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Pcr-restriction Endonuclease Analysis for Strain Typing of *Mycobacterium avium* Subsp. Paratuberculosis Based on Polymorphisms in IS 1311

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Abstract: Point mutations in the IS 1311 sequences from sheep and cattle strains of *Mycobacterium avium* subsp. *paratuberculosis* were targeted to develop a Polymerase Chain Reaction (PCR) that would be useful in the diagnosis and control of Johne's disease. PCR/REA strategy based on amplifying a 268 bp fragment of IS1311 and digestion by *Hinf*I was developed. Results showed that all of positive results were assigned to cattle strain (C). This simple and rapid test can be used on a range of diagnostic samples for the confirmation of Johne's disease and will be of benefit in control and eradication programs for this disease.

Key words: Johne's disease · *Mycobacterium avium* subsp. *paratuberculosis* · PCR/REA · IS 1311

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (*M. paratuberculosis*) is the causative agent of The Johne's disease, a chronic and incurable disease affecting many ruminants. Furthermore, *M. paratuberculosis* has been suggested as an etiological agent of Crohn's disease, a chronic infection of the human intestines [1] but this is controversial. Although there is no proven evidence of transmission of *M. paratuberculosis* from livestock to humans, milk is a potential vector for transmission and *M. paratuberculosis* has been demonstrated by PCR in goat's milk in the United Kingdom, Switzerland and Norway [2].

Isolates of the organism were first classified into cattle and sheep types in 1990 [3] on the basis of restriction fragment length polymorphisms (RFLPs) of the insertion sequence IS900 and this largely correlates with the difficulty of primary isolation of sheep types [3, 4]. Sheep strain appears to infect sheep and goats, whereas the cattle strain is known to infect many species including cattle, goats, sheep and man. In humans, C type is demonstrated most frequently, indicating the close association between bovine strains and Crohn's disease [4, 5]. C strains are relatively easy to culture from tissues and feces of animals with paratuberculosis but S strains are difficult to culture [6]. Infection with *M. paratuberculosis*, as for other slowly growing mycobacteria, is difficult to diagnose due to the long incubation times required to culture and identify the organism [7]. Strain identification is currently performed by RFLP analysis on DNA extracted from the cultured organisms, an expensive and time-consuming process. The ability to rapidly differentiate the sheep and cattle strains would be of great benefit in voluntary eradication and control programs for Johne's disease. Current management practices assume that cattle are not susceptible to infection with the sheep strain and can safely graze on pasture after the removal of sheep. A rapid, sensitive test that confirms *M. paratuberculosis* and differentiates between the sheep and cattle strains would help to ensure that management practices are scientifically sound.

Mobile genetic elements known as Insertion Sequences (IS) are popular targets for Polymerase Chain Reaction (PCR) tests for many mycobacterial disease. As IS are generally present in multiple copies in the genome of an organism they make useful targets to develop highly sensitive PCR assays. At present the IS900 gene, a member of the IS116 family, is the only IS known to be unique to *M. paratuberculosis*. The IS1311 element was identified in *M. paratuberculosis* and was subsequently characterized from that species. IS 1245 is present in *M. avium* [8, 9]. Although IS1311 has been demonstrated in both *M. paratuberculosis* and M. avium, five point mutations differentiate the IS1311 sequences of the two species [9]. These point mutations can be targeted by Restriction Endonuclease Analysis (REA) to differentiate *M. paratuberculosis* from *M. avium*. In addition, some copies of IS1311 in the cattle strain of *M.*

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paratuberculosis contain an additional point mutation that can be used to differentiate it from the sheep strain [9]. In this study we used the IS1311 PCR/REA for typing of Map isolates.

MATERIALS AND METHODS

Sampling: A total of 68 feces samples were randomly obtained from keneh Bist Dairy Farm (Mashhad), supposed to have high number of infected animals, according to previous records. Feces were collected from the rectums of animals with high attention on cross contamination. Feces samples were stored at -20°C until DNA extraction.

