A new marker IS1311 L2 PCR-REA for identification of ‘Indian Bison’ type
*Mycobacterium avium* subspecies *paratuberculosis*

J S Sohal1,2, S V Singh1*, P K Singh1,3, AV Singh1,3 and Naveen Kumar1

1Microbiology Laboratory, Central Institute for Research on Goats (CIRG), Makhdoom, Mathura 281 122, India
2Canadian Food Inspection Agency, St. Hyacinthe, Quebec, Canada
3National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Tajganj, Agra 282 001, India

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Previously, a new biotype (Indian Bison type S5) of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), genetically different from so far reported MAP genotypes, was identified. This biotype is widely distributed in different geographical regions in India among various host species including humans. Due to deletion of TG at 64th and 65th of IS1311 element at locus 2 (L2), there is a loss of BsaJI restriction site in Indian Bison type MAP S5. However, in non-Indian MAP (cattle type/sheep type/US Bison type), the site was intact. Taking advantages of these variations in the genome, authors optimized a new marker IS1311 L2 PCR-REA (Restriction Endonuclease Assay), capable of distinguishing Indian Bison type MAP S5 from other biotypes. To test the field applicability, newly optimized assay was applied on 66 DNA samples (27 from cultural isolates and 39 from clinical specimens) where it successfully distinguished the MAP of Indian Bison type from other MAPs. Hence, this assay can be used to characterize MAP isolates in future molecular epidemiology investigations. Also the applicability of this assay on clinical DNA samples gives us freedom to have knowledge of the infecting MAP genotype prior to culture.

**Keywords:** Diagnosis, Indian Bison type S5, molecular marker, *Mycobacterium avium* subspecies *paratuberculosis*

**Introduction**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP), the cause of paratuberculosis or Johne’s disease (JD), is an important pathogen of animals with serious zoonotic concerns1. Disease is worldwide in distribution and severely affects the animal industry and may also pose great threat to human population as MAP may escape pasteurization2. Chemotherapy is not recommended for treating JD and worldwide used ‘test and cull’ policy has failed to control the infection; rather burden of the disease has increased over the years3. Vaccination is the only measure to show some potential in controlling the infection3 with varying efficacy depending on the strain of MAP and species involved4. Hence, knowledge of the infecting genotypes in endemic regions becomes critically important to design disease control measures.

Previous studies by our group identified a new genotype of MAP (India Bison type S5) as predominant cause of infection in wide range of host species including cattle, goat, sheep, buffalo, bison, hog deer, rabbits, blue bull and humans in India5,6. Comparative genomics studies identified that native Bison type isolates have accumulated genomic variations in terms of genetic rearrangements, duplications, deletions and single nucleotide polymorphisms (SNP). Hence, Sohal et al7 have proposed a new nomenclature ‘Indian Bison type’ for these isolates. These studies also identified a molecular signature capable of differentiating Indian Bison type MAP from other MAP isolates8. In Indian Bison type MAP S5, there is a deletion of two base pairs (‘TG’) at 64th and 65th position of IS1311 element at locus 2 (L2) compared with original complement of IS1311 sequence found in other MAP isolates8. Taking advantage of this molecular signature, in the present study, authors have designed and validated a marker IS1311 L2 PCR-REA (Restriction Endonuclease Assay) capable of discriminating the Indian Bison type MAP from other MAP isolates.

**Materials and Methods**

**Primer Design Strategy for Amplification of IS1311 Element at Locus 2**

Strategy to amplify IS1311 element at L2 has previously been described in detail7,8. Briefly, two primers were designed (Fig. 1), one 5’ end
Fig. 1—Primer design strategy to amplify IS1311 L2 (LP, Locus specific primer; IP, Internal primer).

Genomic location specific primer (specific for L2) and other IS1311 internal primer using MAP K10 genome sequence (GenBank Acc. No. NC002944). Online Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design the primers. Primer validation and mapping was done using online OligoAnalyzer (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer) and Dnannotator software (http://sky.bsd.uchicago.edu/primer_compact.htm), respectively.

PCR Optimization

Details on the PCR optimization have been previously described7,8.

Design of Marker IS1311 L2 PCR-REA

IS1311 sequences for L2 from Indian Bison type and non-Indian MAP were subjected to offline restriction analysis using BioEdit v7.0.0 software to have idea of the change in the profile of restriction pattern due to deletion of ‘TG’ in sequence of Indian Bison type. Subsequently, a restriction map was generated and loss of restriction sites for a particular restriction enzyme (RE) was recorded.

