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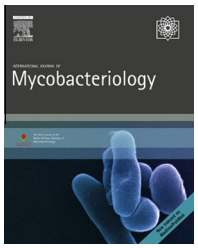

Molecular epidemiology of *Mycobacterium avium* subspecies *paratuberculosis* in ruminants in different parts of India

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ARTICLE INFO

Article history:
Received 7 October 2015
Received in revised form
24 November 2015
Accepted 28 November 2015
Available online 28 December 2015

Keywords:
India
Molecular typing
Mycobacteium avium subspecies *paratuberculosis*
Ruminants

ABSTRACT

Objective/Background: Paratuberculosis is an economically important, chronic, and incurable disease in ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Understanding the genetic variability of MAP strains is important in diagnosis, epidemiological investigation, and the formation of strategies for prevention and control of the disease.
Methods: In the present study, a total of 61 MAP isolates obtained from different parts and species of India were typed using IS*1311* polymerase chain reaction-restriction endonuclease analysis (PCR-REA) to analyze the genetic difference(s), if any, between them and the host adaptation.
Results: Based on PCR-REA results, bison B type was detected in 54 (87%) MAP isolates obtained from cattle, sheep, and goats. Of these, 19 were from sheep of the Rajasthan ($n = 17$) and Bareilly ($n = 2$), North India regions, 28 were from cattle of Chennai, South India ($n = 3$), Bareilly, North India ($n = 3$), and Nagpur, West India ($n = 22$), and seven goat isolates from Bareilly, North India region. The 'C' type strain was detected in only seven cattle isolates obtained from the Bareilly region.
Conclusion: The study revealed that in India, bison B-type MAP strains were prevalent in most of the ruminant species. These results have important epidemiological implications with regard to control and prevention of paratuberculosis in India.

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a fastidious, slow-growing acid-fast organism that causes paratuberculosis (Johne's disease) in domestic and wild ruminants [1,2]. The bacterium has also been implicated in Crohn's disease and is, therefore, now considered a public health concern [2]. Paratuberculosis is widely prevalent in ruminant populations throughout the world, including India, causing significant economic loss to dairy industry [3].

The genetic variability of different MAP strains and their influence on infection and pathogenesis has important implications for diagnosis and control of Johne's disease. MAP strains can be classified into two major groups: Type I (sheep) and Type II (cattle). The third group (Type III) was originally thought to be intermediate between Type S and Type C, but

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Peer review under responsibility of Asian African Society for Mycobacteriology.
http://dx.doi.org/10.1016/j.ijmyco.2015.11.003

whole-genome sequencing confirmed that it is actually a subtype of Type-S strains. The Type I pigmented MAP isolates from sheep in the UK and those from Arabian camels [4] were also found to be sublineages of Type S [5]. The epidemiological information about the disease has been minimal in most the prevalent parts of the world, except for the USA and a few European countries. In India, disease is widely prevalent in small and large domestic ruminants in several states [3]. Strategies for prevention and control require an understanding of the molecular epidemiology of the disease. Specifically, strain genotyping is a valuable tool for epidemiological tracing of pathogenic microorganisms [6].

A number of molecular methods have been developed for the typing of MAP isolates, including IS*900*-restriction fragment-length polymorphism (RFLP), random amplified polymorphic DNA, variable number tandem repeats (VNTRs), large-sequence polymorphisms (LSPs), and single nucleotide polymorphisms (SNPs), but they are technically more demanding. The sheep and cattle isolates were distinguished by RFLP analysis using IS*900* probes, but this method is complex, expensive, and requires large amounts of DNA [7]. VNTR typing often failed to identify closely and distantly related isolates, limiting the applicability of this typing scheme to study the molecular epidemiology of MAP at a national or herd level [8]. The IS*1311* polymerase chain reaction-restriction endonuclease analysis (PCR-REA) technique was found to be useful in distinguishing different MAP strains on the basis of their PCR-REA pattern on agarose gels [9]. The method is very simple, fast, and can be used on a range of diagnostic samples for the confirmation of paratuberculosis infections and strain differentiation [9,10]. In India, molecular epidemiology of paratuberculosis has been rarely studied, and a small number of MAP isolates have been characterized by IS*1311*, LSP, and pulse-field gel electrophoresis (PFGE) methods [10–13]. The present study was aimed at characterizing MAP isolates from different geographical origins obtained from ruminant species of India using the IS*1311* PCR-REA method.

