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Geographic distribution of restriction types of *Mycobacterium bovis* isolates from brush-tailed possums (*Trichosurus vulpecula*) in New Zealand

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SUMMARY

DNA restriction endonuclease analysis was used for intra-specific typing of *Mycobacterium bovis* isolates from 83 brush-tailed possums (*Trichosurus vulpecula*) obtained between 1982 and 1984 from the three major regions in New Zealand with endemic bovine tuberculosis. All the isolates were found to be genetically very similar. Differentiation of the isolates into 33 restriction types was achieved by using high-resolution electrophoresis and the combined results from separate digestions with the restriction enzymes *Bst* EII, *Pvu* II and *Bcl* I. The typing system was entirely reproducible. Isolates of the same type were usually found in adjacent localities and were always limited to one of the three major regions. In some cases, isolates of the same type were found in both 1982 and 1984. The phenotypic significance of the small genetic differences identified between different isolates is unknown. The typing system will be useful for monitoring the transmission of *M. bovis* to other species and the future spread of different *M. bovis* types through possum populations.

INTRODUCTION

Feral brush-tailed possums (*Trichosurus vulpecula*) were introduced into New Zealand from Australia on many separate occasions from 1840 until 1920 (Pracy, 1974). The animals thrived in their new habitat and are now common throughout most parts of the country. The first report of naturally acquired *Mycobacterium bovis* infection in these animals was in 1970 (Eckdahl, Smith & Money, 1970) and since then many infected possums have been discovered (Julian, 1981). In contrast, *M. bovis* infection has never been reported in feral possums in Australia (Corner & Presidente, 1981). It is generally assumed that the disease in possums in New Zealand was originally acquired here from tuberculous cattle, that possums are a maintenance host for *M. bovis*, and that transmission to cattle occurs from infected possums entering grazing land. The situation has some similarity to that with badgers (*Meles meles*) in South-West England (Julian, 1981; Collins & Grange, 1983). In both countries the nature of the disease and the limited techniques available for its study have hampered detailed epidemiological investigations. The lack of an adequate intra-specific typing system for *M. bovis* has been one limiting factor.

Table 1. *Pattern designation and restriction type for 83 isolates of* Mycobacterium bovis *from possums*

Restriction type	No. of isolates	Pattern designation		
		Bst EII	*Pvu* II	*Bcl* I
1	1	A	A′	A″
2	6	B	B′	B″
3	6	B	B′	C″
4	4	C	C′	A″
5	1	D	C′	A″
6	1	E	C′	A″
7	3	F	D′	D″
8	11	G	E′	E″
9	3	H	C′	F″
10	2	I	C′	G″
11	2	J	C′	E″
12	1	K	F′	A″
13	3	L	C′	F″
14	1	M	G′	E″
15	3	N	D′	H″
16	2	L	H′	F″
17	1	A	C′	G″
18	1	O	I′	E″
19	1	P	J′	I″
20	2	A	H′	A″
21	1	Q	C′	F″
22	1	A	D′	J″
23	5	A	C′	A″
24	1	R	K′	K″
25	1	A	K′	L″
26	4	A	K′	A″
27	1	A	L′	A″
28	8	A	M′	A″
29	2	S	M′	A″
30	1	T	E′	A″
31	1	U	M′	M″
32	1	A	M′	N″
33	1	A	N′	M″

Recently we developed a reliable system for typing isolates of *M. bovis* based on DNA restriction endonuclease analysis (Collins & de Lisle, 1985). In that study, two restriction enzymes were used to type 24 isolates from various animal species, including three isolates from possums. This system, further refined by the use of a third restriction enzyme, has now been applied to isolates of *M. bovis* obtained from possums in the three main regions of New Zealand with endemic bovine tuberculosis (Fig. 1). These are the regions in which traditional test and slaughter procedures have failed to eradicate tuberculosis in cattle and are also the regions in which nearly all tuberculous possums are found. The study was designed to investigate the geographic distribution of *M. bovis* restriction types. Possum isolates were used because these animals are known to have restricted territorial behaviour (Green & Coleman, 1981; Ward, 1984).

