Molecular Characterization of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) Water Buffalo in Chitwan, Nepal

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ABSTRACT

Mycobacterium avium subspecies paratuberculosis (MAP) causes Johne's disease (JD) in ruminants which is an economically important and endemic in many parts of the globe. This disease regarded as highly prevalent disease of domestic and wild animals, especially ruminants, is manifested as chronic granulomatous enteritis with decreased milk production, progressive emaciation and death. Understanding the genetic variability of MAP strains are important in diagnosis, epidemiological investigations, and formulation of strategies for prevention and control of the disease. However, MAP strains in Nepal have never been characterized. This study was conducted to understand the molecular characterization of MAP isolates of Nepal. A total of 49 MAP isolates obtained from buffalo population of three different dairy pocket areas of Chitwan, Nepal were typed using IS1311 polymerase chain reaction-restriction endonuclease analysis (PCR-REA). The extracted DNA samples (n = 49) were analyzed for the presence of MAP specific sequences (IS900) using PCR. DNA samples were further subjected to genotype differentiation using IS1311 PCR-REA and IS1311 L2 PCR-REA methods. All the DNA samples were positive for the entire three MAP specific sequences based PCRs. This study revealed that 'Bison type' strain is the most prevalent MAP genotype circulating within the riverine buffalo population of Nepal. IS1311 PCR-REA showed that MAP DNA samples of Nepal origin belonged to 'Bison type', whereas, IS1311 L2 PCR-REA method showed similarity with "Indian Bison type" and different restriction profiles of 'Bison type' genotype as compared to non-Indian strains. The study concludes that "Bison type" MAP stains was prevalent in all the MAP samples obtained from riverine buffaloes of Chitwan, Nepal. These results provided important insights in the epidemiological understanding of paratuberculosis in Nepal and will be helpful while designing its control and prevention strategies.

Keywords: Bison type, IS*1311*PCR-REA, MAP, Molecular characterization, buffalo, Nepal

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Paratuberculosis, also known as Johne's disease (JD), which is an economically important and endemic in many parts of the world including Nepal. This disease is considered as highly prevalent disease of domestic and wild ruminants and is manifested as a chronic granulomatous enteritis with decreased milk production, progressive emaciation and death (Yue et al., 2016). MAP is a fastidious, slow growing acid-fast organism (Behr & Kapur, 2008). Clinical symptoms of paratuberculosis include slowly progressive wasting and chronic or intermittent, therapy-resistant diarrhoea (Stabel, 1997), which

are intermittent initially, become progressively more severe and remain constant. Affected animals typically die as a result of dehydration and severe cachexia (Cheng et al., 2020).

Paratuberculosis causes huge economic impact on livestock industry, because of premature culling of animals, reduced weight gain, reduced feed efficiency, high morbidity (Kaur et al., 2011), poor carcass quality, reduced milk yield, increased disease susceptibility to mastitis and reproductive disorders leading to increased calving intervals, poor fertility and additional veterinary costs (Hasonova & Pavlik, 2006). Paratuberculosis is chargeable for considerable economic losses causing disease within the dairy industry within the U.S., estimated about over $200 million each year (Groenendaal et al., 2015), and thus draws significant attention in developing as well as well developed countries. No country claims that it is free from MAP (Yue et al., 2016). The disease is taken into account as one amongst the foremost important diseases of ruminant population (Sivakumar et al., 2005), however, MAP has also been isolated from a large range of non-ruminant including horses, pigs, rabbits, foxes, stoats, weasels (Beard et al., 2001), humans (Chiodini et al., 1984) and non-human primates (McClure et al., 1987). Paratuberculosis is similar to Crohn's disease in humans (McAloon et al., 2019), which reflects that MAP is potential etiology of Crohn's disease (Sechi& Dow, 2015), and thus, has amplified the possible zoonotic threat.

Control of MAP infection is the priority of developed countries to secure animal productivity and to reduce human exposure. Vaccination is considered as the reliable method of choice for the control of JD in animals, however, efficacy of vaccines depends on the genotype of candidate strain used (Singh et. al., 2007). According to Sonawane et al. (2015), the genetic variability of various MAP strains and their impact on infection and pathogenesis has significant implication for diagnosis and control of JD. Therefore, better understanding of the genotypes infecting livestock species is necessary for designing disease control strategies. Based on IS*1311* polymerase chain reaction restriction endonuclease analysis (PCR-REA) method, MAP isolates are grouped into three genetically distinct genotypes, namely, 'Cattle type', 'Sheep type' and 'Bison type' (Sevilla et. al., 2005).

