

Detection of *Mycobacterium avium* Subspecies *paratuberculosis* in Milk and fecal Samples in Dairy Cattle by PCR and Nested-PCR

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Abstract: *Mycobacterium avium* ssp. *paratuberculosis* (MAP) is the etiologic agent of Johne's disease in cattle. The disease causes diarrhea, reduced milk production, poor reproductively, infertility and emaciation. MAP was isolated from intestinal tissue of a human patient suffering from Crohn's disease, a controversial discussion exists whether MAP have a role in the etiology of Crohn's disease or not. Milk may be a potential vehicle for the transmission of MAP to human population. In this study, milk and fecal samples were collected from cows (n = 150) with suspected of Johne's status. Milk and fecal samples were processed for Nested PCR. Nested PCR detected positive samples in milk and feces 68.150 (45.3%) and 74.150 (49.3%), respectively and PCR detected positive samples in milk and feces 41.150 (27.3%) and 48.150 (32%), respectively.

Key words: Cattle, *Mycobacterium avium*, subsp. *paratuberculosis*, single PCR, nested-PCR, milk, feces

INTRODUCTION

Mycobacterium paratuberculosis is an organism which can cause chronic inflammation of the intestine in cattle, known as Johne's disease. Clinically infected animals may shed large numbers of *M. paratuberculosis* organisms in their feces and smaller numbers in their milk (Committee on Diagnosis and Control of Johne's Disease, National Research Council, 2003). Sub clinically infected animals also shed the organism, though usually in smaller amounts. Generally, cattle are infected early in life by ingestion of *M. paratuberculosis* through colostrums, milk, fecal contaminated teats, water, feeds or surfaces (Larsen *et al.*, 1975; Stabel, 1998). Johne's disease also is one of the most economically important diseases of dairy cattle, costing over \$250 per cow in inventory per year in highly infected herds.

Dairy herds with clinical Johne's disease in $\geq 10\%$ of cull cattle have been estimated to lose USD\$ 227-245 cow year⁻¹ (Ott *et al.*, 1999). There are strong incentives for dairy farmers to control *M. paratuberculosis* in their herds. According to the USDA, MAP is estimated to have a herd prevalence of 21.6% (NAHMS, 1997) and causes an estimated loss of \$1.5 billion to the agricultural industry every year (Harris and Barletta, 2001). For example, it has been compulsory to notify the Department of Agriculture and Food (DAF) of any incidence of Johne's disease since 1955 in the Republic of Ireland. All confirmed infected animals and their immediate progeny are removed from herds and slaughtered. The National

Animal Health Monitoring System (NAHMS) Dairy 1996 study in the United States found that about 40.6% of US dairy operations had at least 1 cow that was seropositive for *M. paratuberculosis* while 16.8% had 2 or more seropositive cows.

Van Leeuwen *et al.* (2001) found that 43.3% of dairy herds in Maritime Canada had at least 1 cow that was seropositive for *M. paratuberculosis* while 16.75% had 2 or more seropositive cows. The disease causes enteritis, weight loss, reduced milk production (Stabel, 1998) and premature culling in dairy cattle and other ruminant species. Transmission occurs primarily through, the fecal-oral route and most herds are infected through, introduction of sub clinically infected cattle. Crohn's disease is characterized by a relapsing inflammatory process in the digestive tracts of humans. Crohn's disease in humans has features that resemble Johne's disease in animals and the question has been raised of whether the causal organism of Johne's disease, *M. paratuberculosis* could have the same role in the etiology of Crohn's disease.

Contaminated milk may be a source of exposure to the organism in human and need to be further investigated (Chiodini, 1989; Bull *et al.*, 2003). There is no effective treatment for Johne's disease; therefore, identification and culling of infected cows in conjunction with on-farm biosecurity are vital to developing and maintaining a *M. paratuberculosis*-free herd (Wells and Wagner, 2000).

The long delay between infection and the appearance of Johne's disease (Chiodini *et al.*, 1984; Manning and Collins, 2001) may result in cows being culled from the

herd before clinical signs develop, leaving the farmer unaware of the presence of *M. paratuberculosis* in the herd.

Control programs for Johne's disease have been established in a number of countries. One goal is to prevent the spread of the disease through, the sale of infected replacement stock from infected herds. Several methods are reported for isolation of MAP from milk and feces in the past decades (Beard *et al.*, 2001; Streeter *et al.*, 1995). A basic procedure for isolation of MAP from milk includes centrifugation to collect pellet fraction, chemical decontamination and culture on slants containing antibiotics and other supplements. Development of methods to detect MAP contamination in clinical, food and environmental samples has posed a number of challenges.

