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Molecular Typing of *Mycobacterium bovis* Isolates in Argentina: First Description of a Person-to-Person Transmission Case

I. Etchechoury[1], G. Echeverría Valencia[1], N. Morcillo[2], M. D. Sequeira[3], B. Imperiale[2], M. López[3], K. Caimi[1], M. J. Zumárraga[1], A. Cataldi[1] and M. I. Romano[1]

[1] Instituto de Biotecnología, INTA, Castelar, Buenos Aires, Argentina
[2] Hospital ''Dr. Antonio Cetrángolo'', Vicente López, Buenos Aires, Argentina
[3] Instituto Nacional de Enfermedades Respiratorias Dr. Emilio Coni, Santa Fe, Argentina

Impacts

- Bovine tuberculosis could be acquired by and transmitted between humans.
- Estimates of the incidence of zoonotic bovine tuberculosis in Argentina may be useful to improve clinical diagnosis.
- Molecular markers can contribute to typify and differentiate pathogens responsible for emerging and zoonotic diseases.

Keywords:
Mycobacterium bovis; human TB; MDR-*M. bovis;* spoligotyping; variable number tandem repeat

Correspondence:
María Isabel Romano. Instituto de Biotecnología, CICVyA, INTA, Los Reseros y Las Cabañas, s/n Castelar, Argentina.
Tel.: 54 11 4621 1447;
Fax: 54 11 4621 0199;
E-mail: mromano@cnia.inta.gov.ar

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Summary

Bovine tuberculosis is caused by *Mycobacterium bovis*, a mycobacterium highly similar to *M. tuberculosis* that belongs to the *M. tuberculosis* complex. The main host of *M. bovis* is cattle but it also affects many other mammalians including humans. Tuberculosis in humans caused by either *M. bovis* or *M. tuberculosis* is clinically hard to distinguish. During 2004–2005, samples from 448 patients with diagnosis of TB were collected from different regions of Argentina. The PRA technique identified 400 isolates with representative patterns of mycobacterium. The predominant ones were the *M. tuberculosis* complex, the *M. avium–M. intracellulare* complex and *M. gordonae*. Samples with *M. tuberculosis complex* PRA restriction profiles were analyzed with a multiplex PCR to differentiate between *M. tuberculosis* and *M. bovis*. Multiplex PCR identified nine *M. bovis*. The results allowed the possibility to establish that 2% of pulmonary tuberculosis was due to *M. bovis*. Isolates of *M. bovis* from humans were examined using spoligotyping. These isolates presented five different spoligotypes. The main spoligotype was also the most frequently one found in cattle. The remaining human spoligotypes (grouped in clusters) are occasionally found in cattle. Variable number tandem repeat (VNTR) analysis identified five different patterns. By combining the results of spoligotyping and VNTR analysis, we were able to differentiate seven *M. bovis* isolates. The remaining two *M. bovis* samples showed the same spoligotype and VNTR profile and belonged to household contacts. An MDR-*M. bovis* was isolated from the samples of these household contacts. The identification of two epidemiologically linked cases of human *M. bovis* infection suggests person-to-person transmission of an MDR-*M. bovis*.

Introduction

Bacteria belonging to the genus *Mycobacterium* are Gram-positive, acid-alcohol resistant and include pathogens that affect both humans and animals. The major pathogens of this genus pertain to the *M. tuberculosis* complex, which comprises: *M. tuberculosis, M. bovis, M. microti, M africanum and M. canettii* and the vaccine strain Bacille Calmette-Guérin (BCG). New members of the *M. tuberculosis* complex are *M. bovis* subsp. *caprae*, primarily infecting

goats in Spain and humans and cattle in Central Europe (Kubica et al., 2003; Niemann et al., 2002), and *M. pinnipedii,* infecting seals in Australia and Argentina (Cousins et al., 2003; Romano et al., 1995).

