Molecular characterization of mycobacteria isolated from seals

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Tuberculosis (TB) was diagnosed in 10 seals from three species (*Arctocephalus australis*, *Arctocephalus tropicalis* and *Otaria flavescens*) found in South America. The mycobacteria isolated from these cases belonged to the *Mycobacterium tuberculosis* complex, as determined by RFLP using an IS6110 probe, spoligotyping, analysis of the 16S rRNA gene sequence and by PCR-restriction analysis of *hsp65*. Polymorphisms in *gyrA*, *katG*, *oxyR* and *pncA* were investigated in some of the isolates, as well as the presence of the MPB70 antigen. The insertion sequence IS6110 was present in three to seven copies in the genome of the mycobacteria isolated from seals. Using the IS6110 probe, six patterns (designated A, B, C, D, E and F) were identified from 10 different isolates. Patterns A and B were found for the mycobacteria isolated from two and four seals, respectively, indicating an epidemiological relationship between isolates grouped according to their IS6110 RFLP. The mycobacteria isolated from seals shared the majority of their IS6110 DNA-containing restriction fragments, and nine isolates had an identical spoligotype; only one isolate showed a minor difference in its spoligotype. In addition, none of these spoligotypes were found in other *M. tuberculosis* complex strains. These results suggest that the isolates from seals constitute a unique group of closely related strains. The mycobacteria isolated from seals showed polymorphisms at *gyrA* codon 95 and *katG* codon 463, as do group 1 *M. tuberculosis*, and *M. bovis*. Group 1 mycobacteria are associated with cluster cases. The spoligotypes found in the mycobacteria isolated from seals lack spacers 39–43, as does *M. bovis*, but the MPB70 antigen, which is highly expressed in *M. bovis* and minimally expressed in *M. tuberculosis*, was not detected in these mycobacteria. The mycobacteria isolated from seals also showed *oxyR* and *pncA* polymorphisms specific to *M. tuberculosis*. In conclusion, the mycobacteria that cause TB in seals in the South-Western Atlantic are a related group, and based on the combination of genetic characteristics, belong to a unique genotypic group within the *M. tuberculosis* complex.

Keywords: tuberculosis, *Mycobacterium tuberculosis* complex, spoligotyping, RFLP

INTRODUCTION

During the period 1986–92 the first cases of tuberculosis (TB) in wild seals were registered on the Australian Pacific coast (Cousins *et al.*, 1990; Forshaw & Phelps,

1991; Cousins *et al.*, 1993; Thompson *et al.*, 1993) and the South-Western Atlantic coast (Bernardelli *et al.*, 1993, 1996; Romano *et al.*, 1995). These isolates were characterized as belonging to the *Mycobacterium tuberculosis* complex, and several genetic markers were used to confirm the relatedness between the mycobacteria isolated from seals (Cousins *et al.*, 1993; Romano *et al.*, 1995). The most widely used genetic

Abbreviations: DR, direct repeat; PZA, pyrazinamide; Pzase, pyrazinamidase; TB, tuberculosis.

marker for molecular epidemiology of TB is the insertion sequence IS6110 (Hermans *et al.*, 1992; van Soolingen *et al.*, 1993; Romano *et al.*, 1996; Samper *et al.*, 1997), which is a mobile genetic element present exclusively in the genome of members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. microti*). *M. bovis* isolates have often been found to harbour only a single copy of IS6110 (Romano *et al.*, 1996), while mycobacteria isolated from seals, as well as *M. tuberculosis*, show several copies of this element (Cousins *et al.*, 1993; Romano *et al.*, 1995; Bernardelli *et al.*, 1996).

A new technique called spoligotyping was designed as a tool for molecular epidemiology of the *M. tuberculosis* complex (Kamerbeek *et al.*, 1997). The method is based on the *in vitro* amplification of the DNA sequence of the highly polymorphic direct repeat (DR) locus in the chromosome of members of the *M. tuberculosis* complex. This region flanks an IS6110 copy, and has a characteristic organization, with conserved 36 bp DR sequences interspersed with variable spacers (Hermans *et al.*, 1991). The polymorphism is due to these spacers, which are variable in length, sequence and number. The spoligotyping technique detects the spacers present in the strains, and allows each to be characterized by its spacer content (Kamerbeek *et al.*, 1997). Spoligotyping has been used to type isolates of *M. tuberculosis* (Goguet de la Salmonière *et al.*, 1997) and *M. bovis* (Aranaz *et al.*, 1996; Blázquez *et al.*, 1997; Cousins *et al.*, 1998; Zumárraga *et al.*, 1999).

