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Insertion Sequence IS*900* Revisited

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Many studies investigating *Mycobacterium avium* subsp. *paratuberculosis* in Crohn's disease have used molecular detection of IS*900* in clinical samples, but some have described polymorphisms in IS*900* as variants of this organism. Analysis of 23 *M. avium* subsp. *paratuberculosis* isolates revealed that IS*900* is highly conserved, with only two sequevars distinguishing sheep and cattle lineages. Amplification of IS*900*-like sequences is not sufficient as a proxy for *M. avium* subsp. *paratuberculosis*.

Mycobacterium avium subsp. *paratuberculosis* is a pathogen of many mammals, in which it causes a severe inflammatory bowel disease called Johne's disease (8). Similarities between this and Crohn's disease of humans have fueled investigations on the zoonotic potential of *M. avium* subsp. *paratuberculosis*. Because of the limitations of culture-based diagnostics, including slow growth and the presumed low abundance of the organism, PCR-based testing of a multicopy insertion element, IS*900*, has been used in a number of studies as the sole criterion for the detection of *M. avium* subsp. *paratuberculosis* in clinical and environmental samples. This approach raises two critical issues: first, false-positive results may be obtained if isolates other than *M. avium* subsp. *paratuberculosis* bear sequences with similarity to the IS*900* element (5, 6), and second, there is the risk of laboratory cross-contamination, which is of special concern in PCR-based assays.

Sequencing of IS*900* amplicons has been performed in different studies to address both of these concerns (1, 4, 11, 13, 14), but predictably, this has led to confusion. The determination that IS*900* sequences from different samples are invariant and identical to a reference sequence would suggest the presence of *M. avium* subsp. *paratuberculosis* rather than another species but cannot address the issue of contamination. Conversely, the finding of polymorphisms within IS*900* would suggest that cross-contamination did not occur (11); however, this raises the possibility that some amplicons may not represent *M. avium* subsp. *paratuberculosis*.

Sequence comparisons have further been confounded by the fact that several different IS*900* sequences are deposited in GenBank, with no clear indication of which should be the referent sequence. The original IS*900* sequence (GenBank accession no. X16293) is a 1,451-bp sequence (7) and differs by 4 single-nucleotide polymorphisms from the 15 IS*900* elements of the sequenced strain, *M. avium* subsp. *paratuberculosis* strain K10 (GenBank accession no. NC002944) (10). Additionally, several groups have described unique variants of IS*900* with sequences that differ from the original sequence and from the *M. avium* subsp. *paratuberculosis* strain K10 sequence by one or more single-nucleotide polymorphisms, with a total of eight such variants at the time of this writing (2, 12, 17).

Our goal was to determine the IS*900* sequence conservation in a panel of isolates confirmed to be *M. avium* subsp. *paratuberculosis* by the use of IS*900*-independent markers. The use of strains classified as *M. avium* subsp. *paratuberculosis* by other markers permitted us to overcome the inherent tautology of IS*900* and *M. avium* subsp. *paratuberculosis* (since the former has been used as a proxy for the latter) and formally study the IS*900* sequences from clinical isolates. We selected 23 isolates which reflect a broad host range (including human isolates), broad geographic origins, different genomic clades (16), different *hsp65* sequevars (18), and different molecular fingerprints. The isolates had been cultured and the DNA was extracted by standard methods (16, 19). The isolates were identified as *M. avium* subsp. *paratuberculosis* based on (i) PCR for large sequence polymorphism A8 (LSP^A 8), a region that is specifically missing from *M. avium* subsp. *paratuberculosis* (15), and (ii) *hsp65* sequencing (18). Furthermore, they were determined to be sheep ($n = 10$) or cattle ($n = 13$) types by using a three-primer PCR for LSP^A 20 (which is missing from sheep strains) (16) and by determination of the *hsp65* sequevars (code 5 for cattle, code 6 for sheep types) (18) (Table 1). Most of the isolates were also characterized by restriction fragment length polymorphism analysis with IS*900* and IS*1311* and were determined to bear nonidentical fingerprints.

