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Milk ELISA in Diagnosing Paratuberculosis in Cattle and Buffaloes

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A B S T R A C T

Paratuberculosis or Johne's disease is a chronic debilitating disease of ruminants caused by *Mycobacterium avium paratuberculosis.* In dairy cows, paratuberculosis has been associated with production losses such as decreased milk yield, pre-mature culling, reduced carcass value etc. A possible mode of transmission for the ruminant pathogen *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) from cattle to humans is via milk and dairy products. Although, MAP has been suggested as the causative agent of Crohn's disease and its presence in consumers' milk might be of concern. During the active stage of infection and prior to the onset of clinical disease, animals generally develop antibodies to MAP antigens. Uninfected animals lack specific antibodies to MAP. In this study a total of 128 milk samples from cattle and buffaloes (irrespective of shedding of acid fast bacilli in the faeces) were screened for the presence of antibodies against MAP. A commercially available *in vitro* diagnostic kit for detection of antibodies against MAP was used. Seven out of 128 milk samples (5.46%) were positive by ELISA. The percent positivity of > 15% was considered as positive sample. The percent positivity of positive control was considered as 100%. The percent positivity ranged from 20% to 100%. However, none of the milk sample was positive for the presence of MAP by using conventional PCR and real time PCR using Taq Man assay. The presence of MAP specific antibodies in the milk is an indication of prior exposure of the animal to the organism.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP), the recognized pathogen of Johne's disease (JD), causes chronic granulomatous enteritis in cattle, sheep and other ruminants (Beard *et al.,* 2001) and results in significant economical loss to the dairy industry (Ott *et al.,* 1999). It has also been suspected as a causative agent of Crohn's disease in humans (Hermon *et al.,* 2000). Given its global coverage, it was included by the World Organisation for Animal Health (OIE 2004) in the list of diseases with particular economic importance and importance for public health (OIE, 2008). Johne's disease occurs in the majority of European countries (Nielsen and Toft, 2009), in both Americas (Manning and Collins, 2001; Hori-Oshima *et al.,* 2007), Asia (Singh *et al.,* 2008) as well as in Australia and New Zealand (Ridge *et al.,* 2010).

The 'gold standard' for *Mycobacterium avium* subsp. *paratuberculosis* identification is still based on bacterial culture on solid media of

faecal samples (Ayele *et al.,* 2001). The slow growth (up to 16 weeks) and false negatives in samples that have low concentrations of MAP makes it difficult to implement efficient protective strategies in an animal population when the MAP identification is solely based on bacterial cultivation (Collins, 1996). Due to the chronicity and preclinical nature of the disease, no single assay at a time will be able to detect all infected cows within a herd (Kalis *et al.,* 2003).

Furthermore, even when more sensitive molecular assays are used, fewer than one third of infected preclinical cows in a herd will be identified (Whitlock, 2009). Several diagnostic tests involve detection of an immune response to the bacteria, with ELISA being most commonly used. ELISA kits can be used to detect a quantitative optical density (OD) reading that correlates to the amount of MAP-specific antibody in milk samples (Nielsen, 2010).

ELISA is a much more time and cost-efficient assay than direct pathogen detection methods. However, sensitivity of ELISA is generally poor (29 to 61%), with specificity between 83 to 100% (Nielsen and Toft 2008). Efforts have been made in the last few decades to develop protocols for the detection of MAP in feces milk, tissue, food and environmental samples using various methods. Serology and fecal culture, however, are the most commonly used tests in the field (Stable *et al.,* 2002). Polymerase chain reaction (PCR) is an ideal method for rapid and accurate detection (Vander Giessen *et al.,* 1992).

Progress has been made recently to improve the sensitivity of PCR-based tests for MAP in milk (Gao *et al.,* 2007). The present study was planned with an objective to detect antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk samples using ELISA.

