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Application of IS*1311* locus 2 PCR-REA assay for the specific detection of 'Bison type' *Mycobacterium avium* subspecies *paratuberculosis* isolates of Indian origin

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***Background & objectives*: Of the three major genotypes of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), 'Bison type' is most prevalent genotype in the domestic livestock species of the country, and has also been recovered from patients suffering from Crohn's disease. Recently, a new assay based on IS*1311* locus 2 PCR- restriction endonuclease analysis (REA) was designed to distinguish between 'Indian Bison type' and non-Indian genotypes. The present study investigated discriminatory potential of this new assay while screening of a panel of MAP isolates of diverse genotypes and from different geographical regions.**

***Methods*: A total of 53 mycobacterial isolates (41 MAP and 12 *Mycobacterium* other than MAP), three MAP genomic DNA and 36 MAP positive faecal DNA samples from different livestock species (cattle, buffaloes, goat, sheep and bison) and geographical regions (India, Canada, USA, Spain and Portugal) were included in the study. The extracted DNA samples (n=92) were analyzed for the presence of MAP specific sequences (IS*900*, IS*Mav* 2 and *HspX*) using PCR. DNA samples were further subjected to genotype differentiation using IS*1311* PCR-REA and IS*1311* L2 PCR-REA methods.**

***Results:* All the DNA samples (except DNA from non-MAP mycobacterial isolates) were positive for all the three MAP specific sequences based PCRs. IS*1311* PCR-REA showed that MAP DNA samples of Indian origin belonged to 'Bison type'. Whereas, of the total 19 non-Indian MAP DNA samples, 2, 15 and 2 were genotyped as 'Bison type', 'Cattle type' and 'Sheep type', respectively. IS*1311* L2 PCR-REA method showed different restriction profiles of 'Bison type' genotype as compared to non-Indian DNA samples.**

***Interpretation & conclusions*: IS*1311* L2 PCR-REA method successfully discriminated 'Indian Bison type' from other non-Indian genotypes and showed potential to be future epidemiological tool and for genotyping of MAP isolates.**

Key words Genotypes - Indian bison type - IS*1311* L2 PCR-REA - *Mycobacterium avium* subspecies *paratuberculosis* - paratuberculosis

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Mycobacterium avium subspecies *paratuberculosis* (MAP) is a cause of chronic granulomatous enteritis and lymphadinits (paratuberculosis or Johne's disease) in different species of animals[1] including primates[2]. Johne's disease (JD) is responsible for inflicting huge economic losses to the livestock industry worldwide due to reduced milk production, increased premature culling and mortality and reduced fertility[3]. MAP-infected animals (sub-clinical and clinical) shed live bacilli in faeces and milk, thereby increasing the risk of transmission to newborn and other susceptible animals. Recently MAP has also been shown to be associated with Crohn's disease[4,5] in human beings. MAP escapes standard pasteurization temperatures, and, therefore, presence of live MAP bacilli in the milk supplies is of great concern and poses potential risk to human population[6,7].

Control of MAP infection is the priority of developed countries to secure animal productivity and to reduce chances of human exposure. Inspite of the availability of sensitive tests[8,9], 'Test and cull' method is not effective for the control of disease in livestock herds/flocks. Vaccination is considered as the method of choice for the control of JD in animals[10,11]. However, efficacy of vaccines depends on the genotype of candidate strain used[11,12]. Therefore, knowledge of the genotypes infecting domestic livestock species is critical for designing disease control strategies.

