Insertion Sequence IS900 Revisited

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Many studies investigating Mycobacterium avium subsp. paratuberculosis in Crohn’s disease have used molecular detection of IS900 in clinical samples, but some have described polymorphisms in IS900 as variants of this organism. Analysis of 23 M. avium subsp. paratuberculosis isolates revealed that IS900 is highly conserved, with only two sequencings distinguishing sheep and cattle lineages. Amplification of IS900-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, IS900, 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 IS900 element (5, 6), and second, there is the risk of laboratory cross-contamination, which is of special concern in PCR-based assays.

Sequencing of IS900 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 IS900 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 IS900 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 IS900 sequences are deposited in GenBank, with no clear indication of which should be the referent sequence. The original IS900 sequence (GenBank accession no. X16293) is a 1,451-bp sequence (7) and differs by 4 single-nucleotide polymorphisms from the 15 IS900 elements of the sequenced strain, M. avium subsp. paratuberculosis strain K10 (GenBank accession no. NC002944) (10). Additionally, several groups have described unique variants of IS900 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 IS900 sequence conservation in a panel of isolates confirmed to be M. avium subsp. paratuberculosis by the use of IS900-independent markers. The use of strains classified as M. avium subsp. paratuberculosis by other markers permitted us to overcome the inherent tautology of IS900 and M. avium subsp. paratuberculosis (since the former has been used as a proxy for the latter) and formally study the IS900 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 sequences (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^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^8 (which is missing from sheep strains) (16) and by determination of the hsp65 sequences (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 IS900 and IS1311 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 IS900 sequence. Three sets of primers were used to cover nearly the entire length of IS900: IS900L1 (5'-CCT TTC TTG AAG GTT GGT CG-3') and IS900R1 (3'-GTC TTT TTG GCC GTC GTT CG-5') to amplify the sequence from nucleotide positions 7 to 555; IS900L2 (5'-CAG CGG CTG CTG 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 GA-3') and IS900R3 (3'-GGG TGG TGG TAG ACA GCG TGG T-5') to amplify the sequence from nucleotide positions 739 to 1413 of IS900.

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-
TABLE 1.\textit{M. avium paratuberculosis} isolates studied$^a$

| Isolate no.$^a$ | Host    | Origin of isolate | LSP$^b$ A20$^b$ | 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     | +               | 5          | C              |
| (ATCC 43015)    |         |                   |                 |            |                |
| TMC 1613        | Cow     | United States     | +               | 5          | C              |
| 1518            | Cow     | United States     | +               | 5          | C              |
| (ATCC 19698)    |         |                   |                 |            |                |
| 7926            | 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 \textit{M. avium subsp. paratuberculosis} by a three-primer PCR for LSP$^b$ A8 and by sequencing of hsp65.

$^b$ Testing for LSP$^b$ A20 was done by using a three-primer PCR: +, the sequence is present (product size, 197 bp); −, the sequence is missing (product size, 306 bp).

$^c$ Sequences were edited to ensure sequence accuracy and were added to the alignment component of MEGA 3 (9). Sequence comparisons 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 IS900 were obtained for each isolate under study. All 13 isolates of the strain gave uniform sequences, with 100% identity to IS900 elements of the published genome sequence of \textit{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 \textit{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 IS900 in sheep strains have undergone polymorphisms at these two loci.

Comparisons of other sequences deposited in NCBI, notably, the original IS900 sequence (GenBank accession no. X16293) and several others (3, 7), consistently revealed polymorphisms at four different loci in reference to the genome sequence of \textit{M. avium subsp. paratuberculosis} strain K10: positions 36 and 37 (a 2-nucleotide gap, corresponding to GC in the sequences of \textit{M. avium subsp. paratuberculosis} strain K10), positions 122 and 123 (CG changed to GC in the \textit{M. avium subsp. paratuberculosis} K10 sequence), position 726 (a 1-nucleotide gap corresponding to a G in the sequence of \textit{M. avium subsp. paratuberculosis} K10), and position 690 (insertion of a G in these sequences compared to the sequence of \textit{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 \textit{M. avium subsp. paratuberculosis} isolates had highly uniform IS900 sequences, and the polymorphisms described in other IS900 sequences deposited in public databases were not found in our isolates. As many studies have relied solely on the PCR detection of IS900 to identify \textit{M. avium subsp. paratuberculosis}, it is difficult to determine whether these polymorphisms reflect true variants, such as those that have been described in \textit{Mycobacterium} isolates other than \textit{M. avium subsp. paratuberculosis} (5, 6), or result instead from technical sequencing errors.

Based on this analysis we propose that future IS900 sequence comparisons be made in reference to the sequenced strain, \textit{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 \textit{Mycobacterium} organism other than \textit{M. avium subsp. paratuberculosis} until proven otherwise through conventional microbiologic or alternate genetic methods.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Sequence electropherogram of two sections of the IS900 element from \textit{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).}
\end{figure}
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