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APPLICATION OF IS900 NESTED-PCR FOR DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* DIRECTLY FROM FAECAL SPECIMENS

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Summary

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Mycobacterium paratuberculosis (MAP) is known to cause a chronic inflammation of intestine in cattle, called Johne's disease. The symptoms are very similar to Crohn's disease in humans. The feasibility of coupling a nested-PCR was investigated as a mean to improve turnaround time and detect MAP in the presence of contaminants.

The aim of this study was to use nested-PCR as an accurate and rapid method to detect MAP in bovine faeces. Faecal samples from 120 dairy cattle were collected and the extracted DNA was evaluated by PCR test for the MAP-specific IS900 gene. Of the 120 specimens, 96 were identified positive by direct microscopy, whereas 4 were positive by single PCR. Nested-PCR showed a detection rate of 70.8% (68 out of 96 samples) as compared to direct microscopy. Nested PCR also identified many positive samples that were not detected by either Ziehl Neelsen staining or single PCR. The results show that IS900 nested-PCR assay may be applied to detect *M. paratuberculosis* directly from faecal sample of dairy cows and therefore could be a valuable diagnostic or screening test for herds with Johne's disease.

Key words: Johne's disease, *Mycobacterium paratuberculosis*, nested-PCR

INTRODUCTION

Bacteria of the genus *Mycobacterium* are Gram-positive, acid-fast bacteria that include a number of significant human and animal pathogens (Harris & Barletta, 2001). The genus *Mycobacterium* is a large group with more than 70 species (Shinnick & Good, 1994). Although the best known species are *M. tuberculosis* and *M. bovis*, which are associated with human and bovine tuberculosis respectively, other species may cause disease in animals or exist in the environment and rarely or never cause infection in otherwise healthy humans (Cocito *et al.*, 1994; Falkinham, 1996). Paratuberculosis or Johne's disease, is a chronic, progressive enteric disease of ruminants caused by *Mycobacterium avium subsp. paratuberculosis* (MAP). Clinical disease in cattle is characterized by weight loss, diarrhoea, reduced milk production, and ultimately death. Animals are most likely infected by ingestion of contaminated food or milk before 6 months of age. Because of the slow progression of the disease, clinical signs are often not observed until the ani-

mal is at least 3 years-old (Huntley *et al.*, 2005).

Although animals with clinical disease are often culled from the herd, animals with subclinical paratuberculosis may cause economic losses because of reduced milk production and poor reproductive performance (Stabel, 1998). During the preclinical incubation stage bacteria are shed through the milk, faeces and semen at varying levels, serving as a potential source of infection for other animals (Slana *et al.*, 2008).

Some animals can recover spontaneously. In other animals, MAP survives and multiplies in the intestinal tract mucosa. Having no clinical symptoms, these animals (often highly productive) become dangerous vectors of infection (sources of infection for other susceptible animals), shedding MAP into the external environment in their milk and faeces. Other animals may become carriers of MAP which survives in the intestinal tract (Clark *et al.*, 2006; Hasonova & Pavlik, 2006).

There is no substantiated causal link between Johne's disease and Crohn's disease. MAP may however have a role in the development of Crohn's disease in humans via the consumption of contaminated milk and milk products (Donald *et al.*, 2005; Slana *et al.*, 2008). Therefore, the assays for its reliable detection, especially in subclinical cases, are particularly important.

The culture method for MAP detection holds the advantage of specificity. However, the disadvantages of this method are the long time necessary for culture (six weeks or more) and the insufficient effectiveness of decontaminating methods (Harris & Barletta, 2001; Ayele *et al.*, 2005).

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 (Green *et al.*, 1989). The sequencing of the entire MAP genome (strain K-10) also significantly contributed to the precise detection and identification of MAP specific sequences (Li *et al.*, 2005). With regard to the fact that various modifications of the PCR method are usually employed for MAP detection.

The aim of this study was to assess the potential of nested PCR for the rapid detection of *Mycobacterium avium* subsp. *paratuberculosis* in faecal samples from dairy cows and compare its performance with single PCR.