Based on IS*1311* polymerase chain reaction-restriction endonuclease analysis (PCR-REA) method, MAP isolates have been grouped into three genetically distinct genotypes ('Cattle type', 'Sheep type' and 'Bison type)[13,14]. Studies reported host preferences of these MAP geno-groups, however, host adaptation is not absolute and inter-species sharing of MAP genotypes has been reported[15]. 'Bison type' genotype was first reported from wild bison of Montana, USA[13], and later similar genotypes have been reported as major genotypes infecting domestic livestock, wild ruminants and human population in India[16]. Recently, 'Bison type' genotype has also been reported from other regions of Asia (Korea)[17] and Africa (Uganda)[18]. We have earlier identified genomic variations in terms of genetic rearrangements, in-del polymorphisms and locus polymorphisms in 'Bison type' genotype of Indian origin when compared with genotypes reported from other parts of the world[19,20]. Therefore, new nomenclature 'Indian Bison type' was assigned to 'native Bison type' genotype[20]. Another molecular signature (sequence variations of native genotype) was deletion of two base pairs (TG) at 64th and 65th positions of IS*1311* element particularly at locus 2 as compared to non-Indian isolates[19,20]. Taking the advantage of this molecular signature, a new IS*1311* locus 2 specific PCR-REA (IS*1311* L2 PCR-REA) assay was optimized for discrimination of 'Indian Bison type' from other isolates[21]. However, validation of this newly optimized assay was not done on a large panel of MAP isolates from different geographical regions of the world. The present study was carried out to investigate applicability of IS*1311* L2 PCR-REA assay on samples of diverse genotypes (Cattle type, Sheep type and Bison type) and different geographical regions (India, Canada, USA, Spain and Portugal).

Material & Methods

A total of 53 mycobacterial isolates (41 MAP and 12 mycobacteria other than MAP), three MAP genomic DNA and 36 MAP faecal DNA samples from different hosts and geographical regions were processed for the investigation of genomic variations using IS*1311* PCR-REA and IS*1311* L2 PCR-REA. The study was performed between August 2010 and July 2013 at the Department of Microbiology and Molecular Biology, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (NJIL&OMD) located in Agra, Uttar Pradesh, India. Of the 41 MAP isolates, 25, 3, 2 and 11 were from India, Canada, Spain and USA, respectively (Table). Three MAP genomic DNA samples of Portugal origin (provided by Dr Maria Gazouli, School of Animal Science, Agricultural University, Athens, Greece) were also included in this study.

MAP isolates (n=25) and faecal DNA samples (n=36) of Indian origin were collected from epidemiological studies conducted in Agra, Mathura, Jhansi and hilly regions of northern India (data not shown). To check the specificity of IS*1311* L2 PCR-REA, a total of 12 mycobacterial isolates other than MAP [*M. smegmatis, M. vaccae, M. marinum*, *M. chelonae, M. flavescens, M.fortuitum, M. kansasii, M. bovis, M. bovis (BCG), M. avium, M.gastri, M. indicus pranii*] were obtained from mycobacterial repository, NJIL&OMD, Agra. All the mycobacterial isolates (n=53) were subjected to isolation of genomic DNA as per the method of van Soolingen *et al*[22]. DNA samples recovered from isolates and faecal samples were subjected to IS*900*[23], IS*Mav2*[24] and *HspX*[25] specific PCRs for the molecular identification of MAP.

IS1311 PCR-REA (Genotyping of isolates/DNA): Genotyping of each MAP DNA obtained from

Table. Genotyping of MAP isolates/ DNA using IS*1311* PCR-REA and locus 2 specific IS*1311* PCR-REA method

Country	Isolates / DNA	Host	Source	No. of isolates/ DNA	Genotypes (IS1311 PCR-REA)	No. and size of detectable band (IS*1311* L2 PCR-REA)
India	MAP isolates	Cattle	Faeces	5		
		Buffaloes	Faeces	1		
		Goat	Faeces	13	Bison type	Three (67, 78 & 262 bp)
			Intestine	4		
		Sheep	Faeces	2		
	Faeces MAP DNA*	Cattle	Faeces	16	Bison type	
		Buffaloes	Faeces	2		
		Goat	Faeces	10		
		Sheep	Faeces	8		
Canada	MAP isolates	Cattle	Faeces	1	Cattle type	
		Sheep	Faeces	1	Sheep type	
		Bison	Faeces	1	Bison type	
USA	MAP isolates	Cattle	Faeces	5	Cattle type	
			Intestine	2	Bison type-1, Cattle type-1	
			Lymph node	1	Cattle type	Four (34, 67, 78 & 230 bp)
		Goat	Mesenteric lymph node	1	Cattle type	
			Lymph node	2	Cattle type	
Spain	MAP isolates	Cattle	Faeces	1	Cattle type	
		Sheep	Ileocecal lymph node	1	Sheep type	
Portugal	MAP genomic DNA	Sheep	Culture isolates recovered from faeces	3	Cattle type	

