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Identification of Two Groups of *Mycobacterium paratuberculosis* Strains by Restriction Endonuclease Analysis and DNA Hybridization

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Genomic DNA was prepared from four reference strains of *Mycobacterium paratuberculosis* and 46 isolates of this organism from New Zealand, Australia, Canada, and Norway and also from two mycobactin-dependent "wood pigeon" strains. The DNA was characterized by restriction endonuclease analysis, both with and without DNA hybridization, with a probe specific to a repetitive DNA sequence in *M. paratuberculosis*. Both techniques differentiated *M. paratuberculosis* strains into two groups, but DNA hybridization revealed more differences between strains within the larger group. All the strains from cattle and many strains from other animals belonged to this group. The second group of nine strains included the Faroe Islands strain, all New Zealand sheep strains, and one New Zealand goat strain. Primary isolation of strains belonging to this group was difficult to achieve. DNA from acid-fast organisms harvested directly from intestinal tissues of sheep with Johne's disease was shown to have restriction and hybridization patterns identical to those of DNA obtained from *M. paratuberculosis* isolates cultured from the same tissues. Two Norwegian goat strains and the wood pigeon strains did not hybridize to the *M. paratuberculosis* probe and had restriction patterns very different from those of other *M. paratuberculosis* strains. The wood pigeon strains had restriction patterns very similar to those of strains of *Mycobacterium avium*, indicating that they should be classified as that species. The presence of two distinct groups of *M. paratuberculosis* strains and their predominant distribution in different host animals may be significant in management of mixed-animal farming operations.

Mycobacterium paratuberculosis causes paratuberculosis or Johne's disease, a chronic wasting enteropathy, in ruminants. The definitive method of diagnosis is culture of the organism from intestinal tissue or fecal specimens. *M. paratuberculosis* is differentiated from other mycobacteria by its characteristics in culture, particularly its extremely slow growth and its dependence on added mycobactin for in vitro culture. These criteria and the use of biochemical tests and antibiotic sensitivities are not adequate to distinguish *M. paratuberculosis* from some *Mycobacterium avium* strains or from the so-called "wood pigeon" strains of mycobacteria (2). Studies of strains of these three groups of organisms by genomic DNA-DNA hybridization have shown that they are all very closely related (10, 18, 19, 28), and it has been suggested that they should be regarded as a single species (18). While these studies have been helpful in characterizing the organisms, their emphasis on the closeness of the relationship has tended to focus attention away from strain differences which may be epidemiologically important.

In an earlier study (4), we examined cattle strains of *M. paratuberculosis* from New Zealand by a sensitive restriction analysis technique and showed that, with one very minor exception, they were all identical to each other and to the type strain. In a separate study (26), we found that 31 reference serotype strains of the MAIS (*M. avium-M. intracellulare-M. scrofulaceum*) complex had restriction patterns very different from those of *M. paratuberculosis*. While genomic DNA-DNA hybridization shows that there is a high degree of genetic similarity between *M. paratuberculosis* and *M. avium*, restriction analysis is sufficiently sensitive for the patterns of DNA fragments from the two species to appear completely different. Therefore, restriction analysis appeared to be an ideal method to characterize a greater variety of *M. paratuberculosis* strains and also to compare wood pigeon strains to strains of *M. paratuberculosis* and *M. avium*.

Recently, we identified a repetitive DNA sequence in the type strain of *M. paratuberculosis* and isolated from it a 0.22-kilobase-pair (kbp) fragment (6). When a restriction digest of *M. paratuberculosis* DNA was separated on an agarose gel, blotted onto nitrocellulose, and hybridized with this fragment, a reproducible pattern of lines was obtained. We also showed that this 0.22-kbp sequence was not present in strains of 19 other species of mycobacteria, including 31 reference serotype strains of the MAIS complex (6). Since this fragment was highly specific for *M. paratuberculosis*, it was used to search for genomic variations between *M. paratuberculosis* strains. For the present study, we examined strains of *M. paratuberculosis* from cattle, goats, and sheep from New Zealand and other countries. Four reference strains of *M. paratuberculosis* and two strains of wood pigeon mycobacteria were also examined. All strains were characterized both by restriction analysis and by hybridizing restriction digests with the DNA fragment specific to *M. paratuberculosis*.