Materials and methods

Bacterial isolates and DNA extraction

DNA extracted from 61 MAP isolates and clinical samples from different host species and locations of India were used (Tables 1–3). DNA was extracted from ileal/mesenteric lymph node tissues, and MAP-culture isolates of paratuberculosis-positive animals from different parts of India as per the method described previously [14]. Five reference strains, EU139 (*Mycobacterium bovis*), M120/04 (*M. avium*), K-10 MAP, and ATCC19698 MAP were received from the Moredun Research Institute, UK, and the 316F vaccine strain from the Biological Product Division of IVRI, Uttar Pradesh, was also included in the study to support and check integrity of the assay.

IS900 PCR

An IS*900* gene PCR assay was performed to confirm MAP identity using the primers BA5 (F) and BA6 (R) [15]. A 50 μL IS*900* gene PCR mix containing 2 μL of the DNA solution, 1 × PCR buffer, 1.5 mM $MgCl_2$, 250 mM of each of the nucleotides (dATP, dTTP, dGTP, and dCTP), 2 U *Taq* polymerase (MBI; Fermentas, Hanover, MD, USA), and 1 μM of each primer was prepared for each sample. Amplification was achieved using the following conditions: one cycle of denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final elongation at 74 °C for 4 min. PCR products were evaluated by electrophoresis in 1.5% agarose gels containing ethidium bromide (0.5 μg/mL). DNA bands of 314-bp sizes were considered positive results for the IS*900* gene (Fig. 1).

Molecular typing of MAP by IS1311 PCR-REA

MAP strains isolated from different species were characterized by molecular typing for the IS*1311* gene as described previously [16,10]. Briefly, DNA isolated from different species ($n = 61$) were amplified using oligonucleotide primers M56 (Forward: 5′-GCG TGA GGC TCT GTG AA-3′) and M119 (Reverse: 5′-ATG ACG ACC GCT TGG GAG AC-3′) [9] flanking 608 bp of the IS*1311* gene (Fig. 2). The amplified product was digested with restriction enzymes *HinfI* and *MseI* (New England Biolabs, Inc., Ipswich, MA, US), and the digested products were electrophoresed in 2% agarose gels to identify the MAP strain on the basis of DNA-band patterns as reported previously [16]. MAP 'S' (sheep) strains were defined by two bands of 285 bp and 323 bp. MAP 'C' (cattle) strains were characterized by four bands of 67 bp, 218 bp, 285 bp, and 323 bp. MAP B (bison) strains were identified by three bands of 67 bp, 285 bp, and 323 bp. *M. avium* subsp. *avium* (Maa) isolate was identified by three bands of 134 bp, 189 bp, and 285 bp.

Results

All MAP and non-MAP strains were tested by IS*900* (Fig. 1) and IS*1311* PCR (Fig. 2) before proceeding to PCR-REA. All 61 MAP isolates from India were found to be positive by IS*900* and IS*1311* PCR. The reference strain *M. avium* was negative according to IS*900* PCR and positive according to IS*1311* PCR. The reference MAP strains K-10, ATCC19698, and 316F (vaccine) were found to be positive by both PCR methods. A non-MAP reference strain (*M. bovis*) was found to be negative according to both PCR methods. The summarized results of IS*1311* PCR-REA are shown in Tables 1–3. The PCR-REA patterns of representative isolates of B type and 'C' type are shown in Figs. 3 and 4.

All sheep isolates ($n = 17$) from the semi-arid zone of Rajasthan (North India) produced three bands by REA (67 bp, 218 bp, and 323 bp) and were identified as "bison" B-type strains. MAP isolates from cattle ($n = 3$), sheep ($n = 2$), and goat ($n = 7$) from Bareilly region (North India) were also identified as "bison" B-type strains. Similarly, all the MAP isolates from cattle from the Nagpur region (West India; $n = 22$) and Chennai (South India; $n = 3$) were also found to be B type. The cattle 'C' type was detected only in seven cattle isolates from the Bareilly region (North India) and produced four bands by PCR-REA (67 bp, 218 bp, 285 bp, and 323 bp).

Table 1 – Molecular typing of MAP strains obtained from cattle by IS1311 PCR-REA.