The culture method takes 8-16 weeks due to the slow growth of this organism (Whipple *et al.*, 1991). Although, molecular methods offer the advantage of rapid turn around time for test results they suffer from low sensitivity of detection (Giese and Ahrens, 2000). But other investigators found higher detection rate by IS900 PCR than the culture method (Gwozdz *et al.*, 1997; Green *et al.*, 1989; Pillai and Jayarao, 2002). Thus the reported protocols require an investigation with well designed experiments. A common procedure used in many studies for detection of MAP in milk by PCR includes centrifugation of milk sample to collect pellet fraction, lysis of cells and extraction of template DNA (Millar *et al.*, 1996). Nested PCR is one method that can be used to overcome some of the disadvantages of the testing methods currently available.

This method can be used as a diagnostic tool to detect the presence of MAP in animals suspected of having Johne's disease. To date studies have focused on the Nested PCR detection of MAP from feces, milk. In this study, we used IS900 sequence to investigate the presence of MAP in bovine feces and milk samples.

MATERIALS AND METHODS

Sampling: Milk and fecal samples were collected from 150 cows known to have or suspected of having Johne's disease in Tehran province. Feces were collected directly from rectum with sterile gloves with high attention on cross contamination and milk samples from aseptic teats (equally from each teat). Feces and milk samples then were stored at -20°C until DNA extraction.

Extraction of DNA from milk samples: For crude extraction of *M. paratuberculosis* DNA from milk samples,

0.5 mL from each samples was added to 1.5 mL Eppendorf tubes. Tubes were centrifuged at 15,000 rpm for 5 min and supernatants were discarded. To each cell pellet, 1 mg of proteinase K (Sigma Chemical Co.) was added and samples were vortex mixed vigorously to resuspend pellets. Samples were incubated overnight at 50°C in a shaking water bath. To each sample, 22 µL of TEN buffer (0.1 M NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; Sigma Chemical Co.) and 10 µL of 0.4 M NaOH was added and samples were vortex mixed. Tubes were then placed in a boiling water bath for 30 min.

Samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1; Amresco, Solon, OH) and vortex mixed, followed by immediate centrifugation at 10,000 rpm for 5 min. The aqueous layer was transferred to a new tube and the DNA precipitated by adding 2.2 volumes of cold 100% ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2). Samples were mixed gently and put in a -20°C freezer for at least 1 h. Tubes were centrifuged at 15,000 rpm for 15 min and supernatants were aspirated off. Pellets of DNA were air-dried for 2-5 min and then pellets were resuspended in 10 µL of sterile water. The DNA was either used immediately or frozen at -20°C for later.

Extraction of DNA from fecal samples: Fecal 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⁻¹ 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:isoamylalcohol (25:24:1 v/v/v) was added, vortexed for 30 sec 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 centrifugation 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 PCR: Detection of *Mycobacterium avium* subsp. *paratuberculosis* was performed by amplification with the following: primers: P90 (GAAGGGTGT 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 MgCl₂, 0.25 mM deoxynucleoside triphosphates (from each), 10 pmol of each primers and 1 U oligoTaq DNA

polymerase. 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 extraction at 72°C. the samples were considered as positive if 413 bp amplified band was present.

Analysis of samples with nested-PCR: The DNA isolated from milk and feces samples was amplified by using a nested Polymerase Chain Reaction (PCR) protocol as described (Gwozdz *et al.*, 1997). For the first amplification reaction, forward and backward primers (5'-GTTCG GGGCCGTCGCTTAGG-3'; 5'-GAGGTC GATCGCCCACGTGA-3') to amplify a 400 bp region of the insertion element is 900 which is specific for *M. paratuberculosis*.

A second amplification reaction further amplified the PCR product above by using internal forward and backward primers (5'- GCTTAGGCTT CGAATTGCC-3'; 5'-CTCCGTAACCGTCATTGTCC-3') and resulted in a final product of 194 bp.

After amplification, DNA was electrophoresed in a 2/5% agarose gel containing ethidium bromide in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA) and bands were visualized by using a UV transilluminator.

RESULTS AND DISCUSSION

Milk and fecal samples were collected from cows (n = 150) with suspected of Johne's status. Result of Nested-PCR on feces DNA showed that MAP genome is present in 49.3% (74/150) of samples (Fig. 1). 45.3% (68/150) of milk DNA Nested-PCR were positive (Fig. 2). Result of PCR on feces DNA showed that MAP genome is present in 32% (48/150) of samples (Fig. 3). About 27.3% (41/150) of milk DNA PCR were positive (Fig. 4).

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, feces and semen. The primary method of MAP transmission is believed to be a direct fecal-oral cycle. However, there is still a possibility of indirect transmission.

