JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1997, p. 3328–3330 Vol. 35, No. 12
0095-1137/97/$04.00+0

Identification by Spoligotyping of a Caprine Genotype in *Mycobacterium bovis* Strains Causing Human Tuberculosis

MONTSERRAT GUTIÉRREZ,[1] SOFÍA SAMPER,[2] MARÍA SOLEDAD JIMÉNEZ,[3] JAN D. A. VAN EMBDEN,[4] JUAN FRANCISCO GARCÍA MARÍN,[1] AND CARLOS MARTÍN[2]*

Departamento de Patología Animal, Medicina Animal, Facultad de Veterinaria, Campus de Vegazana, 24071 León,[1] Departamento de Microbiología, Salud Pública y Medicina Preventiva, Facultad de Medicina, Domingo Miral, 59009 Zaragoza,[2] and Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid,[3] Spain, and Unit of Molecular Microbiology and Laboratory of Bacteriology, National Institute of Public Health and Environmental Protection, 3720 BA Bilthoven, The Netherlands[4]

Received 4 June 1997/Returned for modification 4 August 1997/Accepted 29 September 1997

We have used spoligotyping to characterize 18 *Mycobacterium bovis* strains isolated from cattle and 23 *M. bovis* strains isolated from goats. The spoligotypes revealed that caprine strains form a separate and well-differentiated group that we refer to hereafter in this abstract as the caprine genotype. To evaluate the importance of this genotype as a cause of tuberculosis in other animal species, including humans, we applied the spoligotyping method to 112 strains, including to all isolates identified as *M. bovis* by a Mycobacterial National Reference Laboratory (Majadahonda, Madrid) from 1994 to 1996. Eighty-three of these strains were identified in human isolates. In addition to being identified in three goat isolates and two sheep isolates, the caprine genotype was also found in three isolates causing human tuberculosis. Evidence to support the argument that there is a zoonotic risk of caprine tuberculosis was presented by the identification of the caprine genotype in an isolate from a veterinary worker with a recent history of contact with tuberculous goats.

Mycobacterium bovis infection is recognized worldwide as the major agent responsible for tuberculosis (TB) in cattle. It is also considered, in some countries, an important pathogen in other farmed animals, such as goats (2, 7, 16), deer (8), and buffaloes (10). In general, a wide range of animal species can act as hosts for *M. bovis*, including human beings (3, 4, 14, 15, 21). In Spain, no feral reservoirs that play an important role in *M. bovis* transmission have been described, and TB in cattle is controlled through systematic eradication campaigns. However, *M. bovis* infection in goat livestock is widespread, and only minimal control measures are adopted to prevent spread to human and bovine populations (5). Although mixed goat and sheep farming is a common practice in Spain, cases of TB in sheep are seldom found, and evidence of infection is generally only detected in postmortem findings (6). This raises questions as to the ability of TB to spread from goats to other species. Regarding the importance of *M. bovis* as a pathogen for human beings, the frequency of isolation in clinical samples is low (4, 14, 15). In Spain, less than 0.5% of the strains isolated from humans with tuberculosis are *M. bovis* (18). The difficulty in discriminating strains of the *Mycobacterium tuberculosis* complex may be responsible for the underreporting of *M. bovis* cases (3), as *M. bovis* infection is largely clinically indistinguishable from *M. tuberculosis* infection (14). It is considered that human-to-human transmission of *M. bovis* is not of major importance in immunocompetent persons. However, Samper et al. (17) have demonstrated the possibility of transmission of a group of multidrug-resistant *M. bovis* strains among human immunodeficiency virus-infected patients. The development of molecular techniques to differentiate strains of the *M. tuberculosis* complex has helped to provide a greater understanding of the epidemiology of TB. The use of restriction fragment length polymorphism (RFLP) with the IS*6110* probe (20) is convenient and reliable for the detection of *M. tuberculosis* strains but lacks sensitivity for the majority of *M. bovis* strains. A combination of several probes can be used to derive RFLPs for *M. bovis* when detailed differentiation is required (19).