MATERIALS AND METHODS

Mycobacteria. The 29 strains of *M. paratuberculosis* from New Zealand were isolated by us from fecal or intestinal-tissue samples from cattle, sheep, and goats. Strains of *M. paratuberculosis* from other countries were kindly provided as follows: Canadian sheep strains from B. Brooks, Nepean, Ontario, Canada; Norwegian goat strains and a Faroe Is-

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TABLE 1. Epidemiological, restriction analysis, and hybridization data for 50 strains of *M. paratuberculosis* and 2 wood pigeon strains

Host or source	Geographic origin and subgroup	Hybridization type[a]	Restriction type[b]	No. of strains
Cattle	New Zealand			
	1	C1	c1	10
	2	C1	c2	2
	3	C2	c1	1
	Australia			
	1	C1	c1	1
	2	C3	c3	3
	3	C4	c3	1
	4	C5	c4	1
Sheep	Canada			
	1	C1	c1	6
	2	I	s2	1
	New Zealand	S1	s1	7
	Faroe Islands	S2	s1	1
Goats	New Zealand			
	1	C1	c1	8
	2	S1	s1	1
	Norway			
	1	C1	c5	1
	2	None	Unknown	2
Human strain Linda (ATCC 43015)[c]	United States	C5	c6	1
Type strain TMC 1613[d]	United States	C6	c1	1
Vaccine strain 316v (Weybridge, United Kingdom)	United Kingdom	C1	c1	1
Vaccine strain (Merieux Institute, France) (Weybridge strain 316f)	United Kingdom	C7	c1	1
Wood pigeon strains	Norway	None	*M. avium*	2

[a] Hybridization types designated C and S have patterns similar to those of cattle strains and New Zealand sheep strains, respectively. The pattern designated I has an intermediate pattern.

[b] Restriction types designated c and s have patterns similar to those of cattle strains and New Zealand sheep strains, respectively.

[c] ATCC, American Type Culture Collection, Rockville, Md.

[d] TMC, Trudeau Mycobacterial Collection, National Institutes of Health, Bethesda, Md.

lands sheep strain from F. Saxegaard, Oslo, Norway; and Australian cattle strains from N. Skilbeck, Bairnsdale, Victoria, Australia. Four reference strains of *M. paratuberculosis* were obtained from the sources shown in Table 1. Two wood pigeon strains of mycobacteria were kindly provided by F. Saxegaard, Oslo, Norway. The serotype 2 strain of the MAIS complex, *M. avium* P 194, was kindly provided by D. Dawson, Brisbane, Queensland, Australia. Strains were isolated and subcultured for DNA extraction on Herrold egg yolk medium containing 3 mg of mycobactin J per liter (16). In the case of four New Zealand sheep whose intestinal tissues gave positive cultures for *M. paratuberculosis*, preparations of acid-fast organisms were also obtained by direct extraction of washed ileal mucosa by a trypsin digestion method (17).

DNA isolation, restriction analysis, and hybridization. Mycobacterial DNA was extracted by a gentle lysis procedure described previously (3). For restriction analysis, DNA samples (4 μg) were digested with *Bst*EII and the fragments were separated by electrophoresis at 130 V on 400-mm gels of 1% agarose for 24 h. With the exception of the voltage and gel size, conditions were as described previously (4). For hybridization, DNA samples (2 μg) were digested with *Bst*EII and the fragments were separated by electrophoresis at 35 V on 200-mm gels of 1% agarose for 16 h. Fragments were transferred to 140-mm squares of nitrocellulose by Southern blotting using standard procedures (11). The 0.22-kbp sequence used as a probe was labeled with [α-^{32}P]dCTP by the polymerase chain reaction (20). The labeling reaction was done for 25 cycles with 23-mer oligonucleotides homologous to each end of the fragment, a denaturation step at 98°C for 0.5 min, and a combined annealing and extension step at 65°C for 1 min. Filters were hybridized for 16 h at 50°C in 50% formamide and washed by standard methods (11) with a final stringent wash at 80°C in 0.075 M sodium chloride–0.0075 M trisodium citrate (pH 7.0).