Optimization of Marker IS1311 L2 PCR-REA

Based on the results of the above step, a RE was selected capable of discriminating the presence/absence of TG in IS1311 L2 sequence. PCR amplifications were carried out as described previously7,8. Briefly, PCR reaction mix contained primers (10 pmoles), Taq polymerase (1 unit), MgCl2 (1.5 mM), dNTPs (0.2 mM), Buffer 10× (2.5 µL) and template (0.5 ng) in a final volume of 25.0 µL. Cycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C at 30 sec, and extension at 72°C for 1 min, and final extension at 72°C for 7 min. PCR products were visualized on 1.5% agarose gel.

Amplification products of expected size (~425 bp) were further digested with BsaJ1 (Fermentas, USA) restriction enzyme (at 37°C) as per manufacturer instructions. The digested products were analyzed on 4.0% SFR agarose gel (Ameresco, USA).

Validation PCR Amplifications for IS1311 L2 on Field Samples and Marker IS1311 L2 PCR-REA

To further validate the marker IS1311 L2 PCR-REA, 27 genomic DNA specimens from MAP cultural isolates and 39 DNA specimens from clinical materials (originated from blood, feces, milk and tissues of different host species previously characterized as Indian Bison type S5 and were available in authors laboratory) were used for analysis.

Results and Discussion

Genomic differences of Bison type isolates of Indian origin (Indian Bison type) with other MAP isolates have been previously described and characterized5,7,10. These variations include genetic rearrangements, duplication events, gene deletions, SNPs and locus polymorphisms. Phenotypic differences can be correlated with the genetic differences. Indian Bison type is highly pathogenic11 and its isolation (and subsequent subculture) is difficult as compared to Bison type isolates described in US12.

In the present study, BsaJ1 enzyme was selected on the basis of in silico restriction map analysis of targeted genomic region (IS1311 element at L2) of MAP. Due to deletion of the TG at 64th and 65th of IS1311 element at L2, a loss of BsaJ1 restriction site in Indian Bison type was observed; however, (cattle type/ sheep type/ US Bison type) the site was intact in non-Indian MAP (Fig. 2). IS1311 L2 was successfully amplified for MAP S5 (Indian Bison type) and for non-Indian MAP, the amplification yielded ~425 bp product. Further, the amplified product was subjected to restriction digestion with BsaJ1 that clearly distinguished the Indian Bison type from non-Indian MAP (Fig. 3). Thus, restriction profile and band pattern for marker IS1311 L2 PCR-REA were able to clearly distinguish the Indian Bison type and non-Indian MAP. Restriction digestion of 425 bp product (belonging to IS1311 element at L2) with BsaJ1 generated six digestion products (1, 15, 34, 67, 78 & 230 bp) for non-Indian MAP, of which four digestion products were detectable in the gel (34, 67, 78 & 230 bp). However, for Indian Bison type, restriction digestion generated five products (1, 15, 67, 78 & 262 bp), of which three were detectable in the gel (67, 78 & 262 bp).

The simple PCR-REA method is known to have advantage for detection of MAP, which can not be
easily cultured due to different nutritional requirements, and thus prevents false negative results. It can also be applied on clinical DNA extracted from tissues, milk, feces and blood to have knowledge of the infecting MAP genotype and then culture regime can be designed to get rid of false negative results. Our PCR-REA method successfully discriminated the Indian Bison type isolates from non-Indian MAP. Three detectable bands were observed for Indian Bison type isolates (67, 78 & 262 bp), while the fourth band (34 bp), which is the characteristic of non-Indian isolates, was absent from the isolates. All specimen DNA samples from different animal species, previously characterized to be infected with Bison type MAP, gave detectable product for IS1311 L2.

In deciding the disease control measures (treatment & vaccination), knowledge of the infecting strain is also important. For paratuberculosis, the vaccination appears to be the most successful control measure. However, efficacy of a particular vaccine varies in different geographical locations. Moreover, vaccines containing antigen from local strains are found to be more successful for that particular region. Hence, IS1311 L2 PCR-REA can be used to have knowledge of infecting strains before finalizing the control strategy. In summary, polymorphisms in IS1311 element at locus 2 are susceptible to PCR-REA. These variations have been used to optimize marker IS1311 L2 PCR-REA capable of distinguishing Indian Bison type isolates from other MAP isolates. To our knowledge, the present report is the first description of this efficient and fast molecular tool that is able to discriminate Indian Bison type from other MAP isolates.

Fig. 2—Restriction map for IS1311 element at L2 for ‘Indian Bison type’ of MAP (S-5 strain) and non-Indian MAP (K-10 strain; fully sequenced reference ‘cattle type US strain’).

Fig 3—Optimization of marker IS1311 PCR-REA using standard DNA of Indian Bison type (MAP S5) and non-Indian MAP (MAP K10) on 4% agarose SFR gel. [Lane M, 100 bp; lane 1, MAP S5; & lane 2, MAP K10]

References


