JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2002, p. 4760–4762 Vol. 40, No. 12
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.12.4760–4762.2002

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

Desmond M. Collins,* May De Zoete, and Sonia M. Cavaignac

AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand

Received 10 June 2002/Returned for modification 2 September 2002/Accepted 22 September 2002

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 IS*900*, 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 IS*1311* (13). We reasoned that the RFLP differences between sheep and cattle types might indicate differences in their genomic insertion sites for IS*900* 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 for the presence or absence of IS*900* and IS*901* and are given in Table 1. All strains of *M. paratuberculosis* were characterized as sheep or cattle types on the basis of their RFLPs with IS*900*. 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 IS*900*, 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 IS*900* site at the 5′ end. The 342-bp product was thus a result of DMC136 hybridizing perfectly to the terminal part of IS*900* that comprises the 5′ end of the sheep sequence in 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 IS*900* 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 IS*900* in the sheep type, but a truncated version of it might be produced, as

* Corresponding author. Mailing address: AgResearch, Wallaceville Animal Research Centre, P.O. Box 40063, Upper Hutt, New Zealand. Phone: 64-4-922-1310. Fax: 64-4-922-1413. E-mail: desmond.collins@agresearch.co.nz.

TABLE 1. Strains of the MAI complex and *M. tuberculosis* complex subjected to PCR

No. of strains	No. of strains, with insertion sequence:		Description	Source[a]
	IS*900*	IS*901*		
10	0	3	Reference serotypes 1 to 6 and 8 to 11 of MAI complex[b]	D. Dawson (16)
11	0	7	Cattle, deer, and pig isolates of MAI complex[b]	New Zealand
6	0	5	Bird isolates of MAI complex[b]	New Zealand
4	0	0	Human isolates of MAI complex[b]	New Zealand
14	14		*M. paratuberculosis* cattle type	New Zealand
3	3		*M. paratuberculosis* cattle type	Canada
1	1		*M. paratuberculosis* TMC1613; cattle type	USA
1	1		*M. paratuberculosis* 316F; cattle type	UK
7	7		*M. paratuberculosis* sheep type	New Zealand
1	1		*M. paratuberculosis* sheep type	Canada
3	3		*M. paratuberculosis* sheep type	South Africa
1	1		*M. paratuberculosis* sheep type	Iceland
1			*M. tuberculosis* H37Rv	USA
2			*M. bovis*	New Zealand

[a] USA, United States; UK, United Kingdom.
[b] None of these strains of the MAI complex were *M. paratuberculosis*.

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 IS*900* 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. para-*

