Assessing the Fecal Shedding Consistency of *Mycobacterium Avium* Subsp. *Paratuberculosis* by Dairy Cows by qPCR: A Preliminary Study

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ABSTRACT

Paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is predominantly a disease of ruminants. The microorganism is shed principally in the feces of affected animals, even without clinical signs. Young animals, up to the age of 6 months are at the highest risk of infection by oral exposure to contaminated feces. There is no treatment and vaccination is not permitted in Israel. The standard method of reducing farm infection rates is by improving management hygiene and culling shedding cows, especially super-shedders (more than 10^4 copies of the *hsp*X gene). Consequently the prompt identification of animals shedding MAP is of cardinal importance in eradicating the infection. The aim of this preliminary study was to devise a fecal sampling protocol that would maximize the likelihood of detecting MAP shedding cows and assessing the influence of parturition induced stress on shedding. Sixteen cows and 15 heifers, raised on a commercial dairy farm of about 350 lactating cows, with 10% milk MAP ELISA positive cows were included in the survey. Rectal feces, sampled for 5-7 consecutive days, before and after calving, were examined by quantitative PCR (qPCR). Results indicate that the microorganism was shed by positive animals regularly and quantitative variations were minimal. Consequently one sample is a good indication of the animal's shedding status. MAP shedding was found not be influenced by parturition associated stress.

Keywords: Feces, *Mycobacterium avium* subsp. *paratuberculosis*, quantitative PCR, shedding.

INTRODUCTION

Paratuberculosis (Johne's disease), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is predominantly a disease of ruminants but may affect other animals as well (1).

MAP causes significant economic damages due to the necessity to cull shedding or clinically ill animals and by the infection's indirect actions, such as reduced fertility and production (2).

The disease has a worldwide distribution, increasing in time, with only 26 countries reported free of the disease (3). In Israel, out of 189 farms examined, about 66% tested positive (KO, unpublished data).

Following the isolation of MAP from human patients suffering from Crohn's Disease (CD), the microorganisms involvement in the syndrome as causative agent or secondary invader aggravating its symptoms has been suggested, originating an ongoing controversy (for a recent review see 4).

Young animals aged up to six months are at the highest risk (5) and typically only about 10% of those infected will develop clinical signs. The most frequent mode of infection is fecal-oral, although other means of transmission have been described (6). The microorganism enters the enteric mucosa mostly but not exclusively through the lymphatic tis-

sue (Payer's patches) (7). Foci of infection are formed, some of which are cleared from the bacteria whereas others act as source of its further dissemination and the formation of new foci (8). At sites of permanent infection, a granulomatous reaction ensues but does not limit the microorganisms' spread in the surrounding tissues (9). The initial cellular immune reaction is replaced after 2 or more years by an inefficient, humoral one. Concomitantly, fecal shedding may increase (6). The intestinal lesions cause the thickening of the intestine's wall and consequently malabsorption.

Clinically 4 stages of the infection have been described (10): a) latent: without clinical signs, shedding or antibodies, b) subclinical: with a cellular immune response and some shedding, c) clinical: characterized by weight decrease (in spite of normal feeding), declining cellular and rising humoral immune response and MAP shedding and d) advanced clinical: with intensive diarrhea, development of edema and massive shedding of MAP. About 10% of infected animals reach this stage.

Recently the classification of cows by shedding intensity has been proposed (11):

a. Negative
b. Passive shedders – cows that shed a few bacteria following their exposure to a contaminated environment without the colonization of the gastrointestinal tract (GIT)
c. Active shedders with colonized GIT
d. High shedders ("super-shedders") – cows that shed more than 10000 bacteria/g feces. This group poses by far the highest risk to act as source of infection of other animals and thus have to be identified and eliminated from the herd.

Laboratory identification of animals infected by MAP is either by methods assessing the presence of an immune reaction or by showing the presence of the bacteria or their subcellular components in feces. Each of these methods suffers from shortcomings: the former have unsatisfactory specificity (for detecting the cellular response) or sensitivity (due to the relative advanced stages of the disease in which antibodies are produced) (12) whereas the latter's sensitivity has been reported to be hampered by the irregularity of the fecal shedding of MAP (6).

In this preliminary study we aimed at identifying an eventual short term pattern of MAP shedding so as to be able to devise the optimal number and timing of fecal sampling to improve the likelihood of identifying shedding cows. Moreover, the two parameters that may influence MAP shedding were assessed: age and parturition induced stress. A qPCR technique aimed at detecting the hspX gene, shown to be specific to MAP (13), was used. The accuracy of this technique was found to be comparable to culturing the microorganism, the latter being significantly longer (6-8 weeks vs. 1-2 days) (14). To the best of our knowledge, this is the first publication of a MAP shedding study, using qPCR.

