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Molecular Epidemiology of Multidrug-Resistant *Mycobacterium bovis* Isolates with the Same Spoligotyping Profile As Isolates from Animals†

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PCR-based characterization techniques have been adopted in most laboratories for *Mycobacterium bovis* typing. We report a molecular characterization of human multidrug-resistant *M. bovis* isolates and three bovine isolates that share the spoligotyping profile. The analysis of the direct repeat region showed that both groups differed in the presence of spacers not included in the current membrane. They were also distinguished by two out of the nine mycobacterial interspersed repetitive unit variable-number tandem repeat loci tested, indicating that the human infection was not acquired from the cattle from which isolates were obtained. These results highlight that a combination of techniques is required for appropriate discrimination, even for those spoligotypes that have a low frequency.

*Mycobacterium bovis* causes tuberculosis in cattle and other animals, and its implication in human tuberculosis has been recognized since the beginning of the twentieth century (21). In industrialized countries, human infection with *M. bovis* has been largely controlled through pasteurization of cow’s milk, abattoir inspection, and culling of cattle reacting to compulsory diagnosis (tuberculin test). In spite of this, several cases of human tuberculosis caused by *M. bovis* have been described in recent years (6–8, 12, 17, 20), although some human *M. bovis* infections could be attributed to a possible endogenous reactivation of a historically acquired infection (11). On the other hand, some new cases of human *M. bovis* tuberculosis have also been associated with the consumption of dairy products, for instance, unpasteurized cheeses on the United States-Mexico border (7, 17, 35). Thereby, cases of human *M. bovis* have been related to immigrants or to people who have contracted the infection in countries where eradication programs are patchy or nonexistent (35). Moreover, several of these studies also found an association between human *M. bovis* disease and human immunodeficiency virus coinfection, showing tuberculosis to be an opportunistic disease in immunodepressed patients (5, 7).

In Spain, less than 1% of clinically diagnosed cases of tuberculosis that are subsequently proven bacteriologically are attributed to *M. bovis* (28). From December 1993 to February 1995, 19 cases of primary *M. bovis* tuberculosis were detected in two big hospitals in central Spain (12). Afterwards, the outbreak spread to other cities and countries (18, 23, 27). These human isolates were resistant to 11 drugs (12). These isolates were characterized by spoligotyping (direct variable repeat [DVR] spacer oligonucleotide typing [15] and restriction fragment length polymorphism-IS6110 [RFLP-IS6110]) (32), showing two different profiles, group A and group B, by both techniques (3).

Advances in molecular characterization have provided tools to enhance our knowledge of *M. bovis* dissemination and tuberculosis control. PCR-based characterization techniques such as spoligotyping (15) and mycobacterial interspersed repetitive unit (MIRU) variable-number tandem repeat (VNTR) typing (9, 31) have been adopted in most laboratories for *M. bovis* typing because they are faster, simpler, and cost-effective, with the added advantage that results are qualitative (present or absent or in the form of a number). The degree of differentiation and reproducibility is good, but spoligotyping has a lower discriminatory power than MIRU-VNTR (1, 16).

The target of this study was to search for the existence of *M. bovis* isolates of animal origin with the same spoligotyping pattern and, if found, to determine whether or not human and animal *M. bovis* isolates are clonal using molecular characterization. This finding may indicate direct or indirect cattle-to-human transmission.

**Surveillance and monitoring system of animal tuberculosis.** As part of the surveillance and monitoring system of animal tuberculosis employed by the Spanish Ministry of Agriculture, Fisheries, and Food, we maintain a Spanish database of spoligotyping profiles of *M. bovis* of domestic and wild animal origin, which consists of around 3,500 strains grouped in 200 spoligotypes. We identified the same profile involved in the multidrug-resistant (MDR) human outbreak (3) in only three *M. bovis* isolates from three cattle in a farm in central Spain (Hexacode 63-5F-5E-7F-FF-60, SB0426; http://www.mbovis.org).

