Comp Clin Pathol (2012) 21:755–760
DOI 10.1007/s00580-010-1170-7

ORIGINAL ARTICLE

PCR-restriction endonuclease analysis of *Mycobacterium avium* subsp. *paratuberculosis* isolates from goats, sheep, and cattle in Jordan

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Received: 13 October 2010 / Accepted: 16 December 2010 / Published online: 11 January 2011

Abstract Paratuberculosis is an endemic disease and induces high economical losses in Jordan. There is no information available on genotypic variation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolated from animals in Jordan. In this study, we investigated 150 fecal samples from sheep, goats, and cattle for the presence of paratuberculosis using bacterial culture and polymerase chain reaction (PCR)-RFLP analysis of insertion sequence IS*1311*. Analysis of the results revealed that genotypic information from sheep, goat, and cattle could classify them into cattle or sheep strains. All culture isolates from cattle, 12.5% of the isolates from sheep, and 50% of the isolates from goats were cattle strain, while 87.5% of the isolates from sheep and 50% of the isolates from goats were sheep strain. Sequencing of the IS*1311* 268 bp PCR product from the three animal species confirmed the different MAP patterns.

Keywords Paratuberculosis · PCR · Sheep · Goats · Cattle · Jordan

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Introduction

Paratuberculosis (Johne's disease) is chronic granulomatous enteritis in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Lilenbaum et al. 2007). *Mycobacterium avium* subsp. *paratuberculosis* is a gram-positive, acid-fast, and facultative anaerobic intracellular bacterium (Buergelt et al. 2004). The disease has been reported in domestic and wild ruminants around the world such as cattle, sheep, goat, bison, and species of camelides, bighorn sheep, Rocky Mountain goat, deer, and elk (Sivakumar et al. 2006).

Analysis of the molecular diversity within MAP strains from various animal species will increase understanding of the host range, distribution, and natural history of MAP infections (Bull et al. 2000). The most commonly used typing method is the analysis of restriction fragment length polymorphism (RFLP) using IS*900*-specific probes (IS*900* RFLP) (Djønne et al. 2005). Bacterial isolates of MAP were first classified into cattle and sheep types in 1990. IS*900* RFLP classified isolates from cattle, sheep, and goats into two major groups, these were called sheep (S) and cattle (C) types (Collins et al. 1990). A new IS*1311* band pattern in bison isolate from Montana (USA) was discovered and considered as bison (B) type (Whittington et al. 2001). In India, the same B type was found in 5 ovine MAP isolates and 6 caprine MAP isolates (Sevilla et al. 2005), and 24 buffalo MAP isolates (Yadav et al. 2007). The sheep strain appears to infect sheep and goats, whereas the cattle strain is known to infect many species including cattle, goats, sheep, and man (Pavlik et al. 1995 and Collins et al. 1993).

Polymerase chain reaction (PCR) amplification of IS*1311* followed by restriction endonuclease analysis (PCR-REA) has been used to distinguish between cattle or sheep strains of MAP. The method is based on a point

mutation that has created an extra *Hinf*I site, which is found in some copies of IS*1311* in cattle strains but not in sheep strains of MAP. IS*1311* PCR-REA is rapid and provides information similar to IS*900* RFLP (Marsh et al. 1999). Other methods have been used to distinguish between isolates of MAP include amplified fragment length polymorphism analysis and random amplified polymorphic DNA analysis. Another promising technique for typing MAP is pulsed-field gel electrophoresis of digested genomic DNA (Sevilla et al. 2007).

Very limited studies on Johne's disease and on the different genotypes of MAP have been conducted in the Middle East. In previous studies, we have found that the disease is prevalent in Jordan and induces high economical losses (Hailat et al. 2010). About 65% of the apparently healthy cattle, 60% of camels, and more than 70% of sheep and goats were found to have lesions compatible with Johne's disease. No previous studies have been carried out to determine the strains of MAP that possibly exist in Jordan.

Therefore, the aim of the present study is to investigate the genotypic variation among MAP isolates from sheep, goats, and cattle in Jordan using IS*1311* PCR-REA.

Materials and methods

Sample collection During a period of 7 months, from July 2007 to January 2008, a total of 150 fecal samples (50 each) were collected from local Awassi sheep (Baladi), goats (Baladi and Shami), and Friesian cattle, older than 2 years. Fecal samples were collected from each animal rectally using a clean, dry examination glove, placed into clean plastic storage containers, transported to the laboratory on ice, and then stored at −80°C until they were processed for culture and PCR-REA.