MATERIALS AND METHODS

Milk or serum antibodies, in cows and heifers respectively, were assessed by ELISA (ID Screen, IDvet, Grabels, France). The survey was conducted in a commercial dairy farm of about 350 lactating cows, 10% of which were MAP milk antibody positive. Fifteen heifers, two of them seropositive, were sampled for 5-7 consecutive days during the last trimester before and during the first two weeks following parturition. Sixteen cows, 15 of which were positive for MAP milk antibodies, were sampled under a similar protocol. None of the animals showed clinical signs of paratuberculosis. The samples were frozen at -18°C and all the samples of a given series were examined at the same time with a commercial quantitative polymerase chain reaction (qPCR) kit, based on detection of the *hsp*X gene (Vetalert, Tetracore, Rockville MD, US).

Means of the samples before and after parturition were statistically compared by the two tailed dependent paired t test (https://statistics.laerd.com/calculators/dependent-t-test-paired-samples-calculator.php) and the resulting t value's significance was evaluated (http://www.danielsoper.com/statcalc3/calc.aspx?id=8).

Antibody levels and qPCR values before and after calving were compared by the Correl() function of Microsoft Excel®. To assess whether the observed variability in the qPCR results for the same series resulted from irregular distribution of the microorganism in the sample or differences in shedding levels, six samples from three cows were examined 5-6 times each.

RESULTS

Results showed that MAP shedding variability was limited (Table 1 for cows and Table 2 for heifers). The comparison of pre- and post-parturient animals showed that three cows each were positive, low shedders or negative respectively, before and after calving. One cow was positive before calving but became negative 3 months later. Five cows, all negative, were available for only one series of testing. Among the

heifers, two were seropositive for MAP. All the heifers were found to be negative or low shedders before calving. Nine months later, one of the seropositive heifers became a high shedder and was removed from the herd.

No significant statistical difference was found between pre- and post-parturient shedding levels (p=0.3374 for heifers and p=0.1482 for cows). Correlation coefficients were low (0.20-0.27).

Ten samples from a historically negative (serologically and clinically) paratuberculosis free herd were examined and resulted in completely negative reading.

DISCUSSION

Results of the qPCR repeat tests (Table 3) showed that sample variability was minimal and consequently the discrepancies probably result from differences in shedding lev-

Table 1: Anti-MAP milk antibody titers and qPCR results (number of hspX gene copies) - cows

No	ELISA	Last trimester of pregnancy							Post-partum				
1	Pos	NE							0.39	1.16	7.61	4.37	0.14
2	Pos	165.21	534.11	151.24	715.00	173.34			130.85	56.01	86.51	257.09	793.22
3	Pos	9431.19	9747.90	4176.75	6965.82	2733.37			0.76	1742.49	23391.85	167.14	6.61
4	Pos	7.47	4.40	37.97	27.80				NE				
5	Pos	24.13	14.97	12.08	11.58	77.15	22.89	28.04	26.13	18.38	14.96	10.04	
6	Pos	7.96	58.46	29.95	69.40	21.38	51.26	59.43	337.47	46.38	31.03	348.26	25.82
7	Pos	1.94	1.53	30.22	54.85	13.41	15.13	0.69*	239.81	76.22	52.80	50.86	84.63
8	Pos	3.04	213.55	0.92	1360.49	1546.96	207.40		0.03	3.18	4.90	5.43	2.76
9	Pos	NE							29.91	50.99	100.36	1.55	5.52
10	Neg	321.52	5.20	1.73	0.01*	1.53	0.04*	2.48	2.98	1.93	0.68*	2.00	90.21
11	Pos	2.99	8.61	10.61	7.53	42.66			2.71	7.47	5.97	0.06	0.13
12	Pos	NE							25.08	1.18	2.82	0.23	0.62
13	Pos	273.71	0.40	0.18	3.60	4.79			16.16	99.88	7.13	130.63	13.30
14	Pos	5.39	4.99	2.79*	4.94	4.21*			NE				
15	Pos	3.24	2.73	29.69	7.86	0.34			1.63	0.95	7.83	12.70	
16	Pos	1025.74	10042.3	2405.10	3885.55	1227.91			69.22	797.25	1493.02	827.61	1340.66

* Samples reexamined. See table 3. Low positive Positive High positive (supershedders)

Table 2: Anti MAP serum antibody titers and qPCR results (number of hspX gene copies) - heifers