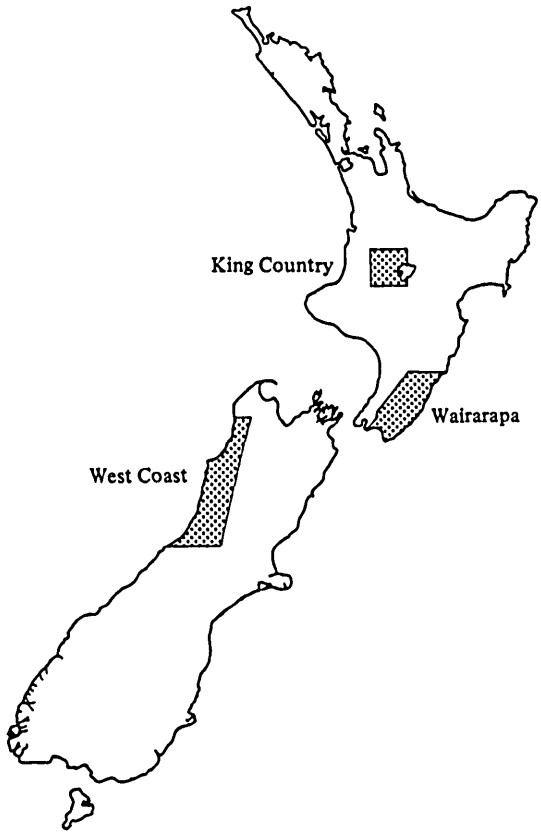

Fig. 1. Major regions of New Zealand with endemic bovine tuberculosis.

MATERIALS AND METHODS

Bacteria

The 83 isolates of *M. bovis* which were examined came from possum tissues submitted to this laboratory during 1982–4. They constituted all viable *M. bovis* isolates from possums originating from the King Country, Wairarapa and West Coast regions, and 94 % of possum isolates found throughout New Zealand during this period. Most of these isolates came from routine submissions by officers of the Ministry of Agriculture and Fisheries who were carrying out surveys of tuberculous possums. The isolates were identified as *M. bovis* by standard microbiological methods (Collins & de Lisle, 1985).

Restriction endonuclease analysis

DNA was prepared as described previously (Collins & de Lisle, 1984). DNA samples (4 μg) were digested separately with 20–40 units of the restriction enzymes *Bst* EII, *Pvu* II and *Bcl* I at the temperature and with the buffer specified by the supplier (New England Biolabs, Beverly, Mass., U.S.A.). Digests were analysed by gel electrophoresis on 330 mm long, 1 % agarose gels run for 24 h at 100 V as described previously (Collins & de Lisle, 1985).

