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Genomic Analysis of *Mycobacterium bovis* and Other Members of the *Mycobacterium tuberculosis* Complex by Isoenzyme Analysis and Pulsed-Field Gel Electrophoresis

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Initially, multilocus enzyme electrophoresis was used to examine genetic relationships among 63 isolates of *Mycobacterium bovis* and 13 other members of the *M. tuberculosis* complex. The isolates were divided into five electrophoretic types, with a mean genetic diversity of 0.1. The strains were genetically homogenous, indicating that members of the complex were closely related. This supported the suggestion that they should be considered as subspecies of a single species. Pulsed-field gel electrophoresis (PFGE) was then used to differentiate these isolates, as well as 59 additional isolates of *M. bovis* from different parts of the world. PFGE differentiated these strains into 63 patterns (53 patterns for *M. bovis*). Isolates of *M. bovis* from Western Australia ($n = 46$) were more homogenous than isolates from other regions. Eight strains were identified in that state, and one predominantly bovine strain was isolated from two human beings and a feral pig. Although *M. bovis* isolates from different parts of the world had distinct DNA patterns, some were very similar. PFGE is a highly discriminatory technique for epidemiological studies of bovine tuberculosis. For example, it allowed differentiation between isolates of *M. bovis* cultured from animals in separate outbreaks of tuberculosis, it suggested the transmission of infection between certain properties, and it demonstrated the existence of multiple infections with different strains at certain farms.

The *Mycobacterium tuberculosis* complex is composed of four species, *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* (16). *M. tuberculosis* infects over one-third of the world's population, and it is estimated that approximately 88 million people will die of this infection by the end of the century (9). *M. africanum* causes an infection similar to that of *M. tuberculosis* in some parts of Africa (33), and *M. bovis* and *M. microti* cause tuberculosis in domesticated and wild animals (28). Bovine tuberculosis caused by *M. bovis* is widespread in many countries and leads to significant economic losses. *M. bovis* also infects feral animals, such as the possum in New Zealand and the badger in the United Kingdom, providing a reservoir for the organism and making it difficult to eradicate this disease from cattle in these countries (2, 19).

In recent years molecular techniques for typing strains of the *M. tuberculosis* complex have been developed to assist with investigating the epidemiology of these organisms (3–5, 8, 29). Restriction endonuclease analysis of mycobacterial DNA produces a large number of DNA bands, and after electrophoresis, the gel is difficult to read and interpret, especially when two or three restriction enzymes have been used (34). However, this technique has been used successfully for typing isolates from different animal species, including possums, badgers, cats, and cattle, and has provided valuable epidemiological information (4–6). Restriction fragment length polymorphism (RFLP) has also been used to type isolates of *M. tuberculosis* and *M. bovis*; however, it requires the use of specific DNA probes and labelling kits (8, 29). An alternative is to cut the whole chromosomal DNA by infrequently cutting restriction enzymes and to separate the fragments by pulsed-field gel electrophoresis (PFGE). PFGE has been applied to type members of the *M. tuberculosis* complex, as well as *M. bovis* BCG (15, 22). Although multilocus enzyme electrophoresis (MEE) has been used for genetic analysis of different species of bacteria and is a powerful tool for studying the population genetics of bacteria (24), it has not previously been applied to members of the *M. tuberculosis* complex.

In this study we used both MEE and PFGE to examine the genetic relatedness of a large number of isolates of *M. bovis*, mainly recovered in Australia, and compared these with a small number of strains of different members of the *M. tuberculosis* complex. PFGE was also used to examine the transmission of *M. bovis* during outbreaks of disease.

MATERIALS AND METHODS

Bacterial isolates. Isolates of *M. bovis* cultured from animals ($n = 108$) and human beings ($n = 13$) and 14 strains of other members of the *M. tuberculosis* complex were examined (Table 1). Isolates of *M. bovis* were cultured from different host species, including cattle, pigs, deer, seals, badgers, possums, and human beings. These isolates originated from different countries, including Australia ($n = 69$), Canada ($n = 28$), Republic of Ireland ($n = 13$), Iran ($n = 6$), New Zealand ($n = 1$), and Papua New Guinea ($n = 1$). Two reference strains of *M. bovis* of uncertain origin were also examined.

The 69 Australian isolates of *M. bovis* consisted of isolates cultured from cases of bovine tuberculosis at 23 farms (A to X [see Table 2]) in Western Australia (WA) ($n = 46$) and 1 farm in Victoria ($n = 2$), isolates from seals in WA ($n = 5$), isolates cultured from feral pigs in the Northern Territory (NT) ($n = 6$), and human isolates ($n = 10$). The latter included eight isolates from patients born outside Australia (six in the United Kingdom, one in Singapore, and one in Italy). The other two isolates were from patients born in Australia. All the Australian isolates, single isolates from New Zealand and Papua New Guinea, and the two reference strains were obtained from the Australian Reference Laboratory for Bovine Tuberculosis in WA.