Several research has identified genomic variations in terms of genetic rearrangements, in-del polymorphisms and locus polymorphisms in 'Bison type' genotype of Indian origin in comparison with genotypes reported from other parts of the world, and thus, new nomenclature 'Indian Bison type' was assigned to 'native Bison type' genotype (Sohal et al., 2010). As contrast, 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 (Sohal et al., 2010) and with advantage of this molecular signature, a brand 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 (Sohal et al., 2013).

The prevalence of MAP in dairy buffaloes of Nepal was detected to be 16.59% by faecal PCR (Singh et. al., 2020). However, these MAP strains have never been molecularly characterized to understand the circulating strains in Nepal. Thus, this present study was conducted to understand the molecular characterization of MAP isolates from water buffalo in different dairy pocket areas of Chitwan, Nepal.

MATERIALS AND METHODS

Sample and sampling

Samples were collected from three major dairy pocket areas namely, Ratnanagar, Gitanagar and Mangalpur of Chitwan district, Bagmati province, Nepal. The buffaloes herds were selected on random basis and sampling were done from individual lactating cows old ≥ 2 years showing diarrhea and poor body score with suspect of the disease. The faecal samples were kept at 4°C until they were transported to the laboratory and [illegible] further processing.

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Bacterial isolates, DNA extraction and IS*900* PCR

Fecal cultures of the samples were done under protocol as recommended by Ristow et al. (2006) and DNA extraction from MAP colonies was done as stated by van Soolingen et al. (1993). Later, PCR using specific IS*900* commercial PCR test primer was performed done for confirmation of DNA samples and standardization. The use of IS*900* in this type of PCR is sensitive to reveal very low numbers of MAP, but insufficient for accurate quantification of CFU within the sample, since its present in many copies within the bacterial genome (Singh et al., 2018).

Molecular characterization of MAP by IS*1311* PCR-REA

A total of 49 MAP isolates (harvested by fecal culture and later confirmed by IS*900* gene PCR) of various locationswere processed for the investigation of molecular genotyping (molecular characterization) using IS*1311* PCR-REA and IS*1311* L2 PCR-REA. Out of the 46 MAP isolates, 16, 15and 15 were from Ratnanagar, Geetanagar and Mangalpur, respectively, of Bagmati Province, Central Plain region of Nepal. Similarly, to examine the specificity of IS*1311* L2 PCR-REA, a set of five mycobacterial isolates aside from MAP [*M. marinum*, *M.fortuitum*, *M. kansasii*, *M. bovis*, *M. bovis (BCG)*] were used for this research. All the mycobacterial isolates were subjected to isolation of genomic DNA as per the method designed by van Soolingen et al. (1993). DNA samples recovered from isolates samples were subjected to IS*900* (Singh et al., 2020)specific PCRs for the molecular identification of MAP.

IS1311PCR-REA (*Genotyping of isolates*): Genotyping of every MAP DNA obtained from mycobacterial isolates was allotted by IS*1311* PCR-REA method (Sevillaet al., 2005).In brief, the reaction was distributed in 30 µl volume, containing 20 µl positive IS*1311* PCR product, 3 µl 10X buffer (Fermentas, USA), 2 units of every endonuclease *HinfI* and *MseI* (Fermentas). Reaction mixture was incubated at 37°C for 1.5 hrs. Band patterns were visualized after electrophoresis on four percent high resolution agarose gel stained with ethidium bromide and genotype profile analysis was done as described by Sevilla *et al* (2005).

IS1311locus 2 PCR-REA (*Sub-genotyping of MAP isolates*): IS*1311* L2 PCR-REA was used to specify all MAP isolates DNA included during this study. Initially, locus 2 of IS*1311* was amplified using the distinct 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) during a final volume of

25.0 µl at thermocycler conditions: denaturation at 95 °C for five minutes followed by 40 cycles of denaturation 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 percent agarose gel. Amplification products (~425 bp) were digested with *BsaJI* enzyme for two hours at 37°C and band pattern was detected on four percent agarose gel.

RESULTS

Molecular detection of MAP isolates by using PCR targeting IS*900* sequence

All the samples were tested by IS*900* PCR before proceeding to Polymerase Chain Reaction - Restriction Endonuclease Analysis (PCR-REA). MAP specific IS*900* PCR showed presence of MAP DNA in all 49 DNA isolates (samples) as shown in Fig. 1, except those extracted from isolates of mycobacteria other than MAP. The reference MAP strains K-10 and ATCC19698 were found positive by PCR method.