Human tuberculosis (TB) is produced mainly by *M. tuberculosis*; however, *M. bovis*, the etiologic agent of bovine tuberculosis, can also be responsible for the disease in humans, which makes this bacterium an important zoonotic species. Tuberculosis transmitted from animals to humans is clinically hard to distinguish from TB caused by *M. tuberculosis*. Before milk pasteurization, *M. bovis* used to be an important cause of human TB, especially of tuberculous lymphadenitis in children. However, after the generalized adoption of pasteurization of milk and other dairy products, zoonotic TB dropped sharply. The medical literature on the incidence of zoonotic TB is marked by numerous clinical descriptions of cases, but there are very few systematic nationwide surveys of *M. bovis* incidence (Anon, 2003; Barrera and De Kantor, 1987; de Kantor et al., 2008; Pavlık et al., 1998).

At the global level, the incidence of bovine TB is heterogeneous. In many developed countries bovine TB was eradicated 30–40 years ago thanks to strong campaigns based on the tuberculin skin test and mandatory sacrifice of animals at slaughterhouses. Currently, human TB caused by *M. bovis* in developed countries accounts for around 1% of all TB cases, and sporadic cases occur either in elderly people by reactivation of ancient infections or in immigrants from countries where bovine TB has not been eradicated (de la Rua-Domenech, 2006).

In developing countries *M. bovis* is thought to account for up to 10% of cases of human TB (Cousins et al., 1999). From 1995 through 1998, a nosocomial outbreak of multidrug-resistant (MDR) tuberculosis caused by *M. bovis*, in which most of the patients were HIV-positive, caused special concern because it affected many patients (Rivero et al., 2001). In Argentina, data of TB in cattle was obtained in 1994–1996 by tuberculinization of exported animals to countries of the Mercosur; the percentage of positive reactants ranged from 1.6% to 6.1% (Torres, 2007). A study of zoonotic tuberculosis incidence was performed in Santa Fe province of Argentina, where *M. bovis* was identified in 2.4% of human patients with TB, 64% of which were slaughterhouse or rural workers (Sequeira et al., 1990). The absence of clinical differences between TB caused by *M. tuberculosis* and *M. bovis* is the main reason for the lack of information regarding the *M. bovis* contribution to the global TB burden. Another important factor is the type of culture medium utilized for human pulmonary tuberculosis samples. Most laboratories exclusively use Löwenstein–Jensen (LJ) medium supplemented with glycerol, which does not promote *M. bovis* growth. On the contrary, *M. bovis* grows faster on egg medium supplemented with pyruvate (Stonebrink medium). In addition, for many low-income countries, a mycobacterial culture is always an expensive option in comparison to the cheaper and quicker acid-fast staining. Biochemical differentiation at species level within the *M. tuberculosis* complex is a difficult task. Current methods are tedious and slow as they are based on cultural and biochemical tests, such as sensitivity to thiophene-2-carboxylic acid hydrazine (TCH), sensitivity to pyrazinamide and the niacin test. Telenti et al. have described a method for mycobacterial species identification named PCR-Restriction Analysis (PRA) (Telenti et al., 1993). Several species and subspecies have been identified by this technique. However, PRA is not able to differentiate among members of the *M. tuberculosis* complex.

The sequencing of the complete genomes of *M. tuberculosis* (Cole et al., 1998) and *M. bovis* (Garnier et al., 2003) strains has allowed an important advance in the knowledge of large and single base polymorphisms between these species. The identification of genetic differences between members of the *M. tuberculosis* complex may also lead to a better understanding of the virulence and host range variability displayed by the members of the complex. Several regions of difference (RD) between *M. bovis* and *M. tuberculosis* have been described and reviewed by Mostowy (Brosch et al., 2002; Mostowy et al., 2005). Brosch et al. have proposed a scenario for *M. tuberculosis* complex evolution where the classical *M. bovis* shows the greatest number of RD deletions relative to other members of the *M. tuberculosis* complex (Brosch et al., 2002). Before *M. bovis* sequence was available, Zumárraga et al. demonstrated that *M. bovis* and *M. bovis* BCG lack a 12.7-kb fragment present in the genome of *M. tuberculosis* (Zumárraga et al., 1999). This region was used to differentiate *M. tuberculosis* from *M. bovis* and was later identified as RD7 by Brosch. (Brosch et al., 2002).

