Molecular Analysis of Clinical Isolates of Mycobacterium bovis Recovered from Humans in Italy

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In order to achieve a better knowledge of Mycobacterium bovis epidemiology in Italy, 42 clinical isolates from humans were genotyped. Predominant molecular patterns were found in one cluster of 15 isolates sharing spoligotype (ST482), variable-number tandem repeat (VNTR), and IS6110-based restriction fragment length polymorphism (one 1.9-kb band) profiles and in two clusters of 6 and 3 Mycobacterium bovis BCG isolates differing by one VNTR character. The remaining 18 isolates yielded unique profiles. Our results confirm the potential utility of spoligotyping and VNTR typing as a major typing system of M. bovis isolates.

Mycobacterium bovis can cause tuberculosis in a wide range of domestic and wild animals. The organism can also infect humans, producing a disease indistinguishable from that caused by Mycobacterium tuberculosis. In many industrialized countries, M. bovis has been eradicated from cattle or reduced to very low levels. In Italy as well as in other western European countries, only a few bacteriologically proven human tuberculosis cases have been attributed to M. bovis (12, 17, 26), but in developing countries M. bovis is still a cause of concern for animals and humans (5, 8). M. bovis is transmitted within and between domestic and wild animals as well as from animals to humans or vice versa (19). Contaminated food (especially milk) or direct contact with infected animals is considered to be the primary route by which M. bovis infects humans (9). Direct human-to-human transmission seems to be rare and has been confirmed mainly among immunocompromised patients (2, 19).

Molecular typing of isolates has become a valuable tool in the study of M. bovis epidemiology, allowing investigators to detect outbreaks and achieve better knowledge of transmission and increasing incidence of infection. However, only very few molecular surveys of human populations have been reported (5, 12, 17, 26), and no studies have been reported from Italy. This study, as a contribution to the knowledge of M. bovis molecular epidemiology in Italy, examines the molecular characteristics of 42 M. bovis clinical isolates collected from 34 Italian-born and 8 foreign-born tuberculosis patients hospitalized in Tuscany, Italy, between 1990 and 2005. A total of 13 isolates were from human immunodeficiency virus-negative patients; human immunodeficiency virus serology was not known for 29 patients. The M. bovis isolates were identified by the use of DNA molecular probes specific for the M. tuberculosis complex (Accuprobe; Gen-Probe) and by determining that niacin production and nitrate reductase activity were both negative. The isolates were typed by molecular techniques including (i) the commercial GenoType MTBC assay (Hain Life
cience GmbH, Nehren, Germany), (ii) high-performance liquid chromatography (HPLC) analysis of cell wall mycolic acids, (iii) spoligotyping, (iv) IS6110-based restriction fragment length polymorphism (IS6110-RFLP), and (v) variable-number tandem repeat (VNTR) typing. The overall molecular results are reported in Table 1.

GenoType MTBC assay. The GenoType MTBC assay targets fragments of the rpoB gene; the RD1 region of M. tuberculosis and M. bovis, which is absent from M. bovis bacillus Calmette-Guérin (BCG) (13); and the gyrB polymorphism, characterized by mutations suitable to differentiate most of the species belonging to the M. tuberculosis complex (20). All the isolates hybridized with the rpoB probe, thus confirming that they belonged to the M. tuberculosis complex. The results demonstrate that 41 strains had gyrB mutations typical of M. bovis and BCG; 9 of these strains lacked RD1 and were identified as M. bovis BCG. One isolate presenting RD1 had the gyrB mutations typical of Mycobacterium caprae.

HPLC. HPLC analysis of cell wall mycolic acids, performed according to the work of Butler et al. (4), yielded the specific six-clustered BCG profile for the nine isolates identified as M. bovis BCG by the GenoType MTBC assay. The M. bovis isolates, including the M. bovis/M. caprae isolate, yielded the four-clustered M. tuberculosis profile (10).

Spoligotyping. The spoligotyping (spacer oligonucleotide typing) technique, which probes the variability of spacer sequences interspersed with direct repeat (DR) sequences in the polymorphic chromosomal loci DR, was performed by use of the standard spoligotype assay described by Kamerbeek et al. (15). Spoligotyping detected 12 individual patterns that were assigned to one of the share types (ST) contained in the SpolDB4 (3) and the Mbovis.org (http://www.Mbovis.org) databases (the ST designation of the SpolDB4 database is used throughout the text). The prevalent spoligotype was ST482, detected in 29 isolates (69.0%). This spoligotype was found in all the M. bovis BCG isolates as well as in the reference strains M. bovis BCG Pasteur and M. bovis BCG Tice (data not shown). The “BCG-like” strain ST482 has been shown to be

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### TABLE 1. Molecular characteristics of M. bovis clinical isolates from humans

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>GenoType test HPLC pattern</th>
<th>Spoligotyping</th>
<th>IS6110 - RFLP fingerprint</th>
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**Spoligotyping** was performed according to the definition in SpolDB4 and in the Mbovis.org databases. **IS6110 - RFLP fingerprints** were analyzed by the Gelcompar 4.1 software package (Applied Maths, Kortrijk, Belgium). **VNTR-MIRU profile** is expressed as a string of 12 numbers, each representing the number of tandem repeats at a given VNTR position (see text for more details).
prevalent in animals in Italy (24), France (14), Germany (17), and Spain (1) and has been isolated in many other countries but apparently never in the United Kingdom or Ireland. In the very few reports on M. bovis isolates from humans, the ST482 spoligotype was prevalent in Germany (ca. 20%) (17) but rarely found in the United Kingdom (1%) (12), thus paralleling the spoligotype distribution in animals. Spoligotypes ST820 and ST1932, found in two isolates, and spoligotypes ST1842, ST777, ST672, and ST665, found in individual isolates, have previously been detected in several different geographical areas among humans (3) and animals (6, 14). Five individual spoligotypes detected among our human isolates had not been described previously; these isolates, generally referred to as “orphan,” are labeled as ST0, according to the rules of the SpolDB4 database.