The isolates thus assembled as well as two negative control isolates (*M. avium* subsp. *avium* strain 104 and a water isolate) were subjected to PCR amplification of the IS*900* sequence. Three sets of primers were used to cover nearly the entire length of IS*900:* IS900L1 (5′-CCT TTC TTG AAG GGT GTT CG-3′) and IS900R1 (3′-GTC TTT GGC GTC GGT C-5′) to amplify the sequence from nucleotide positions 7 to 555, IS900L2 (5′-CAG CGG CTG CTT TAT ATT CC-3′) and IS900R2 (3′-ATG CTG TGT TGG GCG TTA-5′) to amplify the sequence from nucleotide positions 472 to 918, and IS900L3 (5′-CGC CTT CGA CTA CAA CAA GA-3′) and IS900R3 (3′-GGG TGG TAG ACA GCG TGG T-5′) to amplify the sequence from nucleotide positions 739 to 1413 of IS*900*.

Amplification reactions were performed as described previously (15). The PCR products were sequenced on a 3730XL DNA analyzer system in a core facility (McGill University and Génome Québec Innovation Centre), using the PCR primers for sequencing of forward and reverse fragments. Chromato-

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TABLE 1. *M. avium paratuberculosis* isolates studied[a]

Isolate no.[a]	Host	Origin of isolate	LSP^A 20[b]	*hsp65* code	Strain type[c]
K10	Cow	United States	+	5	C
17	Bison	Canada	+	5	C
989	Cow	New Zealand	+	5	C
6024	Cow	New Zealand	+	5	C
316	Cow	United Kingdom	+	5	C
6770B	Deer	New Zealand	+	5	C
6354	Deer	New Zealand	+	5	C
1515	Human	United States (ATCC 43015)	+	5	C
TMC 1613	Cow	United States	+	5	C
1518	Cow	United States (ATCC 19698)	+	5	C
7296	Deer	New Zealand	+	5	C
291679	Human	Canada	+	5	C
4531	Cow	Canada	+	5	C
LN 20	Pig	Canada	−	6	S
4857	Sheep	New Zealand	−	6	S
4873	Sheep	New Zealand	−	6	S
6758	Sheep	New Zealand	−	6	S
506c	Sheep	South Africa	−	6	S
575A	Sheep	South Africa	−	6	S
85/14	Sheep	Canada	−	6	S
P465	Sheep	Iceland	−	6	S
6282	Deer	New Zealand	−	6	S
3579	Deer	New Zealand	−	6	S

[a] Each isolate listed was determined to be *M. avium* subsp. *paratuberculosis* by a three-primer PCR for LSP^A 8 and by sequencing of *hsp65*.

[b] Testing for LSP^A 20 was done by using a three-primer PCR; +, the sequence is present (product size, 197 bp); −, the sequence is missing (product size, 306 bp).

[c] Isolates were qualified as C (cattle type) or S (sheep type) based on the results of LSP^A 20 PCR testing and the *hsp65* sequevars.

grams were manually edited to ensure sequence accuracy and were added to the alignment component of MEGA 3 (9). Sequence comparisons of each IS*900* fragment were performed by BLAST analysis against sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/).

Sequences spanning nucleotide positions 26 to 1400 of IS*900* were obtained for each isolate under study. All 13 isolates of the cattle type gave uniform sequences, with 100% identity to IS*900* elements of the published genome sequence of *M. avium* subsp. *paratuberculosis* strain K10 (GenBank accession no. NC002944) (10), as well as to a sequence obtained by an independent group from a bovine field isolate of *M. avium* subsp. *paratuberculosis* from New Zealand (GenBank accession no. AF305073). Sequences obtained from sheep type isolates exhibited two ambiguities with respect to those of the cattle type, manifested by double peaks on the electropherograms at two separate loci. At position 216, all 10 sheep strains showed a predominant G peak and a smaller A peak, in contrast to cattle strains, for which only an A peak was noted. At position 169, a C was present for all cattle strains, whereas for 5 of the 10 sheep strains a predominant T peak with a smaller C peak was seen at the same locus (Fig. 1). No other ambiguities or differences were noted. This suggests that some copies of IS*900* in sheep strains have undergone polymorphisms at these two loci.