MAP culture To culture and isolate MAP, fecal samples were processed by sedimentation method described by Stabel (1997). Briefly, 2 g of fecal sample was measured into 50-ml sterile, conical centrifuge tubes containing 35 ml sterile water. Tubes were placed in a rack and shaken on a horizontal shaker for 30 min at 22°C. Tubes were returned to an upright position and the sample suspension allowed to settle for 30 min. Five milliliters of the upper portion of the supernatant was transferred to a new 50-ml centrifuge tube containing 25 ml 0.9% hexadecylpyridinium chloride (HPC) (Acros Organics, New Jersey, USA). Tubes were vortexed thoroughly to mix the sample with the HPC and then maintained in an upright position at room temperature for overnight decontamination of 16–24 h. Tubes of Herrold's egg yolk agar with mycobactin J and amphotericin B, nalidixic acid, and vancomycin (BD, USA) were then inoculated with 0.1 ml of the sediment at the bottom of the sample tubes. Inoculated tubes were incubated at 37°C (Binder GmbH, Germany) in a horizontal position for 1 week with loose caps to permit evaporation of residual moisture on the surface of the medium. Caps were then tightened, and tubes were returned to an upright position and were examined every week.

DNA extraction from feces DNA was extracted from fecal samples using the MAP Extraction System (Tetracore, USA). One gram of fecal material was transferred into a 15-ml conical tube. Two milliliters of 1× TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) was added to each fecal sample. The samples were vortexed for approximately 15 s to disrupt large particles and then incubated at room temperature without agitation for 5 min to allow larger particulate matter to settle in the bottom of the tubes. For bacterial lysis, fecal samples were transferred into disruption tubes containing glass beads. Disruption tubes were taped to the vortex and left for 10 min at high speed. The samples were centrifuged in the disruption tubes for 10 min at 13,200×g (Centrifuge 5415 R; Eppendorf, Germany). The supernatant was removed from the sample and placed into a 2-ml microcentrifuge tube. NAB buffer (provided with the kit, 100 μl) was added, and the tubes were inverted five times to mix and then centrifuged at 1,200×g for 3 min. Gently, the supernatant was removed and discarded. Binding buffer (provided with the kit, 560 μl) was added to the pellet, vortexed for 5 s to loosely disrupt the pellet, and then incubated at room temperature for 10 min. Absolute ethanol (560 μl) was added to the sample and mixed by vortexing for a few seconds. The sample (630 μl) was added to a spin column placed in a clean 2-ml collection tube and centrifuged at 5,200×g for 1 min. The last two steps were repeated. After that, 500 μl of the wash buffer A (provided with the kit) was added to the spin column which was placed into a clean collection tube and then centrifuged at 5,200×g for 1 min. Five hundred microliters of wash buffer B (provided with the kit) was added to the spin column placed into a clean collection tube. The spin columns were centrifuged at 12,000×g for 3 min then at 13,200×g for 1 min without buffers to remove any residual wash buffer B. The spin columns were placed into 1.5-ml Eppendorf microcentrifuge tubes. DNase free water (50 μl) was added to the spin column to elute the DNA, incubated at room temperature for 1 min, and centrifuged at 5,200×g for 1 min. DNA was stored at −20°C to be used for PCR analysis.

DNA extraction from culture media One or two colonies of MAP were taken from the positive culture tubes using a sterile loop and mixed with 1 ml of 1× TE buffer, then transferred into a disruption tube containing glass beads. Tubes were taped to the vortex for 10 min at high speed. The samples were

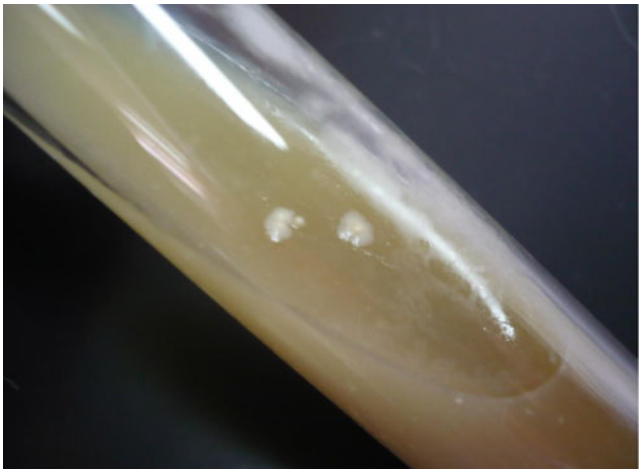

Fig. 1 Smooth colonies of cattle strain of MAP from cattle after 8–10 weeks of culture on Herrold's egg yolk agar

then centrifuged for 10 min at 13,200×g. The supernatant which contains extracted MAP DNA was transferred to a new 2-ml microcentrifuge tube and stored at −20°C.