The Canadian isolates ($n = 28$) were cultured from various animals, including bison, cattle, cougars, deer, eland, and elk, in four outbreaks of tuberculosis on 12 premises in different provinces of Canada (24). These isolates were provided by Elizabeth Rohonczy of the Animal Disease Research Institute, Nepean,

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TABLE 1. Isolates of the *M. tuberculosis* complex used in PFGE analysis

Species	Origin	No. of isolates	No. of PFGE types[a]
M. bovis	Australia	69	27
	Canada	28	7
	Ireland	13	10
	Iran	6	5
	Other[b]	4	4
M. tuberculosis	Australia	8	6
M. tuberculosis H37Rv		1	1
M. bovis BCG		1	1
M. microti	United Kingdom	4	1
M. africanum TMC 12		1	1
Total		135	63

[a] Includes polymorphisms obtained with *Dra*I, *Xba*I, *Vsp*I, and *Spe*I.
[b] Includes two reference strains and single isolates from New Zealand and Papua New Guinea.

Ontario, Canada. Thirteen isolates of *M. bovis* cultured from cattle ($n = 8$), humans ($n = 2$), and badgers ($n = 3$) in seven different counties in the Republic of Ireland were supplied by Louis O'Reilly, Veterinary Research Laboratory, Dublin, Ireland. The Iranian isolates included one from a human being and five cultured from cattle in the outer suburbs of Tehran. These were provided by H. Hedayati of the Razi Research Institute, Tehran, Iran.

Other strains of the *M. tuberculosis* complex examined included isolates of *M. tuberculosis* cultured from humans in WA ($n = 8$), reference strains of *M. microti* ($n = 4$), *M. tuberculosis* reference strain H37Rv, *M. africanum* reference strain TMC 12, and *M. bovis* BCG, all received through the Australian Reference Laboratory for Bovine Tuberculosis.

Bacterial culture and enzyme extraction. The isolates used in the MEE study were grown and identified by standard bacteriological procedures (32). Growth from a fresh subculture of each isolate was inoculated onto 10 slopes of an agar-based solid medium, E8, supplemented with egg yolk extract and activated charcoal, prepared as described previously (8). Cultures were incubated for 2 weeks at 37°C, and cells were harvested by centrifugation at 2,500 × *g* at room temperature for 20 min, washed twice in phosphate-buffered saline (PBS), and then stored overnight at −20°C. Cells were disrupted in 1.5 ml of sonication buffer (10 mM Tris, 1 mM EDTA, 0.5 mM NADP [pH 6.8]) containing 500 mg of glass beads (catalog no. G-4649; Sigma, St. Louis, Mo.) in an ice bath for four 1-min cycles with a 50-W sonic probe (Lab Sonic 1510). They were centrifuged at 13,000 × *g* and 4°C for 20 min, and the supernatant was dispensed into 100-μl aliquots and stored at −70°C until used. All steps for growth, harvesting, and sonication of cells were carried out inside a class II biological safety cabinet in a containment area within a specialized mycobacterial reference laboratory.

Electrophoresis and running conditions for MEE. Supernatants were electrophoresed on horizontal 11.4% starch gels, and the electrophoretic mobilities of the following 26 enzymes were determined by staining for specific enzyme activity as recommended by Selander et al. (24), except for peroxidase (PER) and catalase (CAT), which were assayed according to the method of Lygren et al. (18): aconitase, adenylate kinase, arginine phosphokinase, creatine phosphokinase, CAT, three esterases (EST 1, EST 2, and EST 3), isocitrate dehydrogenase, fructose 1-6 diphosphate dehydrogenase, fumarase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, leucyl-glycyl-glycine peptidase, leucyl-proline peptidase, leucyl-tyrosine peptidases (LT1 and LT2), leucine aminopeptidase, PER, maleate dehydrogenase, mannose phosphate isomerase (MPI), nucleoside phosphorylase, phosphoglucose isomerase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, and superoxide dismutase. Four different buffer systems, formulated according to Selander et al. (24), were used for electrophoresis as follows: buffer A for aconitase, CAT, phosphoglucose isomerase, phosphoglucomutase, MPI, nucleoside phosphorylase, isocitrate dehydrogenase, maleate dehydrogenase, leucine aminopeptidase, fumarase, glutamate dehydrogenase, PER, and superoxide dismutase; buffer B for glucose-6-phosphate dehydrogenase and 6-phosphoglyconate dehydrogenase; buffer D for adenylate kinase, arginine phosphokinase, creatine phosphokinase, fructose 1-6 diphosphate dehydrogenase, and EST 1, 2, and 3; and buffer G for leucyl-glycyl-glycine peptidase, leucyl-proline peptidase, LT1, and LT2.