Strain no.	ID no.	Host	Source	Sample	Primary culture	IS900 PCR	IS1311PCR-REA type
1	C-132	Cattle	North India	Feces	7H9MB	Positive	B
2	C-143	Cattle	North India	Feces	7H9MB	Positive	B
3	C-123	Cattle	Chennai, South India	Feces	7H9MB	Positive	B
4	C-139	Cattle	Chennai, South India	Feces	7H9MB	Positive	B
5	C-126	Cattle	North India	Feces	7H9MB	Positive	B
6	C-128	Cattle	Chennai, South India	Feces	7H9MB	Positive	B
7	C1 A	Cattle	Bareilly, North India	Tissue	HEYM	Positive	C
8	C287 A	Cattle	Bareilly, North India	Tissue	–	Positive	C
9	C337 A	Cattle	Bareilly, North India	Tissue	–	Positive	C
10	C148 A	Cattle	Bareilly, North India	Tissue	–	Positive	C
11	C306 A	Cattle	Bareilly, North India	Tissue	–	Positive	C
12	C680 A	Cattle	Bareilly, North India	Tissue	–	Positive	C
13	C111 A	Cattle	Bareilly, North India	Tissue	–	Positive	C
14	C16	Cattle	Nagpur, Maharashtra	Tissue	7H9MB	Positive	B
15	C17	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
16	C27	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
17	C42	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
18	C43	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
19	C54	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
20	C56	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
21	C57	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
22	C60	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
23	C70	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
24	C85	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
25	C86	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
26	C90	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
27	C93	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
28	C98	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
29	C100	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
30	C250	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
31	C259	Cattle	Nagpur, Maharashtra	Tissue	HEYM	Positive	B
32	C260	Cattle	Nagpur, Maharashtra	Tissue	HEYM	Positive	B
33	C349	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
34	C352	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B
35	C388	Cattle	Nagpur, Maharashtra	Tissue	–	Positive	B

Note: 7H9 MB = Middlebrook 7H9; HEYM = Herold's egg yolk with sodium pyruvate; ID = identification; MAP = *Mycobacterium avium* subspecies *paratuberculosis*; PCR = polymerase chain reaction; REA = restriction endonuclease analysis.

Table 2 – Molecular typing of the MAP strains obtained from goats by IS1311 PCR-REA.

Strain no.	ID no.	Host	Source	Sample	Primary culture	IS900 PCR	IS1311 PCR-REA type
1	163A	Goat	Bareilly, North India	Tissues	7H9MB	Positive	B
2	12A	Goat	Bareilly, North India	Tissues	7H9MB	Positive	B
3	750A	Goat	Bareilly, North India	Tissues	7H9MB	Positive	B
4	18A/2k	Goat	Bareilly, North India	Tissues	7H9MB	Positive	B
5	88A	Goat	Bareilly, North India	Tissues	7H9MB	Positive	B
6	1168A	Goat	Bareilly, North India	Tissues	7H9MB	Positive	B
7	352/06	Goat	Bareilly, North India	Tissues	7H9MB	Positive	B

7H9MB = Middlebrook 7H9; ID = identification; MAP = *Mycobacterium avium* subspecies *paratuberculosis*; PCR = polymerase chain reaction; REA = restriction endonuclease analysis.

All MAP reference strains (K10, ATCC 19698, and 316F) were 'C' type, while *M. avium* (M120/04 UK) showed an *Maa*-type REA pattern as expected.

Discussion

India is a tropical country, with the animal rearing system and husbandry practices vary between different states in comparison to other countries. Therefore, molecular epidemiology of MAP infection in India is expected to be different from that of other countries. There are ample opportunities for interspecies transmission of infection, making it necessary to analyze the genotype of MAP strains prevalent in this country. The factors affecting MAP transmission and the associated risk factors for herd- and animal-level infection are not well studied [6]. Assessment of variations among

Table 3 – Molecular typing of the MAP strains obtained from sheep by IS1311 PCR-REA.

Strain no.	ID no.	Host	Source	Sample	Primary culture	IS900 PCR	IS1311 PCR-REA type
1	987A	Sheep	Bareilly, North India	Tissues	7H9MB	Positive	B
2	H615	Sheep	Bareilly, North India	Tissues	7H9MB	Positive	B
3	CS2484	Sheep	Semi-arid zone, Raj. India	Tissues	HEYM	Positive	B
4	CS2028	Sheep	Semi-arid zone, Raj. India	Tissues	HEYM	Positive	B
5	CS2280	Sheep	Semi-arid zone, Raj. India	Tissues	HEYM	Positive	B
6	CS1700	Sheep	Semi-arid zone, Raj. India	Tissues	HEYM	Positive	B
7	CS2625	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
8	CS2819	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
9	CS1882	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
10	CS2498	Sheep	Semi-arid zone, Raj. India	Tissues	HEYM	Positive	B
11	CS2946	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
12	CS2836	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
13	GC4818	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
14	GC4608	Sheep	Semi-arid zone, Raj. India	Tissues	HEYM	Positive	B
15	CS2820	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
16	CS2750	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
17	T2131	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
18	CS2681	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B
19	CS2829	Sheep	Semi-arid zone, Raj. India	Tissues	–	Positive	B

Note: 7H9 MB = Middlebrook 7H9; HEYM = Herold's egg yolk with sodium pyruvate; ID = identification; MAP = *Mycobacterium avium* subspecies *paratuberculosis*; PCR = polymerase chain reaction; REA = restriction endonuclease analysis; Raj India = Rajasthan, India.