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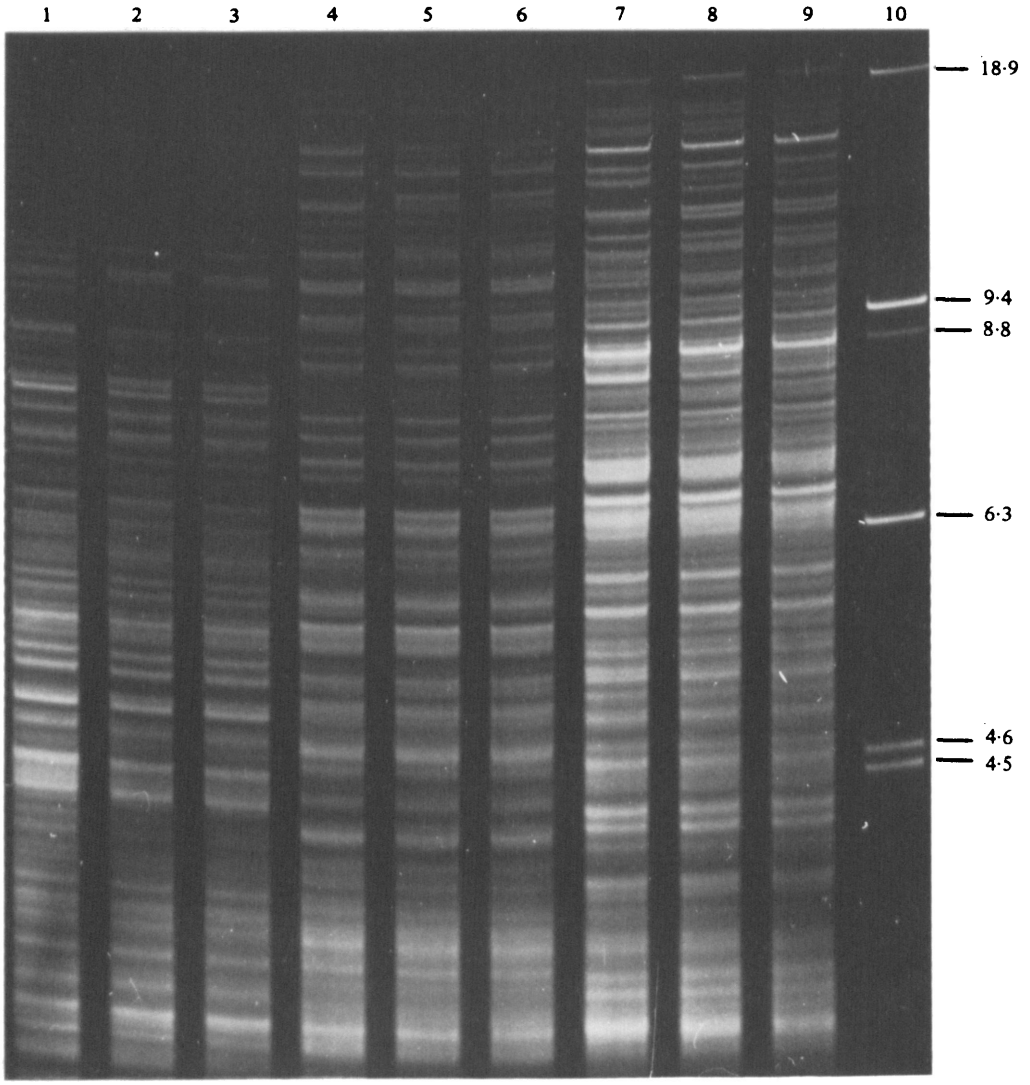

Fig. 2. Fragment patterns after digestion of DNA from isolates of types 1–3 respectively with the restriction enzymes *Bst* EII (lanes 1–3), *Pvu* II (lanes 4–6), and *Bcl* I (lanes 7–9). The sizes of the λ phage fragments (lane 10) are given in kilobase pairs.

RESULTS

Under the electrophoretic conditions employed, each of the three restriction enzymes gave DNA fragment patterns in which the larger molecular size fragments were well resolved. All of the 83 isolates had very similar patterns when their DNA was digested with the same enzyme but for each enzyme there were a large number of slightly different patterns. A total of 21 different patterns (designated A-U) for *Bst* EII, 14 (A′–N′) for *Pvu* II, and 14 (A″–N″) for *Bcl* I could be distinguished (Table 1). Depending on the enzyme and the isolate from which the DNA was extracted, the number of discrete fragment lines that could be distinguished on the original gel photographs ranged from 60 to 70 and the number of fragment differences between patterns ranged from 1 to 6. Repeat culture, DNA extraction, and

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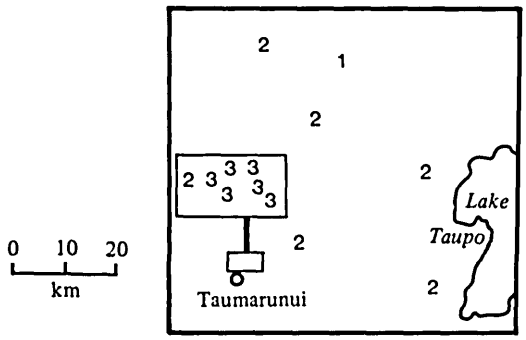

Fig. 3. Geographic distribution and restriction type of *M. bovis* isolates from possums in the King Country region.

