International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (11), pp. 001-003, November, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Nested PCR on semen samples for the detection of *Mycobacterium avium* subsp paratuberculosis

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Accepted 8 November, 2018

Mycobacterium paratuberculosis **(MAP) is a gram positive, acid-fast bacterium and cause of Johne's disease in some animals. The important signs of this disease in bovine are diarrhea, weight loss, bowel inflammation, fever and reduce of milk production. The symptoms of this disease are very similar to Crohn©s disease in humans. The aim of this study was to use nested -PCR as an accurate and fast method to trace MAP in bull semen. Semen samples from 112 bulls were collected and DNA was extracted. Then, nested-PCR was performed by specific primers for IS900 gene of MAP. The PCR products with 230 bp length were estimated as a positive. The frequency of MAP in semen samples were 12.50%. The results were showed nested-PCR is a good procedure with high efficiency for detection of intracellular bacteria such as *M. paratuberculosis* in bull©s semen samples. Thus, despite this abundance more attention to this disease in bulls to identify MAP quickly is essential.**

Key words: MAP, nested-PCR, John's disease, IS900 gene, semen, bull.

INTRODUCTION

Mycobacteria are rod-shaped, acid fast, Gram- positive aerobes or facultative anaerobes. The genus Mycobacterium is a large group with more than 70 species (Shinnick et al., 1994). *Mycobacterium avium* subspecies paratuberculosis (MAP) causes Johne's disease or paratuberculosis, a gastro intestinal inflammatory condition in ruminants and other animals, MAP may also be a cause of Crohn's disease in human (Donaghy et al., 2003; Nebbiaa et al., 2006).

Although animals with clinical disease are often culled from the herd, animals with sub clinical paratuberculosis may cause economic losses because of reduced milk production and poor reproductive performance (Kalis et al., 1999).

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Abbreviations: MAP, *Mycobacterium avium* subspecies paratuberculosis.

Johne's disease causes major economic losses to the dairy herds. The level of infection in a herd increases over time and if the disease is left unmanaged, the economic effect of bovine Johne's disease becomes increasingly significant (Pence et al., 2003). Diarrhea and rapid weight loss are the two main symptoms of Johne's disease. Diarrhea may be less common in certain species of animals such as goats and sheep (Nebbiaa et al., 2006; Stephan et al., 2007). In general, the larger the herd the more likely it is to have animals infected with Johne's disease. After the long incubation period, the main clinical signs seen in infected animals are profuse, long-term watery diarrhea, marked weight loss and, sometimes, intermittent fever. Johne's infected cows continue to eat even with severe diarrhea. The diarrhea usually has no blood or mucous in it. So the clinical symptoms of Johne's disease are largely non-specific and may be caused by several other agents. Often, even in severe clinical cases, Johne's disease is not recognized and the animals are simply sent to slaughter without concern for the underlying cause of the disease. Within any infected herd, only a very few infected cows

will have diarrhea at any one time (Nebbiaa et al., 2006; Stephan et al., 2007). The seroprevalence of Johne's disease in Georgia beef and dairy cull cattle in United States in 2000 was estimated and in dairy cattle was 9.58%, in beef cattle it was 3.95% and in cattle of unknown breed it was 4.72% (Pence et al., 2003). Serologic tests, such as ELISA, agar gel immunodiffusion (AGID) test and fecal culture are recommended to confirm the diagnosis of paratuberculosis in a clinically affected animal or in an infected herd. Indirect diagnostic methods based on immunological techniques such as skin testing with johnin, -interferon test, complement fixation test, and enzyme linked immmunosorbent assay have shown low sensitivity or specificity (Buergelt et al., 2004).

The disease is principally confined to the small intestinal tract and its draining lymph nodes. Infection may disseminate to extra intestinal sites as evidenced by successful cultural isolation of the organism from milk, fetus, lung, and semen. Unlike the intestinal tract, these other organs do not elicit a typical inflammatory response to the presence of the organism (Buergelt et al., 2004). Earlier reports describe the isolation of MAP from male accessory genital organs and semen in bulls and from semen in rams. The use of molecular biological methods for the detection of MAP in milk and other matrices were made possible by the discovery of specific DNA sequences, particularly IS900 (Larsen et al., 1981) . The discovery of the IS900 insertion sequence in the MAP genome has offered an alternative for the rapid detection of the bacterium DNA in clinical samples (Taddei et al., 2004). The purpose of this study was to distinguish the potential of nested PCR for the rapid detection of *M. avium* subsp. paratuberculosis in semen samples from bulls and determine the frequency of Johne's disease infection in bulls.

MATERIALS AND METHODS

A total of 112 semen samples were collected randomly from Animal Science Research Institute, Iran in summer of 2010. Semen was stored at –70°C until use. Genomic DNA was isolated from semen samples with DNA extraction kit (Qiagen, Germany), according to the producer's instructions. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001).

