Mycobacterium avium subsp. paratuberculosis Strains from Cattle and Sheep Can Be Distinguished by a PCR Test Based on a Novel DNA Sequence Difference

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A DNA sequence differing between sheep and cattle types of Mycobacterium avium subsp. paratuberculosis was identified and used to develop a PCR test. The test unequivocally distinguished all sheep types from cattle types and was negative for a wide range of other strains from the Mycobacterium avium-Mycobacterium intracellulare complex. The test will be useful for epidemiological purposes, particularly in hosts such as deer that can be easily infected with either type.

Paratuberculosis, or Johne's disease, is a chronic granulomatous enteritis that affects domestic and wild ruminants, causing reduced food intake, weight loss, and death. The disease is present in most countries and results in significant production losses. The causative organism, Mycobacterium avium subsp. paratuberculosis (basonym M. paratuberculosis) (11), has also been implicated as the etiologic agent of Crohn's disease in humans and is a member of the M. avium-Mycobacterium intracellulare (MAI) complex which includes M. intracellulare and all subspecies of M. avium. Two recent discoveries have shown that the spread of M. paratuberculosis may be more complicated than previously believed and emphasize the need for the development of new diagnostic tools. First, the organism has been reported to survive normal milk pasteurization (10), and second, it has also been isolated in the United Kingdom from common wild nonruminant animals such as rabbits, foxes, stoats, and crows (2). Isolates of the organism were first classified into cattle and sheep types in 1990 (4) on the basis of restriction fragment length polymorphisms (RFLPs) of the insertion sequence IS900, and this largely correlates with the difficulty of primary isolation of sheep types (4, 14). The distinction into cattle and sheep types is epidemiologically useful, as cattle and sheep are preferentially infected with their named types while other ruminant species such as deer and goats appear to be equally susceptible to either type (4, 7, 14, 17). Recently, a two-step method for distinguishing between cattle and sheep types of M. paratuberculosis was developed based on polymorphic differences in the insertion sequence IS900 (13). We reasoned that the RFLP differences between sheep and cattle types might indicate differences in their genomic insertion sites for IS900 that could be used for constructing a simple PCR assay to distinguish between the two types. This study describes the successful development of such an assay.

The strains of the MAI complex used for this study were all characterized as sheep or cattle types on the basis of their RFLPs with IS900. Strains were cultured with standard mycobacterial media (3). Purified DNA was extracted as described previously (4). When DNA from strains of each type was subjected to PCR at an annealing temperature of 50°C with primers directed outward from each end of IS900, only DNA from sheep types gave a product between 300 and 400 bp. Subsequently, it was observed that the same 342-bp product was obtained if only one PCR primer (DMC136, Fig. 1) was used. The PCR product was cloned into pBluescript KS II (Stratagene) and sequenced. Comparison of this sequence to the homologous region of a cattle type of M. paratuberculosis (National Center for Biotechnology Information database [http://www.ncbi.nlm.nih.gov/]) with BLAST revealed that the cattle type was not homologous to DMC136 at the 5’ end. Further investigation with a range of PCR primers (data not shown) indicated that the two types have similar sequences at the 3’ end but that only the sheep type has an IS900 site at the 5’ end. The 342-bp product was thus a result of DMC136 hybridizing perfectly to the terminal part of IS900 that comprises the 5’ end of the sheep sequence (Fig. 1) and partially to the last 10 nucleotides at the 3’ end of this sequence. DNA from the cattle type has a similar sequence at the 3’ end, but it gives no product with DMC136 because it lacks a sequence matching DMC136 at the 5’ end. The site of insertion of IS900 is just downstream of the start codon for a putative gene on the complementary strand shown in lowercase letters in Fig. 1. This gene has high homology to a Mycobacterium smegmatis gene whose product is involved in phage attachment (1). The other major difference in sequence between the two types is that the sheep type has a tandem repeat of a 12-bp sequence followed by a 4-bp linker, which together contain a 14-bp palindromic sequence (Fig. 1). Such sequences are commonly found in bacterial chromosomes, often as part of a promoter region (8), a terminator (15), or a phage attachment site (12). Analysis of the sequence around the tandem repeat site showed that it is inserted at the stop codon of a hypothetical gene in the cattle type that encodes a putative protein of 97 amino acids with no good homology to other proteins. This hypothetical gene is interrupted by insertion of IS900 in the sheep type, but a truncated version of it might be produced, as...
there are other putative start codons. The tandem repeat is just 19 bp before an open reading frame encoding a putative protein of 134 amino acids with good homology to two hypothetical proteins of *Mycobacterium tuberculosis* and a hypothetical protein encoded by a small *Yersinia pestis* virulence plasmid. The palindromic sequence might therefore be involved in controlling gene expression of this second gene, either by limiting read-through from the truncated first gene to the second gene or by acting as part of an independent promoter region for the second gene. These sequence differences between sheep and cattle types may therefore be important in determining the host preference of the two types.