Currently, strains of *M. bovis* and *M. tuberculosis* are distinguished by several biochemical parameters, including niacin accumulation, pyrazinamide (PZA) susceptibility, pyrazinamidase (Pzase) activity, nitrate reduction and thiophenecarboxylic acid hydrazide susceptibility (Collins *et al.*, 1985). An immunological method for differentiating between *M. bovis* and *M. tuberculosis* based on detection of the protein antigen MPB70 in *M. bovis* has been reported (Harboe & Nagai, 1984), but its use and reliability are limited because this protein is also present in *M. tuberculosis*. This protein is highly expressed in *M. bovis* and minimally expressed in *M. tuberculosis* (Harboe & Nagai, 1984). The *mtp40* gene, considered to be present in *M. tuberculosis* but absent in *M. bovis*, was used to differentiate *M. tuberculosis* from *M. bovis* (Del Portillo *et al.*, 1991). However, this method has recently been invalidated, as the gene has been shown not to be present in all *M. tuberculosis* strains, and not absent in all *M. bovis* strains (Weil *et al.*, 1996). In many mycobacteria, 16S rRNA and the heat-shock protein gene (*hsp65*) contain variable sequences that allow mycobacterial differentiation at the species level. However, within the *M. tuberculosis* complex, these genes are not variable. (Kirschner *et al.*, 1993; Telenti *et al.*, 1993). *M. tuberculosis* complex organisms have multiple mutations in the *oxyR* gene, a homologue of the extensively studied central regulator of peroxide stress response in enteric bacteria (Deretic *et al.*, 1997). Because *oxyR* in *M. tuberculosis* complex isolates probably does not

encode a functional protein, it is referred to as a pseudogene. In *M. bovis* the *oxyR* pseudogene has an adenine residue at nucleotide 285, while the remaining *M. tuberculosis* complex strains have a guanine residue (Sreevatsan *et al.*, 1996). In addition, the Pzase gene (*pncA*) has a guanine instead of a cytosine residue at nucleotide position 169 in *M. bovis*. This results in production of an inactive Pzase, thus conferring resistance to PZA in these strains (Scorpio & Zhang, 1996). Therefore, polymorphisms in the *oxyR* and *pncA* genes allow differentiation of *M. bovis* from the other mycobacteria of the complex.

M. tuberculosis can be assigned to one of three distinct genotypic groups, based on the combinations of polymorphisms at the genes encoding catalase-peroxidase (*katG*) and the A subunit of gyrase (*gyrA*). *M. tuberculosis* group 1 has the allele combination *katG* codon 463 CTG (Leu) and *gyrA* codon 95 ACC (Thr); group 2 has *katG* codon 463 CGG (Arg) and *gyrA* codon 95 ACC (Thr); group 3 has *katG* codon 463 CGG (Arg) and *gyrA* codon 95 AGC (Ser). All isolates of *M. bovis*, *M. microti* and *M. africanum* studied have the combination of polymorphisms of *M. tuberculosis* group 1. The isolates of *M. tuberculosis* grouped in clusters were mainly of genotypic groups 1 and 2 (Sreevatsan *et al.*, 1997).

The aim of this study was to evaluate the epidemiological relationship and genetic characteristics of the mycobacteria that cause TB in seals in South America.

METHODS

Mycobacterial isolates. A total of 10 cases of TB in seals, with bacteriological confirmation, were included in this study. Nine of these cases (Table 1, cases 2 to 10) were from wild seals (the South American fur seal *Arctocephalus australis* and Subantarctic fur seal *Arctocephalus tropicalis*) found on the Argentine coast during the period 1991–96. These animals were caught for medical treatment at the Rehabilitation Centre of the Foundation Marine World (San Clemente del Tuyú, Argentina). One case (Table 1, case 1) was from an outbreak of TB occurring in 1987 in captive South American sea lions (*Otaria flavescens*) at the Montevideo Zoo (Uruguay) (Castro Ramos *et al.*, 1998). Only one isolate from this outbreak was included in the present study.