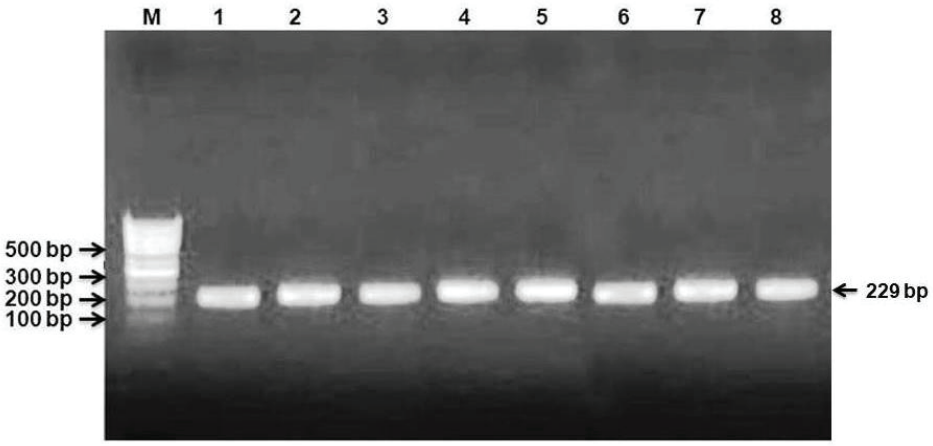

Figure 1. Molecular detection of MAP isolates by using PCR targeting IS*900* sequence (specific product size 229 bp). Lane M: 100 bp marker; Lane 1: Positive Control (Indian Bison type MAP S5); Lane 2: Sample no. R09 (MAP positive); Lane 3: Sample no. R13 (MAP positive); Lane 4: Sample no. R16 (MAP positive); Lane 5: Sample no. G06 (MAP positive); Lane 6: Sample no. G10 (MAP positive); Lane 7: Sample no. M03 (MAP positive); Lane 8: Sample no. M07 (MAP positive).

Molecular typing of MAP by IS*1311* PCR-REA and IS*1311* L2 PCR-REA

IS*1311 PCR-REA (Genotyping)*: All 49 MAP DNA samples showed the pattern of 'Bison type'genotype (Figure2). Thus, it was confirmed that all MAP DNA samples of three different regions of Nepal belonged to 'Bison type'.

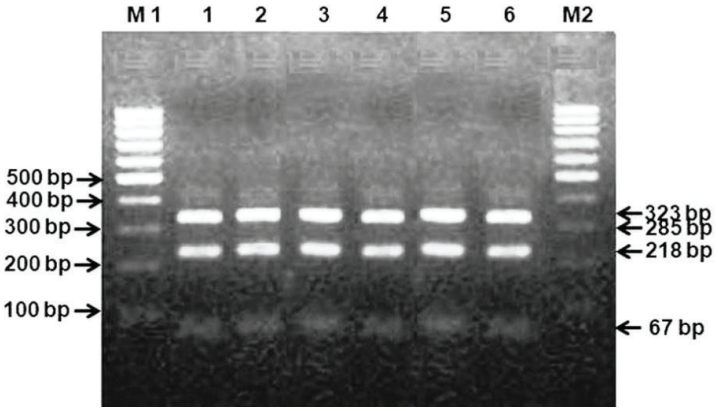

Figure 2. Genotyping of MAP isolates by using IS*1311* PCR-restriction endonuclease analysis. Lane M1: 100 bp marker; Lane 1: Isolate no. R07 (Bison type MAP positive); Lane 2: Isolate no. R11 (Bison type MAP positive); Lane 3: Isolate no. G06 (Bison type MAP positive); Lane 4: Isolate no. G09 (Bison type MAP positive); Lane 5: Isolate no. M07 (Bison type MAP positive); Lane 6: Isolate no. M11 (Bison type MAP positive); Lane M2: 100 bp DNA ladder/marker.

***IS1311 L2 PCR-REA (Sub-genotyping)*:** Restriction profile and band pattern for marker IS*1311* L2 PCR REA results similar with genotype of 'Indian Bison type' MAP from all the samples (Figure3). 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 percent high resolution agarose gel (34, 67, 78 & 230 bp) for other MAP isolates. 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).