PFGE. Cells for PFGE analysis were grown under the same conditions described above for MEE. Cells were harvested by centrifugation at 2,500 × *g* for 20 min at room temperature in PBS, and pellets were frozen overnight. Cells were suspended in 1 ml of PBS and killed by heating at 80°C for 30 min. They were washed twice in 50 mM EDTA, and DNA was extracted by the methods of Lévy-Frébault et al. (17) with modifications. Briefly, 30 μl of cells was suspended in 200 μl of prelysing solution (6 ml of 50 mM EDTA, 6 ml of 10 mM Tris, 0.1 M sodium citrate, 150 μl of β-mercaptoethanol, 2 mg of lyticase [Sigma]), and the cell suspension was mixed with an equal volume of 1% low-melting-point agarose (Bio-Rad Laboratories, Hercules, Calif.) prepared in 125 mM EDTA (pH 8) and cooled at 50°C. This mixture was poured into plug molds. Agarose plugs were kept at 4°C for 30 min, submerged in a 2-ml solution containing 0.5 M EDTA and 7.5% β-mercaptoethanol, and incubated for 24 h at 37°C in a water bath. Agarose plugs were then washed four times with TE buffer (10 mM Tris, 1 mM EDTA [pH 8]) for 10 min each and incubated for 5 h in 3 ml of TE buffer containing 1 mg of lysozyme per ml. The solution was changed to 0.5 M EDTA containing 2 mg of proteinase K (Boehringer GmbH) per ml and 1% sodium lauryl sarcosine (Sigma) and incubated at 55°C for 48 h. Then plugs were washed three times with TE for 30 min each at room temperature and incubated at 55°C in TE plus 0.04 mg of phenylmethylsulfonyl fluoride (Sigma) per ml to inactivate proteinase K. They were washed three times with TE, stored in 0.5 M EDTA at 4°C, and then washed extensively with TE before being subjected to restriction enzyme digestion.

Restriction enzyme digestion. Agarose plugs were cut with a scalpel to fit the size of the combs of the gel casting (4 by 3 mm). They were washed in restriction buffer at 4°C for 30 min and digested with 25 U of either *Dra*I, *Xba*I, *Spe*I (Boehringer GmbH), or *Vsp*I (Promega) in the appropriate restriction buffer recommended by the suppliers plus 2.5 μl of bovine serum albumin (10.27 mg/ml; Pharmacia) for 24 h.

PFGE. Plugs containing digested DNA were loaded onto a 1% agarose gel, prepared, and run in 0.5× TBE buffer (1× TBE contains 0.025 M Tris, 0.5 mM EDTA, and 0.025 M boric acid). PFGE was carried out with a contour-clamped homogenous electric field-DR II system (Bio-Rad) at 14°C for 24 h at 180 V and 20 A. After *Dra*I digestion, the pulse time was ramped from 5 to 15 s for 16 h and then from 60 to 70 s for 8 h. After *Xba*I and *Spe*I digestion, the pulse time was ramped from 5 to 15 s for 16 h and then from 1 to 20 s for 8 h. Gels were stained with 0.5 μg of ethidium bromide per ml for 30 min and photographed with Polaroid film. Bacteriophage lambda or *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad) was used as markers. The photographs of the *Dra*I digests were scanned with a OneScanner apparatus (Apple, Inc., Cupertine, Calif.), and the patterns were analyzed by using a gel manager program (BioSystematica, Pod Rovinou, Czech Republic), which created a dendrogram from the matrix of coefficients by the unweighted-pair group method of arithmetic (UPGMA) average clustering fusion strategy.

Analysis. The genetic diversity for each enzyme locus examined by MEE was calculated from the formula $h = 1 - \Sigma P_i^2[n/(n\text{-}1)]$, where h is genetic diversity, P_i is the frequency of the ith allele, and n is the number of electrophoretic types (ETs) or isolates in the sample (21). Genetic distances between ETs were calculated as the proportion of fixed loci at which dissimilar alleles occurred, and the UPGMA clustering fusion strategy was used to create a phenogram to show the relationships between isolates (1).

