more suitable procedure for typing and identifying *M. bovis* strains (4, 8). We cloned and sequenced the *rpoB* region responsible for the rifampin resistance phenotype for several representative strains of each identified MDR *M. bovis* group. The spoligotype patterns were almost identical between the groups, suggesting that the strains that we studied and that caused the nosocomial outbreak are members of an MDR *M. bovis* family of clones.

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**MATERIALS AND METHODS**

*Microbiological data on the Mycobacterium strains*. The *Mycobacterium* isolates were identified as *M. tuberculosis* complex strains with nucleic acid probes (GenProbe, San Diego, Calif.). All isolates had the same microbiological characteristics: they were slowly growing mycobacteria, nitrate negative, niacin negative, sensitive to thioin-2-carboxylic acid, and resistant to pyrazamide. Drug susceptibility testing was performed by Cant hits (Promega Corp., Madison). Hybrid plasmid DNAs prepared with specific primers, we showed that the IS6110-RFLP. Extraction of mycobacterial DNA was performed as described previously (10, 17). DNA (1 to 4 μg) from each sample was digested for 1 h with *Pvu*II for analysis with the IS6110 probe. Digests were electrophoresed in 20-cm gels of 0.8% agarose. Southern blotting and hybridization studies were performed as described previously (11).

(ii) Spoligotyping. The oligonucleotides DRAs (5′-biotinylated) and DRbs were used as primers for PCR amplification (8). Approximately 10 ng of purified mycobacterial DNA was used as the target. The DNA was resuspended in 50 μl of a reaction mixture containing Taq buffer, 200 mM each (deoxy)nucleoside triphosphate, 20 pmol (each) of primers DRAs and DRbs, and 0.5 U of *Taq* polymerase (Perkin-Elmer). Thermal cycling was performed as described before (8). Twenty microliters of the reaction mixture was hybridized with 43 different spacer oligonucleotides, which were covalently linked to a filter (8).

**RESULTS**

Characterization of *M. tuberculosis* complex isolates. The strains belonged to the *M. tuberculosis* complex, as determined in clinical samples by positive PCR amplification with both IS6110-specific (3) and *pab*-specific (12) primers.

From the end of 1993 to 1995, 19 *Mycobacterium* complex-positive cultures were obtained from 19 HIV-positive patients. All isolates were identified as *M. bovis* by standard procedures, as described in Materials and Methods. By PCR amplification with specific primers, we showed that the *mpt40* sequence was present in chromosomal DNAs from several *M. tuberculosis* strains but not in DNAs from the strains from the HIV-positive patients, supporting the fact that they were *M. bovis*. All strains were resistant to isoniazid, rifampin, pyrazinamide, ethambutol, streptomycin, *para*-aminosalicylic acid, clarithromycin, cyloserine, ethionamide, ofloxacin, capreomycin, and amikacin.

**RFLP analysis of the strains**. IS6110 fingerprinting by RFLP analysis resulted in two different patterns (designated groups A and B). Two copies of IS6110 of identical size (2.45 and 1.95 kb) were found in 16 isolates, and three copies (2.45, 1.8, and 1.5 kb) were found in 3 isolates. Figure 1 presents the results of RFLP analysis for two strains representative of each pattern.

**Spoligotyping**. The strains were studied by spoligotyping, a technique developed to distinguish among *M. bovis* strains and also to distinguish them from *M. tuberculosis* strains (8). In all cases, the results confirmed the previous identification as *M. bovis*. In addition, spoligotyping showed only 1 of 43 spacer differences between RFLP groups A and B (Fig. 2).

Characterization of *rpoB* mutations responsible for rifampin resistance. Substitution of a limited number of highly conserved amino acids of the β subunit of the RNA polymerase results in high-level resistance to rifampin (15, 16). By using the oligonucleotides described previously (7), we amplified a fragment of DNA of 159 bp that contained the cited region of *rpoB* from two of each of the two different IS6110 clusters and the susceptible strain H37rv. The amplified fragments were ligated to plasmid pGEM-T and were introduced by transformation into *E. coli* TG1. Two transformant clones from each RFLP type and one from H37rv were sequenced. Analysis of the sequence showed that the same change appeared in the two clusters, Leu-531 (codon TTG), with respect to the wild type, Ser-531 (codon TCG).