RESULTS

The results for both restriction and hybridization analyses are shown in Table 1. Most of the strains were clearly separated into two groups. To avoid confusion, hybridization types have been assigned uppercase letters and restriction types have been assigned lowercase letters. One group was designated as the cattle group (C and c in Table 1) because all strains found in cattle belonged to this group. This group also included some strains isolated from sheep and goats. The second group of strains was designated the sheep group (S and s in Table 1), as these strains were isolated only from sheep and one New Zealand goat. Within the cattle group of *M. paratuberculosis* strains, there were a number of minor pattern differences; these are shown for the hybridization patterns in Fig. 1 and 2 and are indicated by arrowheads alongside the restriction patterns in Fig. 3. One Canadian sheep strain had a hybridization pattern (Fig. 2, lane 6) intermediate between those of the two groups and a restriction pattern (Fig. 3, lane 8) that appeared more similar to that of the sheep group than to that of the cattle group. DNA from the two wood pigeon strains and two of the Norwegian goat strains did not hybridize to the 0.22-kbp sequence. The two wood pigeon strains had restriction patterns that were very different from those of *M. paratuberculosis* strains and very similar to that of a reference strain of *M. avium* serotype 2 (Fig. 4). The restriction patterns of the two Norwegian goat strains (Fig. 4) were very different from those of all *M. paratuberculosis* strains.

Strains belonging to the sheep group were particularly difficult to isolate on primary culture by either our standard method or other established techniques. Typically, an inoculum from sheep intestine containing 10^8 to 10^9 acid-fast organisms would yield no colonies or a few colonies. On subculture such strains grew almost as well as cattle strains. This raised the possibility that the culture medium was highly selective for a tiny minority of the organisms present

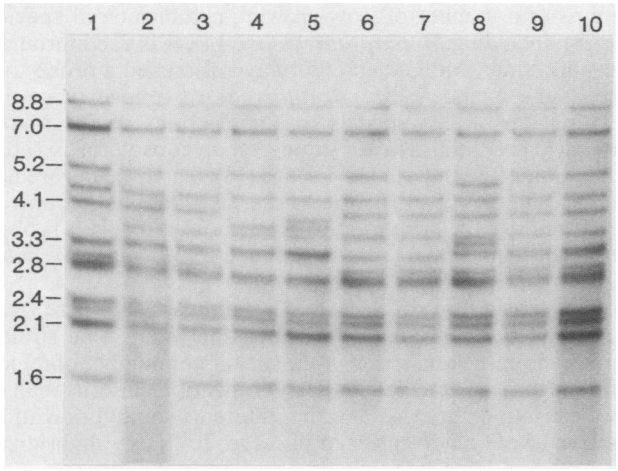

FIG. 1. Hybridization patterns of DNA from selected *M. paratuberculosis* strains after digestion with *Bst*EII, transfer to nitrocellulose, and probing with a ^{32}P-labeled sequence specific to *M. paratuberculosis*. Lanes, strains, and hybridization types (with the same designations as in Table 1) are as follows: 1, type strain TMC 1613, C6; 2, vaccine strain 316v, C1; 3, Australian cattle strain 1, C1; 4, Australian cattle strain 2, C3; 5, Australian cattle strain 3, C4; 6, Australian cattle strain 4, C5; 7, human strain Linda (ATCC 43015), C5; 8, vaccine strain 316f, C7; 9, Norwegian goat strain 1, C1; 10, New Zealand cattle strain 1, C1. Molecular size markers (in kilobase pairs) are given on the left side of the figure.

in sheep samples and that these few cells were substantially different genetically from the vast majority of the organisms. To investigate this possibility, bacteria were prepared directly from sheep tissues. DNA obtained from these acid-fast organisms had the same restriction (Fig. 3, lanes 9 and 10) and hybridization (Fig. 2, lanes 7 and 8) patterns as DNA obtained from *M. paratuberculosis* strains cultured from the same animal tissues.