PCR amplification was performed with two sets of primers: the outer oligonucleotide primers were ISo-1 F: 5´-GTTCGGGGCCGTCGCTTAGG-3´ and ISo-1R: 5´GAGGTCGATCGATCGCCCACGTGA-3´ and the inner oligonucleotide primers were ISi-2F: 5´-CCGCTAATTGAGAGATGCGATTGG-3´ and ISi-2R:5´-AATCAACTCCAGCAGCGCGGCCTCG-3´.

The target sequence was amplified in a 50 µl reaction volume containing 100 ng of genomic DNA, 0.2 mM dNTPs, 1X Taq buffer, 2 mM $MgCl_2$, 100 ng of each primer and 1 unit of Taq DNA polymerase (Fermentas, Germany).

The first round of PCR was carried out by applying a step-up program as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final extension for 5 min at 72°C. Two to five l from the first round amplicon was used as a template for the second round PCR with the identical PCR program by inner oligonucleotide primers. Amplified samples were analyzed by electrophoresis (120 V/208 mA) in 1.5% agarose gel. Positive and negative PCR controls were run with each series of amplifications. The gel was stained with 0.1% ethidium bromide (0.4 g/mL) and viewed on UV transilluminator.

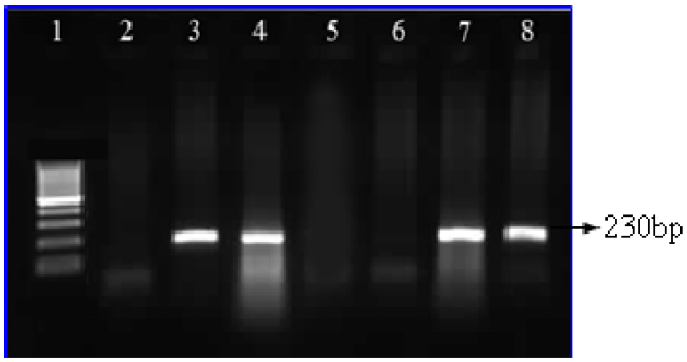

Figure 1. Identification of *M. paratuberculosis* by nested-PCR amplification of the IS900 insertion element. Lane 1: 100 bp DNA ladder. Lanes 2 and 3: negative and positive controls respectively. Lanes 4, 7 and 8 are positive samples of *M. paratuberculosis*. Lanes 5 and 6 are negative samples.

RESULTS

In this study, a total of 112 semen samples of bulls were tested for MAP using a nested PCR assay. The nested PCR assay used in this study enabled the detection of IS900 gene of MAP. Nested PCR amplification of the *M. avium* subsp. paratuberculosis in specific insertion sequence IS900 and subsequent agarose gel analysis of the amplified products showed a single band of 230 bp for each of the positive semen samples (Figure 1). The presence of MAP DNA was detected by nested PCR in semen samples were from 14 out of 112 specimens (12.50%).

DISCUSSION

M. avium subsp. paratuberculosis (MAP) is a bacterium that is the cause of Johne's disease, was isolated from the feces of a donor bull in an artificial insemination stud (Larsen et al., 1981). MAP can live in animals for years without necessarily causing clinical disease (Ayele et al., 2005). MAP may have a role in the development of Crohn's disease in humans via the consumption of contaminated milk and milk products (Pillars et al., 2009; Doosti and Moshkelani, 2010). Milk and milk products derived from cows with clinical or suspected paratuberculosis are not consumable even after pasteurization (Ayele et al., 2005).

The signs of this infection in human are a chronic

inflammatory and bowel disease that can be severe, prolonged and debilitating (Donaghy et al., 2003; Kalis et al., 1999).

There are two methods for transmission of *M. avium* subsp. paratuberculosis is considered and include direct faecaloral cycle and indirect transmission, such as through manure contamination of water bowls and machinery used for feed delivery (Doosti and Moshkelani, 2010).

PCR based on IS900 has been used for direct detection of MAP, without primary culture, from milk, faecal specimens, semen and human intestinal tissue and workers have been able to identify the presence of paratuberculosis DNA in intestinal tissue from patients with Crohn's disease and semen samples from bulls. Because the clinical symptoms of Crohn's disease closely mimic those found in animals with Johne's disease (Donaghy et al., 2003; Doosti et al., 2010; Herthnek et al., 2006).

Many studies have been focused on the association of Crohn's disease with MAP. Publications dealing with the culture detection of MAP in milk and milk products have also been increasing in number over the last decade (Rademaker et al., 2007; Stephan et al., 2007).

Larsen et al. (1981) separated these bacteria from the genital organs and the semen of bulls (Larsen et al., 1981). Larsen et al. (1981) study for tracing *M. paratuberculosis* in the semen and genital organs of a semen-donor bull showed 8 of 31 semen samples are infected (Larsen et al., 1981). The prevalence of *M. avium* subspecies paratuberculosis in bulk-milk samples via tracing of IS900 gene in Switzerland was 19.7% and indicated MAP can therefore often be transmitted to humans by raw milk consumption (Corti et al., 2002). These results largely same to the results of current study.