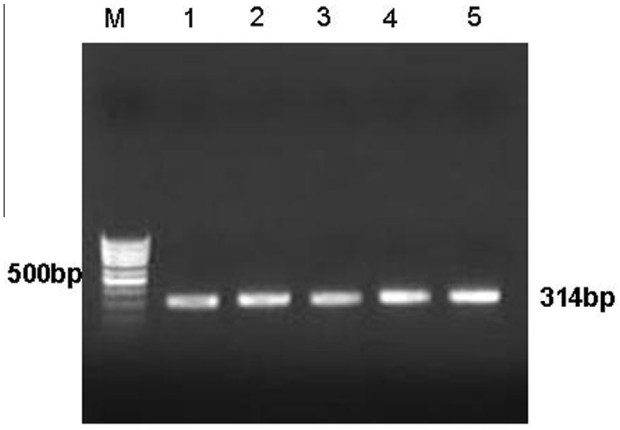

Fig. 1 – *IS900* PCR. Lane M, marker; lane 1, C-132; lane 2, C1A; lane 3, 163A; lane 4, CS2028; lane 5, K10 MAP. *Note:* MAP = *Mycobacterium avium* subspecies *paratuberculosis*; PCR = polymerase chain reaction.

MAP isolates affecting different ruminant species is an essential requirement for developing diagnostic methods and immunoprophylaxis for control and eradication of Johne's disease in India. Small numbers of MAP isolates characterized in previous studies suggested that a different strain could infect ruminant populations in India [10,12].

Johne's disease presents differently in cattle and sheep with respect to clinical, pathological, and epidemiological features [17]. Early epidemiological studies identified that sheep and cattle were infected by different MAP strains, and strains isolated from sheep were difficult to grow on primary cultures [18]. DNA-based studies using RFLP of genomic DNA from MAP isolates from a range of hosts confirmed the existence of two strains referred to as either sheep (S) or cattle (C) [19], and one MAP strain referred to as bison (B type) [16,10], each with different PFGE profiles in comparison to European strains [11,12].

In the present study, all sheep isolates from the semi-arid zone of Rajasthan and Bareilly, all cattle isolates from Nagpur and Chennai, and the majority of MAP isolates from cattle and goats of Bareilly were found to be bison B-type strains. Sevilla et al. [11] typed a few Indian sheep ($n = 5$) and goat ($n = 6$) strains from Mathura (U.P.) region and found all of them to be B type. Another study in Mathura and Ajmer regions of North India [20] also detected B type as the most prevalent type, whereas the C-type MAP genotype predominated in the New Delhi and Agra regions (North India) [20]. Studies in the Punjab region (North India) revealed B type as the most prevalent (82%) MAP genotype, infecting all domestic ruminants, including sheep, whereas C type was present in a minority of cases (15%) from cattle, buffalo, and goats [13,21].

The origin of Indian MAP isolates included in this study were from different geographic regions, e.g., cattle MAP isolates were from South and West India, goat isolates were from North India, and sheep isolates were from the Northwest (the semi-arid zone of Rajasthan). These results suggested that in most parts of India, B-type strains might be circulating among the domestic ruminant population, which is quite interesting, given that bison strains have been reported only in bison from Montana, USA. Most other domestic animals in the USA were infected with the cattle strain [11,16]. The Indian B-type isolates were found to be similar to that of B-type isolates from Montana, however, the genomic RFLP patterns of these isolates were different from each other. It was proposed that the Indian MAP isolates represented a new MAP biotype not seen outside of India. The "Indian Bison type" isolates from animals in India were different from those isolated from US bison [10]. In a recent study, it was found that the "Indian Bison type" strains were a sublineage of Type C strains [22]. Another interesting observation of the present study was that