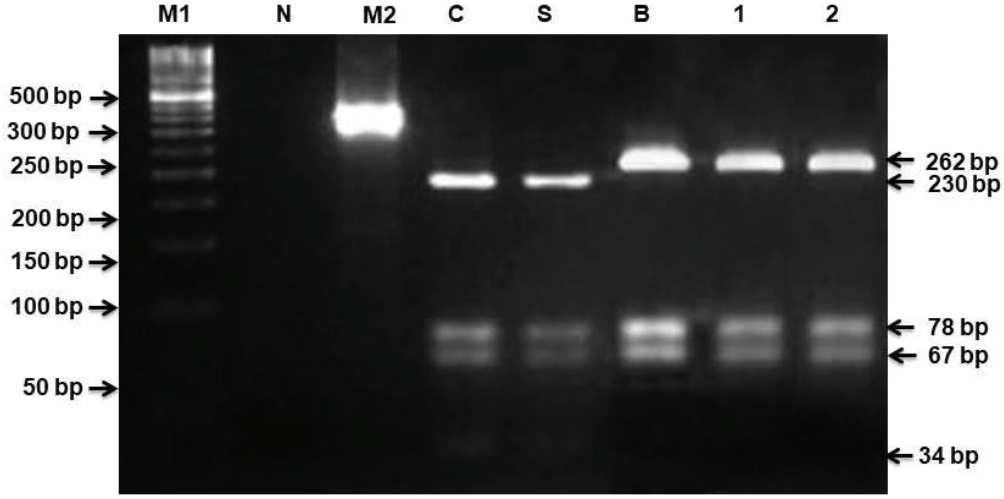

Figure 3. Discriminatory restriction pattern of typical MAP isolates using IS*1311* PCR locus 2-REA method. Lane M1: Marker (50bp); Lane N: Negative control, Lane M2: Undigested PCR product (~425bp); Lane C: Cattle type MAP positive; Lane S: Sheep type MAP positive; Lane B:

Bison type MAP positive); Lane 1: Isolates no. R16 ('Bison' type Indian origin MAP positive); Lane 2: Isolates no. M09 ('Bison' type Indian origin MAP positive). Note: MAP = *Mycobacterium avium* subspecies *paratuberculosis*; PCR = polymerase chain reaction; REA = restriction endonuclease analysis.

DISCUSSION

The molecular epidemiology of MAP has been neglected due to slow growing nature of bacteria in artificial medium. Thus, rapid strain differentiation methods are important 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 from different dairy pocket areas of Nepal and MAP positive faecal DNA samples for determining their genotype. DNA based studies using 'Restriction Fragment Length Polymorphism (RFLP)' of genomic DNA from MAP isolates from a range of hosts confirmed the existence of two strains referred to as either sheep (S type) or cattle (C type) (Whittington & Sergeant, 2001), and one MAP strain referred to as bison (B type) (Sevilla et al., 2005). According to Thibault et al. (2012), it was found that the "Indian Bison type" strains were a sublineage of Type C strains.

All DNA samples obtained from MAP infected animals of Nepal were genotyped as 'Bison type' which is the first report to identify the strain of MAP present in Nepal. In present study, we could not detect any ‘Cattle type’ and ‘Sheep type’ genotype of MAP in animals. These results suggested that in Nepal, "Bison type" strains might be circulating among the buffaloes population, which is quite interesting, given that bison strains have been reported only in bison from USA (Sonawane et al., 2015). These findings were similar to that reported in previous epidemiological investigations conducted in north India (Singh et al., 2009). Similarly, Yadav et al., 2008 has published first report of the infection of buffaloes with MAP ‘Bison type’ strain. Contrary to the present finding, 'Bison type' was found to be the foremost prevalent MAP genotype whereas "Cattle type" was present in minority of cases from cattle, buffalo, and goats of India (Kaur et al., 2010). Likewise, studied done in India reveled that ‘Cattle type’ genotypeof MAP was found as predominant genotype infecting domestic livestock, wild ruminant and non-ruminant species in other countries (Motiwala et al, 2006). ‘Sheep type’ strains arerarely associated with paratuberculosis in species otherthan sheep (Sevilla et al., 2007).

The results showed that new IS*1311* L2 PCR-REA assay successfully discriminated genotype exactly similar to ‘Bison type’genotype of Indian origin (‘Indian Bison type’) from MAP isolates of other genotypes (Cattle type, Sheep type and Bison type). The test was found to be very specific as all mycobacterialisolates (except *M. avium*) apart from MAP could notbe 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*1restriction enzyme were different among both species.This study confirmed that ‘TG’ gap deletion at 64^{th} and 65^{th} 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 (Singh et al., 2015).

This study confirms that MAP strains "Bison type" found in Nepal has similarity exactly with the "Indian Bison type" but different with other strains. This fact has significant epidemiological

implications and support the scientific aspects that diagnostics and vaccines prepared from foreign MAP isolates might not be very effective, except vaccine prepared from "Indian Bison type" strain.

Thus, in this study, only 46 isolates obtained from dairy buffaloes of three locations from Chitwan districts were considered, however, large numbers of isolates from different ruminant species and geographic regions have to be characterized so as to assess a national scenario about the foremost prevalent MAP genotype in Nepal.

CONCLUSION

In Nepal, MAP isolates infecting buffaloes have been first time genotyped as 'Bison type' causing Johne's disease. Based on PCR-REA patterns of the MAP isolated obtained from riverine buffaloes of various locations of Nepal, we conclude that the "Bison type MAP strain" was the sole circulating strain, which has important epidemiological implications in controlling paratuberculosis in Nepal. The current study validates that IS*1311* L2 PCR-REA assay is a rapid and simple method for the differentiation of 'Bison type' MAP. MAP is an important livestock pathogen worldwide, which besides inviting trade restrictions, may be a potential human pathogen.

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