Mycobacterial DNA fingerprinting allows the tracing of transmission chains between infected hosts. This could confirm conventional epidemiological links and extend our understanding of the dynamics of TB transmission in the community. As strains of *M. bovis* from cattle usually have few copies of IS*6110,* RFLP-IS*6110* is not the best method for distinguishing strains of *M. bovis* (Allix et al., 2006). Spoligotyping has proven to be a practical and discriminatory method for large-scale studies of epidemiology of *M. bovis* as well as for the differentiation of *M. bovis* from *M. tuberculosis*, because the former bacteria lack of the spacers 39 to 43 (Kamerbeek et al., 1997). The main disadvantage of spoligotyping is that all genetic polymorphism is restricted to a single genomic *locus*, the DR cluster, which limits resolution. Recently, the variable number tandem repeat (VNTR) method has been introduced for typing of *M. tuberculosis* complex bacteria

(Frothingham and Meeker-O'Connell, 1998; Mazars et al., 2001; Roring et al., 2002). The VNTR *loci* change faster than the spoligotype and hence VNTR typing provides a greater resolution than spoligotyping alone (Roring et al., 2002).

The aim of the present study was the molecular typing of *M. bovis* isolates from patients with diagnosis of tuberculosis in Argentina and to establish their epidemiological relationship.

Materials and Methods

Bacterial strains and media

During 2004–2005, a total of 448 respiratory samples from patients with diagnosis of tuberculosis were obtained from different regions of Argentina: 161 were clinical isolates from Buenos Aires province, obtained at the Cetrángolo Hospital, and 287 were from other 13 provinces of Argentina, collected at the "Emilio Coni" National Institute of Respiratory Diseases. The samples were cultured either in Lowenstein–Jensen medium or in Middlebrook 7H9 supplemented with OADC (Oleic acid-albumin-dextrose-catalase, Sigma, St Louis, MO, USA) or MGIT 960 (Becton Dickinson, Franklin Lakes, NJ, USA).

PRA (PCR-restriction analysis)

The isolates were characterized by PRA (Telenti et al., 1993). Briefly, genomic DNA was used as a target to amplify a polymorphic gene, *hsp65*, present in the genome of all mycobacteria. The polymorphisms in the sequence were detected by digesting the amplified fragments with two restriction enzymes: *Bst*EII and *Hae*III (Promega, Madison, WI, USA). Restriction analysis was performed comparing the obtained bands with molecular mass standards in 3% agarose gels embedded in 5% ethidium bromide. Samples were visualized in a Gel Doc XR documentation system (Bio-Rad, Hercules, CA, USA). An Internet Database (PRASITE) (http://app.chuv.ch/prasite/index.html) was employed for interpretation of PRA results.

Multiplex PCR for further differentiation between *Mycobacterium tuberculosis* and *Mycobacterium bovis*

The samples with a *M. tuberculosis* complex PRA restriction profile were further analyzed by subjecting them to multiplex PCR (Fig. 1).

Two of the primers were designed to anneal in the immediate flanking regions of RD7:

1566up 5′ GCGTGGCGTGAATACCTACTT 3′

14930low 5′ CGGGTGTAGCTCGAGGATTTT 3′

The third primer anneals within the RD7 sequence:

1960low 5′ TGAGAAACACCGAGCAAAAGA 3′ (Fig. 1).

Amplifications were performed in a final volume of 50 μl containing: 10 μl of 5× PCR buffer (Green GoTaq Buffer, Promega), 4 μl of 20 mM dNTPs mix, 0.25 μM of each primer, 1–10 ng of template DNA, 1 Unit of GoTaq polymerase (Promega), and sterile double distilled water to reach the final volume. Thermal cycling was performed on a PTC-100 (MJ Research, Cambridge, MA, USA) with an initial denaturizing step of 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 52°C, and 1 min at 72°C, and a final step of 5 min at 72°C. Primers 1566up and 14930low amplify a single 635-bp fragment (*M. bovis*-specific amplification). Primers 1960low and 1566up amplify a 394-bp fragment in *M. tuberculosis* (*M. tuberculosis*-specific amplification) (Fig. 1).