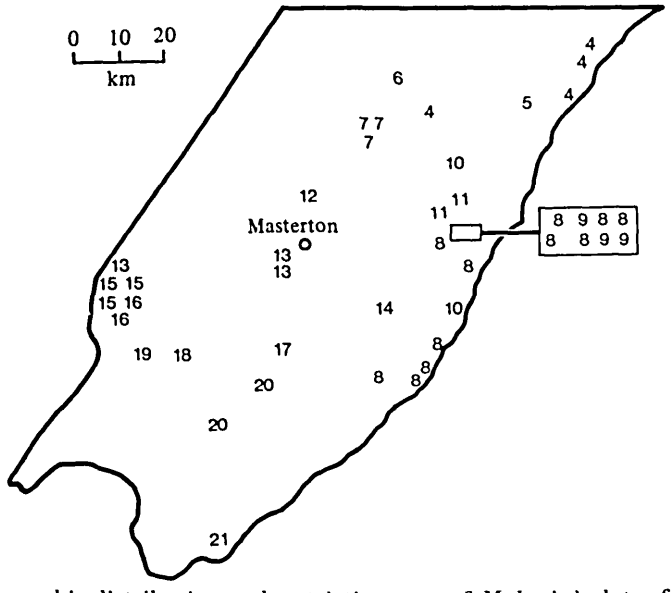

Fig. 4. Geographic distribution and restriction type of *M. bovis* isolates from possums in the Wairarapa region.

restriction enzyme digestion of a representative isolate from each of the 33 different restriction types gave identical fragment patterns to those obtained originally.

Fig. 2 shows the DNA fragment patterns produced when representative isolates of each of the three restriction types found in the King Country region were digested with the three restriction enzymes. A *Bcl* I digest of λ phage was run in parallel to indicate fragment sizes. Digests of DNA from isolates of restriction types 2 and 3 differed at only one fragment line in their *Bcl* I patterns (the fragment with the second largest molecular size) and had identical patterns when digested with the other two restriction enzymes. In contrast, DNA patterns for the isolate of restriction type 1 differed from the type 2 and 3 patterns at 4–6 fragment lines with all three enzymes.

The geographic distribution of the isolates by restriction type is shown for each region in Figs. 3–5.

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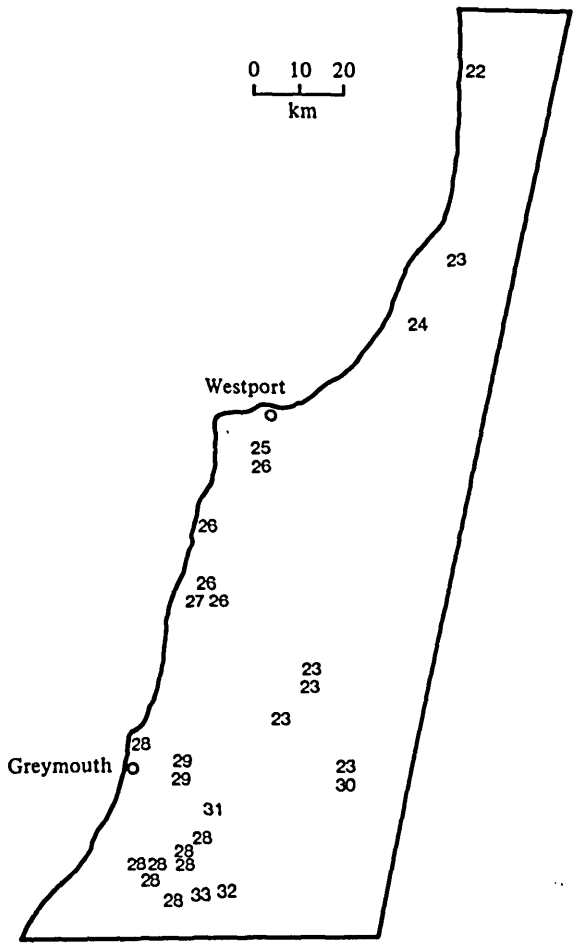

Fig. 5. Geographic distribution and restriction type of *M. bovis* isolates from possums in the West Coast region.

