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## Chapter 8: Tuberculosis caused by Other Members of the *M. tuberculosis* Complex

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### 8.1. *Mycobacterium bovis* disease in humans

Bovine tuberculosis (TB) is caused by *Mycobacterium bovis*, a mycobacterium highly similar to *Mycobacterium tuberculosis* and belonging to the *M. tuberculosis* complex. The main host of *M. bovis* is cattle (*Bos taurus*) but it affects many other mammals including man. In man, it is the most frequent cause of zoonotic TB, i.e. TB transmitted from animals to humans, which is clinically indistinguishable from TB caused by *M. tuberculosis*. Before milk pasteurization, *M. bovis* was an important cause of human TB, especially intestinal TB in children. After the generalized adoption of pasteurization of milk and other dairy products, the occurrence of zoonotic TB dropped sharply.

Very few studies are published on zoonotic TB. In the last 50 years, research on zoonotic TB was influenced by scientific trends, societal worries such as human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and contaminated food, as well as by the availability of tools for the identification of the bovine TB bacillus. For example, the development of the polymerase chain reaction (PCR) and other molecular tools to identify *M. bovis* and differentiate it from other members of the *M. tuberculosis* complex have allowed the discovery of more cases in retrospective studies and have suggested new forms of transmission. The medical literature on the incidence of zoonotic TB is marked by numerous clinical descriptions of cases of *M. bovis* at the regional or nosocomial level, but there are very few systematic surveys of *M. bovis* diagnosis on a national level (Anon 2003, Barrera 1987, Cousins 1999, Pavlik 1998). There are three main explanations for the absence of an accurate and methodical estimation of the contribution of *M. bovis* to the global TB burden. First, at the clinical or radiological level, there is no difference between TB caused by *M. tuberculosis* or that of *M. bovis*. Second, most laboratories use Löwenstein-Jensen culture medium with glycerol, which does not promote *M. bovis* growth. Furthermore, cultivation is always an expensive option for many low-income countries compared to the cheaper and faster acid-fast staining. Third, and perhaps most important, in most cases the treatment of TB caused by *M. tuberculosis* or *M. bovis* was the same; therefore, there was no clinical interest in differentiating the causative agent.

There is a direct correlation between the prevalence of human TB of bovine origin

and that of TB in livestock (Cosivi 1998). At the global level the situation of bovine TB is disparate. In many developed countries bovine TB was eradicated 30-40 years ago by strong campaigns based on tuberculin skin testing (TST) and mandatory sacrifice of animals at the slaughterhouse. In these countries, human TB caused by *M. bovis* accounts for around 1 % of all TB cases, and sporadic cases occur in elderly people by reactivation of ancient infections or in immigrants from countries where bovine TB has not been eradicated. Importantly, some developed countries, including England or New Zealand, could not completely eliminate bovine TB, or worse, there is a re-emergence of the disease (Thoen 2006). The persistence of *M. bovis* in wildlife is frequently indicated as the main cause of this re-emergence. On the other hand, in many low-income countries, bovine TB continues to be an important animal health problem. Different epidemiological scenarios can be observed. Meat and bovine products are important resources in some countries, such as Argentina, Brazil, Mexico, and Venezuela, where the number of cattle equals or exceeds that of the human population, and the risk of zoonotic TB could be higher. Less clear is the situation in countries where livestock industry is less developed and intensive, and cattle farming is a family affair for milk consumption or retail commercialization; on the other hand, if the total number of cattle is highly reduced, people live near the animal folds and sometimes consume milk raw. In Central American and African countries, as well as in China, cows are preserved for milk production and meat is consumed from other species such as sheep and swine that are less susceptible to *M. bovis*. Finally, in India, a high proportion of people do not eat cattle meat but do consume milk and are in close contact with cattle, increasing the risk. Some low-income countries, including Cuba, Mongolia, and Costa Rica are remarkable exceptions, because they have eradicated bovine TB, probably because the cattle population is relatively small.

The epidemiology of zoonotic TB, was recently examined by Thoen *et al.* (Thoen 2006), who reviewed publications from 1966 on. In this chapter we will concentrate on the most recent findings.

In Africa, there are several reports about the incidence of zoonotic TB. Many of these studies involved research on pre-existing mycobacterial collections or in limited clinical settings, such as in Egypt, Nigeria, Madagascar, Zaire, and Tanzania (Table 8-1). Other authors looked for *M. bovis* in cattle, as in Ghana and Zambia, where high incidences were described. Another study demonstrated the presence of *M. bovis* in milk in Tanzania (Kazwala 1998). An excellent review about bovine TB was published by Ayele (Ayele 2004). Genotyping analysis demonstrates different clonal populations depending on the geographical region under study. Due to the consumption of raw milk in regions where AIDS is highly

prevalent, many studies concentrated on patients having lymphadenitis. One study in Djibouti showed a low prevalence of *M. bovis* in those patients (Koeck 2002), while others in Ethiopia (Kidane 2002) and Tanzania (Mfinanga 2004) demonstrated 17 % and 10 % prevalence respectively (Table 8-1).

In Asia, where a policy of bovine TB control was adopted in few countries, there are very few publications on zoonotic TB (Table 8-1). Clearly, a more active search for *M. bovis* is needed on the Asian continent, where the burden of TB is high.

In Latin America, most of the studies were published in Argentina describing incidence ranging from 0.7 % to 6.2 % in a main milk region, with a much lower national prevalence. In Brazil and Mexico, only one publication was available per country (Table 8-1). In a small collection of human isolates from Chile, *M. bovis* was not found (Mancilla 2006), a finding that merits a more extended survey in that country (Table 8-1). Genotyping of *M. bovis* from humans in Argentina showed that the predominating genotype in cattle also predominates in humans, strongly indicating that infection is transmitted from cattle. Furthermore, the majority of patients are related to the farming or meat industry (Zumárraga 1999). In contrast, Romano *et al.* recently selected isolates with scarce growth in Löwenstein-Jensen media from different hospitals in Argentina, and many of them were *M. bovis*. Genotyping of these unsuspected *M. bovis* isolates showed that they belong to a spoligotype that is not predominant in cattle, suggesting that a clone circulates among humans (M. Romano, unpublished observations). The situation of zoonotic TB in Latin America has recently been reviewed by Ritacco *et al.* (Ritacco 2006).

In the United States, the description of an outbreak of *M. bovis* TB cases in San Diego was of special interest (Table 8-1). The ingestion of raw milk products by immigrant children was suspected as the source of the infection (Dankner 1993, Dankner 2000). No recent cases were informed from Canada. Other reports also describe a higher incidence of *M. bovis* among third world immigrants residing in industrialized countries (Cousins 1999, Jalava 2007).

