# *Mycobacterium paratuberculosis* Shedding Into Milk: Association of ELISA Seroreactivity With DNA Detection in Milk

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## ABSTRACT

The objective of this study was to analyze the association between ELISA seroreactivity and *Mycobacterium paratuberculosis* DNA presence in bovine milk as detected by nested PCR. An irregular pattern of detection was observed for milk PCR outcomes along with fluctuations in serial ELISA results. Cows testing positive by milk PCR had negative and inconclusive ELISA results in 23.5% and 11.8% of the cases, respectively. A kappa coefficient of 0.012 indicated a slight agreement between both tests; Fisher's Exact Test did not indicate a significant association between tests outcomes ($P = 0.55$). Ability of serum ELISA as indicator of the likelihood of milk shedding of *Mycobacterium paratuberculosis* in dairy cows is questionable.

## INTRODUCTION

*Mycobacterium avium* subspecies *paratuberculosis* (Map) is the etiologic cause of a chronic granulomatous intestinal disease (Johne's disease) in ruminants, characterized by progressive weight loss and profuse diarrhea.[1] The disease has a worldwide distribution, and it is categorized by the Office International Des Epizooties as a list B disease, which is a serious economic or public health concern.[2] Paratuberculosis represents a significant problem for the dairy industry, and one of the main issues relates to the efficiency of subclinical diagnosis.

Map isolation from milk was first reported in 1935, in association with advanced clinical paratuberculosis.[3] More recent studies have found Map isolation rates in milk of up to 45% in clinically affected animals[4] and of up to 22% in colostrum or 8% in milk in subclinical cases.[5]

The concern about Map in milk and milk products centers on its apparent heat resistance and in the controversial role that the bacteria could play in Crohn's disease in human.[6-7] In testing units of whole pasteurized milk from retail outlets throughout central and southern England, it was found that, over 3-month periods, up to 25% of commercial units sampled were affected by the presence of Map DNA.[8] In a study in the Czech Republic, Map was cultured from 1.6% of commercially pasteurized retail milk.[9] A United States' study found viable Map in 2.8% of milk samples taken from grocery stores in 3 states.[10]

ELISA testing for Map has been used as a herd tool from which producers could make management decisions.[11] Using nested PCR, Buergelt and Williams[12] showed a positive correlation between high Map ELISA readings in blood and increased probability of detection of Map DNA in milk of clinical cows. However, a clear association was not found when comparing a sub group of subclinically infected animals. These latter findings bring into question whether detection of subclinical infection by ELISA is an effective tool for identification of cows shedding Map into milk as a first step of protecting the food chain.

The purpose of this report was to analyze the association between ELISA seroreactivity and the presence of Map DNA in milk based upon a nested PCR. The information reported here is retrospective and based on data from dairy cows tested concurrently by serum ELISA and milk PCR, or with prolonged serial observations to detect Map DNA in milk.

## MATERIALS AND METHODS

### Study Population

Blood and milk samples were derived from cows belonging to the University of Florida's Dairy Research herd (USA), composed of 500 Holstein cows, and known to be infected with Map. As a routine, cows were tested for Map by serum ELISA annually and, in some cases, milk samples were obtained for PCR analysis. Research data on 98 adult cows, tested between October 2003 and September 2004, were selected on the sole basis of their having had a PCR analysis for Map in milk concurrent with the routine ELISA test. No formal randomization in the selection of animals was attempted and the samples analyzed do not represent a particular status of paratuberculosis infection. Thirteen animals were considered for the serial analysis. One particular animal (cow Id#3900) was successively tested for ELISA and milk and blood PCR for about 9 months and finally submitted for necropsy. All the procedures involving animal handling were in agreement with the animal care protocols of the University of Florida.