Comparisons of other sequences deposited in NCBI, notably, the original IS*900* sequence (GenBank accession no. X16293) and several others (3, 7), consistently revealed polymorphisms at four different loci in reference to the genome sequence of *M. avium* subsp. *paratuberculosis* strain K10: positions 36 and 37 (a 2-nucleotide gap, corresponding to GC in the sequences of *M. avium* subsp. *paratuberculosis* strain K10), positions 122 and 123 (CG changed to GC in the *M. avium* subsp. *paratuberculosis* K10 sequence), position 726 (a 1-nucleotide gap corresponding to a G in the sequence of *M. avium* subsp. *paratuberculosis* K10), and position 690 (insertion of a G in these sequences compared to the sequence of *M. avium* subsp. *paratuberculosis* K10). Our isolates did not manifest these or other polymorphisms described in unusual isolates from water buffaloes, goats, sheep, and cows (2, 17).

We therefore observed that a small but diverse panel of *M. avium* subsp. *paratuberculosis* isolates had highly uniform IS*900* sequences, and the polymorphisms described in other IS*900* sequences deposited in public databases were not found in our isolates. As many studies have relied solely on the PCR detection of IS*900* to identify *M. avium* subsp. *paratuberculosis*, it is difficult to determine whether these polymorphisms reflect true variants, such as those that have been described in *Mycobacterium* isolates other than *M. avium* subsp. *paratuberculosis* (5, 6), or result instead from technical sequencing errors.

Based on this analysis we propose that future IS*900* sequence comparisons be made in reference to the sequenced strain, *M. avium* subsp. *paratuberculosis* strain K10, shown here to be identical to a broad range of clinical isolates. Furthermore, we suggest that sequence discrepancies in comparison to this reference sequence should prompt further investigations and should be interpreted as suggestive of a *Mycobacterium* organism other than *M. avium* subsp. *paratuberculosis* until proven otherwise through conventional microbiologic or alternate genetic methods.

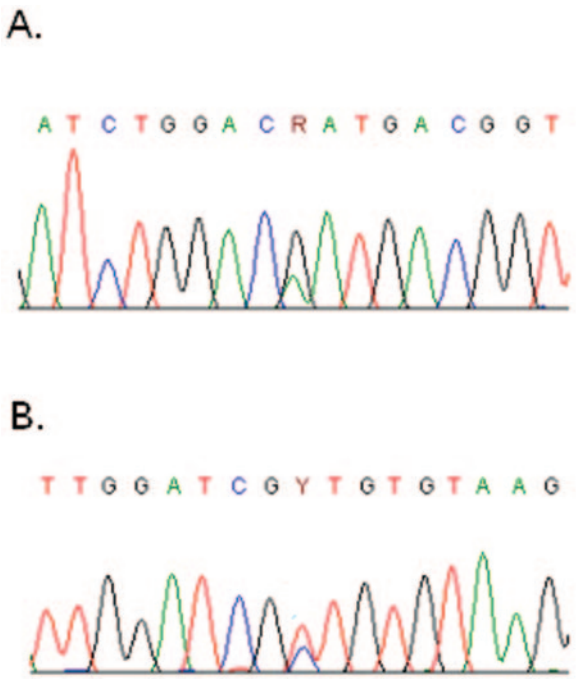

FIG. 1. Sequence electropherogram of two sections of the IS*900* element from *M. avium* subsp. *paratuberculosis* of the sheep type showing sequence ambiguities (depicted by the codes R and Y) at base pair position 216 (A) and base pair position 169 (B).

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