In Europe, in the last decades, *M. bovis* in humans was reported sporadically (Thoen 2006). The prevalence of TB cases caused by *M. bovis* is around 1.0 % of all TB cases in the United Kingdom. A similar figure is found in Germany (where *Mycobacterium caprae* is relevant) and in Spain (Table 8-1). A recent paper described that in Lyon, France, there was no genetic relatedness among nine *M. bovis* isolates collected from patients over five years, strongly indicating that there is no active transmission (Mignard 2006). In contrast, another typing study in Italy described spoligotyping and Mycobacterial Interspersed Repetitive Units (MIRU) patterns in a collection of 42 isolates, with one genotype accounted for 32 % of all

isolates, while the others were unique (Lari 2006). Importantly, a study in the United Kingdom showed that there is no increase in *M. bovis* disease in humans in spite of an important increase in the incidence of bovine TB (Jalava 2006).

In New Zealand, *M. bovis* accounts for 2.7 % of laboratory-confirmed human TB cases. Many of the genotypes were identical to patterns from farmed and wild animals (Baker 2006). In contrast, there has been no publications on zoonotic TB in Australia during the last six years.

Table 8-1: Bovine TB in humans

Country	Target of study	Main findings	Reference
Egypt	Identification of <i>M. bovis</i> in humans using cultures.	6 % of the total TB cases were caused by <i>M. bovis</i>	Elsabban 1992
Egypt	Identification of <i>M. bovis</i> in humans using cultures	High incidence of <i>M. bovis</i>	Nafeh 1992
Nigeria	Identification of <i>M. bovis</i> in humans using cultures	3.9 % of TB cases caused by <i>M. bovis</i>	Idigbe 1986
Madagascar	Identification of <i>M. bovis</i> in humans using cultures	1.25 % of TB cases caused by <i>M. bovis</i>	Rasolofo-Razanamparany 1999
Madagascar	Genotyping of <i>M. bovis</i> collection	A genotype is prevalent in humans and cattle	Rasolofo-Razanamparany 2006
Zaire	Identification of <i>M. bovis</i> in humans using cultures	High incidence of <i>M. bovis</i>	Mposhy 1983
Zambia	Large field diagnostic test of cattle	33 % of positive herds	Cook 1996
Tanzania	Detection of <i>M. bovis</i> in milk	6 % of samples positive for <i>M. bovis</i>	Kazwala 1998
Burundi	Culture of mycobacteria from human and cattle samples.	No <i>M. bovis</i> in humans, 38 % in cattle.	Rigouts 1996
Ghana	Large field diagnostic test of cattle	13.8 % of positive animals.	Bonsu 2000
Tanzania	Screening of TB patients from rural communities	16 % of TB cases caused by <i>M. bovis</i>	Kazwala 2001

Country	Target of study	Main findings	Reference
Tanzania	Genotyping of <i>M. bovis</i> collection	Low clustering of cases in humans and cattle	Kazwala 2005
Djibouti	Biopsies of lymph nodes from TB patients	Low prevalence of <i>M. bovis</i>	Koeck 2002
Ethiopia	Biopsies of lymph nodes from TB patients	17.1 % of samples positive for <i>M. bovis</i>	Kidane 2002
Tanzania	Biopsies of lymph nodes from TB patients	10 % of samples positive for <i>M. bovis</i>	Mfinanga 2004
Nigeria	Genotyping of <i>M. bovis</i> collection	No common patterns of <i>M. bovis</i> from cattle and humans	Cadmus 2006
China	Clinical report	Case of disseminated TB due to <i>M. bovis</i>	Wei 2004
India	Screening of CSF from patients	Molecular evidence of <i>M. bovis</i>	Prasad, 2005
Argentina	Identification of <i>M. bovis</i> in humans using cultures	8 % of <i>M. bovis</i> in extrapulmonary TB	Peluffo 1982
Argentina	Identification of <i>M. bovis</i> in humans using cultures	<i>M. bovis</i> identified in 0.47 % of sputum samples	Barrera 1987
Argentina	Identification of <i>M. bovis</i> in humans using cultures	Annual variations of <i>M. bovis</i> going from 0.7 % to 6.2 % of human TB	Sequeira 1990
Argentina	Identification of <i>M. bovis</i> in humans using cultures	7 % of extrapulmonary TB due to <i>M. bovis</i>	Solda 2005
Brazil	Mycobacterial cultures from children	<i>M. bovis</i> provoked 3.5 % of cases of pediatric TB	Correa 1974
Chile	Identification of <i>M. bovis</i> in humans using cultures	No <i>M. bovis</i> isolation	Mancilla 2006
Mexico	Identification of <i>M. bovis</i> in humans using cultures	3/19 isolates were <i>M. bovis</i>	Toledo Ordoñez 1999
Argentina	Genotyping of <i>M. bovis</i> collection	A genotype is prevalent in humans and cattle	Zumarraga 1999

Country	Target of study	Main findings	Reference
USA	Diagnostic of TB in workers from a dairy farm	Risk factor for zoonotic TB, but no cases demonstrated.	Winthrop 2005
USA	Diagnostic of TB in immigrant children	Pediatric TB due to <i>M. bovis</i>	Dankner 1993, 2000
Australia	Identification of <i>M. bovis</i> in humans using cultures	<i>M. bovis</i> present in immigrant workers	
United Kingdom	Identification of <i>M. bovis</i> in humans using cultures	No increase of zoonotic TB in spite of increase in cattle.	Jalava 2007
United Kingdom	Identification of <i>M. bovis</i> in humans using cultures	<i>M. bovis</i> provoked 1.0% of TB cases	Yates 1988
France	Genotyping of <i>M. bovis</i> collection	No genetic relatedness among <i>M. bovis</i> isolates collected from patients	Mignard 2006
Italy	Genotyping of <i>M. bovis</i> collection	A genotype accounts for 32 % of human isolates	Lari 2006
Germany	Identification of <i>M. bovis</i> in humans using cultures	<i>M. bovis</i> represents 1 % of all TB cases. 31 % of the isolates were <i>M. caprae</i>	Kubica 2003
United Kingdom	Clinical report	An intrafamilial spread of <i>M. bovis</i>	Smith 2004
United Kingdom	National survey for <i>M. bovis</i>	<i>M. bovis</i> represents between 0.5 % and 1.5 % of TB cases	de la Rua-Domenech 2006
Spain	Identification of <i>M. bovis</i> in humans using cultures	9 <i>M. bovis</i> cases in patients in 4 years	Remacha 2006
Spain	Identification of <i>M. bovis</i> in humans using cultures	0.95 % of all cases of tuberculosis due to <i>M. bovis</i>	Esteban 2005
New Zealand	Identification of <i>M. bovis</i> in humans using cultures	2.7 % of TB cases due to <i>M. bovis</i> . Many of the genotypes were identical to patterns from animals	Baker 2006

In the last 10 years, human disease due to drug-resistant *M. bovis* has been described (Blazquez 1997, Hughes 2003, Sechi 2001). One case was of special concern because it affected many patients, most of them HIV-positive (Blazquez

1997). This strain spread over Europe and into Canada, and affected 141 patients (S. Samper, personal communication). This fact highlights the high risk of spread of MDR *M. bovis*, especially in parts of Africa where *M. bovis* animal disease and HIV human infection co-exist.