A novel method called spoligotyping that is highly effective for differentiating strains of the *M. tuberculosis* complex has been developed. It is based on DNA polymorphisms that are found in the direct repeat (DR) regions of the mycobacterial genome (12). The results achieved so far indicate that spoligotyping offers a superior alternative when rapid identification is required, and it is also more suitable for the differentiation of strains with few copies of IS*6110*, such as *M. bovis* (12). In a recent study using spoligotyping of strains isolated from different animal species in Spain, Aranaz et al. (1) showed that goats and cattle were host to different clusters of strains. This observation was in agreement with the results previously obtained in a similar study using RFLP analysis (9, 13).

In the present study, we used spoligotyping analysis on bovine and caprine strains of *M. bovis* that were previously subjected to RFLP typing methods (9). After we had established characteristic spoligotypes for each of these hosts, we carried out spoligotyping on another group of *M. bovis* strains, isolated from human beings and different animal species. We wanted to determine the types of strains present and to evaluate the zoonotic importance of caprine TB.

A total of 153 *M. bovis* strains were analyzed. We initially tested a group of 41 isolates from 18 cattle and 23 goats in herds from the Aragón and Valencia regions of Spain. These isolates were previously subjected to DNA fingerprinting with IS*6110*, PGRS, and the DR region (9). Another group consisted of 103 isolates, previously identified as *M. bovis* on the basis of growth characteristics and biochemical tests, and corresponding to the total number of *M. bovis* strains deposited at the Mycobacterial National Reference Center of Majadahonda, Madrid, Spain, between January 1994 and November 1996. They originated from different geographical areas of

* Corresponding author. Mailing address: Dpto. de Microbiología, Salud Pública y Medicina Preventiva, Facultad de Medicina, Domingo Miral, 59009 Zaragoza, Spain. Phone: 34 976 761759. Fax: 34 976 761664. E-mail: Carlos@posta.unizar.es.

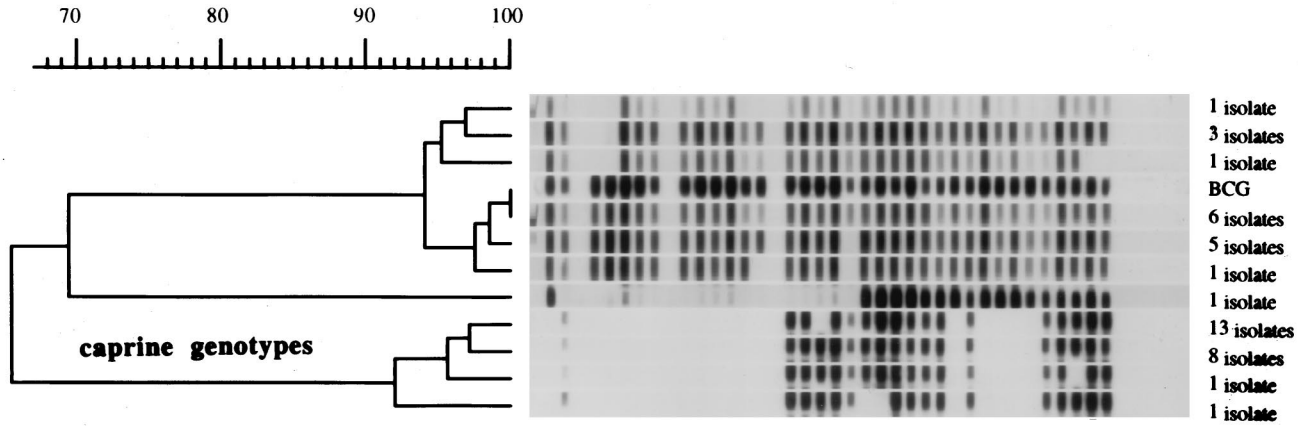

FIG. 1. Dendrogram with the spoligotypes from *M. bovis* bovine and caprine isolates and BCG. Top three lanes and the four lanes below BCG, bovine isolates; bottom four lanes, caprine isolates. The main cluster pattern characteristic of caprine hosts is noted as the caprine genotype.

Spain and from different host species as follows (number of isolates in parentheses): humans (83), bovines (12), caprines (3), an ovine (1), a domestic feline (1), and llamas (3). The remaining 9 strains, obtained from a regional laboratory (Servicio de Investigación y Mejora Agroria, Derio, Madrid, Spain), were isolated from hosts as follows (number of isolates in parentheses): cattle (6), a sheep (1), and llamas (2). As controls in our study, *M. bovis* BCG and *M. tuberculosis* H37Rv were used as reference strains.