*Of these faecal DNA samples, 13 belonged to hilly regions of Himachal Pradesh, remaining 23 were from Agra, Mathura and Firozabad regions of Uttar Pradesh (north India)

mycobacterial isolates or faecal samples was carried out by IS*1311* PCR-REA method[14]. Briefly, reaction was carried out in 30 µl volume, containing 20 µl positive IS*1311* PCR product, 3 µl 10X buffer (Fermentas, USA), 2 units of each endonuclease *HinfI* and *MseI* (Fermentas). Reaction mixture was incubated at 37^{0}C for 1.5 h. Band patterns were visualized after electrophoresis on 4 per cent high resolution agarose gel stained with ethidium bromide and genotype profile interpretation was done as described by Sevilla *et al*[14].

IS1311 locus 2 PCR-REA (Sub-genotyping of MAP isolates): IS*1311* L2 PCR-REA optimized by Sohal *et al*[21] was used to characterize all MAP isolates/faecal DNA included in this study. Firstly, locus 2 of IS*1311* was amplified using the specific primers (P1: CACCAACCATGCAGAGGTAA; P2: GGAATCCGCAACTCCAAAT) and then amplicons were subjected to restriction digestion using *BsaJ1*. PCR reaction mix contained primers (10 pmoles), *Taq* polymerase (1 unit), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), buffer 10X (2.5 µl) and template DNA (5 ng) in a final volume of 25.0 µl at thermocycler conditions: denaturation at 95 °C for 5 min followed by 40 cycles of de-naturation at 95 °C for 30 sec, annealing at 55 °C at 30 sec, extension at 72 °C for 1 min followed

final extension at 72 °C for 7 min. PCR products were visualized on 1.5 per cent agarose gel. Amplification products (~425 bp) were digested with *BsaJI* enzyme for 2 h at 37°C and band pattern was observed on 4 per cent agarose gel.

The study protocol was approved by the institute's ethical committee.

Results

MAP specific IS*900*, IS*Mav* 2 and *HspX* PCRs showed presence of MAP DNA in all samples (Figs. 1-3) except those extracted from isolates of mycobacteria other than MAP.

IS1311 PCR-REA (Genotyping): Of the 80 MAP-DNA samples, 63, 15 and 2 showed the pattern of 'Bison type', 'Cattle type' and 'Sheep type' genotypes, respectively (Table, Fig. 4). All MAP DNA samples of Indian origin belonged to 'Bison type'. Of the three isolates of Canada, one each was genotyped as 'Cattle type', 'Sheep type' and 'Bison type'. Of the 11 isolates of USA origin, one and 10 were genotyped as 'Bison type' and Cattle type, respectively. Of the two isolates from Spain, one was 'Cattle type' and another was identified as 'Sheep type', whereas, all three isolates of Portugal origin were identified as 'Cattle type' (Table).

IS1311 L2 PCR-REA (Sub-genotyping): Restriction profile and band pattern for marker IS*1311* L2 PCR-REA distinguished 'Bison type' DNA of Indian origin from all non-Indian MAP samples (Table, Fig. 5). After restriction digestion of ~425 bp product (belonging to IS*1311* element at locus 2) with *BsaJI* enzyme, four digestion products were visualized in the 4 per cent high resolution agarose gel (34, 67, 78 & 230 bp) for non-Indian MAP. However, digestion of IS*1311* locus 2 amplicon of MAP DNA of Indian origin resulted into only three detectable bands in the gel (67, 78 & 262 bp).