Claus et al. (2004) showed that *M. avium* subsp. paratuberculosis may disseminate hematogenously to the male reproductive tract and semen as an extra intestinal site and agent reservoir (Buergelt et al., 2004). Nebbia et al. (2006) performed molecular diagnosis pathway for detection of mycobacterium in goat and sheep milk. MAP DNA was intermittently recovered in milk samples from 13 out of 29 animals (44.8% prevalence). Furthermore, MAP was particularly found in 9 out of 15 seropositive animals, and in 4 out of 14 seronegative (Nebbiaa et al., 2006). According to the results of this study indication of IS900 gene by nested PCR assay is useful to detect *Mycobacterium* paratuberculosis directly from semen samples of bulls and that it could become a valuable diagnostic or screening test for herds with Johne's disease.

ACKNOWLEDGEMENTS

We would like to thank head and deputy of research of Islamic Azad university of Shahrekord branch in Iran and microbiological laboratory for this sincere support.

REFERENCES

Ayele WY, Svastova A, Roubal P, Bartos M, Pavlik I (2005). *Mycobacterium avium* subspecies paratuberculosis cultured from locally and commercially pasteurized cow's milk in the Czech Republic. Applied and Environment. Microbiol., 71(3): 1210-1214.

Buergelt CD, Donovan GA, Williams JE (2004). Identification of *Mycobacterium avium* subspecies paratuberculosis by polymerase chain reaction in blood and semen of a bull with clinical paratuberculosis. Intern. J. Appl. Res. Vet. Med., 2(2): 130-134.

Corti S, Stephan R (2002). Detection of *Mycobacterium avium* subspecies paratuberculosis specific IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. BMC Microbiol., 2: 1-7.

Donaghy JA, Totton NL, Rowe MT (2003). Evaluation of culture media for the recovery of *Mycobacterium avium* subsp. paratuberculosis from Cheddar cheese. Lett. Appl. Microbiol., 37(4): 285-291.

Doosti A, Moshkelani S (2010). Application of IS900 nested-PCRfor detection of *mycobacterium avium* subsp. Paratuberculosis directly from faecal specimens. Bulgarian J. Vet. Med., 13(2): 92-97.

Herthnek D, Englund S, Willemsen PTJ, Bo Lske G (2006). Sensitive detection of *Mycobacterium avium* subsp. paratuberculosis in bovine semen by real-time PCR. J. Appl. Microbiol., 100: 1095-1102.

Kalis CHJ, Hesselink JW, Russchen EW, Barkema HW, Collins MT, Visser IJR (1999). Factors influencing the isolation of *Mycobacterium avium* subsp. paratuberculosis from bovine fecal samples. J. Veterin. Diagnos. Investig., 11: 345-351.

Larsen AB, Stalheim OH, Hughes DE, Appell LH, Richards WD, Himes EM (1981). *Mycobacterium* paratuberculosis in the semen and genital organs of a semen-donor bull. J. Am. Vet. Med. Assoc., 179(2): 169-171.

Nebbiaa P, Robinoa P, Zoppib S, De Meneghi D (2006). Detection and excretion pattern of *Mycobacterium avium* subspecies paratuberculosis in milk of asymptomatic sheep and goats by Nested-PCR. Science Direct., 66(1): 116-120.

Pence M, Baldwin C, Carter Black C (2003). The seroprevalence of Johne's disease in Georgia beef and dairy cull cattle. J. Vet. Diagn. Invest., 15: 475-477.

Pillars RB, Grooms DL, Woltanski JA, Blair E (2009). Prevalence of Michigan dairy herds infected with *Mycobacterium avium* subspecies paratuberculosis as determined by environmental sampling. Preventive Vet. Med., 89: 191-196.

Rademaker JL, Vissers MM, Te Giffel MC (2007). Effective heat inactivation of *Mycobacterium avium* subsp. Paratuberculosis in raw milk contaminated with naturally infected feces. Appl. Environ. Microbiol., 73: 4185-4190.

Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual. 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shinnick TM, Good RC (1994). Mycobacterial taxonomy. Eur. J. Clin. Microbial. Infect. Dis., 13(11): 884-901.

Stephan R, Schumacher S, Tasara T, Grant IR (2007). Prevalence of *Mycobacterium avium* subspecies paratuberculosis in Swiss raw milk cheeses collected at the retail level. J. Dairy Sci., 90: 3590-3595.

Taddei S, Robbi C, Cesena C, Rossi I, Schiano E, Arrigoni N, Vicenzoni G, Cavirani S (2004) . Detection of *Mycobacterium avium* subsp. paratuberculosis in bovine fecal samples: comparison of three polymerase chain reaction–based diagnostic tests with a conventional culture method. J. Vet. Diagn. Invest., 16: 503-508.