**DISCUSSION**

AIDS is changing dramatically the epidemiology of tuberculosis (14). *M. bovis* was recognized as a relatively common cause of tuberculosis, being implicated in a number of cases, ranging from 0.1% in France to 5% in the United Kingdom (2). The classical observations supported the conclusion that transmission of *M. bovis* between humans was a very infrequent event (20).

The application of strain-specific markers for differentiating *M. tuberculosis* strains is a useful tool for epidemiological studies of tuberculosis. At present the most extensively used method for differentiating among *M. tuberculosis* strains is the stan-
dardized RFLP analysis (17), but the use of alternative techniques to distinguish \( M. \) \( \text{tuberculosis} \) complex strains within the group having low IS\( 6110 \) copy numbers remains necessary (19). Most \( M. \) \( \text{bovis} \) isolates carry only a single IS\( 6110 \) element and are difficult to differentiate by IS\( 6110 \) RFLP analysis.

Spoligotyping has been a useful tool for the detection of an MDR \( M. \) \( \text{bovis} \) outbreak in our hospital. The results presented strongly suggest that the strains characterized here are members of an MDR \( M. \) \( \text{bovis} \) clone family. They share almost identical spoligotypes (only 1 difference in more than 40 positions) both for the two-band and for the three-band IS\( 6110 \) RFLP clusters. Since the clones containing two bands were isolated before those harboring three bands, the transposition of IS\( 6110 \) during the course of the outbreak could be responsible for the new three-band RFLP. New experiments are being developed in order to determine if both clones belong to a unique outbreak. The detection of a particular \( \text{rpo} \beta \) mutation (Ser-531 to Leu), although identical to the mutation most frequently found in rifampin-resistant \( M. \) \( \text{tuberculosis} \) isolates (7), again supports the clonality of the isolates.

The present work confirms at the molecular level that \( M. \) \( \text{bovis} \) was responsible for a hospital MDR outbreak involving transmission between AIDS patients. To our knowledge this is the first characterization at the molecular level of an MDR \( M. \) \( \text{bovis} \) outbreak in a hospital. Spoligotyping, a PCR amplification technique, makes possible the accurate typing of mycobacteria directly from pathological material in only 2 days. In addition, this technique is able to discriminate \( M. \) \( \text{bovis} \) from \( M. \) \( \text{tuberculosis} \).

A comparison of the different spoligotypes in a database containing MDR strains from different hospitals and countries could facilitate the early detection of interhospital or intercountry outbreaks.

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FIG. 1. RFLPs of \( M. \) \( \text{bovis} \) DNA digested with \( \text{Pvu} \)II and probed with the right arm of IS\( 6110 \). Results for two strains representative of each group are shown. The letters A and B indicate the respective patterns. RFLP of \( M. \) \( \text{tuberculosis} \) 14323 is shown at the right. Arrows indicate IS\( 6110 \)-containing \( \text{Pvu} \)II fragments of approximately 14.4, 7.2, 7.0, 4.4, 3.6, 3.0, 2.3, 2.0, 1.8, and 1.5 kb.

FIG. 2. Spoligotyping of MDR \( M. \) \( \text{bovis} \) DNA. The spoligotype of one strain representative of each pattern (A and B) is shown. The spoligotypes of \( M. \) \( \text{bovis} \) BCG (Pasteur) and \( M. \) \( \text{tuberculosis} \) H37rV are also shown.


AUTHORS’ CORRECTION
(2 articles)

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Allele-Specific PCR Method Based on pncA and oxyR Sequences for Distinguishing Mycobacterium bovis from Mycobacterium tuberculosis: Intraspecific M. bovis pncA Sequence Polymorphism
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The following correction pertains to both of the above articles.
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