**IS6110-RFLP.** The IS6110-RFLP assay was performed according to the standardized method described by van Embden et al. (27). IS6110-RFLP analysis is known to provide the best discrimination for M. tuberculosis isolates but is considered poorly discriminatory for M. bovis isolates from cattle, which usually have a single copy of IS6110 (7, 16). In the present study, the 42 isolates yielded four distinct IS6110 banding patterns, each consisting of one or two IS6110 bands, as expected. Similar to the reference BCG Pasteur and Tice strains, 35 isolates, including all the BCG isolates and 26 M. bovis isolates, showed a single restriction fragment of 1.9 kb; 2 M. bovis isolates yielded a single band slightly smaller than 1.9 kb. The two-band profile was found for two isolates yielding the 1.9-kb band and an additional smaller band as well as for one isolate yielding the 1.9-kb band and a larger band. Two M. bovis isolates did not show any IS6110 restriction fragment.

**VNTR typing.** VNTR typing is aimed at displaying variations in the copy number of tandem repeats located in mycobacterial interspersed repetitive units (MIRU) in the chromosome. This technique has been successfully used to differentiate M. tuberculosis strains (25) and to discriminate between M. bovis strains (11, 21). VNTR typing was performed by PCR amplification of 12 MIRU loci (i.e., loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40) as described by Supply et al. (25). The PCR fragments were analyzed by gel electrophoresis using 2% NuSieve agarose (Cambrex Bio Science Rockland). For each locus, sizes of amplicons were estimated by comparison with 20-bp and 100-bp markers (Superladder-low; GenSura, California), and the numbers of repetitive units were calculated.

**VNTR-MIRU analysis** yielded 10 different profiles among the isolates. The nine M. bovis BCG isolates showed, at locus 4, two alleles not found in any other isolate of the collection: in particular, six isolates, as well as the reference strains BCG Pasteur and Tice (not shown in Table 1), displayed two copies of a 77-bp repeat sequence that is commonly found at the 3′ terminus in all the strains of the M. tuberculosis complex. This allele is usually indicated as 3′, as the symbol ‘designates alleles devoid of the 53-bp sequence that is commonly found at the 3′ terminus in all the strains of the M. tuberculosis complex. Allelic diversity was calculated using the equation $h = 1 - \frac{\sum x_i^2}{n(n-1)}$, where $n$ is the number of isolates and $x_i$ the frequency of the $i$th allele at the locus (23).

### TABLE 2. Determination of heterogeneity of VNTR at each MIRU locus of M. bovis clinical isolates

<table>
<thead>
<tr>
<th>No. of TR copies $^a$</th>
<th>No. of isolates at VNTR-MIRU locus</th>
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<tbody>
<tr>
<td>0</td>
<td>42</td>
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<tr>
<td>1</td>
<td>42</td>
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<tr>
<td>2</td>
<td>42</td>
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<td>3</td>
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<td>7</td>
<td>41</td>
</tr>
<tr>
<td>8</td>
<td>41</td>
</tr>
</tbody>
</table>

$^a$ TR, tandem repeat. The symbol ‘ indicates alleles devoid of 53-bp sequences at the 3′ terminus of the MIRU locus.

**Allelic diversity** ($h$) was calculated using the equation $h = 1 - \frac{\sum x_i^2}{n(n-1)}$, where $n$ is the number of isolates and $x_i$ the frequency of the $i$th allele at the locus (23).
found in one cluster of 15 isolates sharing spoligotype, VNTR-MIRU loci, and IS6110-RFLP profile. The two other clusters included six and three M. bovis BCG isolates that differed by one VNTR-MIRU locus. The remaining 18 isolates yielded unique profiles.

The results of this study, which provides a first description of the population structure of M. bovis in Italy, will be useful for further molecular surveys aimed at achieving a better knowledge of moving and expanding strains in our country. Moreover, the results confirm the potential utility of spoligotyping and VNTR-MIRU typing, although with the limits outlined above, as a major typing system for M. bovis isolates. On this last subject, we agree with the statement of Roring and co-workers (21, 22) that for M. bovis typing the number and combination of informative VNTR markers selected for epidemiological studies need to be determined empirically with reference to locally prevalent strains.

This work was supported by Italian MIUR (PRIN-2004) and partly by the “Istituto Superiore di Sanità” (National Research Programme included six and three M. bovis BCG isolates that differed by one VNTR-MIRU locus. The remaining 18 isolates yielded unique profiles.

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REFERENCES