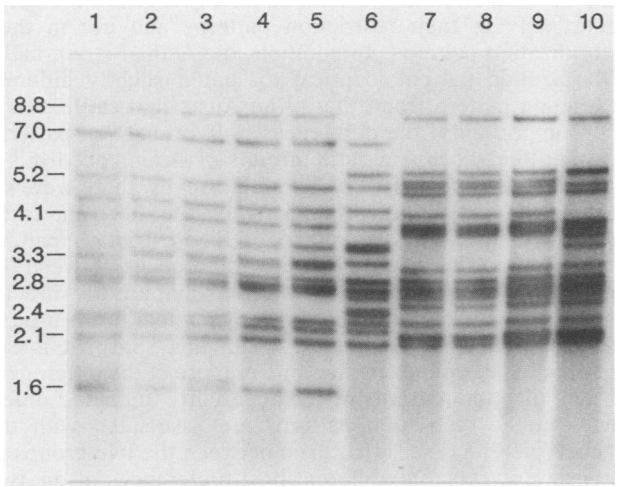

FIG. 2. Hybridization patterns of DNA from selected *M. paratuberculosis* strains after digestion with *Bst*EII, transfer to nitrocellulose, and probing with a ^{32}P-labeled sequence specific to *M. paratuberculosis*. Lanes, strains, and hybridization types (with the same designations as in Table 1) are as follows: 1, type strain TMC 1613, C6; 2, New Zealand cattle strain 2, C1; 3, New Zealand cattle strain 3, C2; 4, New Zealand goat strain 1, C1; 5, Canadian sheep strain 1, C1; 6, Canadian sheep strain 2, I; 7, New Zealand sheep strain, S1; 8, DNA from acid-fast organisms extracted directly from intestinal tissues of New Zealand sheep with Johne's disease, S1; 9, New Zealand goat strain 2, S1; 10, Faroe Islands sheep strain, S2. Molecular size markers (in kilobase pairs) are given on the left side of the figure.

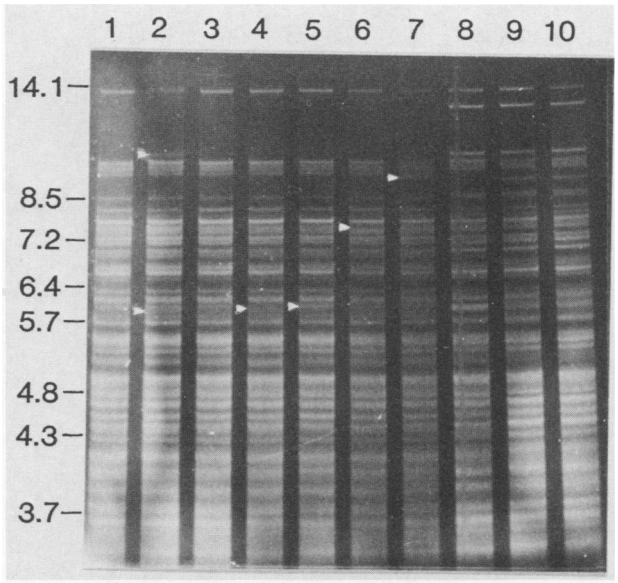

FIG. 3. Restriction fragment patterns after *Bst*EII digestion of DNA from selected *M. paratuberculosis* strains. Lanes, strains, and restriction types are as follows: 1, type strain TMC 1613, c1; 2, New Zealand cattle strain 2, c2; 3, Canadian sheep strain 1, c1; 4, Australian cattle strain 4, c4; 5, Australian cattle strain 2, c3; 6, human strain Linda (ATCC 43015), c6; 7, Norwegian goat strain 1, c5; 8, Canadian sheep strain 2, s2; 9, New Zealand sheep strain, s1; 10, DNA from acid-fast organisms extracted directly from intestinal tissues of New Zealand sheep with Johne's disease, s1. The arrowheads indicate the minor differences between the strains shown in lanes 2 through 7 and the type strain (lane 1). Molecular size markers (in kilobase pairs) are given on the left side of the figure.

By the methods used for hybridization with the 0.22-kbp sequence, 12 to 15 fragment lines were resolved for each strain. When allowance was made for incompletely resolved double fragments (e.g., Fig. 1, lane 1, at 2.8 kbp) and triple fragments (e.g., Fig. 2, lane 7, at 4.1 kbp), the actual number of copies of the repeated fragment in the genome was probably 15 to 18, with strains from sheep having the most copies. Approximately one-half of the fragment lines were shared between the two groups of strains, while within each group any two strains had 75% or more of their fragment lines in common.

DISCUSSION

The results showed that the genomic DNA of *M. paratuberculosis* strains which were isolated from various animal hosts in different countries contained many copies of a specific DNA sequence. In addition, genomic DNA from these strains gave *Bst*EII restriction patterns with many fragment lines common to all strains. The 48 strains that contained the repeated sequence were clearly subdivided by both restriction and hybridization patterns into two groups, and these two groups had different animal host distributions.