RESULTS

MEE. The 76 isolates of the *M. tuberculosis* complex were divided into five ETs, and their genetic relatedness is illustrated in Fig. 1. These ETs had different alleles for only one (MPI) or two (CAT and PER) enzymes. ET 1 contained one human isolate of *M. bovis* from Australia and *M. bovis* BCG and differed from the other ETs at the locus for the enzyme CAT. ET 2 consisted of 60 isolates of *M. bovis*, 2 isolates of *M. tuberculosis*, and the *M. africanum* strain. ET 3 contained five isolates of *M. tuberculosis*, including the reference strain H37Rv, and these differed from the isolates in the other ETs in their alleles for the enzymes CAT and PER. ET 4 contained

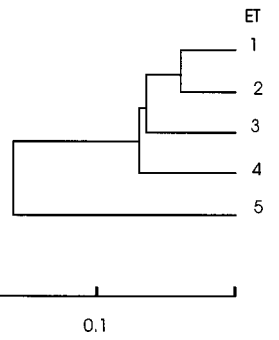

FIG. 1. Genetic variation among strains of the *M. tuberculosis* complex. The scale shows the genetic distance (expressed as percent fixed allelic differences) among five ETs, clustered by the UPGMA strategy.

two human isolates of *M. bovis* cultured from patients in the United Kingdom and differed from ETs 2 and 3 for the enzyme MPI. Finally, ET 5 contained all four strains of *M. microti* and was different from other ETs for the enzyme peptidase.

PFGE. Of the four enzymes used, *Dra*I digestion yielded the fewest DNA fragments (n = 17). These were large, ranging from 50 to 700 kbp, and therefore suitable to scan for computer analysis (see Fig. 3 and 4). *Spe*I, *Vsp*I, and *Xba*I yielded smaller fragments, and they were used only to confirm the results obtained with *Dra*I. By using *Dra*I, 38 patterns (B1 to B38) were found for these 135 isolates of the *M. tuberculosis* complex. Of these patterns, 28 belonged to 122 isolates of *M. bovis* (see Fig. 3 and 4). Seven major patterns (B8, B13, B14, B22, B26, B27, and B31), each containing six or more isolates of *M. bovis*, were obtained with *Dra*I. The total number of patterns for each group of isolates, including those obtained with other endonucleases, is shown in Table 1. Except for pattern B31, which consisted of several isolates from four countries, isolates from each country produced a distinct *Dra*I pattern. However, isolates with pattern B31 were differentiated by *Spe*I according to the country of origin. Each of the other members of the *M. tuberculosis* complex had its own *Dra*I pattern.

Genetic diversity. The dendrogram produced by comparing bands from digests of *Dra*I is shown in Fig. 2, and information about each group is presented in Table 2. Percentages of dissimilarity were used to indicate genetic distances between types. At a genetic distance of 0.4, five groups (A to E) were obtained. Group A contained patterns B1 through B7, including an isolate recovered from a feral pig in the NT (B1), three *M. tuberculosis* isolates from WA (B2, B3, and B7), and five isolates from seals in WA (B4 and B6). Group B contained patterns B8 and B9, made up of 12 isolates of *M. bovis* from Canada (B8), and 1 from New Zealand (B9). Group C was the largest, comprising patterns B10 through B34. It contained most of the *M. bovis* isolates (n = 101), including all 13 recovered from humans, together with 3 isolates of *M. tuberculosis* (patterns B11, B12, and B32), *M. bovis* BCG (Bio), and the *M. africanum* strain (B34). A single bovine isolate from WA (B35) made up group D. Group E contained three patterns. All four isolates of *M. microti* had pattern B36, two isolates of *M. tuberculosis* had pattern B37, and the reference strain of *M. tuberculosis*, H37Rv, had pattern B38. All *M. bovis* isolates were distinct from other members of the complex and were divided into 24 groups at a genetic distance of 0.04. They were included in all groups except for group E.