Polymerase chain reaction PCR was used to discriminate between the sheep and cattle strains of MAP. The forward primer was M56 (5′-GCG TGA GGC TCT GTG GTG AA), and the reverse primer was M94 (5′-CAG CGA TCG TCG ACA GTG TG) (Marsh et al. 1999). GoTaq Green Master Mix (Promega, Madison, USA) was used, 1 μl of each primer (10 pM/μl) and 1 μl of the isolated fecal DNA sample were mixed in PCR tubes. Amplification was performed with a MyCycler Thermal Cycler (Bio-Rad, Hercules, California, USA) using the following conditions: 1 cycle of 94°C for 3 min followed by 37 cycles of 94°C

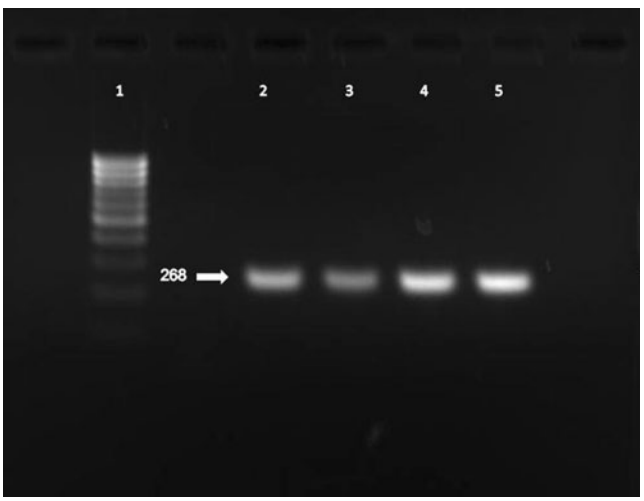

Fig. 2 IS*1311* PCR assay for MAP. *Lane 1* 100 bp DNA ladder. *Lane 2* PCR product of MAP from sheep (DNA from feces). *Lane 3* PCR product of MAP from goat. *Lane 4* PCR product of MAP from cattle. *Lane 5* PCR product of MAP from sheep (DNA from culture)

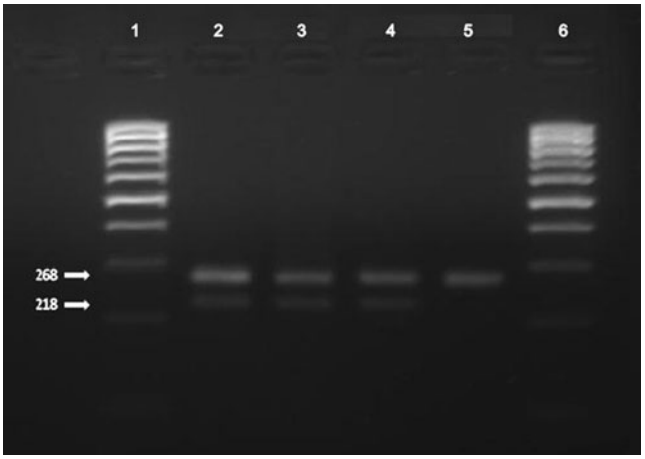

Fig. 3 *Hinf*I restriction endonuclease analysis of reaction IS*1311*. *Lanes 1* and *6* 100 bp DNA ladder. *Lane 2* a C-type pattern from sheep isolate. *Lane 3* a C-type pattern from goat isolate. *Lane 4* a C-type pattern from cattle isolate. *Lane 5* an S-type pattern from sheep isolate

for 30 s, 62°C for 15 s, and 72°C for 1 min. Polymerase chain reaction results were assessed by electrophoresis in 2% agarose gel stained with ethidium bromide.