In humans, the disease caused by *M. bovis* or *M. tuberculosis* is clinically indistinguishable. However, if the physiopathology of TB in humans and cattle are compared, some differences are observed. In humans, the apical lobes of the lungs are most affected. In cattle, lesions are most frequently observed in the dorsal caudal lung regions (Cassidy 2006). This part is the most distant from the mouth and nostrils, meaning that the droplets must travel the longest possible route. In bovines, on the other hand, the lesions are frequently located in lymph nodes associated with the respiratory tract, and not in the lung parenchyma. This observation may be related to the fact that the detection of infected cattle is made in the early stages of disease progression, before the presentation of advanced cavitory lesions. At the histological level, the differences are related to the cell types intervening in the immune response and granuloma formation. For example, the content of  $\gamma\delta$  T cells is much higher in cattle and these cells, as well as neutrophils, participate in granuloma and lesion formation (Cassidy 2001). Nowadays, and due to control campaigns, large liquefied lesions are less frequently observed in cattle in contrast with findings in wildlife where it is possible to observe advanced lesions (Cassidy 2006).

Sheep and horses are rarely infected. The infection in goats shows extreme variation according to the geographic location. There are less reports of TB in domestic than in feral pigs. The direct transmission of *M. bovis* from wildlife animals to humans is much less frequent. Transmission from deer to humans has been reported in Canada (Long 1999). Cats, but not dogs, have been reported in several countries as the source of human TB (Fernandez 1999, Underwood 1999, Monies 2000). Importantly, there are no reports of human infection by *M. bovis* coming from a direct environmental source (Biet 2005). The fact that in situations where the prevalence of *M. bovis* in cattle is high but does not seem to be associated with a higher incidence in humans may suggest that humans are less susceptible to *M. bovis* than to *M. tuberculosis* (de la Rúa-Domenech 2006).

After the introduction of milk pasteurization, there was a clear impact on the death rate of children under five years of age (Thoen 2006). A recent review (de la Rúa-Domenech 2006) described the survival of *M. bovis* in different foods. *M. bovis* survives well in cows' milk. Viable bacilli can be found in yogurt and cream cheese made from unpasteurized milk for up to 14 days after preparation, and in butter for up to 100 days. The consumption of unpasteurized raw milk or milk

products is still allowed in many European countries. In low-income countries, consumption of raw milk or dairy products is common in rural areas.

The detection of *M. bovis* in milk from infected cattle is problematic because *M. bovis* is usually present in low amounts. As contaminating microbiota exist in raw milk, other bacteria and fungi overgrow *M. bovis*. Decontamination methods applied to other clinical samples with higher bacillary loads, such as sputum or necropsy samples, kill the few *M. bovis* that may exist in tested milk. This has led to a worrying situation in which there are no validated methods for its detection in milk or milk products. The main problem is the failure of culture as a gold standard. PCR methods for the detection of members of the *M. tuberculosis* complex in clinical specimens were developed in the mid-90s. Although the first developed PCR method used primers directed at a *M. bovis* specific sequence (Rodriguez 1995), most PCR protocols use primers derived from the insertion sequence IS6110 insertion sequence, present in all members of the *M. tuberculosis* complex. The detection limit in artificially contaminated milk is generally low: 10-1,000 colony forming units (cfu) (Zanini 1998, Zumárraga 2005, Antognoli 2001). The sensitivity among tuberculin skin test (TST)-positive cows also varies in different studies, from 11-50 % (Cornejo 1998, Romero 1999, Sreevatsan 2000, Zumárraga 2005). One study in Argentina did not find *M. bovis* in cattle milk (Perez 2002). This variation is expected, as excretion of *M. bovis* in milk is sporadic and not all infected animals excrete bacilli. There are no published studies on the detection of *M. bovis* by PCR in cheese. In summary, PCR is powerful in detecting *M. bovis* in milk but there is an urgent need to validate this technique on a wider level.

## 8.2. The BCG vaccine: adverse reactions

The bacille Calmette-Guérin (BCG) is a live, attenuated vaccine derived from *M. bovis*. BCG is known to cause local reactions consistent with primary infection with an attenuated strain (i.e. a small localized ulcer and possible regional lymphadenopathy), and more severe reactions are thought to be rare. Deep ulcers, prolonged drainage, lymphadenitis (1 %), abscess (2 %) (Turnbull 2002), osteitis (0.04 %) (Kroger 1995), and rarely disseminated infection have all been reported (Albot 1997).

The age of the recipient and the dose of vaccine affect the incidence of local complications. Disseminated disease is thought to be rare, in the order of 1/1,000,000 doses and directly related to immune dysfunction (Turnbull, 2002). The major worldwide concern about the risk of disseminated infection has been connected to the risk of HIV-related immunosuppression in the recipient. BCG is given routinely

to newborns in many countries. However, this practice is under active review because of concerns that the vaccine's problems may outweigh its efficacy. Some authors recommend that BCG vaccination should be confined to groups of infants with a high risk of TB infection, and should be given at six months of age, in order to reduce severe disease and deaths among infants with immunodeficiency disorders (Romanus 1993).

From 1993 to 2001, 20 adverse events of BCG vaccination were reported in members of TB-endemic Aboriginal communities in Canada. Six of these were disseminated disease and five were in children from Aboriginal communities (the sixth one was vaccinated as an infant outside Canada). All of these cases were confirmed as being caused by BCG. One of these children was HIV-infected and the other four had congenital immunodeficiencies, which presented for the first time as disseminated BCG infection. All of these children died as a result of their underlying immunodeficiency. This rate of disseminated infection indicates a higher rate of underlying congenital immunodeficiency in this population and an unanticipated serious risk in this population of BCG recipients (Hutmacher 2002). Health Canada recommends administration of BCG vaccine to all newborn infants who are members of TB-endemic Aboriginal communities because of the high rate of TB infection and the high risk of serious disease in young children after primary infection. The debate about BCG vaccine in Canada has accelerated as a result of concerns about adverse events (Clark 2006).