The sheep group of strains was difficult to study because of the problems of primary culture. However, since DNA from these cultures had restriction and hybridization patterns identical to those of DNA obtained from organisms

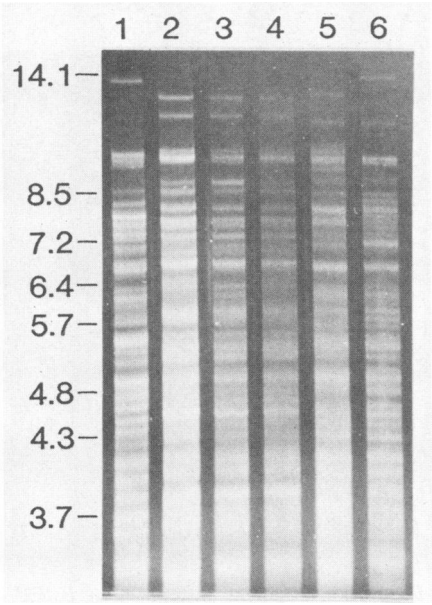

FIG. 4. Restriction fragment patterns after *Bst*EII digestion of DNA from selected strains. Lanes: 1, *M. paratuberculosis* TMC 1613; 2, *M. avium* serotype 2; 3 and 4, wood pigeon strains; 5 and 6, Norwegian goat strains that did not hybridize to the probe of the repetitive sequence in *M. paratuberculosis*. Molecular size markers (in kilobase pairs) are given on the left side of the figure.

extracted directly from sheep tissues, any genetic differences unique to the cultured organisms must be very subtle. More importantly, this work confirmed that the acid-fast organisms present in very large numbers in sheep with Johne's disease are *M. paratuberculosis* and that these sheep strains have a number of clear genetic differences from strains found in cattle. The existence of different sheep and cattle strains has been previously proposed on the basis of epidemiological evidence from Scotland (22), Australia (21), and New Zealand (1) and also because of earlier difficulties in culturing some sheep strains (9).

Although the present results support the conclusion that the sheep group of strains are host adapted, this adaptation is probably relative rather than absolute. Previous studies (23) with strains of *M. paratuberculosis* isolated from sheep, strains whose cultural characteristics suggest they belong to the sheep group of strains described here, have shown that such strains can cause Johne's disease in calves under experimental conditions. Similar results have been reported for wood pigeon strains (12, 25). In those studies the organisms were administered orally, the normal route of natural disease transmission. However, the doses of organisms given were so large that the results of the studies probably have little relevance to natural disease transmission. Certainly in some countries, Johne's disease-infected sheep do not appear to be a common source of disease for cattle grazing in the same pasture (1, 21, 22).

The 0.22-kbp DNA sequence used as a probe in this study was part of a larger repetitive element that was identified in the genome of the type strain of *M. paratuberculosis* (6). The absence of this sequence from genomic DNA of strains of the MAIS complex was particularly important, as genomic DNA-DNA hybridization has shown that *M. paratuberculosis* is very closely related to some of these strains (10, 18, 28). The 0.22-kbp sequence does not contain a *Bst*EII site, so this enzyme, which has given reliable digestion of DNA from strains of a number of slow-growing mycobacterial species (3, 26), including *M. paratuberculosis* (4), was used throughout this study. McFadden et al. have also used a probe to a repeated sequence in *M. paratuberculosis* to investigate the relationship of this species to some strains of the MAIS complex (14) and also to strains of mycobacteria isolated from patients with Crohn's disease (13). Their results are not comparable to those reported here, as they studied only a few strains of *M. paratuberculosis* and used for their probes DNA sequences that were not unique to *M. paratuberculosis*.