Samples obtained from necropsies were processed by the Petroff decontamination method, and inoculated on Löwenstein–Jensen and Stonebrink media.

The mycobacteria isolated from seals were susceptible to PZA, isoniazid, streptomycin, rifampicin, ethambutol and thiophene-2-carboxylic acid hydrazide, and relatively resistant to *p*-aminosalicylic acid. A few strains were positive for niacin production. Biochemical, drug-susceptibility and biological tests were performed by standard procedures (Wayne & Kubica, 1986).

RFLP and spoligotyping techniques. The RFLP technique using IS6110 and *mtp40* as genetic markers was described previously (Romano *et al.*, 1995). Spoligotyping was performed according to Kamerbeek *et al.* (1997). Clustering analysis using UPGMA and the Dice coefficient of spoligotypes was performed with the aid of the computer program

Table 1. Origin of the mycobacteria isolated from seals and their spoligotypes and IS6110 RFLP types

Clinical case	Host species	Origin	Date of isolation	Spoligotype	IS6110 RFLP pattern
1*	<i>Otaria flavescens</i>	Uruguay	1987	1	B
2	<i>Arctocephalus australis</i>	Argentina	1991	1	B
3	<i>Arctocephalus australis</i>	Argentina	1992	1	B
4	<i>Arctocephalus australis</i>	Argentina	1992	1	A
5	<i>Arctocephalus australis</i>	Argentina	1992	1	C
6	<i>Arctocephalus australis</i>	Argentina	1995	1	D
7	<i>Arctocephalus australis</i>	Argentina	1996	1	E
8	<i>Arctocephalus tropicalis</i>	Argentina	1996	1	A
9	<i>Arctocephalus australis</i>	Argentina	1996	1	B
10	<i>Arctocephalus australis</i>	Argentina	1996	2	F

* This case was from an outbreak of TB in captive sea lions.

GelCompar, version 2.1 (Applied Maths, Kortrijk, Belgium). The spoligotypes found in the mycobacteria isolated from seals were compared with the spoligotypes found in over 609 *M. bovis* isolates that originated from different sources (human, cattle, cat, goat, llama, buffalo and deer) and from different countries in Latin America and Europe.

Production of MPB70 antigen. To prepare mycobacterial culture supernatant and sonic extracts, cultures were centrifuged for 30 min at 10000 g. For cell extracts, bacteria were resuspended in distilled water and sonicated for 10 cycles of 30 s followed by a 30 s interval. The culture supernatant was precipitated with trichloroacetic acid (10%) and resuspended in loading buffer (2% SDS, 0.125 M Tris/HCl pH 6.8, 1% 2-mercaptoethanol, 0.02% bromophenol blue, 10% glycerol).

Proteins (50 µg) were analysed by electrophoresis in 12% polyacrylamide-SDS gels and electrotransferred onto a nitrocellulose sheet by the semidry method. Transfer yield was visualized by transient staining with Ponceau Rouge. The membranes were incubated with monoclonal antibody 4C3/17 against MPB70 (CSL Laboratories, Victoria, Australia) overnight at 4 °C and with an alkaline-phosphatase-conjugated goat-antimouse antiserum (Sigma) for 2 h at 37 °C.