Restriction endonuclease analysis of PCR product Restriction endonuclease analysis was performed as described previously (Marsh et al. 1999). Briefly, in a total volume of 20 μl, 10 μl of the PCR-purified product, 2 μl of buffer B, 2 μl of diluted bovine serum albumin, and 2 μl of 20-unit *Hinf*I restriction endonuclease (Promega, Madison, USA) were mixed and incubated at 37°C for 3 h, and then the enzyme was inactivated at 65°C for 20 min. The digested DNA was assessed by electrophoresis in 3% agarose gel stained with ethidium bromide.

DNA sequencing Insertion sequence IS*1311* PCR products from ovine, caprine, and bovine isolates were sent to sequencing by the ABI BigDye Terminator Sequencing Kit at the genomics facility in Princess Haya Biotechnology Center (Jordan University of Science and Technology, Irbid, Jordan). PCR product sequencing was done confirming the point mutation between strains and comparing the sequence from the three species. Jemboss software (European Molecular Biology Open Software Suite) was used to analyze and compare between these three sequences.

Results

Culture For the isolation of MAP, fecal samples were processed and cultured on Herrold's egg yolk agar. After 8–10 weeks of incubation, white colonies scattered on the media

Table 1 IS*1311* restriction endonuclease analysis of MAP from feces and culture of sheep, goats, and cattle

Host	Source of DNA	Number of samples	IS*1311* PCR-REA type
Cattle	Culture	2	C
Cattle	Feces	0	–
Sheep	Culture	1	C
Sheep	Feces	7	S
Goat	Feces	4	S
Goat	Culture	4	C

C cattle strain pattern, *S* sheep strain pattern

started to appear (Fig. 1). Out of 150 fecal samples cultured, seven (4.6%) were positive for MAP. From the positive samples, one from sheep, four from goats, and two were from cattle. According to these results, we expected the existence of two major groups of MAP strains based on the growth characteristics; the sheep strain which was very difficult to isolate using solid media and the cattle strain which started to grow in 8–10 weeks. When the culture positive samples were analyzed using IS*1311* PCR-REA, it was found that they were cattle strain and those that could not be isolated were sheep strain. Only two (1.3%) of the cultured tubes were contaminated by mold growth and were discarded.

IS1311 polymerase chain reaction All extracted DNA samples from fecal and culture were tested by using PCR amplification of the insertion sequence IS*1311*. Analysis of the results revealed that only nine samples of the sheep, five of the goats, and four of the cattle yielded positive results in the IS*1311* PCR assay with a PCR product band of approximately 268 bp (Fig. 2).

Restriction endonuclease analysis of reaction IS1311 Eighteen samples containing MAP DNA from different sources (feces and culture) and hosts (sheep, goats, cattle) were analyzed using IS*1311* PCR-REA. These samples gave a clear band pattern in restriction endonuclease (Fig. 3). IS*1311* PCR-REA from cattle samples confirmed the presence of the polymorphism at base position (223) in this strain. This was demonstrated by the presence of an extra band of 218 bp in the cattle strain digestion pattern compared to the sheep strain digestion pattern. The extra *Hinf*I restriction site also gives a 50-bp band that is difficult to see by electrophoresis in 3% agarose gels. The 218-bp band is consistent with the presence of a *Hinf*I site in copies of IS*1311* in the cattle strain. The 218-bp band in the cattle strain digestion pattern was also demonstrated in sheep and goat isolates. All tested samples from cattle, 12.5% (1) from sheep and 50% (4) from goats were cattle strain, while 87.5% of the samples from sheep and 50% (4) from goats were sheep strain. Table 1 shows IS*1311* PCR-REA results.