Several studies have shown clear benefits of BCG vaccination when the risk of tuberculous infection is higher than 1 % per year (Rouillon 1965, Immunization Practices Advisory Committee 1988, Health Canada 2002). These benefits become less clear when the risk of infection is lower than 0.1 %, as rates of severe TB disease and deaths are quite low, regardless of the BCG vaccination policy. Results of these studies are therefore consistent with recommendations of the IUATLD and World Health Organization (WHO), which state that discontinuation of BCG can be considered in populations with an annual risk of tuberculous infection lower than 0.1 % (IUATLD 1994; World Health Organization 2001). The vaccine may be considered in select situations where exposure to TB infection cannot be readily controlled with anti-tuberculous chemotherapy, particularly where multidrug resistance is documented. The recipients in this situation may include household contacts as well as laboratory personnel and travelers (National Advisory Committee on Immunization 2002). A study in which the vaccine was administered to high risk newborn infants before environmental exposure to mycobacteria could have occurred, showed an overall efficacy of 73 % (range 59 % to 80 %) for disease and 87 % for death (Rosenthal 1961, Fordham von Reyn 2002). The overall trends are

that newborns are better protected and primary disease, miliary TB, and TB meningitis are better prevented.

Another concern about the administration of BCG is its effect on the TST. Because administration of BCG induces a positive skin test of variable size in a large proportion of vaccinated individuals, this reaction will affect the interpretation of TST results in contact tracing, thus, jeopardizing the use of a valuable tool in the control of TB transmission in the community.

The place of BCG vaccination in TB control programs is being carefully reassessed because of the significant risk of dissemination in immunocompromised patients. BCG has to be administered to newborns from endemic countries in Africa, Asia, and Latin America, because the vaccine is effective for the prevention of disseminated TB and meningitis. However, a careful review and identification of underlying risks for immunodeficiency should also be performed. This should include a careful family history for immunodeficiency and prenatal HIV screening.

On the other hand, in non-endemic countries, BCG could be discontinued, but if the vaccine is no longer to be given routinely to newborns from endemic communities in these countries, then possible consequences must be anticipated. The rates of miliary TB and meningitis in these infants will increase if ongoing exposure of young infants continues. In the meantime, if the routine infant BCG vaccine program is abandoned in these communities, this must be compensated for by support of enhanced TB detection and treatment programs. An effective TB prevention and control program requires effective assessment of active disease, effective therapy including DOTS, finding and screening of contacts of infectious cases, and identification and management of latently infected individuals. Otherwise, there is little doubt that infants from these endemic communities will be at increased risk of disseminated primary TB. For example, Sweden moved from the mass vaccination of newborns with BCG to a selective vaccination program for high risk groups. This strategy met with some success, measured at 82 % effectiveness (Romanus 1992). This was accompanied by a higher rate of atypical mycobacterial infection in the non-BCG-vaccinated population (Romanus 1995). In some countries, such as Canada, the high risk population is already being vaccinated, but higher selectivity may be required given the identified risk of the vaccine; possibly limiting newborn BCG use to communities with active cases until the outbreak can be brought under control.

Safer and more effective vaccines for TB prevention may soon be available (Doherty 2005). Alternative vaccines to BCG are on the horizon and it is hoped that they will have better efficacy, be more standardized, and have fewer side effects,

especially in immunocompromised individuals, including the HIV-infected population worldwide, which is at a high risk of TB co-infection. (see chapter 10). One alternative intervention already exists in the form of the early detection and treatment of tuberculous infection. The administration of isoniazid is highly effective in reducing the risk of disease (International Union Against Tuberculosis Committee on Prophylaxis 1982) and protection may last for up to 30 years (Hsu 1984). Treatment of infection is generally well tolerated by children (Kopanoff 1978), and compliance is usually much higher than in adults (Wobeser 1989, McNab 2000). Considering the safety issues outlined in this report, the best course of action may be the removal of the BCG vaccine combined with improvements in TB programming in non-endemic countries (Vaudry 2003). Such improvements must include early case finding in adults to prevent transmission, and early detection and treatment of infection in children through contact tracing and screening in high-risk communities.

### 8.3. *Mycobacterium africanum* subtypes

*M. africanum* is predominantly isolated in Africa and, in certain areas of the continent, it is thought to produce a significant proportion of the cases of pulmonary TB (Frothingham 1999, Haas 1997). Reports on the sporadic isolation of *M. africanum* in Europe and the United States (Desmond 2004) have also been made, including one outbreak of multidrug-resistant (MDR) *M. africanum* (Schilke 1999).

Based on biochemical characteristics, two major subgroups of *M. africanum* have been described, corresponding to their geographic origin in Western (subtype I) or Eastern (subtype II) Africa. Numerical analyses of biochemical characteristics revealed that *M. africanum* subtype I is more closely related to *M. bovis*, whereas subtype II more closely resembles *M. tuberculosis* (Niemann 2002, Sola 2003). *M. africanum* subtype II was classified by its resistance to thiophen-2-carboxylic acid hydrazide (TCH). It is the main cause of human TB in Kampala, Uganda (East Africa).

Spoligotyping does not lead to a clear differentiation of *M. tuberculosis* and *M. africanum*, but all *M. africanum* subtype II isolates lack spacers 33 to 36, differentiating them from *M. africanum* subtype I. In an IS6110 restriction fragment length polymorphism (RFLP)-based dendrogram, *M. africanum* subtype II isolates were clustered into two closely related strain families (Uganda I and II) and clearly separated from *M. tuberculosis* isolates. An additional characteristic of both *M. africanum* subtype II families is the absence of spoligotype spacer 40. In addition, all strains of the *M. africanum* subtype II family Uganda I also lack spacer 43 (Nie-

mann 2002, Viana-Niero 2001). Lack of spacers 40 and 43 are not exclusive markers for the *M. africanum* subtype II family Uganda I, but might represent a useful additional criterion for *M. africanum* subtype identification in combination with biochemical test results (Brudey 2004, Mostowy 2004) (see figure 8-1).

The *gyrB* desoxyribonucleic acid (DNA) sequence allows the differentiation of *M. africanum* subtype I strains from *M. bovis*, *M. caprae*, and *M. microti*. *M. africanum* subtype I and *M. pinnipedii*, however, display identical *gyrB* DNA sequences and the same occurs with *M. africanum* subtype II and *M. tuberculosis* (Niemann 2000). Thus, differentiation of *M. africanum* subtype II from *M. tuberculosis* continues to be based on phenotypic characteristics such as growth on bromocresol purple medium.

In recent studies, based on the regions of difference (RD) to distinguish *M. africanum*, three groups were identified: *M. africanum* subtype II isolates that have deletion of TbD1 and have retained RD9, RD7, RD8 and RD10 intact. Some have suggested that these organisms should be included in the species *M. tuberculosis*. A second group consists of *M. africanum* subtype I with deletion of RD9, RD7, RD8, and RD10, called 1a. Finally, *M. africanum* subtype I with deletion of RD9 but not RD7, RD8, and RD10, and called 1b, forms the third group. Both subtype I branches of *M. africanum* have indistinguishable *gyrB* sequences. In addition, Mostowy *et al.* (Mostowy 2004) have recently described several novel RD loci within *M. africanum* organisms. Among these were RD711 and RD713, deleted in *M. africanum* subtype Ib, and RD701 and RD702, found deleted in a larger study of strains *M. africanum* subtype Ia (Mostowy 2004).