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. Different strategies have been proposed to be suitable for the reliable detection of *M. avium* subsp. *paratuberculosis* by using real-time PCR, including use of conventional IS900 PCR primers

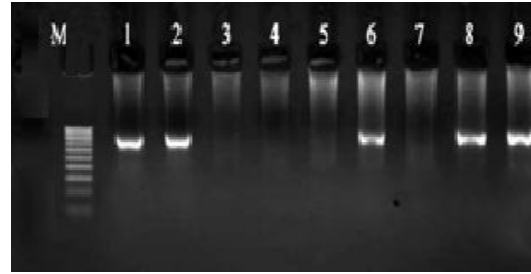


Fig. 1: Result of Nested-PCR on feces DNA



Fig. 2: Result of Nested-PCR on milk DNA



Fig. 3: Result of PCR on feces DNA



Fig. 4: Result of PCR on milk DNA

(Khare *et al.*, 2004), the combination of two separate real-time PCR runs for IS900 and F57 (Herthnek and Bolske, 2006) and the use of F57-derived oligonucleotide primers and hybridization probes restricted to the Light Cycler (Tasara and Stephan, 2005). MAP has also been detected by culture and PCR methods in retail cheeses in the USA (Ayele *et al.*, 2005). In 2002 is 900-nested PCR

was used to determine the specificity and sensitivity of a commercial ELISA test (Stabel *et al.*, 2002). To confirm PCR positives for MAP, one of the most extensively used methods is sequencing of a part of IS900 (Bull *et al.*, 2003; Sechi *et al.*, 2005).

However, there is still a risk for false positive results due to cross-reactions and positive results need to be confirmed. However, with current generally available technology, sequencing may not be the most practical method. Furthermore, sequencing is a laborious and expensive method (Van Belkum *et al.*, 2001) not suitable for confirmation of numerous positive samples.

The running of two consecutive PCR reactions with two different sets of primers, using the product of the first reaction as template in the second, enables a number of potential advantages to be achieved, including specificity and sensitivity. The first set of primers is outside the second set, resulting in progressively shorter amplicons. As all four primers have to match their target in the same DNA region to obtain a final product, specificity is enhanced. In addition because new reagents are added in the secondary reaction, sensitivity is also increased (Englund *et al.*, 2004; Konomopoulos *et al.*, 2004). Issues with the use of conventional PCR include:

- Limited sensitivity; because bands must be visible to the naked eye, sufficient starting DNA must be used in the PCR for a positive result. This is more often a problem with direct PCR (PCR on DNA from prepared clinical samples) than colony PCR
- Relatively high time and labour consumption
- Quantification is unreliable and imprecise
- Risk of cross-contamination
- Inhibition; PCR inhibitors, such as certain salts, ionic detergents, alcohols, etc. can inhibit the reaction. Most direct PCR techniques suffer from this problem to varying degrees

Nested PCR is even more demanding of time and labour than conventional PCR. The transfer of products from the first run to the second is extremely critical and poses an even greater risk for cross-contamination which is the main disadvantage with nested PCR. The first reported use of nested PCR to detect MAP was in a comparison between fecal culture and PCR (Collins *et al.*, 1993). Many of the most cited reports where nested PCR has been used to detect MAP are investigations of Crohn's disease (Lisby *et al.*, 1994; Ryan *et al.*, 2002; Bull *et al.*, 2003; Autschbach *et al.*, 2005). Other important studies include a method comparison (Fang *et al.*, 2002) and a study of MAP in pasteurized milk (Gao *et al.*, 2002). Among recent publications of international veterinary

importance are two method comparisons (Mobius *et al.*, 2008; Pinedo *et al.*, 2008) and a screening of beef carcasses (Meadus *et al.*, 2008). The PCR tests for detection of MAP in fecal samples have vastly improved in recent years, leading to an increased sensitivity of detection of low shedders. This improvement is due to improved DNA extraction and purification procedures and to a lesser extent, the more sensitive PCR systems now available. Recent DNA extraction procedures and PCR systems applied to feces are shown in Table 1. Extraction methods for feces have also been modified for environmental samples (soil and manure) (Cook and Britt, 2007).

Because of the low concentrations of MAP in milk, most DNA extraction methods employ centrifugation of a relatively large volume of milk, typically 10 mL (Slana *et al.*, 2008). IMS which is often applied on milk (Grant *et al.*, 2001; Khare *et al.*, 2004; O'Reilly *et al.*, 2004) can be used on small volumes of non centrifuged milk but more often large volumes are used. It is however, not trivial which fractions to discard after centrifugation. In many methods, including IMS, both whey and cream are discarded (Tasara and Stephan, 2005; Pinedo *et al.*, 2008; Slana *et al.*, 2008).