PCR and DNA sequencing

pncA. The *pncA* gene was amplified by PCR, using primers PNCA-1 and PNCA-2. To determine the sequence of *pncA* (GenBank accession no. U59967) from various strains of mycobacteria isolated from seals, primer PNCA-1 (5'-ATGCGGGCGTTGATCATCGTC-3'), corresponding to bp 1–21 of the *M. tuberculosis* *pncA* gene, and primer PNCA-2 (5'-TCAGGAGCTGCAAACCAA-3'), corresponding to bp 561–544 of the same gene (Scorpio & Zhang, 1996) were used. PCR was performed using PCR buffer composed of 10 mM Tris/HCl, pH 8.8; 50 mM KCl; 1.5 mM MgCl₂; 0.125 µM of the above-mentioned primers; 0.125 mM dNTPs; 1.25 units *Taq* DNA polymerase (Advanced Biotechnologies); and 10–100 ng DNA. PCR amplification was performed in a Perkin-Elmer Cetus DNA thermal cycler, set for 3 min at 94 °C, followed by 40 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min. The *pncA* sequences from different mycobacterial strains were determined by PCR direct sequencing, using a Thermo Sequenase labelled primer cycle sequencing kit (Amersham) with fluorescent 5' Cy 5-labelled PNCA-1 and PNCA-2 primers, in an automatic DNA sequencer (ALFexpress, Pharmacia).

katG. Detection of point mutations in the catalase-peroxidase gene (*katG*) (GenBank accession no. X68081) was performed as described by Uhl *et al.* (1996).

gyrA. To determine the mutations of the quinolone-resistance-determining region of the gene *gyrA* (GenBank accession no. L27512) from *M. tuberculosis* strains, a region of 320 bp was amplified and sequenced. The forward primer GyrA1 (5'-CAGCTACATCGACTATGCGA-3'), corresponding to bp 2383–2402 of *gyrA*, and the reverse primer GyrA2 (5'-GGGCTTCGGTGTACCTCAT-3'), corresponding to bp 2684–2702 of this gene, were used. PCR and sequencing was performed as described above for *pncA* except that an annealing at 60 °C for 1 min was used in the PCR, and GyrA1 and GyrA2 were used as sequencing primers.

16S rRNA. A 1030 bp fragment of 16S rRNA from *M. tuberculosis* (GenBank accession no. X52917) was amplified using 50 pmol each of primers 285 (5'-GAGAGTTTGATCCTGGCTCAG-3'), corresponding to bp 9–30 of the *E. coli* 16S rRNA, and 264 (5'-TGCACACAGCCACAAGGGA-3'), corresponding to bp 1027–1046 of the *E. coli* 16S rRNA (GenBank accession no. U59967) per 50 µl of reaction, as described by Kirschner *et al.* (1993). The cycling parameters were 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min. The reaction was performed in a Perkin-Elmer Cetus DNA thermal cycler.

The 16S rRNA sequence was determined by PCR direct sequencing using a Thermo Sequenase labelled primer cycle sequencing kit (Amersham), with the fluorescent 5' Cy 5-labelled 244 (5'-CCCACTGCTGCCTCCCGTAG-3') primer corresponding to bp 341–361 of the *E. coli* 16S rRNA, in an automatic DNA sequencer (ALFexpress, Pharmacia).

PCR-restriction analysis

hsp65. Specific amplification of a fragment of 439 bp of *hsp65* (GenBank accession no. M15467) from each strain, and digestion by *Bst*II and *Hae*III of the amplified fragments, were performed according to the procedure described by Telenti *et al.* (1993). Following digestion, 10 µl of the mixture was loaded onto a 4% agarose gel. The 10 bp DNA ladder (Gibco-BRL) was used as the molecular marker.

oxyR. A 548 bp segment of the *oxyR* pseudogene of the *M. tuberculosis* complex (GenBank accession no. U16243) was amplified with the forward primer 5'-GGTGATATATCAC-

ACCATA-3' and the reverse primer 5'-CTATGCGATCAG-GCGTACTTG-3', and restriction fragment length polymorphism of the amplified product with restriction endonuclease *AluI* was analysed as described by Sreevatsan *et al.* (1996), to differentiate *M. bovis* from other complex members. PCR was performed as described above for *pncA* except that the cycling conditions used were 30 cycles at 96 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

RESULTS

Bacteriology and biological assays

All the mycobacteria isolated from seals grew in Stonebrink medium. None of the Argentine strains grew in Löwenstein–Jensen medium, while the strain from Uruguay grew very slowly in that medium.

Experimental inoculation of guinea pigs with the mycobacteria isolated from seals produced significant and generalized lesions, and the intradermal tuberculin test with *M. bovis* purified protein derivative (PPD: 50 IU), of the experimentally inoculated guinea pigs gave a strong positive reaction. There was an increase of 6 mm or more in skinfold thickness, and these reactions were 6–16 times greater than with *M. avium* PPD.