A PCR assay was developed with a GeneAmp PCR System 9600 (Applied Biosystems) and the three primers DMC529, DMC531, and DMC533 (Fig. 1) under the following conditions: 1 cycle at 95°C for 3 min; 25 cycles at 60°C for 30 s, 72°C for 30 s, and 94°C for 30 s; and 1 cycle at 72°C for 7 min. DNA from all strains of the cattle type gave the expected product of 310 bp, while DNA from all strains of the sheep type gave the expected product of 162 bp (Fig. 2). A PCR product was not observed for any of the wide range of strains of the MAI complex (Table 1) that did not contain IS900 and were not *M. paratuberculosis*. The PCR was also negative for two isolates of *Mycobacterium bovis* and an isolate of *M. tuberculosis*.

The MAI complex covers a relatively broad group of genetically related mycobacteria that, with the exception of *M. paratuberculosis*...
tuberculosis, are found in many environmental niches and are occasional mammalian pathogens. Because of the potential of these organisms to confuse the diagnosis of paratuberculosis, strains of the MAI complex tested in this study were weighted toward those that had been isolated from humans or from a range of different animal hosts and that might be expected to be most closely related to \textit{M. paratuberculosis} (3). It is highly encouraging that none of these strains was positive in the PCR assay. In the case of \textit{M. paratuberculosis}, the inclusion of 10 strains from five other countries enabled a cross section of strains with different IS900 RFLP types to be tested. This included sheep strains from Canada and Iceland, previously described as having an “intermediate” IS900 RFLP type (4, 7), which we now regard as variant sheep types because their RFLP patterns are more similar to those of sheep types than to those of cattle types (7, 14). In New Zealand, paratuberculosis is an emerging disease of farmed deer (6), and since this host can be infected with either the sheep or the cattle type (7), it was important to establish whether the PCR test could be used for epidemiological studies. For this reason, nine strains of the cattle type and three strains of the sheep type tested were deer isolates. For all \textit{M. paratuberculosis} strains in Table 1, the PCR results were consistent with the RFLP division into sheep and cattle types. These results demonstrate the utility of the test particularly for those hosts such as deer that can be infected with either type. At present, PCR testing for paratuberculosis where both cattle and sheep types are potentially present involves a PCR assay based on IS900 to confirm the presence of \textit{M. paratuberculosis} followed by a PCR test based on IS1311 whose product is then subjected to restriction endonuclease analysis (17). The final step is necessary because, while the presence of IS1311 is not specific for \textit{M. paratuberculosis}, some copies of IS1311 in \textit{M. paratuberculosis} have polymorphisms that are specific for the cattle and sheep types (13). The results of the present study indicate that this three-step process might be replaceable by a single PCR assay which appears specific both for \textit{M. paratuberculosis} and for distinguishing between sheep and cattle types. Since the PCR is based on a single-copy sequence, it may not be as sensitive as PCR assays based on IS900 (5, 9), which has 14 to 18 copies in the genome. Confirmation of these conclusions will require investigation of a wider group of strains of the MAI complex and other mycobacterial species. Nevertheless, the assay described here has the potential to make a useful contribution to epidemiological studies of paratuberculosis and possibly Crohn’s disease.

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FIG. 2. PCR products from cattle and sheep types of \textit{M. paratuberculosis} amplified with DMC529, DMC531, and DMC533. Lanes: 1 and 11, molecular size markers; 2 to 5, cattle types; 6 to 9, sheep types; 10, negative control.