Genotyping of *Mycobacterium bovis* isolates

The *M. bovis* isolates were typed by Spoligotyping following procedures described by Kamerbeek (Kamerbeek et al., 1997).

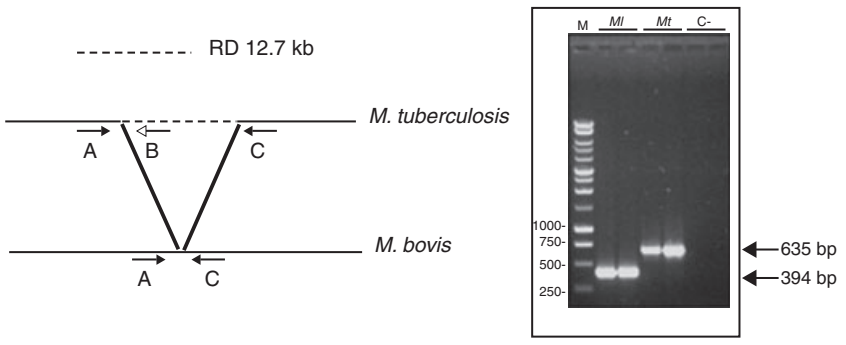


Fig. 1. Scheme of the multiplex PCR for differentiation between *Mycobacterium bovis* and *M. tuberculosis*. Primers 1566/14130 are used for the selective amplification of *M. bovis*, and primers 1566/1960 for the selective amplification of *M. tuberculosis*. The gel shows the PCR products obtained. The 635-bp band belongs to the specific amplification of *M. bovis,* and the 394-bp band to the specific amplification of *M. tuberculosis.* A, primer 1566up; B, primer 1960low; C, primer 14130low.

Variable number tandem repeat typing was performed using the primer sets directed to the following exact tandem repeat (ETR) *loci*: ETR-A, ETR-B, ETR-C, ETR-D, ETR-E and ETR-F described by Frothingham and Meeker-O'Connell (Frothingham and Meeker-O'Connell, 1998). Primers (ETR-A through ETR–F, both up and low) were purchased from Operon Biotechnologies, Inc. (AL, USA). ETR-A-up and ETR-D-low were labelled with FAM fluorochrome, ETR-F-up and ETR-B-low with JOE and ETR-E-up and ETR-C-low with hexachlorofluorescein (HEX). The primer pairs were designed to anneal upstream and downstream of each tandem repeat *locus*. Polymerase chain reaction was performed in a total volume of 50 μl. The PCR mix contained 5 μl of 10× HotStart Taq buffer (Qiagen, Hamburg, Germany), 0.25 μM of each primer, 4 μl of 20 mM dNTPs mix, 1 U HotStart Taq DNA Polymerase (Qiagen), 3–10 ng of template DNA and sterile double distilled water to reach the final volume. Thermal cycling was performed on a MyCycler (Bio-Rad) with an initial denaturizing step of 15 min at 95°C, followed by 30 cycles of 30 s at 95°C, 1 min at 64°C, and 2 min at 72°C, and a final elongation step of 10 min at 72°C. Negative reaction controls were included each time PCR was performed. The positive control was 500 pg of DNA from *M. tuberculosis* H37Rv. The presence and size of each PCR product was determined by electrophoresis on a 2% agarose gel in 1× TAE (Tris-Acetic acid-EDTA) buffer followed by staining with 5% ethidium bromide. Polymerase chain reaction amplifications were also subjected to capillary electrophoresis analysis on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The molecular marker used was GS500-250 (ROX).