### Milk Samples

Before collection, the teats were thoughtfully cleansed with alcohol to avoid sample contamination from skin. Milk (30-40 mL) was collected in a sterile 50-mL centrifuge tube from the 4 quarters by hand milking, discarding the first 10-15 mL. The milk samples were centrifuged at 1000 g for 15 min and the supernatant discarded. The resultant pellet was washed thrice in phosphate buffered saline (PBS, pH 7.3) and centrifuged at 500 g for 15 min. The pellet was resuspended in 1 mL of PBS, centrifuged and resuspended in 100 µL of 0.2 N NaOH. After boiling at 110°C for 20 minutes to extract DNA, the material was centrifuged at 500 g for 3 minutes. The final product was stored at -20°C for subsequent PCR.

### Blood Samples

After cleansing with alcohol, 10 mL of blood per cow were collected from the coccygeal vein into Vacutainer® tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) with and without EDTA. For the blood PCR procedure (cow Id#3900), 3 mL of EDTA blood was added to 4 mL of Ficoll-Isopaque® Plus Gradient (Amersham Pharmacia, Piscataway, New Jersey, USA) and centrifuged for 40 minutes at 500 g at 18°C. The buffy coat was collected, then washed twice with PBS, and centrifuged at 500 g for 15 minutes. Cells from the pellet were resuspended in 100 µL of 0.2 N NaOH,

boiled at 110°C for 20 minutes to extract DNA, and centrifuged at 500 g for 3 minutes. The final product was stored at -20°C for subsequent PCR.

**Map-Nested Polymerase Chain Reaction (PCR)**

After DNA extraction, 1 µL of the previously described product (milk and blood) was submitted for PCR. A commercial reaction mix (Hot Master Mix®, Eppendorf North America, Westbury, New York, USA) was used according to the company's specification. Samples were tested with primers P90, P91 for IS900, which specifically recognize a 413 bp sequence of Map. The reaction was followed by the nested PCR, where 1 µL of previously amplified product was tested with second set of primers J1, J2 overlapping and spanning a 333 bp region within the insertion sequence.[12,13]

A volume of 10 µL of the PCR product was run on 1.5% Agarose gel by electrophoresis in TAE running buffer (Continental Lab Products, San Diego, California, USA). Extracted DNA from the laboratory strain #295 was used as positive control and sterile water was used as negative control for the PCR assay. Gel inspection was done using ultraviolet light and recorded with a computerized digital camera (UVP Transilluminator System). Positive and negative controls were used in each of the reactions.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Serotesting of samples was done by use of the ELISA developed by Allied laboratories Inc. (Ames, Iowa, USA) with crude, soluble Map 18 protoplasmic antigen (Allied Monitor, Fayette, Missouri, USA), based on a previously documented protocol.[14] Antigen was diluted to a concentration of 0.1 mg/mL in 0.05M sodium carbonate buffer at pH 9.6. This dilution (100 µL per well) was incubated over night at 4ºC. A suspension of *Mycobacterium phlei* was prepared by adding 5 g of dry, heat-killed *M phlei* to 1 L of phosphate-buffered saline solution containing 1% gelatin and 0.05% Tween 80 (PBS-TG). Three mL of this base solution were added to 97 mL of 0.85% NaCl solution for use. Test sera (200 µL) including positive and negative controls were preabsorbed over night with this suspension (200 µL) to reduce nonspecific reactions. Samples were centrifuged at 2,000 rpm for 10 minutes, and 20 µL of supernatant were added to 1 mL of PBS-TG. The sensitized plates were washed 3 times with a 0.85% saline solution containing 0.05% Tween 80, allowing 3 minutes/wash. Diluted samples (100 µL) were added to 3 wells followed by incubation at room temperature (2 hours). The wells were then emptied and washed 3 times with PBSS-TG as before. Horseradish peroxidase conjugated with antibovine IgG was diluted to 1:2000 in PBS-TG. Diluted conjugate (100 µL) was added to each well followed by incubation at room temperature (2 hours). The wells were then emptied and washed 3 times with PBS-TG as before. Substrate was prepared by adding 125 µL of a 40-mM solution of 2,2'-azino-bis(3-ethilbenzthiazolinesulfonic acid) and 100 mL of a 1-M solution of hydrogen peroxidase to 25 mL of citrate buffer (10.5 g of citric acid monohydrate/L). Substrate (100 µL) was added to each well. ELISA results were calculated as ELISA ratios (ER) from wavelength readings (optical density [OD] at 405 nm) in triplicates as sample OD divided by a value equivalent to one quarter of the OD of the positive control.[15] This value was typically in the range of 0.13 to 0.14. Results were recorded as negative (<1.5), suspicious (1.5 to 1.9), low positive (2.0 to 2.5), and high positive (>2.5) as reported previously.[12]