The extraction of DNA from *M. bovis* strains was carried out according to the standardized method of fingerprinting for *M. tuberculosis* (20). Spoligotyping relies on the amplification of the polymorphic DR region containing 36-bp DRs and interspersed 35 to 41-bp variable spacer sequences, and this amplification was carried out as previously described (12, 22). A computer analysis of the results was obtained by using the GelCompar computer system (11) (version 3.1; Applied Maths, Kortrijk, Belgium).

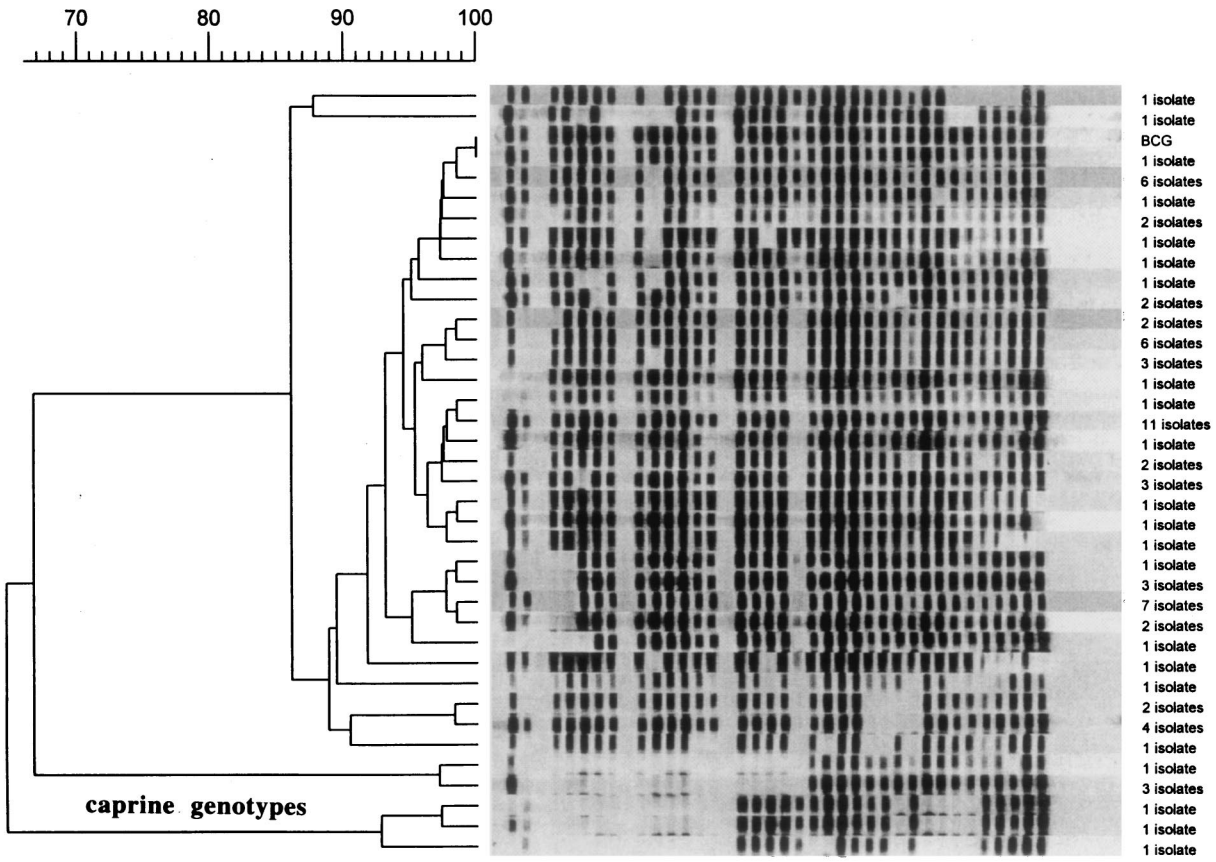

FIG. 2. Dendrogram with the spoligotypes from BCG and all *M. bovis* isolates of human origin used in this study. The total number of isolates identified as *M. bovis* at the Mycobacterial National Reference Center of Majadahonda in Madrid between 1994 and 1996 was 83. Three of the isolates were eliminated from the study since their spoligotypes showed them to be *M. tuberculosis*. The three bottom lanes correspond to the caprine spoligotype, as indicated on the dendrogram.