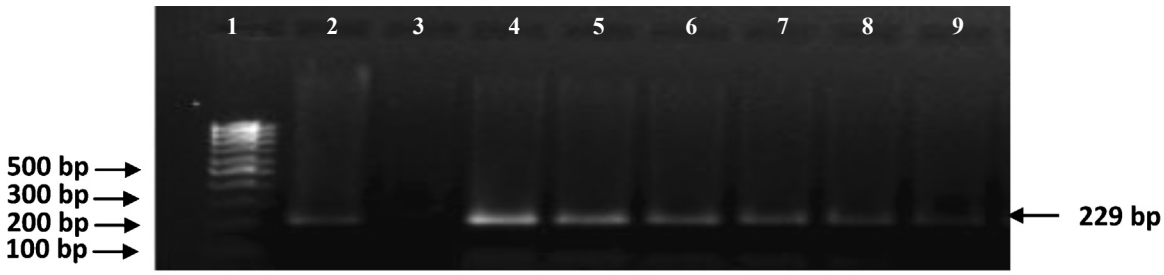

Fig. 1. Molecular identification of MAP isolates by using PCR targeting IS*900* sequence (specific product size 229bp). Lane 1: 100bp marker; Lane 2: Positive control (Indian Bison type MAP S5), Lane 3: Negative control (deionized distilled water); Lane 4: isolates no. T7 (MAP positive); Lane 5: isolates no. 22G (MAP positive); Lane 6: isolates no. R27 (MAP positive); Lane 7: isolates no. MAP 16 (MAP positive); Lane 8: isolates no. MAP 18 (MAP positive), Lane 9: isolates no.W213 (MAP positive).

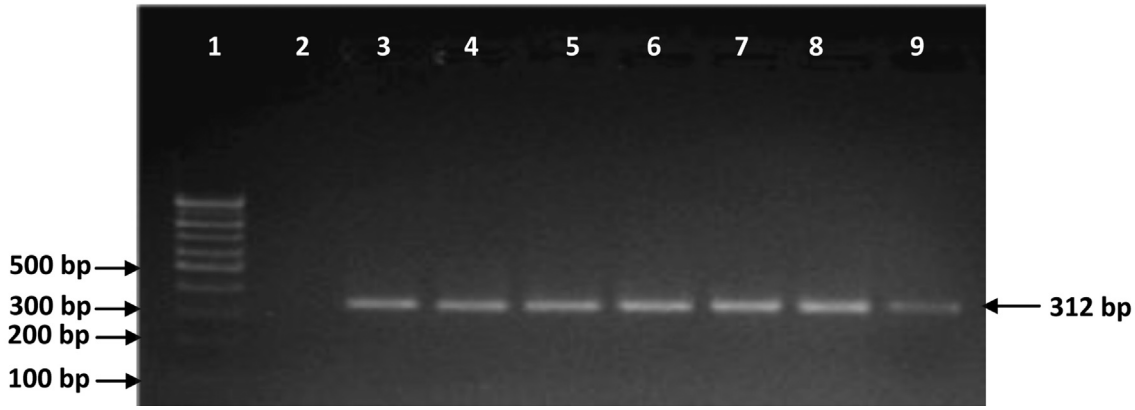

Fig. 2. Molecular identification of MAP isolates by using PCR targeting IS*Mav02* sequence (specific product size 312 bp). Lane 1: 100bp marker; Lane 2: Negative control (deionized distilled water); Lane 3: Positive control (Indian Bison type MAP S5); Lane 4: isolates no.T7 (MAP positive); Lane 5: isolates no.22G (MAP positive); Lane 6: isolates no.R27 (MAP positive); Lane 7: isolates no.MAP 16 (MAP positive); Lane 8: isolates no. MAP 18 (MAP positive), Lane 9: isolates no.W213 (MAP positive).