MATERIALS AND METHODS

Samples

120 faecal specimens were collected from dairy cows known to have or suspected of having Johne's disease. Faeces were collected from the rectums of animals while they were on the farm or during postmortem examination of cows that were killed immediately before sample retrieval. Faeces were stored at –20°C until use.

Acid-fast staining

Smears were prepared from faeces and dried in an oven at 65°C, and stained by a Ziehl-Neelsen (ZN) technique (Cruickshank *et al.*, 1973).

Extraction of DNA from faecal samples

Faecal samples were suspended in 500 µL of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 30 mM DTT, 0.5% SDS) supplemented with 0.4 mg/mL proteinase K (Boehringer, Mannheim, Germany). The samples were kept overnight in a heating block set at 55°C. After the lysis, samples were heated at 95°C for 10 min. Then equal volume of phenol:chloroform:iso-

amylalcohol (25:24:1 v/v/v), was added, vortexed for 30 seconds and centrifuged at 12000×*g* for 10 min. The aqueous phase was transferred to new clean tube and equal volume of ice cold isopropanol was added. The DNA was pelleted by centrifigation at 12000×*g* for 15 min, the pellet washed with 70% ethanol and dried at 65°C for 5 min. The DNA was finally resuspended in 50 μL sterile distilled water. The isolated DNA was quantified on agarose gel.

Analysis of samples with single PCR

Detection of *Mycobacterium avium subsp. paratuberculosis* was performed by amplification with the following primers: Mp3: 5'-CTGGCTACCAAACTCCCGA-3' (forward) and Mp4: 5'-ACTCAGCGCC CAGGA-3' (reverse), derived from the published DNA sequence of IS900 of *Mycobacterium avium* subsp. *paratuberculosis* resulting in a 314 bp product (Bauerfeind *et al.*, 1996). PCR reactions were performed in a total volume of 25 μL, including 2.5 mM $MgCl_2$, 0.05 mM dNTPs, 200 μM of each primer, 1U Taq DNA polymerase (Invitrogen), 1× PCR buffer, and 2 μL DNA. Reactions were initiated at 94 °C for 3 min, followed by 40 cycles of 94 °C for 50 s, 55 °C for 3 s, 72 °C for 1 min and a final elongation step at 72 °C for 10 min, with a final hold at 4 °C. A negative control (sterile water) and a positive control DNA from *Mycobacterium avium subsp. paratuberculosis* strain ATCC 19698, were included in each amplification run.

Analysis of samples with nested-PCR

Nested PCR amplification was performed with two sets of primers: the outer oligonucleotide primers (forward, ISo-1F: 5'-GTTCGGGGCCGTCGCTTAGG-3'; reverse, ISo-1R: 5'-GAGGTCGATCGC CCACGTGA-3') were selected to amplify a 400 bp fragment of the insertion element IS900, specific to MAP genome (accession no. X16293 in GenBank). The inner oligonucleotide primers (forward, -2F: 5'-CCGCTAATTGAGAGATGCGATTGG-3'; reverse, ISi-2R: 5'-AATCAACTCCAG CAGCGCGGCCTCG-3') were used in this study. The target sequence was amplified in a 50 μL reaction volume containing 100 ng of genomic DNA, 0.2 mM dNTPs, 1× Taq buffer, 2 mM $MgCl_2$, 100 ng of each primer, and 1 unit of Taq DNA polymerase (Fermentas, Germany)

The first run of PCR was carried out by applying a step-up programme as followed: initial denaturation for 5 min at 94 °C, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, with a final extension for 5 min at 72 °C. Two to five μL from the first run amplicon were used as a template for the second run PCR with the identical PCR programme by inner oligonucleotide primers to amplify a 229 bp fragment inside the first sequence.

Agarose gel electrophoresis

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 μg/mL) and viewed on UV transilluminator.