Materials and Methods

Collection of samples

Milk samples (n=128) from cattle and buffaloes (irrespective of shedding of acid fast bacilli in the faeces) with a history or incidence of chronic intermittent diarrhoea from dairy farms in and around Ludhiana were screened for the presence of antibodies against *Mycobacterium avium* subsp. *paratuberculosis.* The samples were refrigerated at 4º C until further use.

Microscopic examination of milk samples

Milk samples were also subjected to acid-fast staining. 5 ml of the pooled milk was centrifuged at 2500 rpm for 15 minutes and the supernatant was discarded. Smear was prepared from the pellet collected at the bottom of centrifuge tube. The smear was then stained with Ziehl Neelsen Staining.

Isolation of DNA from milk samples

DNA was extracted from milk using MagaZorb®DNA Mini-Prep Kit (Promega).

IS*900* PCR

DNA was amplified by PCR using Primers based on IS*900* sequence (Table 1). For the amplification of the IS*900* sequence a ready to use GoTaq® Green Master Mix, 2X (Promega) (that contains GoTaq® DNA Polymerase, 400µM of each dNTPs, 3mM $MgCl_2$ and two dyes (blue and yellow) that allow monitoring of progress during electrophoresis) was also used. For this the reaction volume of 25 µl was made containing 12.5 µl of GoTaq® Green Master mix, 1 µl of forward primer (10 pmol/ µl), 1 µl of reverse primer (10 pmol/ µl), 0.5µl of nuclease free water and 10µl of DNA template. Along with the test sample DNA, a known positive

control DNA was also amplified. Thermal cycling were performed in research thermal cycler (Eppendorf, Germany) and cycling conditions were as follows, initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 45 sec, annealing of primers at 56°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min. PCR products were run by agarose gel electrophoresis and visualized in Gel Documentation System (Alpha Innotech).

IS*900*MAP TaqMan Real-time PCR Assay

TaqMan real-time PCR assay for this study was done for the detection of *Mycobacterium avium* subsp. *paratuberculosis.* The MAP specific sequence *IS*900 was targeted as this sequence is having the highest copy number. Primer and probe sequences used in the assay are given in Table 2. The probe was labeled with the fluorescent reporter dye 5-carboxyfluoroscein (FAM) on the 5ˈend and the quencher dye$N$_,N_,N_,N_-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3ˈ end. Primers and probes specific for MAP IS*900*sequence DNA were obtained from Applied Biosystem.

ELISA testing

The milk samples were subjected to ELISA using an *invitro* diagnostic kit (Prionics, based on EIA) for detection of antibodies against *Mycobacterium avium paratuberculosis.* The absorbance was read at 450 nm in an ELISA reader. The percent positivity (PP) values were calculated for the samples using the following formula:

$$\text{Sample \% P (PP)} = \frac{\text{O.D}_{Sample} - \text{O.D}_{NC}}{\text{OD}_{PC} - \text{O.D}_{NC}} \times 100$$

O.D$_{Sample}$ = Mean Optical Density of Sample.

O.D$_{NC}$ = Mean Optical Density of Negative Control.

O.D$_{PC}$ = Mean Optical Density of Positive Control.

The Percent Positivity of positive control was considered as 100%. The samples with PP of more than equal to cut off of 15% were considered as positive and samples having PP below 15% were considered negative.

Results and Discussion

IS*900* PCR

Among 128 milk samples, no sample was detected as positive for *Mycobacterium avium* subsp. *paratuberculosis.*

IS*900*MAP TaqMan Real-time PCR assay

IS*900* TaqMan assay measures the amount of target IS*900* DNA produced during each cycle of an amplification reaction in a real-time format.

