Comparative IS900 and IS1311 Direct Fecal *Mycobacterium Avium* Subspecies *Paratuberculosis* Nested PCR Tests: Significance of Disparities

Williams JE, Pinedo PJ, Monif GRG

University of Florida, College of Veterinary Medicine
Department of Infectious Diseases and Pathobiology, Gainesville, FL

ABSTRACT
To challenge the hypothesis of genomic polymorphism, two direct fecal nested polymerase chain reaction (PCR) tests based upon the IS900 and the IS1311 insertion sequences were constructed and tested in parallel in three United States Department of Agriculture (USDA) Laboratory Certification tests.

The sensitivities for P90-P91 and J1-J2 IS900 direct and nested primers were 21.7% and 76.7% whereas those for the IS1-IS2 and IS3-IS4 primers were 38.3% and 86.7%. The ability of IS1311 base insertion sequence primers to better identify Map in the USDA laboratory certification tests over IS900 base insertion sequence primers argues for the existence of a degree of genetic polymorphism among culture-positive isolates of Map used in USDA’s laboratory certification testing.

INTRODUCTION
*Mycobacterium avium* subspecies *paratuberculosis* is theorized to have evolved from *Mycobacterium avium* subsp. *avium* (Ma) (Frothingham, 1999; Turenne et al., 2007). Map and Ma, by genetic criteria, are classified as subsets of the same species (Harris and Barletta, 2001; Thorel et al., 1990). Research on diagnosis and epidemiological findings relative to Map has often denied the existence of closely related Map phenotypic variants more closely related to MA (Cousins et al., 1999). Some mycobacteria, more Ma-like than Map-like, contain the IS900 insertion sequence (Bolske and Johansson, 2002; Cousins et al., 1999; England et al., 2002).

IS1311 is present in the vast majority of pathogenic mycobacterium. A long evolutionary time span is suggested by the presence of mutations in some of the IS1311 elements (Whittington et al., 1998). Genomic polymorphism is to be anticipated within species evolution. IS1311 is present in Ma and Map (9). Primers based upon the IS1311 insertion sequence that identify Ma variants and Map are encompassed in the direct and nested fecal FecaMap® patented primers.

The purpose of this study was to compare the respective abilities of IS1311- and IS900-based PCR primers for identifying Map isolates within three USDA Laboratory Certification Tests.

MATERIALS AND METHODS

**Population studied:** The fecal samples were obtained from two dairy herds that participated in the Florida Johne’s Disease Dairy Herd Prevention Program. The number of fecal samples analyzed was determined by the number in which nested PCR data was available from the Diagnostic Laboratory of the Department of Infectious Diseases, University of Florida College of Veterinary Medicine.

**Fecal culturing:** The fecal culture testing using the Trek® Diagnostic System was done at Purdue University School of Veterinary Medicine in accordance with the manufacturer’s instructions.

**Real-time Map PCR testing:** The direct fecal PCR testing using the Tetracore® Map Diagnostic System were done at Purdue University School of Veterinary Medicine in accordance with the manufacturer’s instructions.
Direct fecal nested Map PCR testing: Direct fecal nested Map PCR tests were done at University of Florida College of Veterinary Medicine using the FecaMap® system in accordance with the manufacturer’s instructions.

Statistical analysis: The Fisher’s Exact Test was used to test whether there was any non-random association between variables of the two direct fecal, nested Map PCR test results and provided culture results. Kappa coefficient, sensitivity was estimated using Win Episcope 2.0 software (Win Episcope 2.0). Ninety-five percent confidence intervals (CI) were constructed for all estimates.

RESULTS
The direct IS\textsubscript{900} and IS\textsubscript{1311} PCR primers had a sensitivity of 21.7 and 38.3\% respectively; whereas the corresponding nested PCR primers had sensitivities of 76.6\% and 86.7\% (Table 1). The P90-P91 primers did not identify the Ma -spiked culture as being positive whereas the direct IS\textsubscript{1311}, nested IS\textsubscript{900} and IS\textsubscript{1311} primers correctly identified 2 of the 3 M. avium spiked cultures as being positive respectively.

The demonstration that the IS\textsubscript{900} nested primers identified the M. avium-spiked fecal specimens in a manner comparable to the IS\textsubscript{1311} nested primers necessitated excluding these samples in the overall calculation of comparative sensitivity. The spiked fecal samples were deleted in the statistical comparison of direct IS\textsubscript{900} and IS\textsubscript{1311} primers. The ability of the nested IS\textsubscript{900} PCR primers to extend the test sensitivity to M. avium was also a confound variable in the comparison of the nested IS\textsubscript{900} vs. IS\textsubscript{1311} nested primers (Table 1).

Table 1. Statistical comparison of IS\textsubscript{900} versus IS\textsubscript{1311} direct and nested primers on fecal specimens with three USDA Laboratory Certification Tests

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<tr>
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<th>IS1-IS2</th>
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<tr>
<td><strong>Sensitivity</strong></td>
<td>21.7% (13+/46-) 38.3%(23+/37-)</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>100% (19/19)</td>
</tr>
<tr>
<td><strong>Kappa Coefficient</strong></td>
<td>0.11</td>
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<th>IS1-IS2/IS3-IS4</th>
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<tr>
<td><strong>Sensitivity</strong></td>
<td>76.7% (46+/18-)</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>94.7% (18/19)</td>
</tr>
<tr>
<td><strong>Kappa Coefficient</strong></td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Interpretation</strong></td>
<td>good agreement</td>
</tr>
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DISCUSSION
Given that the IS\textsubscript{1311} based primers, IS1/IS2, identify only 6-8 copies where as the P90/P91 primers based upon the IS\textsubscript{900} sequence identify 14-18 copies, there should have been no reason to anticipate that the IS\textsubscript{1311} base primers would exhibit superior sensitivity unless the sequences covered had greater antigenic representation.

As expected, fecal specimens spiked with M. avium were not detected by P90-P91 insertion sequence, but were detected by the IS\textsubscript{1311} direct primers. What was not anticipated was that the nested IS\textsubscript{900} based J1/J2 in two of the three Ma spiked samples as well as the IS\textsubscript{1311} nested IS3/IS4 primers identified M. avium.

Clinical relevance is inferred by a herd study of 341 dairy cows in which IS\textsubscript{1311}-basedFecaMap® identified a non-IS\textsubscript{900} mycobacterium from a cow whose feces demonstrated heavy shedding and tested positive with the Tetracore® hspX PCR test and who was an early cull (Williams et al., 2009).
CONCLUSION
The ability of 1311 insertion sequence direct primers to better identify Map in the USDA laboratory certification tests than the IS900 insertion sequence direct primers indicates greater antigenic representation for the 6 to 8 copies covered by the IS1311 insertion sequence

REFERENCES
Whittington R, Marsh I, Chow E, Cousins D, 1998. Polymorphism in IS1311, an insertion sequence common to Mycobacterium avium subsp. paratuberculosis, can be used to distinguish between and within these species. Mol. Cell. Probes12:349-358.