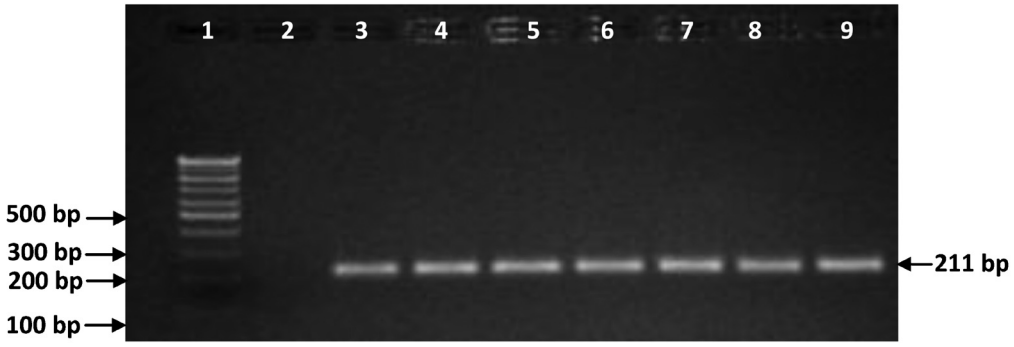

Fig. 3. Molecular identification of MAP isolates using PCR targeting *HspX* sequence (Specific product size 211bp). Lane 1: 100bp marker; Lane 2: Negative control (deionized distilled water); Lane 3: Positive control (Indian Bison type MAP S5); Lane 4: isolates no.T7 (MAP positive); Lane 5: isolates no.22G (MAP positive); Lane 6: isolates no. R27 (MAP positive); Lane 7: isolates no. MAP 16 (MAP positive); Lane 8: isolates no. MAP 18 (MAP positive), Lane 9: isolates no.W213 (MAP positive).

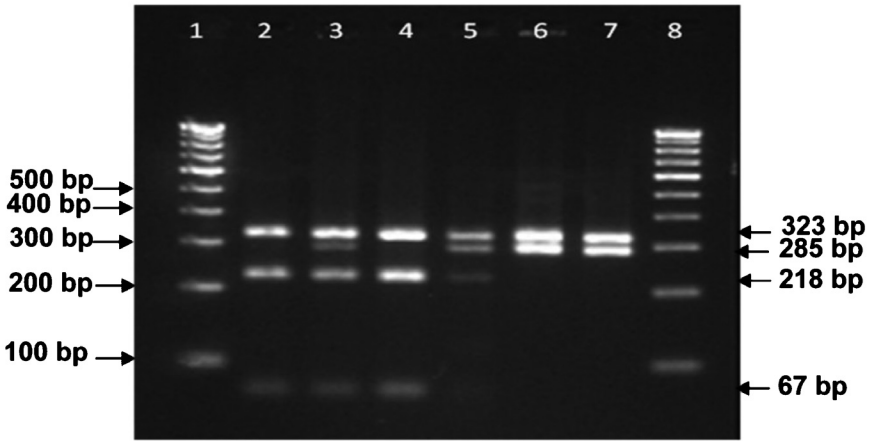

Fig. 4. Genotyping of MAP isolates using IS*1311* polymerase chain reaction-restriction endonuclease analysis. Lane 1: 100bp ladder; Lane 2: isolates no. MAP16 (Bison type of Indian origin); Lane 3: isolates no.429 (Cattle type of Spain origin); Lane 4: isolates no. B1 (Bison type of Canada origin); Lane 5: isolates no.W213 (Cattle type of USA origin); Lane 6: isolates no.22G (Sheep type of Spain origin), Lane 7: isolates no.MAPS1 (Sheep type of Canada origin), Lane 8: 100 bp DNA ladder.

