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A new marker IS*1311* L2 PCR-REA for identification of 'Indian Bison' type *Mycobacterium avium* subspecies *paratuberculosis*

J S Sohal[1,2], S V Singh[1]*, P K Singh[1,3], AV Singh[1,3] and Naveen Kumar[1]

[1]Microbiology Laboratory, Central Institute for Research on Goats (CIRG), Makhdoom, Mathura 281 122, India

[2]Canadian Food Inspection Agency, St. Hyacinthe, Quebec, Canada

[3]National 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 IS*1311* element at locus 2 (L2), there is a loss of *BsaJ*I 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 IS*1311* 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 concerns[1]. Disease is worldwide in distribution and severely affects the animal industry and may also pose great threat to human population as MAP may escape pasteurization[2]. 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 years[3]. Vaccination is the only measure to show some potential in controlling the infection[3] with varying efficacy depending on the strain of MAP and species involved[4]. 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 India[5,6]. 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 al*[7] 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 isolates[8]. In Indian Bison type MAP S5, there is a deletion of two base pairs ('TG') at 64th and 65th position of IS*1311* element at locus 2 (L2) compared with original complement of IS*1311* sequence found in other MAP isolates[8]. Taking advantage of this molecular signature, in the present study, authors have designed and validated a marker IS*1311* 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 IS*1311* Element at Locus 2

Strategy to amplify IS*1311* element at L2 has previously been described in detail[7,8]. Briefly, two primers were designed (Fig. 1), one 5′ end

*Author for correspondence:
Tel: +91-565-2763260 extn 205; Fax: +91 565 2763246
E-mail: shoorvir.singh@gmail.com

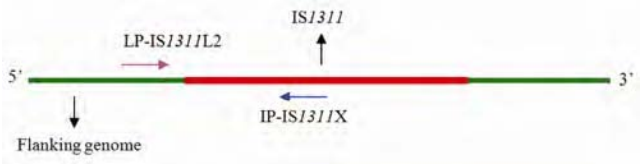

Fig. 1—Primer design strategy to amplify IS*1311* L2 (LP, Locus specific primer; IP, Internal primer).

genomic location specific primer (specific for L2) and other IS*1311* 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 described[7,8].

Design of Marker IS*1311* L2 PCR-REA

IS*1311* 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 IS*1311* L2 PCR-REA

Based on the results of the above step, a RE was selected capable of discriminating the presence/absence of TG in IS*1311* L2 sequence. PCR amplifications were carried out as described previously[7,8]. Briefly, PCR reaction mix contained primers (10 pmoles), *Taq* polymerase (1 unit), $MgCl_2$ (1.5 m*M*), dNTPs (0.2 m*M*), 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 *BsaJ*I (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 IS*1311* L2 on Field Samples and Marker IS*1311* L2 PCR-REA

To further validate the marker IS*1311* 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 characterized[5,7-10]. 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 pathogenic[11] and its isolation (and subsequent subculture) is difficult as compared to Bison type isolates described in US[12].

In the present study, *BsaJ*I enzyme was selected on the basis of *in silico* restriction map analysis of targeted genomic region (IS*1311* element at L2) of MAP. Due to deletion of the TG at 64th and 65th of IS*1311* element at L2, a loss of *BsaJ*I 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). IS*1311* 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 *BsaJ*I that clearly distinguished the Indian Bison type from non-Indian MAP (Fig. 3). Thus, restriction profile and band pattern for marker IS*1311* L2 PCR-REA were able to clearly distinguish the Indian Bison type and non-Indian MAP. Restriction digestion of 425 bp product (belonging to IS*1311* element at L2) with *BsaJ*I 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

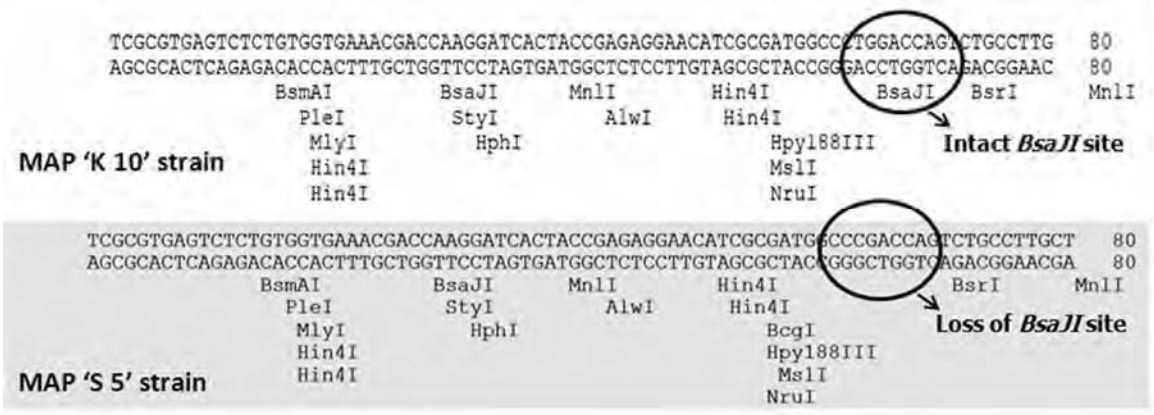

Fig. 2—Restriction map for IS*1311* element at L2 for 'Indian Bison type' of MAP (S-5 strain) and non-Indian MAP (K-10 strain; fully sequenced reference 'cattle type US strain').

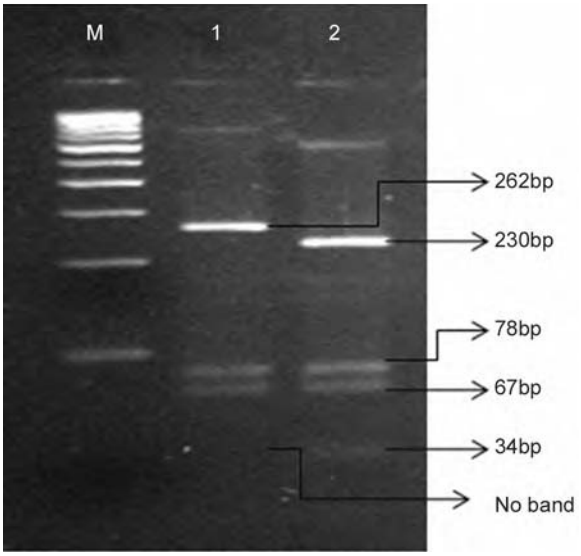

Fig 3—Optimization of marker IS*1311* PCR-REA using standard DNA of Indian Bison type (MAP S5) and non-Indian MAP (MAP K10) on 4% agarose SFR gel. [Lane M, 100 bp; lane 1, MAP S5; & lane 2, MAP K10]

easily cultured due to different nutritional requirements, and thus prevents false negative results[12]. 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 IS*1311* 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[4]. Moreover, vaccines containing antigen from local strains are found to be more successful for that particular region. Hence, IS*1311* L2 PCR-REA can be used to have knowledge of infecting strains before finalizing the control strategy. In summary, polymorphisms in IS*1311* element at locus 2 are susceptible to PCR-REA. These variations have been used to optimize marker IS*1311* 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.

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