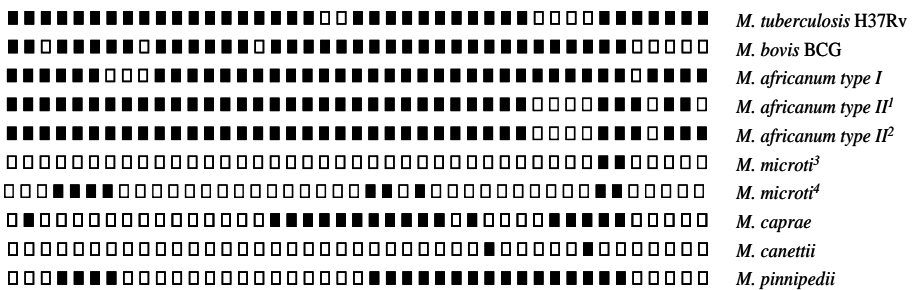


Figure 8-1: Spoligotypes of *Mycobacterium tuberculosis* complex strains. <sup>1</sup>*M. africanum* subtype II Uganda 1; <sup>2</sup>*M. africanum* subtype II Uganda 2; <sup>3</sup>*M. microti* vole type; <sup>4</sup>*M. microti* llama type

## 8.4. *Mycobacterium microti* disease

*M. microti* is a member of the *M. tuberculosis* complex and was first isolated in 1937 as the causative agent of pulmonary TB in the wild vole (*Microtus agrestis*) (Wells 1937). It was considered to be avirulent for humans, cattle and laboratory animals and was therefore proposed as a live vaccine against TB. The efficacy of vaccination with *M. microti* was assessed in clinical trials in the United Kingdom (Hart 1977) and the Czech Republic (Sula 1976), and indeed the strain was used as a vaccine in Africa for more than 15 years (Fine 1995). In all cases, *M. microti* proved to be safe and effective in preventing disease, showing a protective efficacy similar to that of BCG.

However, *M. microti* has been recently identified as the causative agent of pulmonary TB in both immunocompromised and immunocompetent humans (van Soolingen 1998, Horstkotte 2001). Genotypic analysis of *M. microti* showed the existence of two different variants of *M. microti*: vole and llama types. The vole type, isolated from voles, ferrets, and pigs, shows hybridization with only two of the 43 spacers in spoligotyping; whereas in the llama-type, the spoligotype-PCR product hybridizes with nine spacer sequences (figure 8-1). The first four *M. microti* isolates from humans in the Netherlands showed spoligotype patterns of the vole-type. Three of these four human *M. microti* isolates were obtained from immunocompromised patients (two had undergone kidney transplantation; one was HIV-infected). Two of the patients with *M. microti* infection had a history of contact with mice, which was found to be suggestive of zoonotic transmission (van Soolingen 1998, Brodin 2002). The first case of human infection with *M. microti* of the llama-type was reported in Germany: the patient was HIV-infected and presented with pulmonary TB (Horstkotte 2001). The time span required for cultivation of vole-type strains (3 and 4 months) is significantly longer than that required for growth of *M. microti* llama-type strains. The patient with *M. microti* llama-type infection was successfully treated with isoniazid, rifampin, and pyrazinamide, which indicates that the standard TB therapy is sufficient for treatment of patients with *M. microti* infection. A possible source of infection could not be identified in this patient.

Recent data demonstrated that *M. microti* can cause severe pulmonary TB in immunocompetent patients (Niemann 2000a). *M. microti* has been isolated in Germany from two HIV-negative immunocompetent patients with pulmonary TB. According to spoligotype patterns, one of the isolates belonged to the llama type and the other to the vole type. These findings emphasize the relevance of *M. microti* as a pathogen in immunocompromised as well as immunocompetent patients.

The prevalence and clinical importance of the different types of *M. microti* may have been underestimated so far because of difficulties with primary isolation and differentiation. Hence, further studies applying molecular methods are necessary to analyze the epidemiology of *M. microti* more thoroughly.

Genomic differences between *M. microti* and the other strains of the *M. tuberculosis* complex revealed novel deletions specific to *M. microti*. A surprising finding was that one of these deletions overlaps RD1, a locus that is absent from BCG sub-strains but present in *M. tuberculosis* and *M. bovis* and, therefore, assumed to be involved in the attenuation of BCG. The deletion found in *M. microti*, however, was found to be extended further to additional contiguous genes, and was therefore called RD1mic. Subsequent work has shown that complementation of *M. microti* with the RD1 locus increased the virulence of the recombinant strain in the mouse model (Pym 2002), suggesting that the loss of this region may have contributed to the attenuation of *M. microti*. The deletion of RD1 or RD1mic removes the genes *esxA/esxB*, which belong to the early secretory antigenic target 6 (ESAT-6) family and have been shown to be potent T-cell antigens. The ESAT-6 family may play a role in the attenuation of *M. microti*. In a genomic analysis using microarrays to compare *M. tuberculosis* and *M. microti*, 13 deletions were identified in 12 strains of *M. microti*, including regions RD1 to RD10, which are also missing in *M. bovis* BCG. In addition, four new deleted regions, MiD1, RD1mic, MiD2 and MiD3, were identified (Frota 2004). With regard to deleted regions and virulence, this study showed that it is difficult to ascribe virulence to any particular pattern of deletion. We have also used microarrays to extend the analysis of the *M. microti* genome (Garcia-Pelayo 2004). An *M. microti* of the vole-type, another of the llama-type, and a third isolate with an unusual type were used in this study. Using the improved resolution of this technique, a new deletion was described from *M. microti* that removes genes encoding ESAT-6 antigens and PE/PPE proteins, and it was shown that this locus may be prone to deletion. This region, called MiD4, was deleted from all *M. microti* strains tested suggesting that this region was deleted in a common ancestor of the *M. microti* lineage. Intriguingly, MiD4 was also found to be deleted from *M. pinnipedii*. As *M. pinnipedii* is closely related to *M. microti*, it is possible that deletion of MiD4 occurred in a common ancestor to both strains (Cousins 2003, Brosch 2002). The use of deletions as evolutionary markers demands that they are not generated at a hypervariable locus, since if this were the case, the deletion could appear independently in multiple lineages. The highly repetitive nature of the DNA that flanks MiD4 suggests that it may be prone to deletion, hence offering an alternative explanation as to why both *M. microti* and *M. pinnipedii* lack this locus. Sequence analysis of the *M. pinnipedii* junction, however, showed that it is identical to that of *M. microti*, suggesting that the loss of

MiD4 was a unique event that occurred in an ancestor of both strains. PE and PPE genes also appear overrepresented in deletions from *M. microti*, with RD1Mic, RD8, MiD3, and MiD4 removing genes for four PE and five PPE proteins. The genes encoding many PE or PPE proteins show a high degree of variation in the members of the *M. tuberculosis* complex and, indeed, among strains of the same species. Cole *et al.* were the first to speculate that the PE/PPE proteins could be of immunological importance, as a source of antigenic variation (Cole 1998). Further work has shown that some PE\_PGRS proteins are surface-associated and immunogenic (Brennan 2001, Banu 2002). Using a signature-tagged mutagenesis approach, Camacho *et al.* showed that inactivation of PPE46 (Rv3018c) attenuated *M. tuberculosis* for the murine model (Camacho 1999). Interestingly, inactivation of a gene of the MiD4 produces attenuation of *M. tuberculosis*, and lends further evidence to suggest that loss of MiD4 could have attenuated *M. microti*.