**Analysis**

Results are presented in tables to demonstrate the association between the different tests. For the statistical analysis, kappa coefficient was used as a measure of agreement between the 2 tests. The following ranges were considered for interpretation of the kappa coefficient: poor agreement: less than 0.00; slight agreement: 0.00-0.20; fair agreement: 0.21-0.40; moderate agreement: 0.41-0.60; substantial agreement: 0.61-0.80; almost perfect: 0.80-1.00.[16]

**Table 1.** DNA detection of Map in milk by nested PCR grouped by ELISA result categories (N = 98 cows).

| | ELISA (ER) and Interpretation, n (%) | | | | |
|---|---|---|---|---|---|
| | **High Positive** | **Low Positive** | **Suspicious** | **Negative** | **Total** |
| Milk PCR + | 9 (52.9) | 2 (11.8) | 2 (11.8) | 4 (23.5) | 17 |
| Milk PCR - | 31 (38.3) | 17 (21.0) | 13 (16.0) | 20 (24.7) | 81 |
| Total | 40 (40.8) | 19 (19.4) | 15 (15.3) | 24 (24.5) | 98 |

Fisher's Exact Test was used to test whether there was a non-random association between either variable (respective tests results). For both methods of analysis, inconclusive results (suspicious category) for the ELISA test were not considered and ELISA categories "strong" and "low positive" were deemed as a single group (positive).

Data were analyzed using the PROC FREQ procedure of the SAS statistical package for Windows (SAS Systems for Windows® Version 9.00, SAS Institute Inc., Cary, North Carolina, USA).

## RESULTS

Cows tested by serum ELISA and the nested PCR in milk were aggregated by ELISA categories in Table 1. For ELISA categories negative and strong positive the disagreement between tests was 16.6% and 77.5%, respectively. A total of 23.5%, and 11.8% of the individuals that had evidence of Map DNA in milk were negative or inconclusive for ELISA outcomes, respectively (6 animals).

Results for ELISA and milk PCR from 83 cows were the basis for the statistical analysis and are presented in a 2-by-2 contingency table (Table 2).

**Table 2.** ELISA results and DNA detection of Map in milk by nested PCR. Inconclusive ELISA results were removed, and low and strong positive categories are presented as positive (number and % of animals).

| | ELISA (ER) and Interpretation, n (%) | | |
|---|---|---|---|
| | **≥2.0 Positive** | **<1.5 Negative** | **Total** |
| Milk PCR + (%) | 11 (73.3) | 4 (26.7) | 15 |
| Milk PCR - | 48 (70.6) | 20 (29.4) | 68 |
| Total | 59 (71.1) | 24 (28.9) | 83 |

There was agreement between ELISA and milk PCR in 31 of the 83 animals (37.3%) included in the analysis. Four cows were positive for PCR but negative for ELISA, and 48 were positive for ELISA but PCR negative. The kappa coefficient (±1.96 asymmetric standard error) for the association of both tests was 0.0128 (±0.059), which is a slight level of agreement.

Fisher's Exact Test did not result in significant values ($P = 0.55$), indicating that there was not sufficient evidence to reject the null hypothesis of no association between test outcomes.

Serial samples taken for individual cows evidenced a variable pattern of Map shedding into milk, measured as the presence of bacterial DNA by nested PCR. Table 3 presents serial results for a particular cow tested during 9 months (21 times), and confirmed as a clinical case by necropsy. An irregular pattern of detection can be observed for milk and blood PCR results, along with fluctuations in ELISA readings.