Results

Polymerase chain reaction-restriction analysis (PRA) of isolates from patients with tuberculosis was obtained during 2004–2005 from different regions of Argentina. A total of 448 cultures were obtained from patients with pulmonary TB and AFB (Acid fast bacilli) positive smear examination. DNA from these isolates was used to amplify the *hsp65* gene by PCR. Forty-eight samples could not be amplified. With the remaining 400 isolates, 17 different representative PRA patterns were obtained; most of the isolates presented a single pattern (indicating the presence of a single type of mycobacterium in the culture), while two of them exhibited two distinct patterns (indicating two species of mycobacterium coexisting in these samples). In addition, the PRA patterns of five isolates could not be identified in the available databases. The species identified and their frequencies are shown in Table 1. The predominant isolates (n = 333) belonged to *M. tuberculosis* complex, the *M. avium–M. intracellulare* complex and *M. gordonae* (data not shown).

Table 1. Distribution of the different mycobacteria in samples from patients with diagnosis of tuberculosis

Provinces	*Mycobacterium tuberculosis*	*Mycobacterium bovis*	MAC[a]	Other mycobacteria	Total
Buenos Aires	72	4	19	18	113
Chaco	64	1	3	1	69
Corrientes	35	–	2	–	37
Entre Ríos	11	–	–	1	12
Formosa	10	–	–	2	12
La Pampa	4	–	–	–	4
La Rioja	3	–	–	–	3
Mendoza	3	–	–	–	3
Misiones	15	–	–	–	15
Salta	18	–	–	1	19
Santa Fe	55	3	3	5	66
Santiago del Estero	28	–	2	–	30
San Luis	1	–	–	–	1
Tucumán	14	1	1	–	16
Total	333	9	30	28	400

[a]MAC, *Mycobacterium avium* complex.

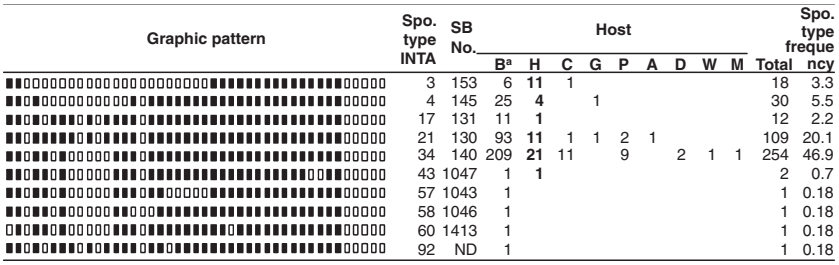
In order to determine which samples showing the *M. tuberculosis* complex PRA restriction profiles corresponded to *M. bovis*, a multiplex PCR was designed. This strategy is based on the amplification of the 12.7-kb genomic region previously characterized in our laboratory (Zumárraga et al., 1999) (Fig. 1). Multiplex PCR identified nine mycobacterial isolates as *M. bovis* (Tables 1 and 2). These data allowed us to establish that 2% of the pulmonary tuberculosis mycobacterial isolates was caused by *M. bovis*.

The nine *M. bovis* samples from different provinces of Argentina had five different spoligotypes (Table 2). The main type observed, arbitrarily designed spoligotype 34,

Table 2. Spoligotypes and VNTRs of *Mycobacterium bovis* strains isolated from humans in this study

Isolate	VNTR number[a]	Procedence	Species	Spoligotype
A	7554*33,1	Buenos Aires	Human	3
B	7554*33,1	Buenos Aires	Human	3
389	7554*33,1	Buenos Aires	Human	4
1934	7654*33,1	Buenos Aires	Human	34
06-642	666433,1	Santa Fe	Human	34
06-644	766433,1	Santa Fe	Human	4
06-645	766433,1	Santa Fe	Human	92
06-646	666433,1	Chaco	Human	43
06-647	566433,1	Tucumán	Human	34

[a]The size of the amplified VNTRs (ETR-A through ETR-F) was converted into a numerical code which refers to the copy number of repetitions.