Genetic characterization

Mycobacterium species can be differentiated by amplification of a fragment of the *hsp65* gene followed by restriction enzyme analysis (Telenti *et al.*, 1993), and by sequencing of a region of the 16S rRNA (Kirschner *et al.*, 1993), because these genes exhibit some internal polymorphism within these regions. Mycobacteria from different seal isolates had DNA fragment sizes generated

Fig. 1. RFLPs of *M. tuberculosis* complex strains. DNAs were digested with *PvuII*, and probed with the right arm of IS6110. Lanes: 1, *M. bovis* isolate; 2, *M. tuberculosis* H37Rv; 3, *M. bovis* AN5; 4, BCG Brazil; 5, mycobacteria isolated from wild seal type C; 6, mycobacteria isolated from wild seal type A; 7, mycobacteria isolated from wild seal type B; 8, mycobacteria isolated from wild seal type D; 9, mycobacteria isolated from wild seal type F; 10, mycobacteria isolated from wild seal type E. The fragment sizes of *M. tuberculosis* H37Rv after digestion with *PvuII* and hybridization with IS6110 are indicated on the left.

after amplification of *hsp65* followed by digestion with *BstEII* and *HaeIII* identical to the organisms belonging to the *M. tuberculosis* complex (data not shown). The

Table 2. Comparison of genetic and antigenic characteristics between mycobacteria isolated from seals, *M. tuberculosis* and *M. bovis*

Gene/antigen	<i>M. tuberculosis</i>	<i>M. bovis</i>	Mycobacteria isolated from seals
16S rRNA	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
<i>hsp65</i> (PRA*)	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
IS6110 element†	+	+	+
<i>mtp40</i> †	+	+	+
Detection of MPB70 antigen	—	+	—
<i>katG</i> codon 463	CTG (Leu) (group 1) or CGG (Arg) (groups 2 and 3)	CTG (Leu)	CTG (Leu)
<i>gyrA</i> codon 95	ACC (Thr) (groups 1 and 2) or AGC (Ser) (group 3)	ACC (Thr)	ACC (Thr)
<i>pncA</i> nt 169 mutation	CAC (His)	GAC (Asp)	CAC (His)
<i>oxyR</i> nt 285 mutation	Guanine	Adenine	Guanine

* PRA, PCR-restriction analysis.

† The IS6110 element and *mtp40* genes are present in most *M. tuberculosis* isolates; however, *M. tuberculosis* isolates without these sequences have occasionally been described. Furthermore, IS6110 is present in several copies in most *M. tuberculosis* isolates, and in a single copy in most South American *M. bovis* isolates. Presence of *mtp40* in mycobacteria isolated from seals was described previously (Romano *et al.*, 1995).

Fig. 2. Spoligotypes of *M. tuberculosis* complex strains. Rows: 1, *M. tuberculosis* H37Rv; 2, BCG; 3, *M. bovis* AN5; 4, *M. africanum*; 5, *M. microti*; 6, mycobacteria isolated from a seal with spoligotype 1; 7, mycobacteria isolated from a seal with spoligotype 2 (case 10). The numbers at the top of the figure correspond to the 43 spacer oligonucleotides numbered as described by Kamerbeek *et al.* (1997).

sequence of the amplified 16S rRNA gene of the different mycobacteria isolated from seals was identical to the sequence of the members of the *M. tuberculosis* complex (data not shown). According to their 16S rRNA gene sequence and the PCR-restriction characteristics of the *hsp65* gene, the seal isolates were identified as belonging to the *M. tuberculosis* complex (Table 2). The IS6110 element and DR region, both sequences characteristic of the *M. tuberculosis* complex, were found in the DNA of the mycobacteria isolated from seals (Fig. 1 and 2, Tables 1 and 2). These mycobacteria had spoligotypes different from those in *M. tuberculosis*, *M. africanum*, *M. microti* and *M. bovis* AN5 (Fig. 2). All the seal isolates lacked the spacers 39–43. The absence of these spacers is characteristic of *M. bovis* isolates. Clustering analysis using UPGMA and the Dice coefficient of spoligotypes was performed to compare the spoligotypes found in the mycobacteria isolated from seals with the spoligotypes of over 609 *M. bovis* isolates from different countries in Latin America and Europe, and with the spoligotypes of BCG and *M. bovis* AN5. The analysis of these spoligotypes showed that the spoligotypes of the mycobacteria isolated from seals were exclusive to these isolates (Fig. 3).