## 8.5. *Mycobacterium caprae* and *Mycobacterium pinnipedii*

The *M. tuberculosis* complex traditionally consisted of four members: *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. More recently, three novel species have been described:

- “*M. canettii*”: less virulent than the classical *M. tuberculosis* H37Rv
- *M. caprae*: a species that occurs primarily in Spanish goats, and also found in humans
- *M. pinnipedii*: responsible for TB in marine hosts.

### 8.5.1. *Mycobacterium caprae*

The names proposed for *M. caprae* are *M. tuberculosis* subspecies *caprae* (Aranaz 1999) and *M. bovis* subspecies *caprae* (Niemann 2002a). This species was originally described as preferring goats to cattle as hosts (Gutierrez 1995, Aranaz 1996) and has been found in Spain, Austria (Prodinger 2002), France (Haddad 2001), Germany (Erler 2003, Erler 2004), Hungary (Erler 2004), Italy, Slovenia (Erler 2004), and the Czech Republic (Pavlik 2002). In addition, *M. caprae* was isolated from humans and wildlife species such as red deer (Prodinger 2002) or wild boar (Erler 2004, Machackova 2004). In Central European regions, where *M. caprae* is the major cause of TB in cattle it is also the predominant agent of TB in humans (Kubica 2003, Prodinger 2002).

The major phenotypic difference between the caprine mycobacterial isolates and *M. bovis* is the sensitivity to pyrazinamide (PZA), which has been used as a major criterion for separation of *M. bovis* from the other members of the *M. tuberculosis* complex. Growth of *M. bovis* is not inhibited by PZA, while other *M. tuberculosis* complex species are susceptible to this antimycobacterial drug. The sequencing of the pyrazinamidase gene (*pncA*) demonstrated a single point mutation at nucleotide 169, a G to C substitution, which appears to be unique to *M. bovis* (Scorpio 1996). The sequence of the *pncA* gene of the *M. caprae* reveals that it has the wild-type *pncA* gene, and it can be used to differentiate between *M. caprae* and *M. bovis*. However, *M. caprae* is similar to *M. bovis* in its preference for pyruvate for growth, which differentiates both species from other members of the *M. tuberculosis* complex. In addition, it is possible to differentiate *M. caprae* from all other *M. tuberculosis* complex members by *gyrB* sequencing or amplification followed by restriction analysis (Chimara 2004). *M. caprae* also has specific fingerprinting patterns obtained by IS6110 RFLP, as well as a spoligotype pattern that is very different from those obtained for other members of the complex. By spoligotyping, *M. caprae* forms a homogeneous cluster easily recognizable by the absence of spacers 1, 3–16, 30–33, and 39–43 (figure 8-1). The lack of spacers 39–43 has also been described in *M. bovis*, *M. microti*, and *M. pinnipedii*. However, the fingerprinting patterns obtained with IS6110 and spoligotyping segregated *M. caprae* isolates from the other members of the complex (Liebana 1996, Aranaz 1998).

As reported in the original description of *M. tuberculosis* subsp. *caprae* (Aranaz 1999), isolates that displayed the caprine spoligotype pattern have also been found in humans, and these clinical cases have been linked with goat farming (Gutierrez 1997). *M. bovis* isolates from cattle and humans, described by Niemann *et al.* (Niemann 2000a, Niemann 2000b), are likely to be caprine isolates, because they share features such as susceptibility to PZA, the substitution described in the sequence of the *gyrB* gene, and the spoligotype pattern defined by the typical absence of spacers. Further evidence for the independence of the caprine mycobacterial isolates from *M. bovis* is derived from two recent studies that have examined the evolution of the *M. tuberculosis* complex (Brosch 2002, Mostowy 2002, see Chapter 2).

In Germany, *M. bovis* subsp. *caprae* has been described (Niemann 2002) as the causative agent of almost one-third (31 %) of the human *M. bovis*-associated TB cases analyzed. This proportion was surprisingly high, especially when compared with the prevalence of *M. bovis* subsp. *caprae* strains in human or animal isolates in other countries: a study on *M. bovis* TB in France revealed no *M. bovis* subsp. *caprae* strains among more than 1,000 animal isolates (Haddad 2000). *M. bovis*

subsp. *caprae* strains were not found in the United Kingdom (Sales 2001, Roring 1998), Ireland (Costello 1999), South America (Zumarraga 1999), and Cameroon (Njanpop-Lafourcade 2001). Outside Germany, small numbers of *M. bovis* subsp. *caprae* strains have been identified only in Spain (3.6 % of *M. bovis* isolates from humans and 12 % of isolates from goats and sheep) (Gutierrez 1997), and in Austria (12 cases in humans and animals in seven years) (Proding 2002). It might be assumed that *M. bovis* subsp. *caprae* represents a newly emerging genotype in Germany and is now spreading to other European countries. However, because the overall mean age for patients in Germany infected with *M. caprae* was 66.1 years, cases are probably due to reactivation rather than recently acquired infection. It is therefore likely that the patients were infected before effective control measures for bovine TB were introduced in the '50s. Consequently, *M. caprae* must have been present in Germany at that time and is not just emerging. Prior to the introduction of molecular tools for the identification and differentiation of *M. bovis* strains, *M. caprae* isolates might have been misclassified due to their susceptibility to PZA, resulting in false low notification rates. Susceptibility to PZA, however, was also observed in three *M. bovis* subsp. *bovis* strains that were obtained from two patients and a cow. These isolates showed no particular spoligotype patterns and were not related in the similarity analysis. From a phylogenetic point of view, these strains may represent ancestral *M. bovis* strains, from which both subspecies might have diverged. The only marked difference between the two patient groups was revealed in the spatial analysis of the inner-German origin of the patients: the regional proportion of *M. bovis* subsp. *caprae* showed a large difference between Southern (up to more than 80 %) and Northern parts of the country (less than 10%).