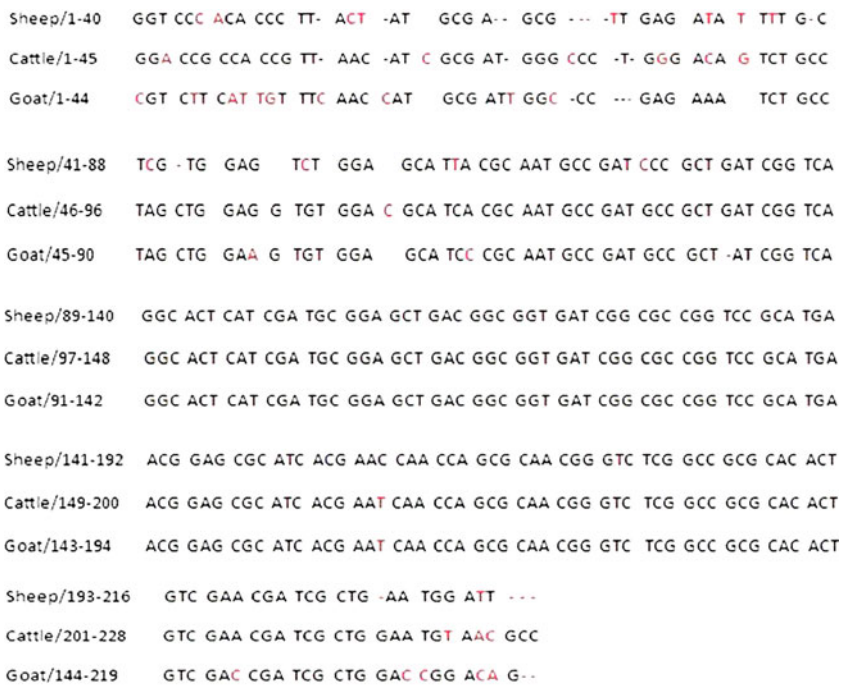

Fig. 4 Partial IS*1311* nucleotide sequence of the local MAP isolates from sheep, cattle, and goat

Sequencing Sequence alignment performed on the DNA sequences obtained from the amplified products shows that similarity between goat (G22) and sheep (S16) (89.1%), between sheep (S16) and cattle (C3) (83.3%), and between goat (G22) and cattle (C3) (84.2%) (Fig. 4). The sheep MAP isolate which was S type in restriction analysis, had a C at base position 159. A mixed C/T signal indicates a heterogeneous mutation was detected at 167 base position in cattle and 161 base position in goat, which were classified as C type by IS*1311* REA because this point mutation causes an extra *Hinf*I restriction site (Fig. 5).

Discussion

Johne's disease is prevalent and induces high economical losses in Jordan. Nevertheless, no studies have been conducted to report the strains of MAP that are present in Jordan. Analysis of the molecular diversity within MAP strains from various animal species would increase the understanding of the host range, distribution, and natural history of MAP infections. DNA-based subtyping techniques such as multiplex PCR and IS*900*-based RFLP analyses have been used in an attempt to reveal the genetic variation in MAP and differentiate among strains infecting different populations (Bauerfeind et al. 2006; Bull et al. 2000; Ellingson et al. 1998; Motiwala et al. 2004). In this study, all of the MAP fecal isolates strains were grouped into two major types according to the growth rate in vitro as well as molecular differences; and the difference in growth rate was compatible with molecular differences which were confirmed by using IS*1311* PCR-REA. Isolates of the organism were first classified into cattle and sheep types in 1990. IS*900* RFLP-classified isolates from cattle, sheep, and goats into two major groups using *Bst*EII endonuclease, these were called S and C strains (Collins et al. 1990). PCR amplification of IS*1311* followed by restriction endonuclease analysis has been used to distinguish between cattle or sheep strains of MAP. The method is based on a point mutation that has created an extra *Hinf*I site, which is found in some copies of IS*1311* in cattle strains but not in sheep strains of MAP. IS*1311* PCR-REA is rapid and provides information similar to Collins' IS*900* RFLP (Marsh et al. 1999). The sheep strain appears to infect sheep and goats,