Thus, the system is able to quantify the amount of target DNA in contrast to the conventional PCR, which measures only the end-point values with qualitative results. C_T values between 20 and 33 were considered positive. In all the milk samples, C_T values were greater than 40 and hence were detected as negative for *Mycobacterium avium* subsp. *paratuberculosis*.
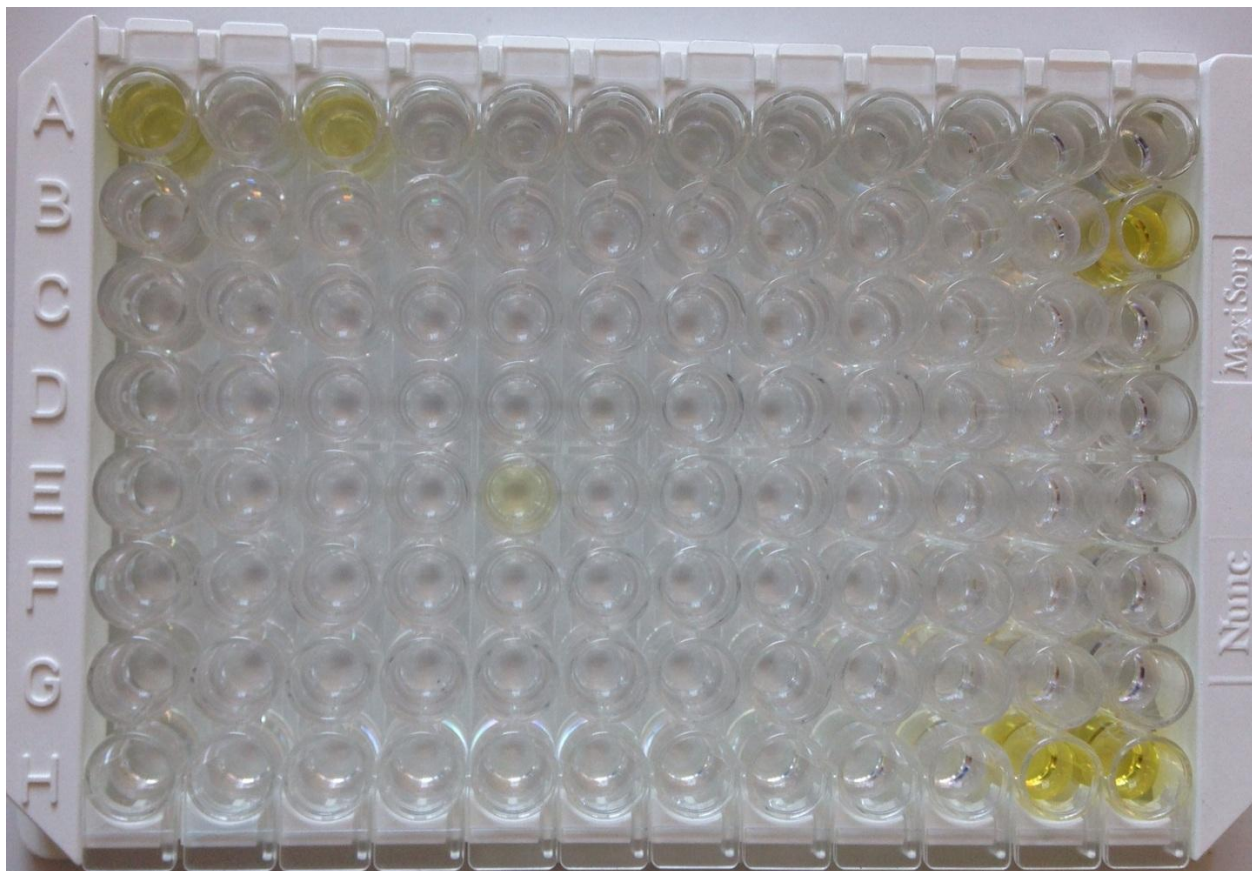
ELISA

Seven out of 128 milk samples (5.46%) were detected positive for MAP by ELISA (Figure 1). The percent positivity ranged from 20% to 100% (Table 1). The percent positivity of > 15% was considered as positive sample and that of positive control was considered as 100%.