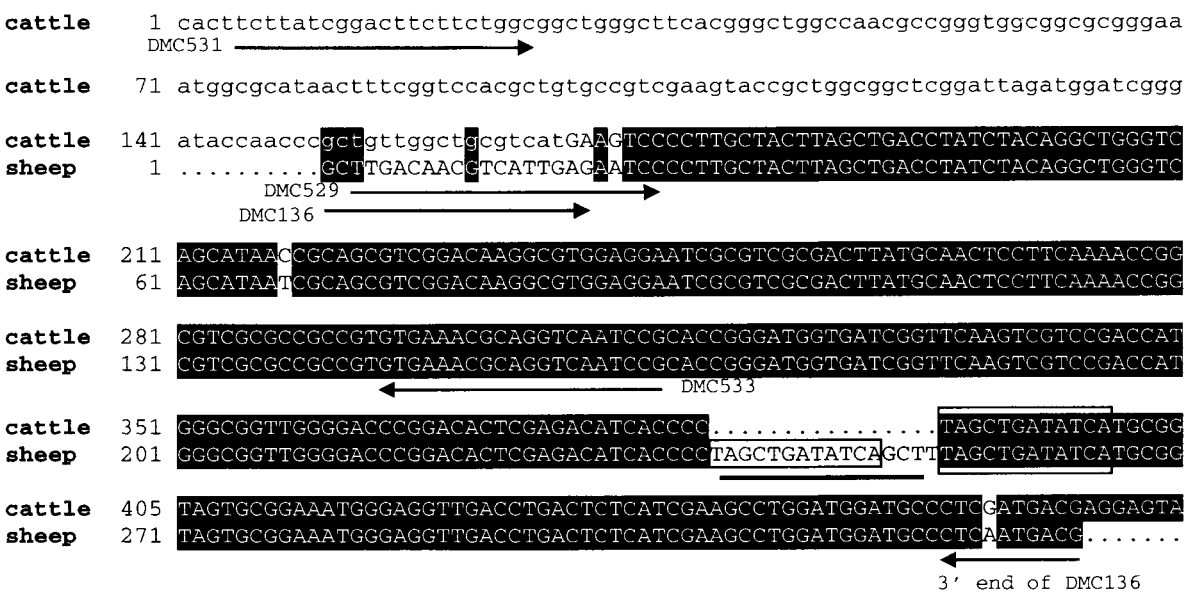

FIG. 1. Alignment of homologous DNA sequences from cattle and sheep types of *M. paratuberculosis*. Nucleotides identical in the two sequences are shown in reverse type, arrows indicate the identities and directions of the oligonucleotide primers used, the tandem DNA sequence present in the sheep type is shown in boxes, the palindromic region is underlined, and the coding sequence of the putative gene involved in phage attachment is shown in lowercase letters.

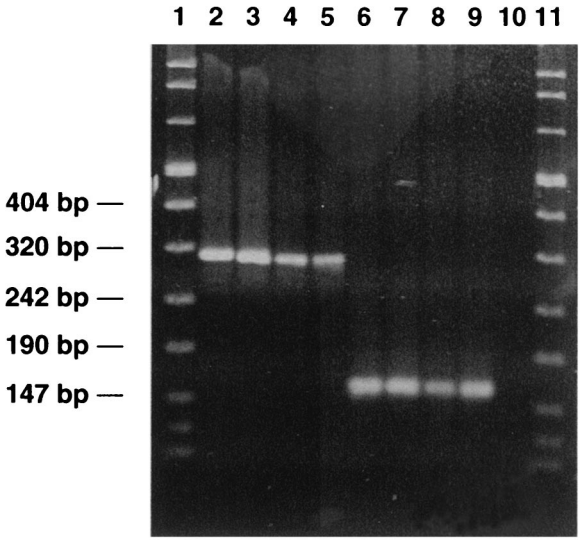

FIG. 2. PCR products from cattle and sheep types of *M. paratuberculosis* amplified with DMC529, DMC531, and DMC533. Lanes: 1 and 11, molecular size markers; 2 to 5, cattle types; 6 to 9, sheep types; 10, negative control.

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 *M. paratuberculosis* (3). It is highly encouraging that none of these strains was positive in the PCR assay. In the case of *M. paratuberculosis*, the inclusion of 10 strains from five other countries enabled a cross section of strains with different IS*900* RFLP types to be tested. This included sheep strains from Canada and Iceland, previously described as having an "intermediate" IS*900* 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 *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 IS*900* to confirm the presence of *M. paratuberculosis* followed by a PCR test based on IS*1311* whose product is then subjected to restriction endonuclease analysis (17). The final step is necessary because, while the presence of IS*1311* is not specific for *M. paratuberculosis*, some copies of IS*1311* in *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 *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 IS*900* (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.

We thank G. W. de Lisle for culturing and helpful advice and D. Dawson, H. F. A. K. Huchzermeyer, B. Brooks, and F. Saxegaard for providing mycobacterial strains.

We thank the New Zealand Foundation for Research Science and Technology for financial support.