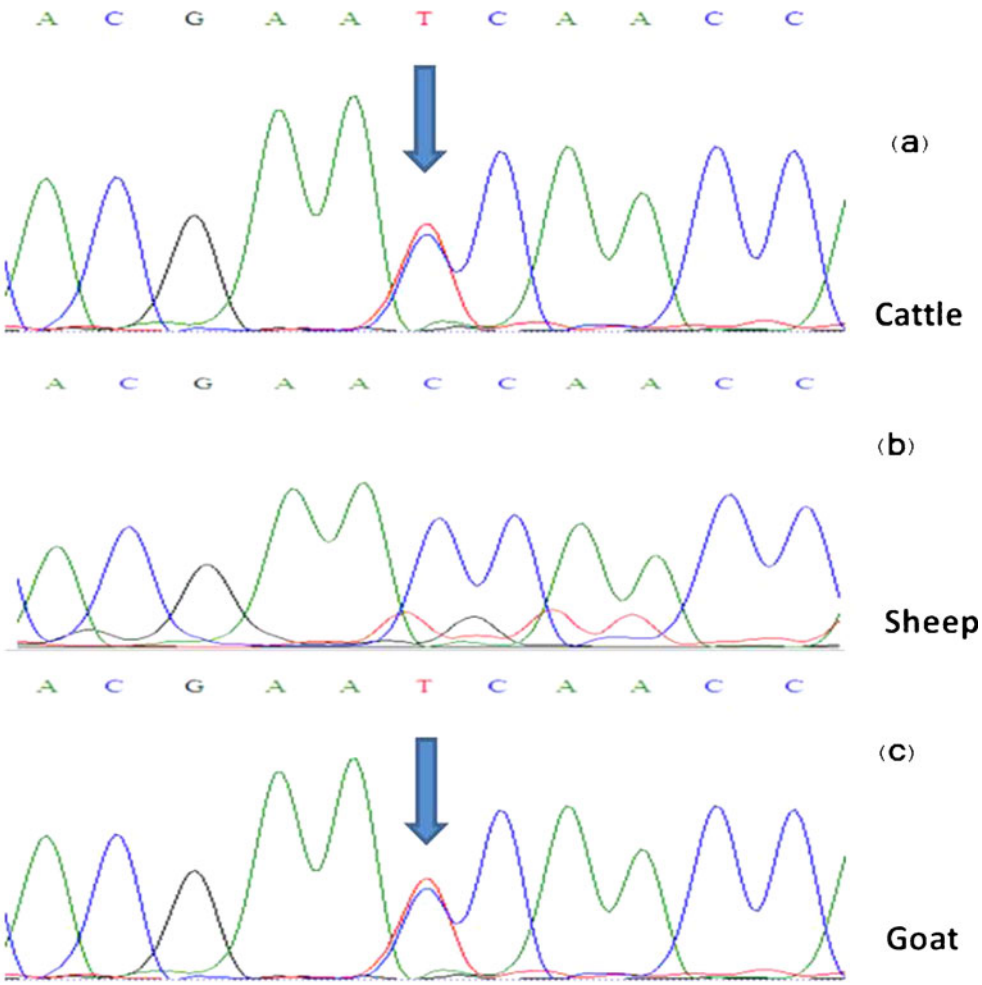

Fig. 5 Partial chromatogram sequence showing the mutation in cattle and goat MAP isolates. The site of the mutation is denoted by the *blue arrow*. **a** Sequence chromatogram from cattle with C/T mutation (C type). **b** Sequence chromatogram from sheep showing the absence of mutation (S type). **c** Sequence chromatogram from goat with C/T mutation (C type)

whereas the cattle strain is known to infect many species including cattle, goats, sheep, and man (Collins et al. 1990; Djønne et al. 2005; Garrido et al. 2000), and in our study, we had similar results. In Australia, cases of paratuberculosis in cattle that were negative in culture were determined to be infected by the S strain. Sheep strain was also identified in archival tissue from paratuberculosis sheep and cattle from Iceland (Whittington et al. 2001). In Spain, it was found that cattle are only affected with C strain, goats and sheep were also infected with C strain, and most of the sheep and cattle isolates were S strain. Our results show that all tested samples from cattle, 12.5% from sheep, and 50% from goats were cattle strain which is readily isolated on culture media, while 87.5% of the samples from sheep and 50% from goats were sheep strain which is difficult to grow in primary culture. Although all cattle isolates were of cattle type, we could not exclude that cattle may also be infected with sheep strain especially those cases that were negative in culture. When the PCR-amplified products were sent to sequencing, a mixed C/T signal indicates a heterogeneous mutation was detected at 167 base position in cattle and 161 base position in goat, which were classified as C type by IS*1311* REA because this point mutation causes an extra *Hinf*I restriction site. Sequence alignment shows that similarity between goat and sheep was 89.1%, between sheep and cattle 83.3%, and between goat and cattle 84.2%. This may suggest that goats may be infected with an intermediate strain that is different from sheep strain and cattle strain. This study confirms the existence of MAP genetic differences related to host factors that would help to explain the variable success rates in the primary isolation of MAP from some host species on culture media. These differences could also prove to be useful for the epidemiological studies of paratuberculosis.

Acknowledgment This work was funded by the Deanship of Research at Jordan University of Science and Technology.

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