Graphic pattern	Spo. type INTA	SB No.	Host										Spo. type frequency
			B[a]	H	C	G	P	A	D	W	M	Total	
	3	153	6	**11**	1							18	3.3
	4	145	25	**4**		1						30	5.5
	17	131	11	**1**								12	2.2
	21	130	93	**11**	1	1	2	1				109	20.1
	34	140	209	**21**	11		9		2	1	1	254	46.9
	43	1047	1	**1**								2	0.7
	57	1043	1									1	0.18
	58	1046	1									1	0.18
	60	1413	1									1	0.18
	92	ND	1									1	0.18

Fig. 2. Diversity, distribution and frequency of *Mycobacterium bovis* spoligotypes found in samples from humans. [a]B, bovine; H, human; C, cat; G, goat; P, pig; A, armadillo; M, monkey; W, Wild Boar; D, deer; ND, Not detected; Spo., spoligotype.

was also the most frequently found in cattle (Zumárraga et al., 1999); (Fig. 2). The other spoligotypes grouped in clusters were spoligotypes 3 and 4 (Fig. 2); these are sporadically found in cattle (Zumárraga et al., 1999). Variable number tandem repeat analysis of the nine *M. bovis* isolated from humans identified five different patterns. Combining the results of spoligotyping and VNTR analysis, seven *M. bovis* patterns were differentiated. The remaining two *M. bovis* samples (A and B, Table 2) showed the same spoligotype and VNTR profile and were isolated from household contacts (father and daughter). The father (A) was native of Buenos Aires province and worked for a cold-storage facility. He was diagnosed as a TB case (1987) based on positive direct smear examination, clinical and radiological findings. He received the standardized treatment with isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), streptomycin (SM), but showed poor adherence to treatment. The patient showed an initial improvement but after 4 months of treatment, he presented positive smear examination again. After that, and by the impossibility to cultivate the mycobacterium causing the disease, all the following therapeutic schemes were empirically designed and contained ofloxacin as well. In the year 2000, three serial samples from sputa of this patient were sent to the Reference Laboratory at Dr. Cetrángolo Hospital where a mycobacterium was isolated and later identified as *M. bovis*. The MGIT 960 and the proportion method on Middlebrook 7H11 supplemented by OADC were used to determine the drug susceptibility to first-line antiTB agents. This strain showed to be MDR (multidrug resistant), with resistance to isoniazid (INH) and rifampin (RIF). The patient dies in 2003 of massive hemoptysis. His daughter was diagnosed as a TB patient in 1999 based also on positive direct smear examination, clinical and radiological findings. Positve bacilloscopy and cultures were obtained from three sputum samples (2005). Slow growing mycobacteria were obtained by the MGIT 960 system. Drug susceptibility testing also performed on MGIT 960 showed a MDR-*M. bovis*. Genotypic identification revealed that this MDR-*M. bovis* had an identical pattern as the one isolated from her father.

Comparison of spoligotypes of *M. bovis* strain isolates from patients of this study with those of *M. bovis* from humans present at our laboratory database (Table 3) revealed that three spoligotypes were repeated. The first was spoligotype 34, the most frequent in cattle in Argentina, and the second and third were spoligotypes 3 and 4 respectively, both less prevalent in cattle. All spoligotypes found in *M. bovis* isolates from humans are shown in Fig. 2. Variable number tandem repeat analysis of *M. bovis* isolates from humans indicated that the most frequent type of ETR was 7554*33,1. ETR-A through ETR–D showed to differentiate the isolates of *M. bovis*, but ETR-F was the same for all the isolates (Table 3).