The results of the spoligotyping of a group of 18 bovine- and 23 caprine-derived strains revealed the existence of two main cluster patterns, with each pattern characteristic of an animal host and with seven and four types identified in each cluster, respectively (Fig. 1). The caprine spoligotypes were characterized by the lack of a large set of oligonucleotides (oligonucleotides 3 to 16, 28, and 30 to 33). These findings confirmed the existence of two genotypes affecting cattle and goats in Spain, as initially reported by Gutiérrez et al. (9) and also demonstrated by Aranaz et al. (1).

Spoligotyping was subsequently performed on the 112 *M. bovis* strains isolated from humans and different animal species. Three of the human isolates were identified as *M. tuberculosis* on the basis of the presence of spacers 39 to 43. This result demonstrated the power of the technique to distinguish *M. tuberculosis* from *M. bovis*. The strains isolated from llamas and cats all showed the genotype characteristic of bovine isolates. With the ovine isolates, two patterns were identified that matched with the group of caprine strains. This agreed with the previous results of Aranaz et al. (1) for ovine isolates showing caprine-like spoligotypes. The 80 *M. bovis* isolates from humans were differentiated into 37 spoligotypes (Fig. 2), with the majority (77 isolates) yielding 34 spoligotypes that were clearly identifiable as belonging to the bovine genotype. However, three of the human isolates displayed three spoligotypes typical of the caprine genotype (Fig. 2). In tracing the origins of these three strains, we found that one of the patients was a resident in a rural area where goat farming was common, the second patient worked in an abattoir, and the third was a veterinary practitioner with a history of contact with goat herds where TB was present. Subsequently, the pattern of this third isolate was found to be identical to that observed for an *M. bovis* goat isolate from one of those herds.

The isolation of the bovine strain type in human populations would indicate that infected cattle constitute the main risk of transmission of *M. bovis* from livestock to humans. This may be because bovine TB is more widespread or because bovine strains are more adaptable in human hosts. However, that fact that our results clearly demonstrate that goats are also a source of infection for humans implies that a zoonotic risk does exist, which could impact on traditional goat farming practices. Although low rates of TB have been demonstrated in sheep, the finding that the *M. bovis* genotype is identical in goats and sheep does not exclude sheep as a possible source of infection. However, our results to date demonstrate the current need for control and eradication policies directed at limiting the spread of TB from goats to other species.

We are indebted to Eamonn Gormley for helpful discussions and preparation of the manuscript and to Ramón Juste who kindly provided us with the strains from the S.I.M.A.

This work was supported by grant FIS97-0042 from the Ministry of Health, Spain (Fondo de Investigaciones Sanitarias de la Seguridad Social), and by grant CT97-2102 from the European Commission Research Program BIOMED2 on Molecular Epidemiology and Control of Tuberculosis.