DNA extraction: For DNA extraction, fecal samples (500 mg or 500 μl from each samples) were transferred to a screw-capped 1.5 micro centrifuge tubes with 20 μl proteinase K enzyme and 1 ml of Lysis Reagent (Guanidine Solution: 6 M GuSCN, 20 mM EDTA, 10 mM Tris-HCl pH 6.5, 40 g/l Triton X-100 and 10 g/l DTT) and vortexed vigorously for 10 minutes. The tubes were incubated in a hot plate incubator at 65°C for 1 hour and vortexed each 5 minutes during incubation. Then, tubes centrifuged for 20 min. at 5000 rpm to pellet debries and then about 1 ml of supernatant was transferred to a clean tube and 400 μl Lysis Reagent was added to each tube and mixed gently to homogenize the tube content. Tubes were incubated at 65°C for 5 min. then tubes vortexed and 30 μl nucleos was added to each tube and the tubes were rotated at room temperature for 10 min. After centrifugation at 5000 rpm for 20 sec. supernatants were discarded and 200 μl Lysis Reagent was added to the pellets and vortexed. 400 μl Saline Buffer solution was added to the homogenized tubes and mixed gently. After centrifugation at 5000 rpm for 20 sec. supernatants were removed and 500 μl Saline Buffer solution was added to each tube and vortexed. Tubes were centrifuged at 5000 rpm for 20 sec and supernatants were removed. To increase the lucidity of the extracted DNA, washing step was repeated once. Tubes containing pellets were placed in dry plate incubator for 5 min until pellets dried completely. 75 μl of Extra Gene solution was added to each tube and vortexed and incubate at 65°C for 10 min. After incubation, tubes were vortexed and then centrifuged at 10000 rpm for 2 min. Finally supernatants containing DNA transferred to 0.5 ml eppendorf tubes. Pure DNA extracts were stored at-20°C for the subsequent analysis.

PCR: For amplification of *M. paratuberculosis* DNA from fecal and milk extracts we used IS1311 specific primers: M56 (GCG TGA GGC TCT GTG GTG AA)and M94 (CAG CGA TCG TCG ACA GTG TG). An aliquot (10 μl) of the DNA samples was added to 10 μl of PCR mixture containing 2 μl PCR buffer. 1.5 mM $MgCl_2$, 0.25 mM deoxynucleoside triphosphates (from each), 10 pmol of each primers and 1 U *OligoTaq* DNA polymerase (IsoGene, Moscow). Amplification condition for IS900 were: 3 min at 94°C, 40 cycles of 35 s at 94°C, 25 s at 62°C, 1 min at 72°C and a final 5 min extension at 72°C. PCR products were analyzed through the electrophoresis of 5 μl of each sample on 2% (W/V) agarose gels and results were recorded by UVidoc Gel Documentation System (Uvitec, UK). The positive samples with 268 bp amplified band were considered for REA (Restriction Endonuclease Analysis).

Restriction endonuclease analysis: Restriction endonuclease analysis reactions were prepared by adding 10 μl of PCR product, 2 U of the *Hinf*I restriction endonuclease enzyme (Sibenzyme, Russia), 2 μl of 10X buffer (supplied with enzyme) and made up to 20 μl with sterile purified water. Restriction digests were incubated for 2 h at 37°C and were assessed by electrophoresis in 4% agarose gel containing ethidium bromide.

RESULTS AND DISCUSSION

The quality of extracted DNA from feces samples by this procedure was good (Fig. 1). Results of PCR amplification on feces DNA showed that Is1311 sequence can be detected in 19 samples (Fig. 2).

As showed in Fig. 3, all of positive results were assigned to cattle strain (C) using REA as described by Marsh *et al.*[10]. This procedure briefly depicted in Fig. 4.

Strain differentiation of pathogens is essential to obtain relevant information in epidemiological studies. In most diagnostic laboratories, molecular characterization of *M. paratuberculosis* isolates still relies on analysis of the restriction patterns of the DNA hybridized with a IS900 probe (IS900-RFLP analysis) [3, 11, 12]. The discriminatory power of this technique is limited by the number of copies of insertion loci of IS900 in the genome and in some countries a single IS900-RFLP type is predominant [5, 13, 14]. The majority of research studies have used RFLP, pulse-field gel electrophoresis or related techniques for Map genotyping. These techniques are laborious, time-consuming and need large amount of genomic DNA from cultured bacteria [3, 4, 11, 15, 16]. The PCR/REA test using