REFERENCES

1. **Barsom, E. K., and G. F. Hatfull.** 1996. Characterization of *Mycobacterium smegmatis* gene that confers resistance to phages L5 and D29 when over expressed. Mol. Microbiol. **21:**159–170.
2. **Beard, P. M., M. J. Daniels, D. Henderson, A. Pirie, K. Rudge, D. Buxton, S. Rhind, A. Greig, M. R. Hutchings, I. McKendrick, K. Stevenson, and J. M. Sharp.** 2001. Paratuberculosis infection of nonruminant wildlife in Scotland. J. Clin. Microbiol. **39:**1517–1521.
3. **Collins, D. M., S. Cavaignac, and G. W. de Lisle.** 1997. Use of four DNA insertion sequences to characterize strains of the *Mycobacterium avium* complex isolated from animals. Mol. Cell. Probes **11:**373–380.
4. **Collins, D. M., D. M. Gabric, and G. W. de Lisle.** 1990. Identification of two groups of *Mycobacterium paratuberculosis* strains by restriction endonuclease analysis and DNA hybridization. J. Clin. Microbiol. **28:**1591–1596.
5. **Collins, D. M., D. M. Stephens, and G. W. de Lisle.** 1993. Comparison of polymerase chain reaction tests and fecal culture for detecting *Mycobacterium paratuberculosis* in bovine feces. Vet. Microbiol. **36:**289–299.
6. **de Lisle, G. W.** 2002. Johne's disease in New Zealand: the past, present and a glimpse into the future. N. Z. Vet. J. **50**(Suppl.)**:**53–56.
7. **de Lisle, G. W., G. F. Yates, and D. M. Collins.** 1993. Paratuberculosis in farmed deer; case reports and DNA characterization of isolates of *Mycobacterium paratuberculosis*. J. Vet. Diagn. Investig. **5:**567–571.
8. **Dussurget, O., J. Timm, M. Gomez, B. Gold, S. Yu, S. Z. Sabol, R. K. Holmes, W. R. Jacobs, Jr., and I. Smith.** 1999. Transcriptional control of the iron-responsive *fxbA* gene by the mycobacterial regulator IdeR. J. Bacteriol. **181:**3402–3408.
9. **Fang, Y., W. Y. Wu, J. L. Pepper, J. L. Larsen, S. A. Marras, E. A. Nelson, W. B. Epperson, and J. Christopher-Hennings.** 2002. Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. J. Clin. Microbiol. **40:**287–291.
10. **Grant, I. R., E. I. Hitchings, A. McCartney, F. Ferguson, and M. T. Rowe.** 2002. Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. Appl. Environ. Microbiol. **68:**602–607.
11. **Harris, N. B., and R. G. Barletta.** 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. **14:**489–512.
12. **Hauser, M. A., and J. J. Scocca.** 1992. Site-specific integration of the *Haemophilus influenzae* bacteriophage HP1. Identification of the points of recombinational strand exchange and the limits of the host attachment site. J. Biol. Chem. **267:**6859–6864.
13. **Marsh, I., R. Whittington, and D. Cousins.** 1999. PCR-restriction endonuclease analysis for identification and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* based on polymorphisms in IS*1311*. Mol. Cell. Probes **13:**115–126.
14. **Pavlik, I., A. Horvathova, L. Dvorska, J. Bartl, P. Svastova, R. du Maine, and I. Rychlik.** 1999. Standardization of restriction fragment length polymorphism analysis for *Mycobacterium avium* subspecies *paratuberculosis*. J. Microbiol. Methods **38:**155–167.
15. **Unniraman, S., R. Prakash, and V. Nagaraja.** 2002. Conserved economics of transcription termination in eubacteria. Nucleic Acids Res. **30:**675–684.
16. **Wards, B. J., D. M. Collins, and G. W. de Lisle.** 1987. Restriction endonuclease analysis of members of the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* serocomplex. J. Clin. Microbiol. **25:**2309–2313.
17. **Whittington, R. J., A. F. Hope, D. J. Marshall, C. A. Taragel, and I. Marsh.** 2000. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: IS*900* restriction fragment length polymorphism and IS*1311* polymorphism analyses of isolates from animals and a human in Australia. J. Clin. Microbiol. **38:**3240–3248.