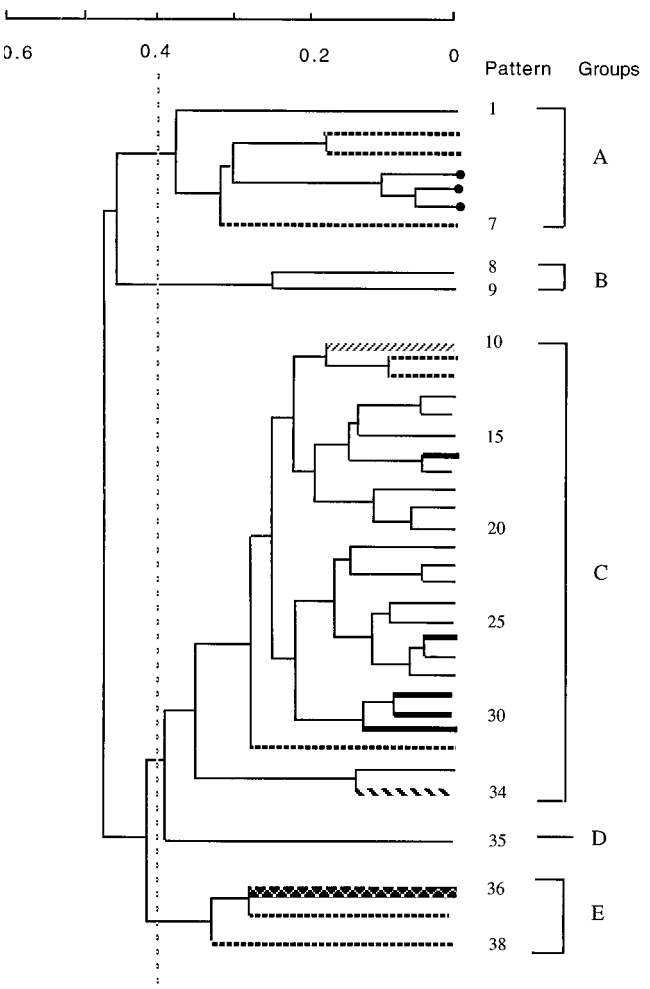

FIG. 2. Percentages of similarity among five groups of isolates belonging to the *M. tuberculosis* complex, obtained by cutting the whole chromosomal DNA with *Dra*I and resolving the fragments by PFGE. The scale shows the percentage of dissimilarity of DNA fragments on the gel, calculated by the UPGMA strategy using the gel manager software program (BioSystematica). Thirty-eight patterns (B1 to B38) are shown. Patterns belonging to each species or its host are demonstrated as follows: ▪▪▪▪▪▪, *M. tuberculosis*; ——, *M. bovis* (animal); ●, *M. bovis* (seal isolates); ////////, *M. bovis* BCG; ▬▬, *M. bovis* (human); ▚▚▚, *M. africanum*; ▩▩▩, *M. microti*.

Distribution of *M. bovis* strains from WA. *Dra*I differentiated 46 *M. bovis* isolates from 23 properties in WA into eight groups (Fig. 3, lanes a to c, e, f, and h to k). Three major patterns (B22, B26, and B27) were demonstrated by more than eight isolates. Pattern B22 was shown by 10 isolates from cattle at four properties in northern WA, and these isolates had identical patterns with other endonucleases. Isolates with pattern B26 were further differentiated by *Spe*I, resulting in six more types being obtained. *M. bovis* isolates from cattle (n = 21), human isolates of *M. bovis* (n = 2), and a single isolate cultured from a feral pig in the NT had type 1 of pattern B26 (Fig. 4, lanes d and j). These 24 isolates were isolated from properties widely dispersed throughout WA and had identical patterns with other endonucleases. The remaining five *Spe*I types of pattern B26 belonged to isolates cultured from feral pigs (n = 4) and a single isolate cultured from a cow in northern WA. Isolates from WA were distinct from two isolates cultured from cattle in Victoria (Fig. 3).

Infection with multiple strains. By using *Dra*I, infection with more than one strain of *M. bovis* was detected among cattle on three properties in WA (properties A, L, and F) (Table 2). *Dra*I and *Spe*I also demonstrated that feral pigs killed on two properties (X and Y) were infected with different strains (B1 and B26). Similarly, three patterns were found among animal isolates on property number one in Canada.

Diversity among human isolates of *M. bovis*. Isolates of *M. bovis* cultured from Australian patients who were born in other countries (n = 8) were clustered into five patterns by *Dra*I. Four of these grouped in B31, and the rest, all single isolates, were grouped in B18, B29, B30, and B33. All were distinct from bovine strains in Australia.

An isolate of *M. bovis* cultured from a patient from Iran was identical to *M. bovis* BCG by using *Dra*I, but it was distinct by *Xba*I. Similarly, isolates cultured from patients in the Republic of Ireland could be differentiated from isolates of animal origin (Table 2).

DNA patterns among seal isolates. Five isolates recovered

TABLE 2. Results of PFGE and MEE typing of 135 isolates of *M. bovis* and other member of the *M. tuberculosis* complex