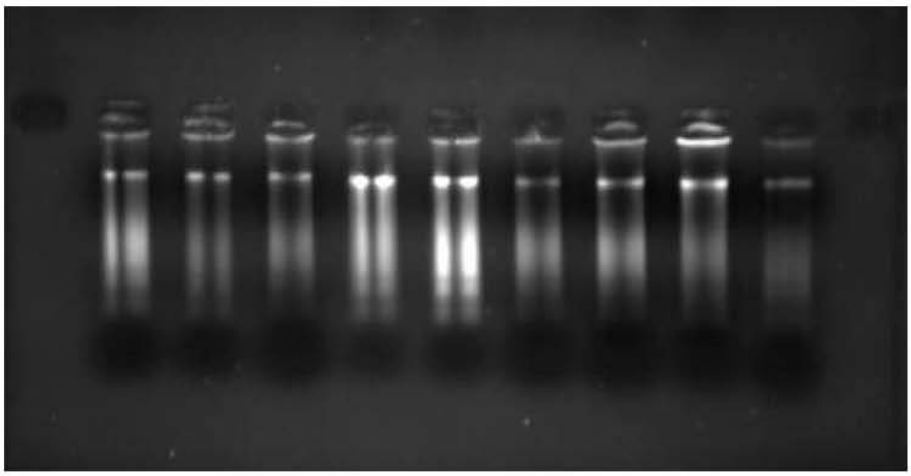

Fig. 1: DNA extracted from feces samples after electrophoresis on 1% agarose

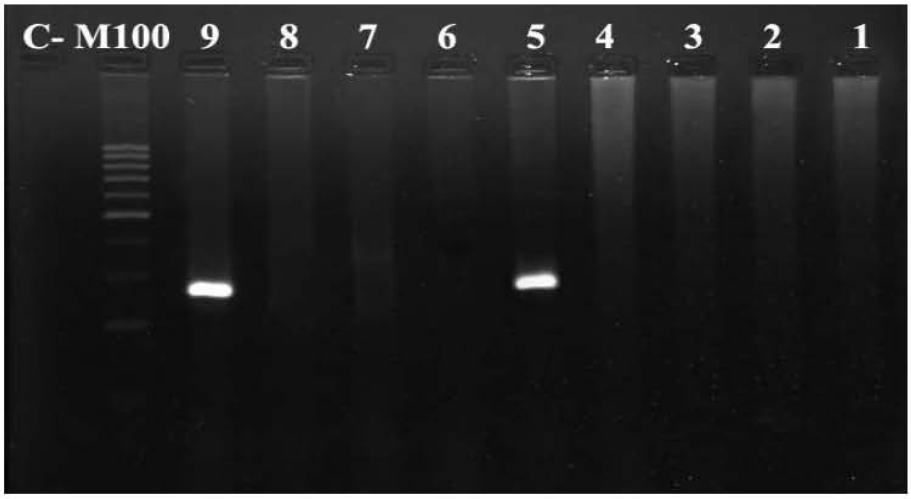

Fig. 2: Results of IS1311 PCR amplification on feces DNA

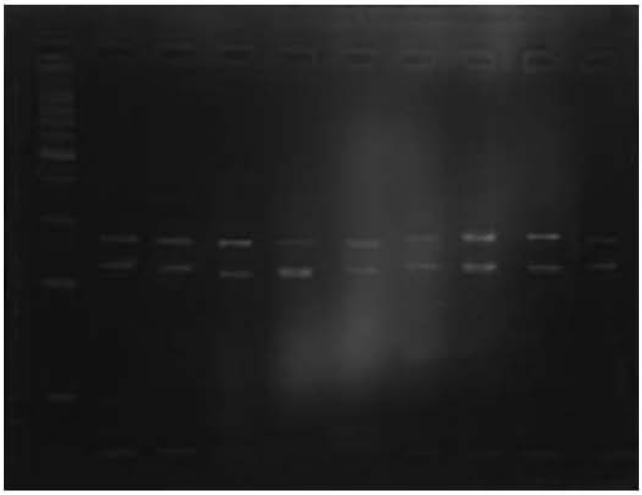

Fig. 3: IS1311 PCR product digested with HinfI restriction endonuclease

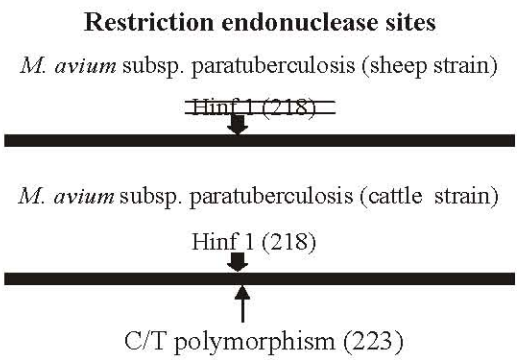

Fig. 4: IS1311 PCR REA by *HinfI* restriction endonuclease

IS1311 sequence was highly sensitive and specific for differentiating *M. paratuberculosis* isolates without the need for IS900 PCR-FFLP. Easily interpretable REA patterns made this test suitable for diagnostic samples.

S strains are uncommonly associated with paratuberculosis in species other than sheep whereas C strains have been recovered from cattle and many other species with paratuberculosis (reviewed in Whittington *et al.* [5]). In Australia there are no published reports of paratuberculosis in cattle due to S strains and several molecular epidemiological surveys have failed to detect S strains in cattle [5, 17]. On the other hand, Whittington *et al.* [6] reported that they isolate S strain from some of infected cows in Iceland. They noted that in all of these animals there had been direct or indirect contact of calves with paratuberculous sheep. These findings confirmed the possibility of transmission of infection from sheep to cattle.

Although we could not detect any S strain in this farm but its detection is possible if sample size increased and sampling is done from different farms.

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