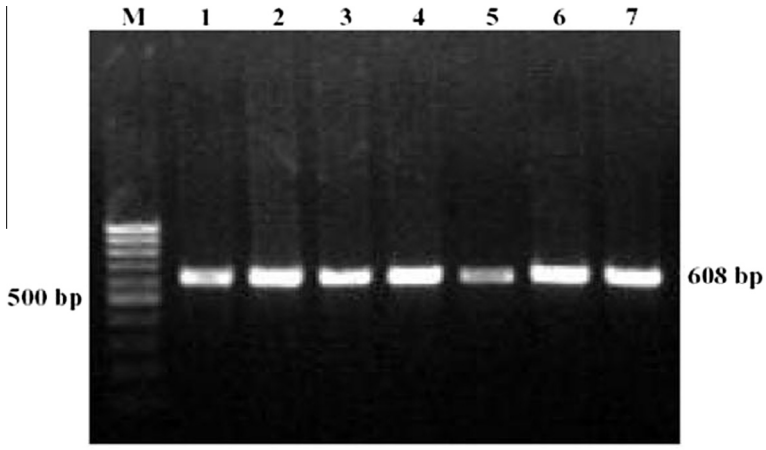

Fig. 2 – *IS1311* gene PCR. Lane M, marker; lane 1, C-132; lane 2, C1A; lane 3, 163A; lane 4, CS2028; lane 5, C16; lane 6, H615; lane 7, K10 MAP. *Note:* MAP = *Mycobacterium avium* subspecies *paratuberculosis*; PCR = polymerase chain reaction.

sheep populations from the semi-arid region of Rajasthan were endemic for paratuberculosis, while the disease was not detected in goat populations, despite the goat farm being situated in the vicinity (2–3 km separation) of sheep farms and the fact that these animals share common grazing areas. The reports from genotyping studies expressed mixed opinions with regard to the host adaptation of MAP. Some studies indicated that MAP strains are host specific and infect their respective host species only [23]. A recent PCR-REA study [24] conducted in the dairy farm region (Mashhad) of Iran reported presence of the C-type strain in all MAP-positive animals. Studies from Australia, England, and New Zealand indicated that cattle were not infected, despite being in contact with paratuberculosis-infected sheep. There was also a failure in the natural transmission of the infection to sheep populations exposed to paratuberculosis-infected cattle [25]. In contrast, at one farm in north India, the disease was endemic in both the species, i.e., sheep and goats, although the disease incidence was slightly lower in sheep [26].

Here, only seven isolates obtained from cattle from the Bareilly region (North India) were found to be C type, suggesting that the C strain was also present in Indian ruminants, but in the minority of cases. However, large numbers of isolates from different ruminant species and geographic regions need to be characterized in order to assess a national scenario about the most prevalent MAP genotype in India.

In a study involving PFGE analysis of Indian and UK strains [12], differences in their profiles were discovered. This study confirmed that UK and Indian Map strains were different from each other based on their IS*1311* PCR-REA patterns. This has significant epidemiological implications, in that diagnostics and vaccines prepared from foreign MAP isolates may not be very effective in the Indian context. Also, since most MAP strains, irrespective of their host and geographic origins, are of similar type based on PCR-REA, the diagnostics and vaccine reagents prepared from one MAP strain (B type) of Indian origin may work for all ruminants. However, this aspect of epidemiology and immunoprophylaxis needs further studies.

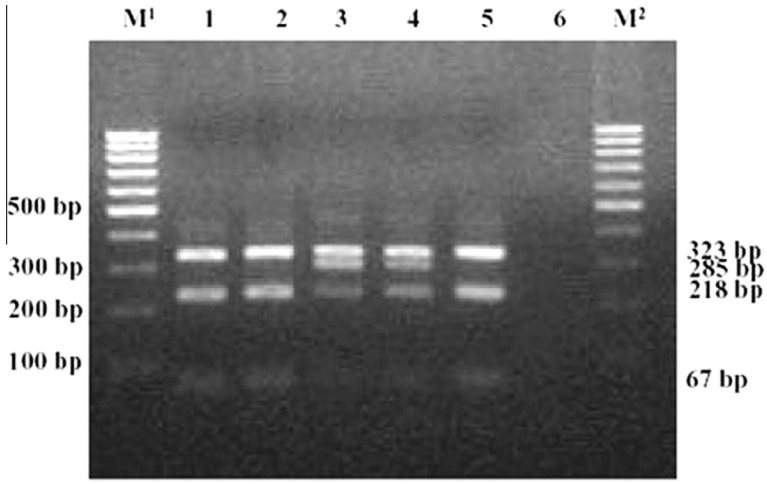

Fig. 3 – PCR-REA analysis of MAP isolates. Lane M1 and M2, markers; lane 1, C-139 (B type); lane 2, C-12A (B type); lane 3, 316FMTCC (C type); lane 4, C-148A (C type); lane 5, 1168A (B type); lane 6, *Mycobacterium bovis* (no band pattern). *Note:* MAP = *Mycobacterium avium* subspecies *paratuberculosis*; PCR = polymerase chain reaction; REA = restriction endonuclease analysis.