Isolate (*n*)	PFGE Group				ET in MEE	Origin[a]
	*Dra*I	*Spe*I	*Vsp*I	*Xba*I		
PMC 221 (1)	B1				2	Feral pig-NT (Y)
19576 (1)	B2				3	*M. tuberculosis*-WA
19893 (1)	B3				1	*M. tuberculosis*-WA
895 (2)	B4	1	1	1	2	Seal-WA
14109 (1)	B4	2	1	1		Seal-WA
14126 (1)	B5				2	Seal-WA
92/1161 (1)	B6					Seal-WA
17683 (1)	B7				2	*M. tuberculosis*-WA
89/203 (9)	B8	1		X 8-1		Canada-outbreak C (V1, V2, S, W, Y)
91/440 (3)	B8	2		X 8-2		Canada-outbreak D (D)
PMC 258 (1)	B9				2	Possum-New Zealand
841 (1)	B10			X 10-1		BCG
IR/1 (1)	B10			X 10-2		Human-Iran
19571 (1)	B11				3	*M. tuberculosis*-WA
17681 (1)	B12				3	*M. tuberculosis*-WA
90/155 (5)	B13			X 13-1		Canada-outbreak B (E, I)
90/345 (1)	B13			X 13-2		Canada-outbreak B (F)
90/307 (7)	B14	1		X 14-1		Canada-outbreak A (1, 2, 4)
93/122 (1)	B14			X 14-2		Canada-outbreak A (1)
19042 (3)	B15	1				Bovine-Ireland
19046 (2)	B16	1	B 21-1	X 21-1		Bovine-Ireland
19048 (1)	B16	2	B 21-2	X 21-2		Bovine-Ireland
19050 (1)	B16	3	B 21-1			Bovine-Ireland
19052 (1)	B16	4	B 21-2			Human-Ireland
12675 (1)	B17				2	Bovine-WA (A)
1126 (1)	B18					Human-Australia
IR/13 (1)	B19					Bovine-Iran
29476 (1)	B20				2	*M. bovis* reference strain
93/080 (2)	B21			X 21-1		Canada-outbreak A
17666 (10)	B22	1			2	Bovine-WA (B, C, D, E)
13816 (1)	B23				2	Bovine-WA (F)
12985 (1)	B24				2	Bovine-WA (G)
136/92V (2)	B25				2	Bovine-Victoria
15416 (21)	B26	1			2	Bovine-WA (F, H, I, J, K, L)
PMC 217 (1)	B26	1			2	Feral pig-NT (X)
6/99A (2)	B26	1			2	Human-WA
PMC 218 (1)	B26	2			2	Feral pig-NT (X)
PMC 222 (1)	B26	3				Feral pig-NT (Y)
17379 (1)	B26	4				Bovine-WA (R)
PMC 219 (1)	B26	5			2	Feral pig-NT
1220 (1)	B26	6				Feral pig-NT (X)
12563 (8)	B27				2	Bovine-WA (A, L, S, T, U)
74/767 (2)	B28				2	Bovine-WA (V)
1134 (1)	B29				4	Human-Australia
1127 (1)	B30				2	Human-Australia
NGI (1)	B31	1	B 15-1			Buffalo-New Guinea
IR/25 (1)	B31	2	B 15-1			Bovine-Iran
IR/9 (1)	B31	3	B 15-1			Bovine-Iran
1131 (1)	B31	4	B 15-1		2	Human-Australia
1128 (1)	B31	5	B 15-1		1	Human-Australia
1129 (1)	B31	6	B 15-1		4	Human-Australia
AN5 (1)	B31	7	B 15-1		2	*M. bovis* reference strain
1125 (1)	B31	8	B 15-1		2	Human-Australia
19043 (2)	B31	9	B 15-1			Badger-Ireland
IR/10 (2)	B31	10	B 15-1			Bovine-Iran
19045 (1)	B31	11	B 15-2	X 15-2		Bovine-Ireland
19051 (1)	B31	12	B 15-3			Human-Ireland
19040 (1)	B31	13				Badger-Ireland
84/768 (1)	B32				2	*M. tuberculosis*-WA
1133 (1)	B33					Human-Australia
TMC 12 (1)	B34				2	*M. africanum*
15145 (1)	B35				2	Bovine-WA (W)
F 63 (4)	B36				5	*M. microti*
19578 (2)	B37	1			3	*M. tuberculosis*-WA
H37RV (1)	B38				3	*M. tuberculosis* H37Rv

[a] Letters in parentheses for Australian isolates indicate the different properties in WA (A to X) and the NT (Y) from which isolates were cultured. All isolates are *M. bovis*, unless indicated otherwise. Canadian outbreaks occurred in Quebec (outbreak A), Alberta (outbreak B), Ontario (outbreak C), and Manitoba (outbreak D) (23). Letters and/or numbers in parentheses for Canadian isolates indicate the different properties from which isolates were cultured.

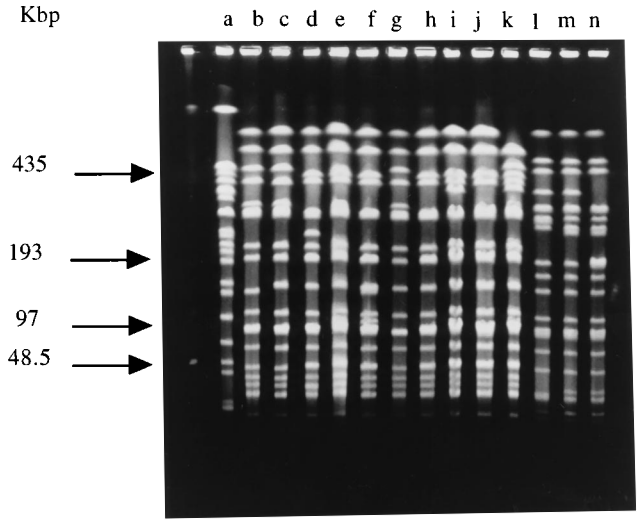

FIG. 3. DNA polymorphisms among Australian isolates of *M. bovis*. Lanes a to c and e to k, bovine isolates from WA; lane d, isolate from Victoria; lane h, the pattern of B26, the predominant type in WA; lanes l to n, seal isolates.

from Australian seals were divided into four patterns (Fig. 3, lanes l to n). These isolates were distinct from bovine isolates by all the restriction enzymes used.