*M. bovis* isolates. The first (93/R1) and the last (95/R4) isolates of the human outbreak of MDR *M. bovis* in the hospital in Madrid, previously classified within the group A (12), and the three isolates from cattle slaughtered in the year 2000 (00/487, 00/491, and 00/583), which shared the same spoligotyping profile, SB0426 (Fig. 1), were studied. Isolates were...
Human and cattle the supplemental material) revealed hidden polymorphisms. The region between nucleotide 681 and the 15th spacer (given in the same group. However, the sequence analysis of the sequence of the BX248343).

The available spoligotyping membrane, as described by the standard proportion method (4) and the number of repeats was calculated (www.ibf.fr/mirus/mirus.html) (9, 25, 29).

The cattle and human isolates differed in a copy at ETR-A (6 and 5 copies, respectively) and at QUB-3232 (7 and 6 copies, respectively) (Fig. 2). However, cattle and human groups did not differ in the number of copies at ETR-B (4 copies), QUB-11a (10 copies), QUB-11b (2 copies), QUB-26 (5 copies), MIRU 4 (3 copies), MIRU 20 (2 copies), and MIRU 40 (2 copies).

The molecular characterization rules out these cattle isolates as direct sources of the MDR human outbreak. Spoligotyping is considered a useful typing method for Mycobacterium tuberculosis complex organisms, at least at the level of a first screening (15, 24), as has been shown in its application in routine epidemiological studies in animal isolates (2, 13). However, its level of discrimination may not be enough to establish the definitive identity. In that sense, these results highlight that a combination of techniques is required for appropriate discrimination, even for those spoligotypes that have a low frequency. Furthermore, the design of a new spoligotyping membrane that complements the available one would be essential to improving the degree of strain differentiation.

Polyorphic MIRU-VNTR loci have been exploited for M. bovis typing (14, 26, 29, 30), although in this study only two out of the nine loci tested were able to distinguish both groups of isolates. VNTR typing could be a good discriminatory typing method, nevertheless, this technique is not yet standardized, and it is necessary to assess the discriminatory power of each polymorphic locus alone and in combination with others before its extended use. Due to the allelic diversity of loci among

MIRU-VNTR analysis. These isolates were also compared by analyzing nine MIRU-VNTR loci (9, 25, 29, 31). PCR was performed as previously described. Amplicon sizes were estimated by electrophoresis on a 2.5% agarose gel at 45 V for 2 h, using a 100-bp ladder (Biotools), and the number of repeats was calculated (www.ibf.fr/mirus/mirus.html) (9, 25, 29).

FIG. 1. Spoligotyping pattern (SB0426) found in two MDR M. bovis isolates from humans (R1/93 and R4/95) and three cattle M. bovis isolates from the same farm (00/487, 00/491, and 00/583). Spoligotyping was performed on a commercial membrane (Isogen Bioscience BV, Maarssen, The Netherlands) following a standard protocol (15). A clinical isolate of M. tuberculosis was used as a control.

The cattle and human isolates were sensitive to the antibiotics tested, unlike the ethambutol (5 and 10 g/ml), rifampin (1 g/ml), streptomycin (2 and 10 g/ml), kanamycin (2.5 μg/ml), and ofloxacin (2.5 μg/ml). The cattle isolates were sensitive to the antibiotics tested, unlike the human isolates, which were described as resistant to these drugs.

