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Detection of *Mycobacterium paratuberculosis* in Feces and Milk Samples from Holstein Dairy Cows by PCR

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Abstract: *Mycobacterium paratuberculosis*, an acid-fast bacterium is the agent of Johne's disease, an intestinal disease that cause poor nutrient intake in ruminants. Because of the heavy economic loses due to this disease, a diagnostic procedure must be defined that could determine the disease in its initial stages. PCR method based on specific IS900 locus primers was used. Feces and milk samples were collected from 68 Holstein dairy cows. 19 feces samples (of 68 samples) were PCR positive whereas 10 milk samples (of 56 samples) were positive. Results of this study showed that PCR might be the preferred method for detection of the disease with feces and milk samples because of its simplicity, sensitivity and rapidity.

Key words: Johne's disease, *Mycobacterium paratuberculosis*, PCR, Holstein dairy cows

INTRODUCTION

Johne's disease affects ruminants worldwide. Due to its long incubation period, mainly subclinical or hidden forms of Johne's disease are noticed. However, after a long subclinical phase, clinical signs can be recorded, such as cachexia and diarrhea. *Paratuberculosis* in livestock causes significant economic losses due to reduced production, increased treatment cost and culling (Ott *et al*., 1999) Herd prevalence of Johne's disease in Europe is reported to be between 7 and 55%, in the United States nearly 40% and in Australia it ranges between 9 and 22% (Manning and Collins, 2001). *Mycobacterium avium* subsp. *paratuberculosis* (Map) may also be a cause of Crohn's disease in human (Gaya *et al*., 2004; Greenstein and Collins, 2004; Romero *et al*., 2005). Map is a slow growing fastidious acid-fast bacillus that requires ferric mycobactin for *in vitro* growth in the culture. Recently, Map has been detected in blood from patients with Crohn's disease (Naser *et al*., 2004). In animals, Map infection can also be systemic (Gwozdz *et al*., 2000), due to extraintestinal infiltration in blood stream, occasionally leading to the presence of the pathogen into milk (Sweeney *et al*., 1992). Research works of Grant *et al*. (2002) and Ayele *et al*. (2005) have confirmed the presence of Map in retail milk (pasteurized as well as raw) supplies suggesting consumer exposure to the pathogen. This may explain why Crohn's disease is moderately prevalent in the developing as well as the developed world, where milk is consumed (Greenstein and Collins, 2004).

Disease control is hampered due to the hidden nature of Johne's disease and ineffective diagnosis, particularly in the subclinically infected animal population. Diagnostic tests, such as ELISA, agar gel immunodiffusion (AGID) test and fecal culture, are being used commonly (Buergelt *et al*., 2004). Though fecal culture is considered the gold standard, the method is time-consuming (6-8 weeks). Serological tests, coupled with apparent clinical signs, can be considered reliable for Johne's disease diagnosis. However, in apparently healthy or subclinically infected animals, tests aimed at antigen or antibody detection frequently give rise to false negative results (Buergelt and Williams, 2004). PCR methods, targeting Map specific insertion sequence (IS900) or other species-specific genes, have been developed to increase specificity and sensitivity of diagnosis, as well as shorten the time required (Millar *et al*., 1995; Englund *et al*., 1999; Djonne *et al*., 2003; O'Mahony and Hill, 2004; Ellingson *et al*., 2005). IS900 belongs to the same family of insertion sequences as IS901 (*M. avium* subsp. *avium*), IS902 (*M. avium* subsp. *silvaticum*) and IS1110 (*M. avium* subsp. *avium*) (Englund *et al*., 2002). IS900 is a 1451 bp segment that lacks inverted terminal repeats and does not generate direct repeats in target DNA (Green *et al*., 1989; Englund *et al*., 2002). IS900-based RT-PCR detection was also used to differentiate the viable Map infection in patients with Crohn's disease (Mishina *et al*., 1996; Naser *et al*., 2004). To date, studies have focused on the PCR-based detection of Map from feces, milk or culture. In this study, we used IS900 sequence to investigate the presence of Map in bovine feces and milk samples.