No	ELISA	Last trimester of pregnancy					Post-partum				
1	Neg	7.11	34.86	1.95	2.15	2.00	4.70	4.41	7.50	7.56	4.18
2	Neg	1.10	1.48	4.41	0.09	1.53	187.12	65.35	20.18	3.09	6.10
3	Pos	39.35	3.35	18.43	28.99	1739.29	39461.14	30402.11	28933.78	34780.57	14401.21
4	Pos	0.10	3.61	65.65	0.47	1.50	2.38	1.64	14.86	105.52	1.80
5	Neg	1.62	0.14	0.10	16.52	1.92	1.27	5.51	10.91	7.67	2.42
6	Neg	0.35	0.01	0.00	0.01	1.27	17.32	5.24	8.34	3.46	33.69
7	Neg	2.84	0.01	0.62	607.64	18.76	4.78	31.56	12.15	7.23	3.69
8	Neg	32.39	3.17	0.82	2.34	0.29	0.87	1.24	16.07	6.34	5.92
9	Neg	11.30	1.47	2.01	6.08	10.91	0.33	20.03	1.94	2.31	0.51
10	Neg	0.50	0.01	0.00	0.88	510.25	19.33	9.46	1.63	25.65	6.08
11	Neg	0.07	0.54	0.04	0.93	1.67	16.74	3.07	86.50	1.32	
12	Neg	6.14	4.60	2.74	3.71	3.97	9.09	32.60	13.83	11.04	14.02
13	Neg	20.82	41.63	73.91	89.12	1.89	19.74	2.70	4.01	3.16	38.41
14	Neg	5.11	3.70	2.86	1.97	0.45	7.57	14.41	10.50	11.42	24.31
15	Neg	5.65	4.66	7.90	14.77	1.39	2.71	0.00	2.04	0.00	5.28

Low positive Positive High positive (supershedders)

els. The conclusions of this preliminary study are that, unlike previously reported (3) fecal MAP shedding levels are, with a few exceptions, constant enough to classify animals as negative, low shedder, positive and high shedder (supershedder), by a single sample. This may be the result of the use of qPCR whereas, to the best of our knowledge, fecal MAP shedding uniformity was thus far assessed by culturing the microorganism. In addition, the results indicate that calving associated stress does not seems to have influenced fecal MAP shedding levels and that the microorganism's distribution in the fecal sample is homogenous enough to allow a single qPCR test to determine fecal MAP shedding levels.

Forty five percent (64/142) and 67% (100/149) (Tables 1 and 2) of the samples from cows and heifers, respectively, resulted in low qPCR results (<10 gene copies). This may be the result of true shedding, passive or incipient active, or due to the inaccuracy of the kit at low reading values.

Table 3: Results of qPCR test replicates of the same sample

Cow no.	**14**	**14**	**10**	**10**	**10**	**7**
Original result	**4.21**	**2.79**	**0.68**	**0.04**	**0.01**	**0.69**
Replicate results	3.51	4.94	1.64	34.49	2.23	2.32
	4.58	2.37	3.79	3.30	1.48	2.10
	0.17	3.02	2.48	9.29	1.40	4.23
	64.90	2.05	2.66	1.56	1.49	3.62
	2.36	0.36	5.08	4.58	0.28	2.67
						1.53

Since the kit correctly identified fecal samples from a historically negative farm, we assume the former assumption to be true.

It is our opinion that low qPCR values should be interpreted in light of the status of the herd: in high prevalence herds these results are more likely to indicate passive shedding due to heavy environmental exposure whereas in low prevalence herds such results may indicate animals in initial stages of shedding that should be tested periodically to identify eventual evolvement into more massive shedders.

This study was conducted in one dairy herd in a limited number of animals. Consequently we recommend that our conclusions should be substantiated further in the future by expanding the number of the examined population.

CONCLUSIONS

Our results indicate that:

a. MAP shedding level variations during 5-7 consecutive days are small and thus one sample is likely to represent the animals shedding status.
b. Parturition stress does not influence shedding levels, neither in pluriparous cows nor in heifers.
c. Low qPCR results indicate low shedding levels and not kit inaccuracies. These results may indicate passive shedding or infection.

Table 4: Two tailed, dependent, paired ttest of pre and postpartum qPCR results (animals sampled during only one period excluded)

Cow no.	**3rd pregnancy trimester mean**	**Postpartum mean**	**Heifer no.**	**3rd pregnancy trimester mean**	**Postpartum mean**
2	306.26	264.74	1	5.67	9.61
3	5836.39	5061.77	2	56.37	1.72
4	15.70	4.95	3	29595.76	365.88
5	22.32	17.38	4	25.24	14.27
6	116.67	190.79	5	5.56	4.06
7	58.84	100.86	6	13.61	0.33
8	279.33	3.26	7	11.88	125.97
10	33.53	19.56	8	6.09	7.80
11	8.87	3.27	9	5.02	6.35
13	54.98	53.42	10	12.43	102.33
15	7.28	5.78	11	26.91	0.65
16	2311.47	905.55	12	16.06	4.23
			13	13.60	45.47
			14	13.64	2.82
			15	2.01	6.87
		p=0.1482			p=0.3374

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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