This observed geographic shift in the regional proportion of both subspecies might have resulted from a similar shift in the animal population, as indicated by the finding that animals infected with *M. bovis* subsp. *caprae* strains were mainly from Southern Germany. This is further supported by the presence of *M. bovis* subsp. *caprae* strains in wild and livestock animals in Western Austria, in a region located at the Southern German border (Proding 2002).

### **8.5.2. *Mycobacterium pinnipedii***

*M. pinnipedii* was first isolated from captive and wild sea lions and fur seals from New Zealand and Australia (Cousins 1993, Cousins 2003). Similar organisms were subsequently recovered from the same mammal species in South America (Bernardelli 1996, Romano 1995, Bastida 1999) as well as from a Brazilian tapir (Cousins 2003). Recently, their ability to cause disease in guinea pigs and rabbits has been

demonstrated by experimental inoculation (Cousins 2003). This fact, together with the finding of a human isolate from a seal trainer, who worked in an affected colony in Australia (Thompson 1993), and a bovine isolate in New Zealand (Cousins 2003), suggests that *M. pinnipedii* can cause infection across a wide host range. Many of the isolates obtained in Australia, Uruguay, and Argentina have been well characterized (Romano 1995, Romano 1996, Cousins 1993, Bernardelli 1996, Cousins 1996, Alito 1999, Zumarraga 1999a, Zumarraga 1999b, Castro Ramos 1998). This information, together with preliminary tests on seal isolates from Great Britain and New Zealand, suggested that the seal bacillus (Cousins 1993), isolated from pinnipeds from all continents, might be a unique member of the *M. tuberculosis* complex.

The results of biochemical tests clearly confirmed that the seal isolates belong to the *M. tuberculosis* complex. The negative reactions in the nitrate reduction and niacin accumulation tests were consistent with the identification of *M. bovis*, a fact that led to their initial identification as such in Australia (Forshaw 1991), Argentina (Bernardelli 1996), and Great Britain. Some seal isolates produced varying amounts of niacin, as do some *M. africanum* isolates. Most seal isolates grew preferentially on media that contained sodium pyruvate, although some also grew on Löwenstein–Jensen medium containing glycerol. In contrast to *M. bovis*, the seal isolates were susceptible to PZA. Isolates inoculated into guinea pigs produced significant lesions or death within six weeks and those inoculated into rabbits caused death within six weeks, confirming that the isolates were fully virulent for both laboratory animals.

Spoligotypes of mycobacteria isolated from seals (Romano 1995) showed the formation of a cluster that is clearly different from those of all other members of the *M. tuberculosis* complex (figure 8-1). All seal isolates lacked spacers 1 to 3, 8 to 22, and 39 to 43. The absence of these latter spacers is a characteristic shared with *M. bovis* isolates.

Mycobacteria isolated from seals were also tested for polymorphisms in the *oxyR* and *pncA* genes. Similarly to *M. tuberculosis*, *M. microti* and *M. africanum*, *M. pinnipedii* was found to contain CAC (His) at codon 57 in the *pncA* gene, and the *oxyR* gene showed G at nt 285. In addition, these mycobacteria had the same sequence polymorphisms of *gyrA* and *katG* as *M. bovis* and as some *M. tuberculosis* (Group 1 of Sreevatsan 1997). The MPB70 antigen, which is always detected in *M. bovis*, was not detected in the mycobacteria from seals. In contrast, their genomes contained the *mtp40* fragment present in the RD5 region described by Brosch *et al.* (Brosch 2002). The RD5 region is present in seal isolates, but is not present in *M. bovis* and BCG.

To extend the repertoire of these deletion markers, we therefore undertook a whole genome microarray analysis of the recently defined *M. pinnipedii* (Bigi 2005). In this study, we evaluated the extent of genetic variability in *M. pinnipedii* by microarray-based comparative genomics. This is a powerful method that allows genomes to be rapidly screened for deletion events. Using a DNA microarray that included both sequenced *M. tuberculosis* strains (H37Rv and CDC1551) and *M. bovis* AF2221/97, we identified two regions exclusively absent from *M. pinnipedii*. The PiD1 deletion was identified in this study for the first time as being absent from all isolates of *M. pinnipedii*. The coding sequences at the junction points are truncated, indicating that it is a deletion. Its bordering genomic regions do not contain repetitive sequences, suggesting that the deletion was the result of an irreversible event in a common progenitor strain. This deletion removes Rv3531c and parts of Rv3530c, encoding a hypothetical protein and possible oxidoreductase involved in cellular metabolism, respectively. The significance of these missing functions, if any, to the seal bacillus host tropism and phenotype is unknown at present. The second specific deletion, PiD2, has been recently defined as RD2seal by Marmiesse et al (Marmiesse 2004), since it overlaps the 10.7 kbp RD2 region. Interestingly, a region encompassing Rv1978 and part of Rv1979 is also missing in some *M. microti* isolates. However this deletion, called RD2mic, maps to a slightly different locus to that of RD2seal. This information, together with the fact that the RD2 region is deleted from some BCG sub-strains, strongly suggests that these deletions have occurred as independent events in an unstable region. These strain-specific deletions could serve as markers for phylogenetic and evolutionary studies, and also as a signature for rapid identification and diagnosis. Thus, these findings, together with previous studies, support the unique taxonomic position of *M. pinnipedii* within the *M. tuberculosis* complex.

## 8.6. Identification of species within the *M. tuberculosis* complex

The high degree of sequence conservation among members of the *M. tuberculosis* complex makes differentiation of species in the clinical mycobacteriology laboratory a difficult task. Routine differentiation is still based on phenotypic characteristics, such as oxygen preference, niacin accumulation, nitrate reductase activity, colony morphology, and resistance to two compounds, TCH and PZA.

*M. tuberculosis* is the most frequent cause of human TB, but some cases are caused by *M. bovis*. It is necessary to differentiate between *M. bovis* and *M. tuberculosis* in order to know the prevalence and distribution of human TB due to *M. bovis*. This may contribute to knowledge about the risk factors associated with the transmission

of *M. bovis* to the human population. *M. bovis* differs from *M. tuberculosis* in having a low growth rate on egg media supplemented with glycerol, but a faster growth on egg media supplemented with pyruvate (Stonebrink medium). *M. bovis* isolates are resistant to PZA, while *M. tuberculosis* strains are generally considered PZA-sensitive.