DISCUSSION

The close similarity between the DNA fragment patterns of the *M. bovis* isolates reflects the high degree of genetic relatedness within this species. Because of this genetic homogeneity, reliable differentiation between isolates was achieved only by using a high-resolution electrophoresis system (Collins & de Lisle, 1985). The phenotypic significance of these small genetic differences is presently unknown.

Fig. 2 is an example of the kind of electrophoretic information on which the restriction typing was based and also illustrates the range of differences that occurred between patterns of different restriction types. In a few cases, such as between restriction types 2 and 3, the only difference detected was a single fragment line on one enzyme pattern. However, even single fragment differences between patterns were entirely reproducible.

The restriction enzymes *Bst* EII and *Bcl* I were chosen after screening 25 enzymes (Collins & de Lisle, 1985) and *Pvu* II was tested concurrently with the

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present study and found to be suitable. The most discriminating of these enzymes was *Bst* EII, which revealed 21 different patterns. The addition of the enzyme *Pvu* II enabled a further eight restriction types to be distinguished, and the enzyme *Bcl* I a further four restriction types. The benefits of using three instead of two enzymes was therefore relatively minor. However, *Bcl* I did enable 12 isolates from the King Country region to be subdivided into restriction types 2 and 3, and also distinguished the single isolate of restriction type 17 in the Wairarapa region from the five isolates of restriction type 23 in the West Coast region (Table 1).

The confined geographic distribution of the different restriction types appears highly significant. In no case was a type found in more than one of the three major regions of endemic tuberculosis and in most cases where more than one isolate of a particular restriction type was found the isolates came from adjacent localities within that region. This close geographic linkage between isolates of the same restriction type in association with the limited territorial range of possums suggests that each type originally came from a single infected possum in that area. It is generally assumed that possums in New Zealand were originally infected from cattle (Julian, 1981; Lepper & Corner, 1983), although the possibility that the disease was transmitted from infected feral deer or pigs cannot be excluded. If possums were infected from cattle the transfer is assumed to have been infrequent largely because tuberculosis used to occur in cattle in many regions of New Zealand whereas the disease has only been found in possum populations in some of those regions. One explanation for the relatively large number of different restriction types found in this present study is that transmission from cattle or other species to possums occurred more frequently than previously assumed. Alternatively, after possums first became infected, the mycobacteria may have undergone sufficient genetic change for numerous different restriction types to have emerged. This latter possibility appears more likely for some of the most similar restriction types such as 2 and 3 in the King Country; 4, 5 and 6 in northern Wairarapa; and 23, 26, 27 and 28 in central Westland which were only differentiated by a single restriction enzyme.

The stability of the restriction patterns used for the typing system is clearly important. While long-term stability can be accurately assessed only after analysing isolates acquired over a much longer period than the 3 years used here, it appears that the types do not undergo rapid change. Isolates of all restriction types were stable to re-culturing in the laboratory. In addition, at least one isolate of each of the restriction types 4, 8, 13, 23, 26 and 28 was obtained in both 1982 and 1984, so these types are maintained naturally over at least that time. This degree of stability indicates that typing based on these DNA fragment patterns provides a useful epidemiological tool for studying transmission of *M. bovis* between animal species and also any future geographic spread of *M. bovis* among feral possums. A similar approach should also assist studies in other countries such as England where different feral hosts are involved in tuberculosis transmission.

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