Canadian isolates. *Dra*I was used to differentiate Canadian *M. bovis* isolates into four major patterns, B8 ($n = 11$), B13 ($n = 6$), B14 ($n = 9$), and B21 ($n = 2$). Isolates of each pattern consisted of isolates which had been cultured during four outbreaks of tuberculosis in different animals. These isolates were further subjected to *Xba*I digestion, resulting in seven patterns being obtained.

Irish isolates. By using *Dra*I, 13 isolates of *M. bovis* cultured from different host species in the Republic of Ireland were differentiated into three patterns (B15, B16, and B31). Analyses with other enzymes resulted in 10 different subtypes being recognized. Two isolates cultured from badgers in different counties, with pattern B15, showed polymorphism by *Spe*I and were distinct from bovine and human isolates. Similarly, three isolates with pattern B16 from cattle were differentiated from a human isolate by *Spe*I and *Vsp*I.

PFGE of other subspecies of the *M. tuberculosis* complex. Pattern B36 was demonstrated by four strains of *M. microti*, which were identical by *Dra*I and *Spe*I digestion (Fig. 4, lane e). Eight isolates of *M. tuberculosis* cultured from humans in WA were differentiated into seven types by *Dra*I and were clustered in groups A, C, and E (B2, B3, B7, B11, B12, B32, and B37). Each subspecies of the *M. tuberculosis* complex had distinct PFGE patterns.

DISCUSSION

MEE is a useful technique for studying the population genetics and taxonomy of bacteria (20). In this study, the 26 enzymes used to analyze members of the *M. tuberculosis* complex were shown to be highly monomorphic, although some polymorphisms were found among certain isolates. The high levels of similarity among *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti* have prompted some authors to classify them as different biovars of *M. tuberculosis* (26). This has been supported by their high degrees of homology in DNA sequence homology studies (3, 13).

In MEE, the DNA sequences of genes which encode metabolic enzymes are compared indirectly. These genes are distributed randomly throughout the genome; therefore, more nucleotides than those in the area of 16S or 16S-23S internal transcribed spacer genes coding for rRNA used for sequencing (24) are considered. Few differences were found in the genomes of these organisms, which were epidemiologically unrelated, showing that isolates of this complex represent a clonal population (25). Despite this clonality, changes in the genomes of certain strains, such as *M. bovis* BCG, were detected by MEE (Fig. 1 [ETs 1 and 2]). The MEE results obtained in the current study support contentions that members of this complex are closely related genetically, form a clonal population, and probably should be considered members of a single species.

The genetic relationships among isolates of *M. bovis* originating from different sources were investigated further by PFGE. In the case of *M. bovis*, PFGE analysis could be complicated by the presence of multiple copies of insertion sequence IS*6110*. Fortunately, *M. bovis* strains contain lower copy numbers of IS*6110* than do *M. tuberculosis* strains (30). Since there is only one restriction site for *Dra*I on IS*6110* (27), there should be fewer polymorphisms obtained with this restriction enzyme for populations of *M. bovis* than for populations of *M. tuberculosis*. Recently, IS*6110* has been used as a probe in RFLP analysis of *M. tuberculosis* (10), and the results were compared with PFGE results (12). Both methods showed high degrees of polymorphism between isolates originating from the same geographical area, with 96% agreement between the two techniques. This supports the validity of using PFGE and computer analysis of banding patterns for *M. bovis* strains, as it is analogous to published studies examining and analyzing RFLP patterns of *M. tuberculosis* strains (31).

Australian isolates of *M. bovis* have previously been analyzed by using the PGRS probe in RFLP (8), and some of these strains ($n = 32$) were also included in this study. A total of 24 patterns were obtained with the PGRS probe, while PFGE with *Dra*I and *Spe*I detected 28 patterns. The latter enzyme detected polymorphisms between isolates which were identical with the PGRS probe. These results again support the use of PFGE for strain identification in the current study.