As first shown by Millar *et al.* (1996), the cream fraction of raw milk can sometimes contain most of the MAP which is why inclusion of the cream fraction should be considered. The pellet and cream fractions can be pooled (Gao *et al.*, 2007) or the complete sample volume can be processed as with peptide mediated capture (Stratmann *et al.*, 2006) and unspecific surface capture by magnetic beads (Donaghy *et al.*, 2008). Other recent publications include a method comparison (Alinovi *et al.*, 2009) and a diagnostic method for screening. The data suggest that the ability of Nested-PCR to detect infected animals is more than single PCR. But in both methods detection rates in milk samples were less than fecal samples (49.3 vs. 45.3% for Nested-PCR and 32 vs. 27.3% for PCR). The sensitivity of Nested-PCR makes it easy to detect MAP in both milk and fecal samples and that it could become a valuable diagnostic or screening test for herds with Johne's disease.

In the past decade, there have been significant improvements in PCR-based detection of MAP. These include improvements spanning sample processing, amplification and detection methods. The precise analytical sensitivity of this method is still difficult to ascertain but some promising data suggest that PCR may achieve a comparable sensitivity to that of culture. While culture may remain the definitive gold standard in research applications, the improved turnaround time with PCR makes this an attractive diagnostic modality for

Table 1: Summary of recently reported direct fecal PCR assays and comparison with fecal culture

Sample (no.) Sample storage	Laysis method	DNA extraction	Target gene	PCR	Faecal culture (Decontamination and media)	PCR results compared with culture (no. pos/ sample size)	References
Bovine faeces (63) No storage information	Bead beating	a) Column purification (HerdChek®) ¹ b) In-house	IS900	qPCR (TaqMan) Nested	DI-C, HEY	a) qPCR (41/63) b) qPCR (43/63) Nested (44/63) Culture (44/63)	Christopher-Hennings <i>et al.</i> , 2003
Bovine faeces (23) No storage record	Bead beating	IMS, solvent extraction	IS900	qPCR (TaqMan)	DI-C, HEY	PCR (17/23) Culture (17/23)	Khare <i>et al.</i> , 2004
Bovine faeces (310) Some frozen -20°C	Boiling/freezing	Spin column (Roche High Pure PCR Kit®) ²	IS900	qPCR (SYBR Green), semi-nested	NaOH, LJ	PCR (31, 32 doubtful/310) Culture (20/310)	Bogli-Stuber <i>et al.</i> , 2005
Bovine faeces (81) No storage record	Heating 100°C for 10 min	Centrifugations and washings in Tris-EDTA buffer	IS900 ISMap02	qPCR (SYBR Green) Nested	DI-DC, HEY	qPCR IS900 (60/81) qPCR ISMap02 (59/81) Nested IS900 (59/81) Nested ISMap02 (59/81) Culture (60/81)	Stabel and Bannantine, 2005
Bovine faeces (1808) Cultured fresh	Bead beating	Spin column (QIAamp® DNA Stool Mini Kit) ³	ISMav2	qPCR (TaqMan)	DI-C, HEY	PCR (7.6% of 1808) Culuter (18.4% of 1808)	Wells <i>et al.</i> , 2006
Bovine faeces (47) frozen -70°C ×2	Bead beating	Spin column (QIAamp® DNA Stool Mini Kit) ³	IS900 F57	qpPCR (TaqMan) IAC for IS900	NaOH, HEY, LJ DI-C, HEY (NVSL)	PCR IS900 (47/47) PCR F57 (47/47) Culture (47/47)	Herthnek and Bolske, 2006
Bovine faeces (143) Cultured fresh	Bead beating	Spin column (Tetracore kit) ⁴	hspX	qPCR	DI-C, HEY	PCR (31/143) Culture (27/143)	Alinovi <i>et al.</i> , 2009

¹Herdchek® *Mycobacterium paratuberculosis* DNA test kit, IDEXX laboratories, Westbrook, Maine, USA; ²High pure PCR template preparation kit, Roche diagnostic, Mannheim, Germany; ³QIAamp® DNA stool mini kit, QIAGEN; ⁴JohnePrep™ kit, Kyoritsu seiyaku Co., Tokyo, Japan; ⁵PowerSoil™ DNA isolation kit, Mo Bio laboratories, Carlsbad, California; ⁶MAP extraction system and VetAlert™ John's real-time PCR kit, Tetracore®, Rockville, Maryland. Abbreviations: IAC: Internal Amplification Control; NVSL: National Veterinary Services Laboratories; USDA, APHIS, Veterinary services, Ames, Iowa; qPCR, real-time PCR; DI-C: Double-Incubation, Centrifugation method by Whitlock and Rosenberger; DI-DC, Double-Incubation, Double-Centrifugation method (Stabel, 1998); NaOH/oxalic acid method by Beerworth; HEY; Herrold's Egg Yolk Medium; LJ: Lowenstein-Jensen medium

clinical applications, especially where adequate safeguards are in place to ensure accurate and reliable results.

CONCLUSION

Results of this study showed that Nested PCR can be as an alternative method for screening herds for diagnosis of MAP in milk and fecal samples because of its simplicity, sensitivity and rapidity.

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