Serial results for serum ELISA and milk PCR in a different group of 5 cows with fluctuations in the milk shedding status are presented in Table 4. For this group, as shown for cow Id#3900, data suggest a poor association between detection of the bacteria in milk and serum ELISA results.

Cows that tested positive for milk DNA had a variable pattern for ELISA ODs over time.

ELISA test results from 29 cows that tested positive

**Table 3.** Results for 21 serial testing (9 months) in cow Id#3900. Milk and blood PCR results are given relevant to ELISA categories in concurrent testing.

| | ELISA (ER) and Interpretation | | | |
|---|---|---|---|---|
| | **Negative** | **Suspicious** | **Low positive** | **High positive** |
| Milk PCR + | 0 | 1 | 0 | 5 |
| Milk PCR - | 0 | 3 | 2 | 10 |
| Blood PCR + | 0 | 3 | 0 | 3 |
| Blood PCR - | 0 | 1 | 2 | 12 |

**Table 4.** Serial results for milk PCR, and serum ELISA in a group of 5 individuals.

| Cow Id | Days From Previous Tests | Milk Nested PCR | ELISA (ER)* |
|---|---|---|---|
| 3475 | - | + | 1.7 |
| | 14 | - | 4.6 |
| 3763 a | - | + | 1.8 |
| | 2 | + | 1.5 |
| | 3 | + | <1.5 |
| | 1 | + | <1.5 |
| | 1 | - | <1.5 |
| | 1 | + | 1.6 |
| 3838 a | - | + | 5.6 |
| | 1 | - | 5.8 |
| | 1 | + | 3.6 |
| | 4 | - | 4.9 |
| | 1 | + | 4.9 |
| | 1 | - | 4.9 |
| | 1 | - | 4.9 |
| 3976 | - | + | 2.6 |
| | 24 | + | <1.5 |
| | 13 | + | <1.5 |
| 6044 | - | + | <1.5 |
| | 27 | - | 1.7 |
| | 35 | + | <1.5 |

*ELISA categories: negative (<1.5); suspicious (1.5 to 1.9); low positive (2.0 to 2.5); high positive (>2.5).

for milk PCR are summarized in Table 5. The data suggest that ELISA seroreactivity may have a negative status despite the fact that the cow is shedding the bacteria in milk, as shown by PCR detection.

Cow Id#6142 was tested by milk PCR using samples taken from separate quarters on 6 different occasions. While milk from all 4 quarters was demonstrated to be positive on 2 instances, milk from 3 of the 4 quarters in a given test was negative on 2 occasions, and from 2 or 1 quarter in 1 sampling each (Table 6), indicating that not pooling milk from all 4 quarters increases the risk of obtaining a false-negative result for the animal.

## DISCUSSION

Serologic tests for Map are most useful in establishing the herd prevalence infection, for presumptive identification of infected animals, and for confirming the diagnosis of Johne's disease in animals presenting compatible clinical signs.[17]

ELISA testing has been advocated as a herd tool from which individual producers could make management decisions, though the ELISA for Map has the disadvantage of moderate to low sensitivity in cows shedding low numbers of bacteria.[11] There are multiple commercial Map ELISA tests available and, despite the fact they are marketed as herd-level diagnostic tools, they are commonly used as cow level.[18] Considering that one of the aims of diagnostic tests in animal production is to help to control the introduction of potential pathogens into the human food chain, the ability of serologic tests as ELISA to detect individuals that are

**Table 5.** Cows that were positive to Map DNA by PCR detection in milk are grouped by their corresponding serum ELISA status.

| ELISA Category | Number of Cows* | ELISA Range (ER) |
|---|---|---|
| Negative | 7 | 0.5-1.4 |
| Suspicious | 8 | 1.5-1.9 |
| Low positive | 2 | 2.2-2.5 |
| High positive | 18 | 2.2-3.9 |

*Six animals with multiple testings

more likely to shed Map into milk is crucial. However, from the data presented, it can be stated that a given ELISA outcome is not conclusive as to whether or not a given cow is shedding Map into its milk (Table 1).