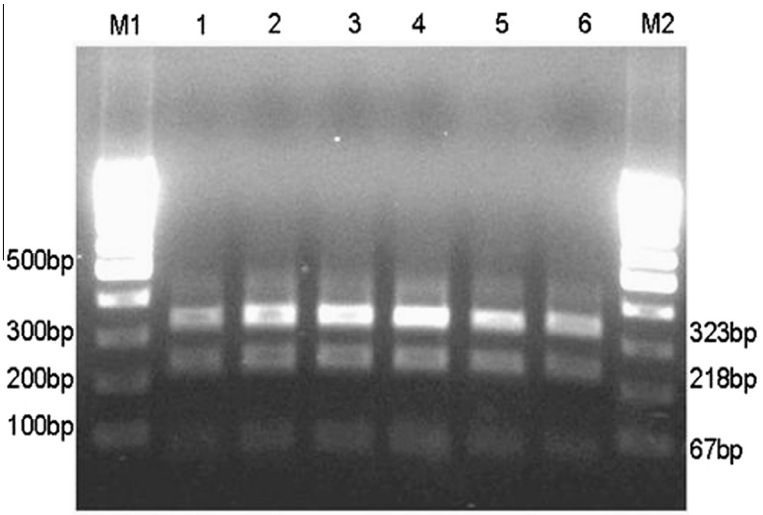

Fig. 4 – PCR-REA analysis of MAP isolates. Lane M1 and M2, marker; lane 1, 163A (B type); lane 2, C-132 (B type); lane3, CS2280 (B type); lane 4, CS1700 (B type); lane 5, C 93 (B type); lane 6, C260 (B type). *Note:* MAP = *Mycobacterium avium* subspecies *paratuberculosis*; PCR = polymerase chain reaction; REA = restriction endonuclease analysis.

In the present study, S-type MAP was not detected from any of the animals. S-type MAP has not been reported in India, however, firm conclusions about the existence of S-type MAP in India require sampling of large numbers of animals from different geographical regions [13]. It was observed that in Australia, C-type strains were also found to infect other species, including sheep [27]. Reports from the UK also suggested that sheep were commonly infected with C strain [28]. S strains, however, predominantly found in sheep, were restricted to this species only [10,27]. There is little evidence of S-type (Sheep strains-BstE II-S) and intermediate-type (BstE II-I) strains being distributed in different ruminant species. S strains have been reported in sheep from Canada, New Zealand, the Faroe Islands, South Africa, Morocco, and Australia [15,23,27,29–32], in goats from New Zealand and the Czech Republic [23,33], in cattle from the Czech Republic [33], and in deer from New Zealand and the Czech Republic [34,35]. Sevilla et al. [11] reported that Spanish cattle were mainly infected with C-type strains, whereas Spanish sheep were infected with mainly S strains and goats were infected with both strains. In New Zealand, Australia, Canada, and Norway, C strains usually infect cattle and S strains usually infect sheep and goats [23,29,32]. Although the S strains could not be isolated from sheep in the present study, it would be premature to speculate about their nonexistence in India.

Conclusion

Based on the PCR-REA patterns of the MAP isolates obtained from different parts and species of India, we concluded that the B-type MAP strain was widely circulating in India, which has important epidemiological implications with regard to control and prevention of paratuberculosis in India. The C-type strain was less prevalent in Indian ruminant populations, as its existence was detected in few cattle. Similar to previous studies, we found that the S strain did not occur in Indian ruminants. It is suggested that newer typing methods, such as VNTR and SNP, which are capable of detecting differences among major types (C, S, or B), may be used to obtain further insight into the epidemiological features of Johne's disease in India.

Conflict of interest

Authors have no conflict of interest.

Acknowledgments

The authors are thankful to the Director, IVRI, Izatnagar, Bareilly (UP), and the Director, CSWRI, Avikanagar, Rajasthan, for providing the necessary help and facilities. Thanks are also due to Dr. Karen Stevenson, Moredun Research Institute (MRI), Edinburgh, UK, for providing DNA of the standard MAP strains.

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