Some of the mycobacteria isolated from seals (clinical cases 2, 4, 5, 6, 7 and 10 from Table 1) were tested for polymorphisms in the *oxyR* and *pncA* genes; they showed *M. tuberculosis*-specific polymorphism in both genes (Table 2). In addition, these mycobacteria had the same sequence polymorphisms of *gyrA* and *katG* as do group 1 *M. tuberculosis*, and *M. bovis* (Table 2).

The MPB70 antigen, which is always detected in *M. bovis*, was not detected in the mycobacteria from seals (Table 2).

Epidemiological implications

Spoligotyping and IS6110-RFLP techniques were used to study the epidemiology of the TB in seals. Six IS6110-RFLP types were found among the 10 seal isolates (Table 1). These isolates contained three to seven copies of IS6110 (Fig. 1). The IS6110-RFLP types were arbitrarily designated as A–F. IS6110-RFLP type A contained three copies of this element (Fig. 1), and was

found in mycobacteria isolated from two seals (Table 1). One of the animals belonged to the species *Arctocephalus australis* and was found stranded on the Argentine coast in 1992 (Table 1, case 4); the second mycobacterial isolate was obtained in 1996 from an *Arctocephalus tropicalis* individual (Table 1, case 8). IS6110-RFLP type B contained five copies of IS6110 (Fig. 1), and was found in mycobacteria isolated from four seals (Table 1): one *Otaria flavescens* individual, involved in an outbreak of TB in 1987 at the Montevideo Zoo, Uruguay (Table 1, case 1), and three wild seals of the species *A. australis* found on the Argentine coast, in 1991, 1992 and 1996 (Table 1, cases 2, 3 and 9, respectively). The remaining four RFLP types (C, D, E and F) were found in mycobacteria isolated from four different animals (Table 1, cases 5, 6, 7 and 10, respectively).

Although there were six different IS6110 fingerprint patterns, they shared many of their IS6110-containing restriction fragments (Fig. 1). Mycobacteria isolated from nine animals had an identical spoligotype. Only one isolate showed a minor difference: it had two missing spacers in its spoligotype (Fig. 2; Table 1, case 10). This individual corresponded to the last isolate (September, 1996), which also showed a unique IS6110 RFLP pattern (F) (Fig. 1; Table 1, case 10).

DISCUSSION

Based on the 16S rRNA and *hsp65* nucleotide sequences, as well as on the presence of the insertion sequence IS6110 and DR region, we have confirmed that the mycobacteria isolated from seals belong to the *M. tuberculosis* complex. These bacteria had some of the genetic characteristics of *M. bovis*, and other genetic and antigenic characteristics identical to *M. tuberculosis*. Several molecular strategies were used to characterize the mycobacterial isolates from seals. Spoligotypes found in these isolates were exclusive to isolates from seals, and lacked spacers 39–43, as does *M. bovis*. In contrast, the restriction analysis of *oxyR*, and the nucleotide sequence of *pncA*, showed polymorphisms characteristic of *M. tuberculosis*. These results indicated that the mycobacteria isolated from seals belong to a

Fig. 3. Dendrogram with 125 spoligotypes identified among 609 *M. bovis* isolates studied of human and animal origin in the authors' laboratories in Spain and Argentina and the spoligotypes from mycobacteria isolated from seals. The spoligotypes of BCG and *M. bovis* AN5 were also included. The numbers on the right correspond to the number of isolates for each spoligotype. Seal spoligotypes are indicated in the dendrogram, and are also indicated on the right with the letters WS.

unique genotypic group inside the *M. tuberculosis* complex. In addition, the mycobacteria isolated from seals, as does *M. bovis*, grew better in Stonebrink than in Löwenstein-Jensen medium and their spoligotypes

lacked the spacers 39–43 (Fig. 2); and like *M. tuberculosis*, they did not produce detectable amounts of MPB70 antigen (Table 2) and contained *mtp40* (Romano *et al.*, 1995). Based on these results, the mycobacteria

isolated from seals can be considered to share genotypic characteristics with different members the *M. tuberculosis* complex.