It has been suggested that the measurable humoral immune response to Map in subclinical cows can vary widely over time, even from day to day.[19] This information is in agreement with our findings (Tables 3 and 4). It is suspected that this variation in ELISA results is due to fluctuation in antibody production, protein enteropathy, variable losses by way of the gastrointestinal tract, or a combination of these.[19]

Further, strong discrepancies between different commercial ELISAs when performed concomitantly on the same animal were found by McKenna et al.[18] In their study, the highest and lowest kappa coefficients for combinations of 3 different commercial ELISA tests were 0.33 and 0.18, which is fair and slight agreement, respectively.

Map has been reported in different tissues and fluids such as blood, milk, semen, lymph nodes, and fetuses suggesting that intermittent bacteremia occurs accompanied by dissemination of Map to body fluids like milk.[3-21]

The ability of IS900 PCR to detect Map in milk has been analyzed in raw bulk tank milk and in individual cows. Pillai and Jayarao[20] reported a detection limit for bulk tank milk of 10 to 100 cfu/mL of Map, which is in agreement with values reported by Giese and Ahrens[4] for cows exhibiting clinical signs. In the same study, Map was detected in 4% and 33% of pooled quarter milk samples, in individuals from infected herds, by culture and IS900 PCR, respectively.[20] According to these authors, the variation in the detection ability for low Map concentration could be due to loss of some organisms in the cream fraction after centrifugation of milk. This could also be the explanation for some of our PCR negative results in cows previously positive or exhibiting high ELISA values.

Because of the apparent intermittent

**Table 6.** Serial results for cow Id#6142 tested by Map PCR on individual quarter milk samples and concurrent serum ELISA.

| | PCR on Milk by Quarter | | | | |
|---|---|---|---|---|---|
| Sample Date | RF | LF | LR | RR | ELISA (ER) |
| 9/24/2002 | - | + | - | + | 2.9 |
| 12/10/2002 | - | + | - | - | 1.5 |
| 12/30/2002 | + | + | - | + | 2.0 |
| 1/21/2003 | + | + | + | + | 2.6 |
| 1/28/2003 | + | + | + | + | 2.5 |
| 2/4/2003 | N/A* | - | - | - | 2.3 |

RF = right front; LF = left front; RR = right rear; LR = left rear.

*Not milk was obtained from RF quarter.

pattern of Map dissemination, shedding into milk may not be ascertained from a single milk sample. As Tables 3 and 4 evidence, Map shedding appears to be irregular over an extended period of time, and herd management decisions based upon a single analysis of milk can not rule out Map shedding into milk at another point in time.

Poor agreement between ELISA results and bacterial DNA detection in blood was previously reported, with kappa values for serum ELISA vs blood PCR results of -0.36, 0.44 and -0.166 for cows, heifers, and the two combined, respectively, suggesting a poor to moderate agreement between tests.[21] The interpretation offered is that each method detects different populations, or stages of Map infection, because their respective targets might not have parallel dynamics. This explanation may apply to our results as well (Table 3), based on the possibility of different temporal patterns for the humoral immune response to Map and the presence of Map in milk. A higher number of individuals positive for Map PCR detection in milk would be desirable in our study to determine, more accurately, the likelihood of having an accompanying negative result for the serum ELISA. One limitation of this study, because of the retrospective nature of the analysis, is that this condition was restricted to only 17 individuals.

An additional factor governing the presence or absence of Map in milk is the means by which a sample is obtained. As demonstrated in Table 6, in order for a milk sample to be deemed adequate for analysis, the milk should be obtained from all 4 quarters (pooled sample).

Based on the results of this study, it is concluded that Map shedding in milk, as detected by PCR, has a slight association with the concurrent ELISA seroreactivity. Ability of serum ELISA as indicator of the likelihood of milk shedding of Map in dairy cows is questionable.

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