The sensitivity of the DNA methods used here was sufficient to reveal minor differences between some strains within the cattle and sheep groups. A larger number of minor differences was detected in the hybridization patterns than in the restriction patterns, and the differences in hybridization patterns were much easier to observe. In theory, hybridization pattern differences should be associated with restriction pattern differences at the same fragment positions. That these restriction pattern differences were not detected was probably due to the difficulty of resolving the much larger number of restriction fragment lines which were very closely spaced for fragments with sizes of 4 or fewer kbp. Most of the hybridization pattern differences within the cattle group of strains occurred below this region. However, the changes in both the copy number and genomic positions of the repetitive DNA sequence which were detected by the changes in hybridization pattern did not account entirely for the genetic differences between strains. This applied both to the minor genetic differences that existed between strains within the cattle group and to the more major differences that existed between the sheep and cattle groups. Three strains, two with an identical restriction pattern obtained from New Zealand cattle (Fig. 3, lane 2) and one with a different restriction pattern obtained from a Norwegian goat (Fig. 3, lane 7), differed from the most common strains of the cattle group only in their restriction patterns and not in their hybridization patterns. In addition, the human strain had a hybridization pattern identical to, but a slightly different restriction pattern from, that of an Australian cattle strain. More importantly, the differences in hybridization patterns between the sheep and cattle groups of strains occurred at and below fragment sizes of 7 kbp. However, there were six restriction fragment differences between the sheep and cattle groups of strains in the region of the restriction pattern above 7 kbp. Thus, there are many differences between the genomes of the sheep and cattle strains that are not directly associated with alterations in the copy number or position of the repetitive DNA sequence. From this study, it is not possible to determine whether one or more of the differences in hybridization patterns or the apparently unrelated differences in the restriction patterns are associated with the phenotypic differences that exist between the two groups of strains. However, the different host preferences of the two groups of *M. paratuberculosis* strains appear to be a real phenomenon, and it is likely that one or more of the restriction or hybridization differences detected between these groups are associated with the change in host specificity. In this connection, the strain of *M. paratuberculosis* from a Canadian sheep is of particular interest. This strain exhibited a hybridization pattern that was intermediate between those of the two groups of strains and a restriction pattern that appeared more similar to that of the sheep group than to that of the cattle group. Investigation of the host preferences of this and other intermediate strains, perhaps with the assistance of some genetic manipulation, may

enable the genetic change or changes that account for host specificity to be ultimately identified.

The species designation of wood pigeon strains has proven difficult. It has been variously proposed that they be classified as *M. avium* (15), as *M. paratuberculosis* (24), or as a distinct group (7) possibly within a single species which includes all three organisms (18). Results of restriction analysis and hybridization of DNA from the two wood pigeon strains studied here indicates that they should not be classified as *M. paratuberculosis*. Previous studies of mycobacterial species have shown that restriction analysis is an excellent method for characterizing different groups of strains within a single species (5, 26) and for allocating strains to closely related species (3). In particular, it has been shown that reference strains of *M. avium* serotypes 2 and 3 have identical *Bst*EII restriction patterns and that these patterns are very different from those of 29 other reference serotype strains of the MAIS complex (26). The finding that wood pigeon strains have very similar restriction patterns to these *M. avium* strains is strong evidence that these organisms should be classified as *M. avium*.

The absence of the repetitive *M. paratuberculosis* sequence from two of the Norwegian goat strains and the finding that their restriction fragment patterns are very different from those of other strains of *M. paratuberculosis* indicate that there are many genetic differences between them and all the other *M. paratuberculosis* strains. While these two strains are slow-growing, mycobactin-dependent mycobacteria and were apparently isolated from goats with Johne's disease, their marked genetic difference from other strains of *M. paratuberculosis* raises considerable doubt about their inclusion in this species. Possibly these strains are uniquely adapted to goats, as strains of mycobacteria causing Johne's disease in Norwegian goats seldom cause disease in sheep or cattle (8).

The strains of *M. paratuberculosis* characterized in this study as belonging to the cattle group came from new Zealand, Australia, Canada, and Norway; the vaccine strains were originally isolated in the United Kingdom, and the type strain was originally isolated in the United States. In a separate study, strains isolated in Argentina; Nova Scotia, Canada; and 23 states of the United States were reported to have similar restriction patterns to those of New Zealand cattle strains (27). From this it appears that variants of the cattle group of strains are responsible for many cases of Johne's disease throughout the world. The present study, together with earlier work (1, 9, 21, 22), indicates that the sheep group of strains is an important cause of Johne's disease in that animal host. Atypical strains such as the Norwegian goat strains appear to be a less common cause of Johne's disease, but they may be important in certain countries or particular animal hosts.

The characterization of two distinct groups of *M. paratuberculosis* strains and their different host distribution is of considerable veterinary significance, particularly in countries such as New Zealand where different animal species often graze the same pastures. The findings that goats can be infected by either group of strains and that, at least in the situation prevailing in Canada, some sheep may also be susceptible to infection by cattle strains are complicating factors that need to be taken into account in designing control strategies for Johne's disease. However, the ability to genetically characterize the different strains provides a sound basis from which complex situations of disease transmission can be investigated.

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