Table.1 The percent positivity of the suspected samples detected by ELISA

Animal No.	Percent Positivity by ELISA	IS*900* PCR	Real time PCR
1	68.91	Negative	Negative
2	64.65	Negative	Negative
3	33.85	Negative	Negative
4	20.00	Negative	Negative
5	122.14	Negative	Negative
6	74.52	Negative	Negative
7	61.64	Negative	Negative

Fig.1 An ELISA Plate showing colour development in positive samples

Mycobacterium avium subsp. *paratuberculosis* is a recognized pathogen that affects many species of ruminant and non-ruminant animals (Stabel *et al.,* 2004). MAP infection of domestic-food-producing animals is associated with significant economic loss to the livestock industry worldwide. At present, several new preventive strategies have been employed to restrict the spread of MAP among animals and subsequently limit the economic losses. This is because of the relatively low sensitivities of the currently available tests, which fail to detect subclinical (first and second stage) MAP-infected animals (Fecteau and Whitlock 2010). Infected animals exhibiting clinical or subclinical infection sheds MAP bacteria in both milk and faeces.

Detectable levels of MAP have been found in milk from both clinically infected cattle (Giese and Ahrens 2000) and asymptomatic carriers (Streeter *et al.,* 1995). Sweeney *et al.,* in 1994 found that MAP is shed into milk by 12% of subclinically infected cows in a concentration of 2 to 8 CFU per 50 ml milk. Serology and faecal culture, however, are the most commonly used tests in the field (Stabel *et al.,* 2002). Efficiency of PCR for paratuberculosis is the challenge faced in extracting DNA from MAP, which can be enhanced by selecting the most accurate methodology for the purpose of testing. Two specific challenges faced during DNA extraction from MAP include inhibitors present in the sample that can hinder DNA amplification and also the thick, waxy cell wall of MAP, which requires more intense lysis techniques, such as bead-beating to extract the DNA. By using a sequence unique for MAP, specificity of real-time PCR is comparable to culture techniques and can reach 100% (Leite *et al.,* 2013). However, sensitivity of real-time PCR for MAP is dependent upon additional factors including technique, bacterial concentration within the sample, bacterial loss during processing or storage, and reference standard accuracy (Bolske and Herthnek, 2010).

The main type of immunological test that is widely available and commonly used is the enzyme linked immunosorbant assay (ELISA), which detects an optical density in serum (Hardin and Thorne, 1996) or milk (Kudhal, 2004) that correlates with an antibody response to MAP. The ELISAs have been desirable tests to use because of their ease of sample collection (blood or milk), rapid test results (within a week), and relatively low cost. The sensitivity of ELISA for detection of high shedders was >90% both for individual milk and serum samples (Van Weering *et al.,* 2007). Specificity for ELISA has been shown to be less than 100%

depending on the kit and method (Nielsen and Toft, 2008). Therefore, ELISA results need to be evaluated in light of the purpose for testing (such as detection or screening) and should be followed up with direct detection methods for absolute confirmation (Collins 2011). The presence of antibodies can be predictive of higher risk for MAP shedding (Lavers *et al.,* 2013). Detection of antibodies using the ELISA test is the most frequently used method for JD diagnosing. However, the concentration of antibodies in milk is related not only to the level of antibodies in serum but also the milk yield, lactation period and even the calving number (Sweeney *et al.,* 2006). Studies so far conducted milk sample testing with ELISA test has shown 12% more positive results than the tests in the serum from the same animals. A recent study in Ontario (Hendrick *et al.,* 2005) reported only moderate agreement between serum and milk ELISAs, and the milk ELISA detected 12% fewer infected cows than did the serum ELISA. These findings make biological sense considering that antibody concentrations in milk depend not only on levels in serum, but also on milk production, parity and days in milk (Nielson *et al.,* 2002). The added variability in antibody levels in milk relative to serum makes interpretation of results from milk ELISA even more challenging than those from serum ELISA, which have inherent laboratory variability (Nielsen, 2002). Further research may identify a role for the milk ELISA as a practical method of monitoring MAP infection at the herd level or instigating interest in controlling JD.

The presence of MAP specific antibodies in the milk is an indication of prior exposure of the animal to the organism.

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