DR region polymorphism. A loopful of each M. bovis isolate was resuspended in 200 μl of sterile purified water, heat inactivated for 10 min, and used as template. The 5′ fragment of this region was amplified with primers DR681 (5′ CCGCTTTG TCAGCCGACAGGAG 3′) and sp15R (5′ GCCAGCCCCGG AGTACTCGCT 3′) (Roche Molecular Biochemical, Berlin, Germany), which are based on known sequences of nucleotides 681 to 701 and genomic spacer 15, nucleotides 1321 to 1340, respectively (GenBank accession no. 929733 [genome number Z48304]; http://www.ncbi.nlm.nih.gov/GenBank). The DNA was inoculated in a 50-μl PCR mixture containing 10× standard reaction buffer including 2 mM MgCl2 (Biotools B&K Labs, Madrid, Spain), 400 μM of deoxynucleoside triphosphate (dNTP mix; Biotools), 0.64 μM of each primer, and 0.2 U of Taq Ultratools (Biotools). Amplification was carried out in a thermal cycler PTC-100 following the conditions as previously described (34).

PCR products were checked on 2% agarose gels and examined under UV light after staining with ethidium bromide. The amplification products obtained were purified with the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced with the DyeDeoxy (dRhodamine) Terminator cycle sequencing kit in an automatic ABI Prism 373 DNA sequencer (Applied Biosystems) (CIB Sequencing Facilities, Madrid, Spain). The sequences from the amplified products were aligned and compared with the CLUSTALW application software (http://www.es.embnet.org/cgi-bin/clustalw.cgi), using the M. bovis AF212297 strain as reference (accession no. BX248343).

The available spoligotyping membrane, as described by Kamerbeek et al. (15), classified the human and animal isolates into the same group. However, the sequence analysis of the region between nucleotide 681 and the 15th spacer (given in the supplemental material) revealed hidden polymorphisms. Human and cattle M. bovis isolates showed differences in the presence of spacers with genomic numbers 6, 7, 8, and 11 (numbering according to van Embden et al. [33]) (Table 1), which are not included in the current spoligotyping membrane.

TABLE 1. Presence or absence of spacers detected by sequencing of a fragment of the 5′ region of the DR locus (accession no. BX248343)

<table>
<thead>
<tr>
<th>Isolate(s) tested</th>
<th>Detection of DVR for genomic order (membrane order)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate(s)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>AF212297</td>
<td>+</td>
</tr>
<tr>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>Cattle</td>
<td>+</td>
</tr>
</tbody>
</table>

* Spacers are numbered according to a previous designation for oligonucleotide (33) and as bound to the membrane (15). +, presence of the DVR (direct repeat and spacer); -, absence of the DVR. The spacers 4, 9, and 10 are absent in the reference strain and studied isolates.
M. bovis isolates in different geographical areas, the most appropriate combination of VNTRs for molecular epidemiological studies should be investigated. As has been established for spoligotyping patterns, a database of VNTR profiles would facilitate the tracking of M. bovis strains.

To date, we have not identified an animal origin for the human outbreak. Risk of infection from livestock has been reduced as a result of eradication programs. Nevertheless, other hazards have emerged that should be controlled, such as consumption of handmade raw-milk products (19, 35) or contact with M. bovis-infected game animals (2, 10, 22).

It is tempting to speculate about the origin of the human isolates. In the 1950s to 1960s the use of INH as a growth promoter in cattle before slaughtering was a common practice in Spain and might have resulted in the development of resistance. We hypothesize that the first human isolate may be a reactivation of an infection acquired at that time. Because diagnosis is commonly based on commercial probes directed to M. bovis, this isolate may have been misdiagnosed as M. tuberculosis. The treatment of the patient with the recommended therapy of INH, RIF, and pyrazinamide (to which all M. bovis isolates are naturally resistant) would have been in fact a monotherapy with RIF that led to multidrug resistance.

In summary, the results obtained with detailed molecular characterization allowed us to distinguish the human and cattle M. bovis isolates and determine that to date, there is no evidence of Spanish animal origin (at least, in recent times) of the multidrug-resistant human outbreak. Therefore, the initial hypothesis of nosocomial infection (12) seems to be the most credible. Spoligotyping may not be useful to determine that two strains are closely related without the support provided by basic epidemiology. Therefore, the application of other techniques is needed. In addition, both human and bovine tuberculosis monitoring are essential in the control and eradication programs of tuberculosis.

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