RESULTS

Direct microscopy on the faecal samples identified acid-fast bacilli compatible with Johne's disease microrganisms in 96 samples. The presence of MAP DNA was detected by single PCR in faecal samples from 4 out of 120 animals (3.33%). The analysis of the MAP DNA amplified

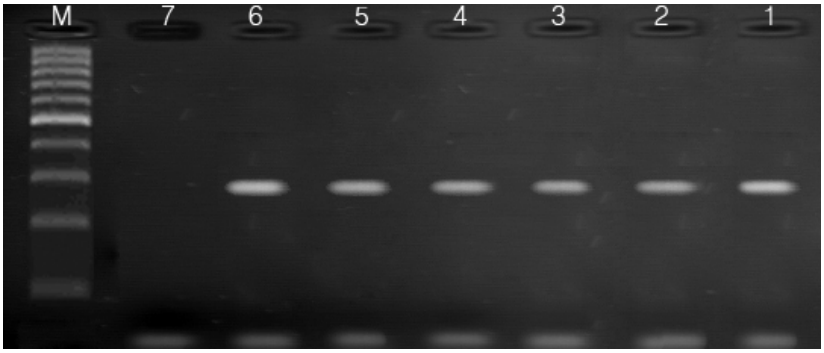

Fig. 1. Identification of *M. paratuberculosis* by nested-PCR amplification of the IS900 insertion element. Lanes 1 to 5: positive samples of *M. paratuberculosis*; lanes 6 and 7: positive and negative controls respectively; M: 100 bp DNA ladder.

314 bp fragments obtained from faecal samples showed that they were homologous to the known MAP IS900 sequence.

The nested PCR assay used in this study enabled the detection of IS900 gene of MAP. Nested PCR amplification of the MAP specific insertion sequence IS900 and subsequent agarose gel analysis of the amplified products showed a single band of 298 bp for each of the positive faecal samples (Fig. 1). MAP was detected in 68 out of 120 studied animals by nested-PCR amplification. Of the 96 faecal samples found positive by ZN staining, three were identified as positive by single PCR representing a detection rate of 3.12% (3/96). On the other hand, nested-PCR showed a detection rate of 70.8% (68/96) as compared to direct microscopy.

DISCUSSION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiologic agent of Johne's disease, a disease with considerable economic impact, principally on dairy cattle herds. Animals with paratuberculosis shed viable MAP especially in their milk, faeces and semen. MAP may have a role in the development of Crohn's disease in humans via the consumption of contaminated milk and milk products.

The primary method of MAP transmission is believed to be a direct faecal-oral cycle. However, there is still a possibility of indirect transmission, such as through manure contamination of water bowls and machinery used for feed delivery. Therefore, any management activities that directly or indirectly lead to exposure of susceptible animals to manure from shedding animals could be considered risk factors of infection. The efficiency of transmission by these pathways depends upon factors such as number of organisms shed in the faeces and the organism's survival characteristics in the environment (McKenna *et al.*, 2006).

Using the IS900 DNA probe, which is specific for *M. paratuberculosis*, workers have been able to identify the presence of paratuberculosis DNA in intestinal tissue from patients with Crohn's disease. Because the clinical symptoms of Crohn's disease closely mimic those found in animals with Johne's disease, a number of laboratories have proposed that *M. paratuberculosis* may be the causative agent of

Crohn's disease (Stabel, 1998).

Many studies have been focused on the association of Crohn's disease with MAP (Hermon-Taylor & Bull, 2002). 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).

Sweeney *et al.* (1992) determined the level of milk contamination in asymptomatic cows as 2 to 8 CFU/50 mL. The level of contamination in clinical cases remains to be determined.

Collins *et al.* (2005) analyzed the commercially available ELISA kits for MAP detection in milk. The tested kits showed high specificity levels in three independent laboratories (> 99%) but very low sensitivity levels (28%). Based on the results of the study, the authors recommended applying ELISA in support of paratuberculosis control programmes in dairy herds, only if data from ELISA are used judiciously and interpreted quantitatively.

MAP has also been detected by culture and PCR methods in retail cheeses in the USA (Ayele *et al.*, 2005). In 2002, IS900-nested PCR was used to determine the specificity and sensitivity of a commercial ELISA test (Stabel *et al.*, 2002).

In summary, the results of this study indicate that IS900-nested PCR assay may be applied to detect *M. paratuberculosis* directly from faecal samples of dairy cows and that it could become a valuable diagnostic or screening test for herds with Johne's disease.

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