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MATERIALS AND METHODS

Sampling: A total of 68 feces samples and 56 milk samples were randomly obtained from keneh Bist Dairy Farm (Mashhad), supposed to have high number of infected animals, according to previous records in 2006. Feces were collected from the rectums of animals with high attention on cross contamination. Milk samples were obtained from aseptic teats (equally from each teat). Feces and milk samples were stored at -20°C until DNA extraction.

DNA extraction from feces samples: 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^{-1} Triton X-100 and 10 g L^{-1} DTT) and vortexed vigorously for 10 min. The tubes were incubated in a hot plate incubator at 65°C for 1 h and vortexed each 5 min 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. Four hundred microliter 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. Seventy five microliter 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.

DNA extraction from milk samples: For DNA extraction, milk samples (2800 μL from each sample) were transferred to 2 screw-capped 1.5 micro centrifuge tubes and centrifuged at 3000 rpm for 5 min. After centrifugation, cream and whey layers were discarded and pellets were transferred to a new micro tube. Twenty microliter proteinase K enzyme and 400 μL of Lysis Reagent was added to each tube and mixed gently to homogenize the tube content and incubated in a dry plate incubator at 65 for 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. Four hundred microliter 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. Tubes containing pellets were placed in dry plate incubator for 5 min until pellets dried completely. Seventy five microliter 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 IS900 specific primers: P90 (GAA GGG TGT TCG GGG CCG TCG CTT AGG) and P91 (GGC GTT GAG GTC GAT CGC CCA CGT GAC). 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 40 sec at 94°C, 30 sec 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 samples were considered as positive if 413 bp amplified band was present.

RESULTS AND DISCUSSION

The quality of extracted DNA from feces and milk samples by this procedure was good (Fig. 1, 2) and revealed that this method is comparable with other methods that used for milk DNA extraction used by Stabel *et al.* (2002) and Giese and Ahrens (2000) because of its rapidity and simplicity.

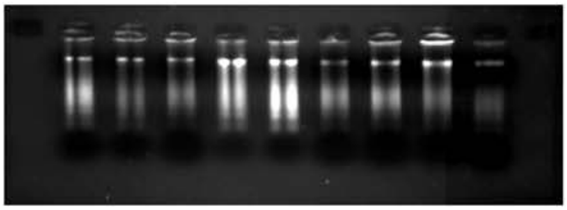

Fig. 1: DNA extracted from feces samples after electrophoresis on 1% agarose

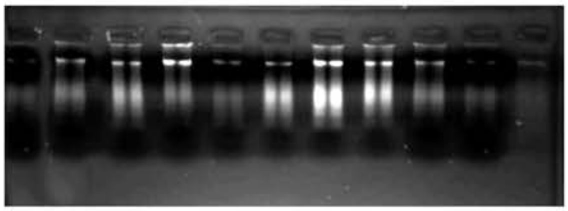

Fig. 2: DNA extracted from milk samples after electrophoresis on 1% agarose

Fig. 3: Results of IS900 PCR amplification on feces DNA

Fig. 4: Results of IS900 PCR amplification on milk DNA

Results of PCR amplification on feces DNA showed that Map genome is present in 28% (19 of 68) of samples (Fig. 3). 18% (10 of 56) of milk DNA PCR amplification results were positive (Fig. 4).

Studies of diagnostic tests for Johne's disease have often been carded out on clinically affected animals which shed very large numbers of Map organisms in their feces. In this study, fecal specimens were selected to include those from clinically normal cattle shedding small numbers of these organisms. Successful control of Johne's disease requires the identification of this group of animals.

Ability of PCR test to detect infected animals by milk samples was less than fecal samples (18% versus 28%). Previously showed that the pattern of bacterial excretion into milk is depend on the number of bacteria exist in the gastrointestinal tract (Nebbia *et al*., 2006). So it can be supposed that bacterial content of gut of some of animals were very low that can't be detected in their milk samples.

Altogether, it can be concluded that PCR test because its high sensitivity and specificity is applicable for detection of john's disease in its initial steps. Also this procedure is useful for detection of Map genome in milk samples depends on the status of the disease.

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