Several molecular techniques were designed to differentiate *M. tuberculosis* complex, including methods to detect mutations in *pncA* and *oxyR* genes (Scorpio 1996), *mpt40*-PCR (Del Portillo 1991, Liébana 1996), and PCR-amplification of regions of difference (RD) (Parsons 2002, Huard 2003), among others. Some techniques are useful for the differentiation of *M. tuberculosis* and *M. bovis*, such as *pncA* and *oxyR*. A species specific mycobacterial DNA element in the *M. tuberculosis* complex has been described by Del Portillo (1991), the *M. tuberculosis mpt40* fragment. Mpt40 protein was originally described as being produced only by *M. tuberculosis*. Now, it is well known that this protein is encoded by the *plcA* gene, contained in RD5. This region is present in most, but not all, isolates of *M. tuberculosis*, *M. africanum*, *M. pinnipedii*, and *M. microti*, and is consistently absent from *M. bovis* and *M. bovis* BCG isolates. Given the high polymorphism in this region, the use of the *mpt40* sequence as a genetic marker for *M. tuberculosis sensu stricto* is very restricted (Viana-Niero 2004).

Spoligotyping can also be used for differentiation of members of the *M. tuberculosis* complex (Kamerbeek 1997). For instance, the spoligotypes of “modern” *M. tuberculosis* strains typically lack spacer sequences 33–36 in the direct repeat (DR) region (see Figure 8-1). Similarly, *M. bovis* and *M. caprae* strains are known to lack spacers 3, 9, and 16. All *M. bovis*, *M. caprae*, and *M. microti* strains are known to lack spacers 39 to 43 in their spoligotypes (Zumarraga 1999b). It should also be noted that all *M. tuberculosis* complex organisms along the *M. africanum* type I to *M. bovis* evolutionary track lack spacers 9 and 39. Therefore, spacers 9 and 39 are potential markers for the differentiation of *M. tuberculosis* from the remaining *M. tuberculosis* complex species by spoligotyping. Although their absence has been noted in *M. africanum* subtype I isolates, they are present in *M. africanum* subtype II.

For more details, see Table 8-2 at <http://www.tuberculosis textbook.com/pdf/Table 8-2.pdf>.

RD analysis is currently used for differentiation between species of the *M. tuberculosis* complex. TbD1 is a deletion found only in *M. tuberculosis*, all other *M. tuberculosis* complex strains, including some *M. tuberculosis* have TbD1. Based on the presence or absence of this *M. tuberculosis*-specific deletion 1 (TbD1), *M.*

*tuberculosis* strains can be divided into ancestral and “modern” strains, respectively; the latter comprise representatives of major epidemics, for example, the Beijing, Harlem, and other epidemics (Brosch 2002). TbD1 is always absent in *M. africanum* type II strains.

Previously, based on *katG* codon 463 (*katG*463) and *gyrA* codon 95 (*gyrA*95) sequence polymorphisms, Sreevatsan *et al.* (Sreevatsan 1996, Sreevatsan 1997) defined three groups among the tubercle bacilli: group 1 with *katG*463 CTG (Leu), *gyrA*95 ACC (Thr); group 2 with *katG*463 CGG (Arg), *gyrA*95 ACC (Thr); and group 3 with *katG*463 CGG (Arg), *gyrA*95 AGC (Ser). *M. tuberculosis* organisms belonging to group 1 have *katG* and *gyrA* sequences indistinguishable from those of *M. microti*, *M. africanum*, and *M. bovis*.

*M. tuberculosis* strains containing the TbD1 region belong to group 1, and are considered ancestral strains. However, *M. tuberculosis* with TbD1 deletion can also be in group 1, although most strains presenting TbD1 deletion belong to groups 2 and 3. This finding suggests that during the evolution of *M. tuberculosis*, the *katG* mutation at codon 463 CTG (Leu) occurred in a progenitor strain that had the region TbD1 deleted. This proposal is supported by the finding that strains belonging to group 1 may or may not have deleted region TbD1, whereas all strains belonging to groups 2 and 3 lack TbD1.

Furthermore, a subsequent loss of DNA, reflected by the deletion of DR9 was identified for an evolutionary lineage that diverged from the progenitor *M. tuberculosis* strains. It is represented by *M. africanum*, *M. microti*, *M. caprae*, *M. pinnipedii*, and *M. bovis* (Brosch 2002). Thus, RD9 allows differentiation between *M. tuberculosis* and the other strains of the *M. tuberculosis* complex. Other regions of difference, such as RD7, also allow differentiation between *M. tuberculosis* and the other species. RD7 deletion was observed in *M. bovis*, *M. microti*, some *M. africanum*, and *M. pinnipedii*.

In a previous report, we described a PCR protocol for the differentiation of *M. tuberculosis* from *M. bovis* (Zumarraga 1999c). This differential strategy is based on the amplification of the region designated as RD7. The deletion removes most of the *mce-3* operon, one of four highly related operons that may be involved in cell entry, and therefore it may contribute to differences in virulence or host specificity within the species of the *M. tuberculosis* complex.

Human beings can be infected by *M. caprae* or *M. bovis* from infected livestock, and infection with both species remains a serious public health problem in some countries. Differentiation of these species is important for epidemiological reasons. *M. pinnipedii*, *M. microti*, *M. bovis*, and *M. caprae* show a single nucleotide poly-

morphism in the TbD1 region at codon 551 (AAG) of the *mmpL6* gene relative to “*M. canettii*”, *M. africanum*, and *M. tuberculosis* strains, which are characterized by codon AAC. This polymorphism, which is associated with deletion of RD12 and RD13 loci, differentiates the group comprised of *M. bovis* and *M. caprae* from other species of the *M. tuberculosis* complex. On the other hand, it is now known that *M. caprae* can be genetically differentiated from *M. bovis* on the basis of a positive amplification of the RD4 locus, as well as SNP analysis of the *gyrB* nucleotide 1311-1410 and *pncA169* (see Table 1). *M. bovis* BCG strains possess a specific polymorphism – the RD1 deletion. This deletion allows the differentiation between BCG and all the other species of the *M. tuberculosis* complex. *M. pinnipedii* and *M. microti* are very closely related microorganisms.

Some deletions that are useful for the differentiation of isolates of the *M. tuberculosis* complex are summarized in Table 8-3. These strain-specific deletions could serve as markers for phylogenetic and evolutionary studies, and also as a signature for rapid identification and diagnosis.

Table 8-3: Differential distribution of some regions of difference (RD) loci among *Mycobacterium tuberculosis* complex

	TbD1	RD1	RD7	RD12 and RD13	RD4	RD2	PiD1
<i>M. tuberculosis</i> <i>ancestral</i>	+	+	+	+	+	+	+
<i>M. tuberculosis</i> <i>modern</i>	-	+	+	+	+	+	+
<i>M. africanum</i>	+/-	+	+/-	+	+	+	+
<i>M. microti</i>	+	RD1mic	-	+	+	RD2 mic	+
<i>M. pinnipedii</i>	+	+	-	+	+	RD2seal	-
<i>M. caprae</i>	+	+	-	-	+	+	+
<i>M. bovis</i>	+	+	-	-	-	+	+

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