In the present study, the spoligotypes of the mycobacteria isolated from seals were identical; only the most recent isolate showed a minor difference in its spoligotype. However, the isolates with the same spoligotype could be differentiated by RFLP analysis with the IS6110 probe. Thus the IS6110 element was more useful than spoligotyping to assess the epidemiological relationship between these mycobacteria. In a previous study, mycobacteria isolated from seals were also typed by RFLP analysis with DR and polymorphic GC-rich repetitive sequence (PGRS) probes. All strains showed the same DR and PGRS patterns, except for one strain which showed a minor difference with the DR probe (Romano *et al.*, 1995), suggesting an epidemiological relationship between these isolates.

The similarities in IS6110-associated RFLPs among mycobacteria isolated from seals suggest that they may have recently diverged from a common ancestor. It is not possible to calculate exactly the time elapsed since the divergence from this putative common ancestor. Another group of strains, which share the majority of their IS6110-containing restriction fragments, are those *M. tuberculosis* strains which have been circulating in the Republic of China (Beijing family); in this case the authors proposed that they have evolved from recent clonal expansion that began less than a century ago (van Soolingen *et al.*, 1995).

The cases of TB reported in this study were in animals that came from the rookeries situated on the Argentine and Uruguayan coasts. Most of the individuals found stranded on the Argentine coast were physiologically depressed, with a higher percentage of diverse illnesses than is generally seen in animals from natural colonies. This situation could contribute to the active transmission of the TB infection and the illness may be endemic among these animals. The results of van Soolingen *et al.* (1991) indicated that IS6110 patterns from *M. tuberculosis* strains from regions where TB is endemic are more related to each other than isolates from countries where the transmission rate is slow. Thus, similar IS6110 types between mycobacterial isolates from seals are a sign of an active transmission of TB between these animals.

IS6110-RFLP type B was found in a mycobacterial isolate, which caused tuberculosis in a colony of captive seals of the species *O. flavescens*, at Montevideo Zoo (Uruguay) (Castro Ramos *et al.*, 1998). These captive seals were collected from wild colonies in Uruguay and at least one of them may have been ill at the time of the capture. This same RFLP type was found in mycobacterial isolates from TB cases in wild seals of the species *A. australis*, found stranded on the Argentine coast. This is probably due to the fact that seals of these two species inhabit the same islands in Uruguay. IS6110-RFLP type A was found in wild seals of the species *A. australis* and *A. tropicalis*, stranded on the Argentine

coast. The presence of the latter species is incidental on this coast, because it comes from subantarctic islands near South Africa. These 'wandering' specimens are swept north by the Benguela current, reach the equatorial current in the direction of South America, and then arrive at the Argentine coast pushed by the Brazil current (Rodríguez *et al.*, 1995).

Cases of TB in other species of marine mammals have also been described in Australia and New Zealand (*Neophoca cinerea*, *Arctocephalus forsteri* and *A. pusillus doriferus*) (Cousins *et al.*, 1990, 1993; Forshaw & Phelps, 1991; Thompson *et al.*, 1993), where these mycobacteria infected a seal trainer (Cousins *et al.*, 1993; Thompson *et al.*, 1993). The restriction endonuclease analysis and RFLP patterns of these seal isolates were different from those of other members of the *M. tuberculosis* complex, and the protein MPB70, present in *M. bovis*, was not detected in these seal isolates (Cousins *et al.*, 1993). Comparisons between Australian and Argentinian isolates would be necessary to determine whether there is a single or multiple origin of this animal disease.

The importance of the results presented here is that they indicate that there are new mycobacteria in the *M. tuberculosis* complex, which cause TB in marine mammals and appear to be specific to these animals, and which are actively transmitted within the animal colonies. It will be necessary to study the prevalence of this disease in the seal colonies of Uruguay and Argentina, in addition to controlling the human population that has contact with these animals.

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