REFERENCES

1. **Aranaz, A., E. Liébana, A. Mateos, L. Domínguez, D. Vidal, M. Domingo, O. González, E. Rodríguez-Ferri, A. E. Bunschoten, J. D. A. van Embden, and D. Cousins.** 1996. Spacer oligonucleotide typing of *Mycobacterium bovis* strains from cattle and other animals: a tool for studying epidemiology of tuberculosis. J. Clin. Microbiol. **34:**2734–2740.
2. **Bernabé, A., M. A. Gómez, J. A. Navarro, S. Gómez, J. Sánchez, J. Sidrach, and V. Menchén.** 1991. Pathological changes of spontaneous dual infection of tuberculosis and paratuberculosis in goats. Small Rumin. Res. **5:**377–390.
3. **Collins, C. H., and J. M. Grange.** 1987. Zoonotic implication of *Mycobacterium bovis* infection. Irish Vet. J. **41:**363–366.
4. **Cotter, T. P., S. Sheehan, B. Cryan, E. O'Shaughnessy, H. Cummins, and C. P. Bredin.** 1996. Tuberculosis due to *Mycobacterium bovis* in humans in the South-west region of Ireland: is there a relationship with infection prevalence in cattle? Tubercle Lung Dis. **77:**545–548.
5. **García Marín, J. F., and M. Gutiérrez.** 1996. Aspectos epidemiológicos y clínicos de la tuberculosis caprina. Ovis **46:**45–60.
6. **García Marín, J. F., L. Luján, and J. J. Badiola.** 1989. Diagnóstico de tuberculosis ovina. Itea **85**(Suppl.)**:**163–165.
7. **García Marín, J. F., B. Peris Palau, and J. J. Badiola.** 1992. Tuberculosis caprina: descripción de casos clínicos, distribución geográfica y formas de presentación en rebaños altamente infectados. Cienc. Vet. **5:**63–66.
8. **Griffin, J. F. T., and G. S. Buchan.** 1994. Aetiology, pathogenesis and diagnosis of *Mycobacterium bovis* in deer. Vet. Microbiol. **40:**193–205.
9. **Gutiérrez, M., S. Samper, J. A. Gavigan, J. F. García Marín, and C. Martín.** 1995. Differentiation by molecular typing of *Mycobacterium bovis* strains causing tuberculosis in cattle and goats. J. Clin. Microbiol. **33:**2953–2956.
10. **Hein, W. R., and A. A. Tomasovic.** 1981. An abattoir survey of tuberculosis in feral buffaloes. Aust. Vet. J. **57:**543–547.
11. **Hermans, P. W. M., F. Messadi, H. Guebrexabher, D. van Soolingen, P. E. W. de Haas, H. Heersma, H. de Neeling, A. Ayoub, F. Portaels, D. Frommel, M. Zribi, and J. D. A. van Embden.** 1995. Analysis of the population structure of *Mycobacterium tuberculosis* in Ethiopia, Tunisia, and the Netherlands: usefulness of DNA typing for global tuberculosis epidemiology. J. Infect. Dis. **171:**1504–1513.
12. **Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. van Embden.** 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J. Clin. Microbiol. **35:**907–914.
13. **Liébana, E., A. Aranaz, L. Domínguez, A. Mateos, O. González-Llamazares, E. F. Rodríguez-Ferri, M. Domingo, D. Vidal, and D. Cousins.** 1997. The insertion element IS*6110* is a useful tool for DNA fingerprinting of *Mycobacterium bovis* isolates from cattle and goats in Spain. Vet. Microbiol. **54:**223–234.
14. **Moda, G., C. J. Daborn, J. M. Grange, and O. Cosivi.** 1996. The zoonotic importance of *Mycobacterium bovis*. Tubercle Lung Dis. **77:**103–108.
15. **O'Reilly, L. M., and C. J. Daborn.** 1995. The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. Tubercle Lung Dis. **76:**(Suppl. I)**:**1–46.
16. **Perrin, G., M. Savey, and E. Baradat.** 1984. La tuberculose de la chèvre: identification et contrôle. Point Vét. **16:**59–63.
17. **Samper, S., C. Martín, A. Pinedo, A. Rivero, J. Blázquez, F. Baquero, D. van Soolingen, and J. D. A. van Embden.** 1997. Transmission between HIV-infected patients of multridrug-resistant tuberculosis caused by *Mycobacterium bovis*. AIDS **11:**1237–1242.
18. **Samper, S., M. J. Iglesias, M. J. Rabanaque, M. A. Lezcano, A. Vitoria, M. C. Rubio, L. Gómez, I. Otal, R. Gómez-Lus, and C. Martín.** The molecular epidemiology of tuberculosis in Zaragoza (Spain): a retrospective epidemiologic study in 1993. Submitted for publication.
19. **Skuce, R. A., D. Brittain, M. S. Hughes, and S. D. Neill.** 1996. Differentiation of *Mycobacterium bovis* isolates from animals by DNA typing. J. Clin. Microbiol. **34:**2469–2474.
20. **van Embden, J. D. A., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick, and P. M. Small.** 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J. Clin. Microbiol. **31:**406–409.
21. **van Soolingen, D., P. E. W. de Haas, J. Haagsma, T. Eger, P. W. M. Hermans, V. Ritacco, A. Alito, and J. D. A. van Embden.** 1994. Use of various genetic markers in differentiation of *Mycobacterium bovis* strains from animals and humans and for studying epidemiology of bovine tuberculosis. J. Clin. Microbiol. **32:**2425–2433.
22. **van Soolingen, D., L. Qian, P. E. W. de Haas, J. T. Douglas, H. Traore, F. Portaels, H. Z. Qing, D. Enkhsaikan, P. Nymadawa, and J. D. A. van Embden.** 1995. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. J. Clin. Microbiol. **33:**3234–3238.