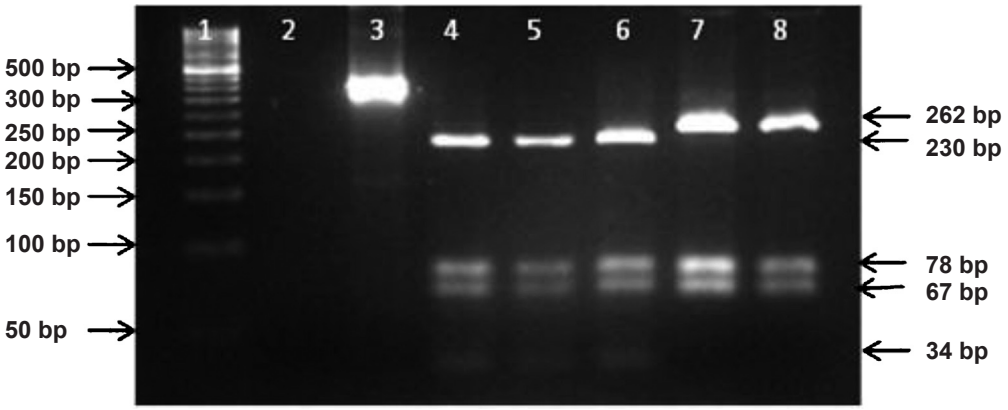

Fig. 5. Discriminatory restriction pattern of representative MAP isolates of Indian and non-Indian origin using IS*1311* PCR locus 2-REA method. Lane 1 Marker (50bp); Lane 2: Negative control, Lane 3: Undigested PCR product (~425bp); Lane 4: isolates no. W213 (Cattle type of USA origin); Lane 5: isolates no. 22G (Sheep type of Spain origin); Lane 6: isolates no. MAP B1 (Bison type of Canada origin); Lane 7: isolates no. MAP 16 (Bison type of Indian origin); Lane 8: isolates no. MAP 18 (Bison type of Indian origin).

On evaluating the specificity of IS*1311* L2 PCR-REA on 12 non-MAP mycobacterial DNA, none were amplified except DNA of *M. avium*. However, the restriction profile of *M. avium* was different to that of MAP.

Discussion

Molecular epidemiology of MAP has been poorly understood due to slow growth of the pathogen in artificial medium. Rapid strain differentiation methods are pre-requisite to understand the origin of infection, disease transmission and to design disease control strategies. In the present study, IS*1311* PCR-REA method was applied on MAP isolates/DNA from India, Canada, USA, Spain and Portugal, and MAP positive faecal DNA samples of domestic ruminants of north India for determining the genotype. Further, potential of recently described IS*1311* PCR L2 PCR-REA method was evaluated for genomic marker based differentiation between Indian and non-Indian strains.

In the present study, genomic and faecal DNA samples obtained from MAP infected animals of north India were genotyped as Bison type. These results were similar to that reported in previous epidemiological investigations conducted in north India[16], and 'Bison type' genotype was identified as the dominant genotype. Contrary to the present study, 'Cattle type' genotype of MAP was found as predominant genotype infecting domestic livestock, wild ruminant and non-ruminant species in other countries[17,26]. 'Sheep type' strains are rarely associated with paratuberculosis in species other than sheep[15,27]. In present study, we could not detect any 'Cattle type' and 'Sheep type' genotype of MAP in animals from north India. Previously 'Cattle type' strains have been reported from cattle and human population of northern India[16].

The results showed that new IS*1311* L2 PCR-REA assay successfully discriminated 'Bison type' genotype of Indian origin ('Indian Bison type') from MAP isolates of other genotypes (Cattle type, Sheep type and Bison type) of non-Indian origin. The test was found to be very specific as all mycobacterial isolates (except *M. avium*) other than MAP could not be amplified by the MAP specific primers. Due to high genetic similarity between *M. avium* and MAP, IS*1311* L2 PCR amplified the DNA of both. However, the restriction profiles of IS*1311* locus 2 by *BsaJ*1 restriction enzyme were different between both species. The present finding confirmed that 'TG' gap deletion at 64th and 65th position of IS*1311* element at locus 2 was a stable marker and could be used in future strain typing as 'Molecular signature' and in epidemiological investigations. This newly optimized tool successfully worked on the clinical DNA samples obtained from faecal samples of MAP infected animals. Further, this assay can give results much faster (1 day) than culture based typing methods (*i.e*. RFLP or PFGE) and is particularly suitable in conditions where we may have culture negative results.

In conclusion, our study demonstrates that IS*1311* L2 PCR-REA assay is a rapid, and easy to perform method for the differentiation of 'Bison type' MAP isolates of Indian origin from non-Indian MAP isolates of different genotypes. MAP is an important livestock pathogen world over which besides inviting trade restrictions, is a potential human pathogen.

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