Discussion

Mycobabterium bovis is the principal agent responsible for TB in domestic and wild mammals. *Mycobabterium bovis* also infects humans, causing zoonotic TB. The disease caused by *M. bovis* is transmissible from cattle to humans by the aerogenous route and by consumption of contaminated milk; person-to-person transmission is an exceptional event. A few cases of drug-resistant *M. bovis* strains

Table 3. Spoligotypes and VNTRs of *Mycobacterium bovis* strains isolated from humans present at INTA's database

ID	VNTR Number	Species	Spoligotype
5	7553*33,1	Human	17
18*2	7554*33,1	Human	34
12038	7554*33,1	Human	58
6812	4354*33,1	Human	57
04-424	7554*33,1	Human	3
04-387	7454*33,1	Human	3
02-2b	7554*33,1	Human	60
436	7554*33,1	Human	4
440	5454*33,1	Human	34
438	7554*33,1	Human	34
435	6354*23,1	Human	21
434	6354*23,1	Human	21
433	7554*33,1	Human	21
432	6354*23,1	Human	21

transmitted between HIV-infected individuals have been reported (Rivero et al., 2001). In some cases, *M. bovis* infection in humans results in extra-pulmonary TB, in particular cervical lymphadenitis. In our laboratory, a lymphadenitis case was confirmed as of animal origin in a woman where the infection came from contact with cats (Colmegna et al., 2004). The estimation of the prevalence of *M. bovis* in human samples requires the use of a culture medium that allows *M. bovis* growth as well as the consideration of extra pulmonary samples.

The use of molecular techniques to determine the type of mycobacterium infecting humans is of vital importance because the accuracy of biochemical tests is limited (Telenti et al., 1993). The development of PCR and other molecular tools has granted the identification of *M. bovis* and has permitted the differentiation of this species from other members of the *M. tuberculosis* complex. Moreover, these tools have allowed the discovery of more cases in retrospective studies and have suggested new forms of transmission. The use of differential multiplex PCR has allowed the detection of mycobacterium strains different from *M. tuberculosis* in isolates from tuberculous patients. Using this approach we showed that 2% of pulmonary TB cases are caused by *M. bovis*. Low numbers of *M. bovis* found in humans are probably due to extra-pulmonary TB among our samples. The method used to find *M. bovis* was based on the amplification of a DNA sequence, called RD7, present in *M. tuberculosis* and absent in *M. bovis*. However, this deletion is also present in *M. microti* and in some *M. africanum* and *M. pinnipedii* (Cole et al., 1998), but these mycobacteria are not a common cause of TB in Argentina. This test could be used for detection of *M. bovis* instead of biochemical tests, which could also indicate resistant *M. tuberculosis* isolates.

In the present study, spoligotyping and VNTR techniques were used to establish the genetic relationship and the epidemiological data of nine *M. bovis* isolates from humans. Most isolates of *M. bovis* were of patients from the central region of Argentina: Buenos Aires and Santa Fe provinces (Table 2). This region contains most of the dairy farms in Argentina and the highest level of bovine tuberculosis. Two of these isolates had an identical spoligotype and VNTR profile, and they were MDR-*M. bovis*. The isolates belonged to relatives (father and daughter), suggesting person-to-person transmission. The father had pulmonary disease and worked in a slaughterhouse, while his daughter had not had any contact with animals.

Most of the *M. bovis* isolated from humans in the present study belong to spoligotypes 34 and 21, prevalent in animals in Argentina (Zumárraga et al., 1999; Zumárraga, 2007). However, other *M. bovis* isolates had spoligotypes that are not predominant in cattle in our country. Indeed, 61% of all the *M. bovis* isolates with spoligotype 3 belong to humans, suggesting a probable relationship between this spoligotype and the human host (Zumárraga, 2007).

Previous findings have shown analysis of VNTR *loci* in *M. tuberculosis* (Frothingham and Meeker-O'Connell, 1998; Mazars et al., 2001). The genetic *loci* analyzed in *M. tuberculosis* strains are fairly well defined, whereas fewer studies have detailed the value of different sets of VNTR *loci* for fingerprinting of *M. bovis* isolates. The results presented here showed that VNTR analysis with ETR-A, ETR-B, ETR-C, ETR-D, and ETR-E was successful to differentiate *M. bovis* isolates, whereas ETR-F was not.

The *M. bovis* detected in humans in the present study and the discovery of the first MDR-*M. bovis* transmission between humans in Argentina show the importance of the application of molecular strategies to identify infected patients with *M. bovis,* especially in regions with high prevalence of bovine tuberculosis.

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