By using *Dra*I, it was demonstrated that cattle populations at different properties ($n = 23$) in WA were infected with eight

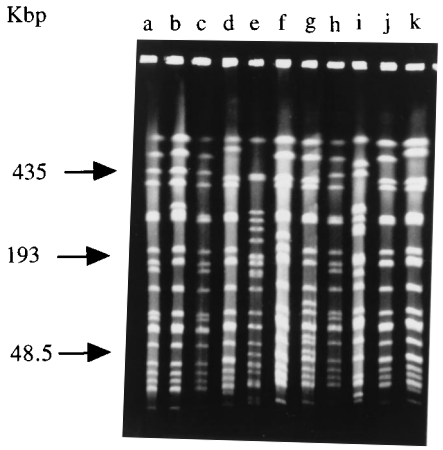

FIG. 4. DNA polymorphisms among isolates of *M. bovis* from different origins, obtained by digestion with *Dra*I. Lanes: a, c, and h, animal isolates from Canada; b, human isolates from Australia (B31); d and k, bovine isolates from properties B to D in WA (B22); e, *M. microti*; f, isolate from Victoria (Australia); g, Irish isolate; i, seal isolate from WA; j, dominant pattern in WA (B26).

different strains of *M. bovis*. Four of these strains were represented by more than one isolate. This suggests that at the time of sampling, there were relatively few sources of infection for cattle in WA.

One major group (B26) ($n = 29$) included isolates from different host species, including cattle from WA ($n = 22$), feral pigs in the NT ($n = 5$), and humans (two isolates from patients who were born in WA). The human isolates and one from a feral pig were also identical to bovine isolates when further analyzed by *Spe*I. This suggests the possibility of cross-species transmission. The other isolates from feral pigs with pattern B26 ($n = 4$) were differentiated by *Spe*I, but all were closely related. Mixed infections in feral pigs at two properties were also detected. The majority of isolates of *M. bovis* cultured from Australian patients who had been born in the United Kingdom ($n = 4$) were identical to Irish strains (B31) and were distinct from WA isolates. This suggests that these patients had been infected with *M. bovis* before they settled in Australia and had probably introduced new strains. There was no evidence that these strains had been transmitted to cattle.

Tuberculosis in seals has been reported in Australia and South America (7, 29). By PFGE, seal isolates from Australia were distinct from bovine strains and were most closely related to *M. tuberculosis* isolates in group A. Furthermore, there was heterogeneity among seal strains, suggesting that infection occurred from a variety of sources other than cattle.

B31 was the only pattern demonstrated by isolates from different countries (Australia, Ireland, Papua New Guinea, and Iran). The majority of isolates in this group were also shown to be identical by *Xba*I, but those originating from different countries were differentiated by *Spe*I. This finding suggests that these isolates originated from a common ancestor.

Isolates from different areas in the Republic of Ireland had different *Spe*I patterns. Similar heterogeneity was reported when restriction endonuclease analysis was used on a different set of Irish isolates (5). None of the animal isolates from Ireland were identical to isolates from humans. However, because of the high degree of heterogeneity among Irish isolates, more isolates need to be examined to determine possible transmission between animal species.

Deer farming has been growing in popularity in Canada, with the importation of breeding animals from the United States, New Zealand, and the United Kingdom (14), and outbreaks of tuberculosis in members of the family Cervidae have become important after eradication of this disease in cattle. Tuberculosis in elk (*Cervus elaphus*) has been reported to be caused by the importation of infected herds from the United States (11). The use of *Dra*I and *Xba*I digestion allowed the differentiation of isolates cultured from four separate outbreaks of tuberculosis in deer and other animals originating from different farms in four provinces of Canada. Isolates from each province yielded different DNA patterns; however, isolates from Ontario and Manitoba were identical by *Dra*I but were differentiated when they were subjected to *Xba*I digestion. In outbreak A, property 1 in Quebec was found to be the source of infection for other properties in that area. Isolates cultured from different animal species at this property were differentiated into three patterns. By using *Xba*I, the infection of cattle with more than one strain of *M. bovis* was detected. In outbreak B in Alberta, four isolates from different tissues of an infected elk at premise E, which had been imported from Montana, were found to be identical by PFGE. This property had sold two elk (both of which were later found to be tuberculous) to properties F and 1. However, by using *Xba*I it was demonstrated that property F was also infected with another strain, which was probably present before importing the elk from Montana. All isolates in outbreak C, cultured from different animal species at properties Y, V, and S in Ontario (23), were identical. The first property, which had purchased a large collection of exotic species from all over North America, sold animals to the other properties and therefore possibly was the point source of infection in this outbreak. Even after the depopulation of property V (V2) and application of quarantine and disinfection requirements, the same type of *M. bovis* (X8-1) was cultured after repopulation with imported deer from New Zealand. In outbreak D, isolates cultured from a wild elk were found to be identical to bovine isolates from that area, supporting the possibility of transmission of infection between these species.

PFGE demonstrated considerable strain variation among isolates of *M. bovis*, but variation among isolates of *M. tuberculosis*, cultured from humans in WA, was greater than that for *M. bovis*. This suggests that the infection of Australians with *M. tuberculosis* is likely to have arisen from a greater number of sources than has the infection of cattle with *M. bovis*.

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