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Short communication

Diagnostic validity and costs of pooled fecal samples and individual blood or fecal samples to determine the cow- and herd-status for *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Two tests are used on a regular basis to detect *Mycobacterium avium* subsp. *paratuberculosis* (*Map*): ELISA and fecal culture. Fecal culture is considered more sensitive and specific but is costly and requires 3–4 months for results. Pooling of fecal samples of individual animals may reduce the high costs of fecal culture. The objective of the study was to investigate the diagnostic validity and costs for pooling of fecal samples in dairy farms relative to culture or an ELISA on individual samples to determine the cow- or herd-status for *Map*.

Fifty fecal and blood samples per herd were collected in 12 Chilean dairy herds. The sensitivity of pooling was estimated given the pool-size, amount of shedding in the pool and the prevalence in the herd.

The sensitivity of the pools relative to individual fecal culture was 46% (95% CI 29–63%) and 48% (28–68%) for pools of 5 and 10 cows, respectively. The sensitivity of the pools was lower in pools with low shedders (26 and 24% for pools of 5 and 10, respectively) than in pools with moderate or heavy shedders (>75% sensitivity). Pools of 10 cows are the better option to determine or monitor the herd status. A whole-herd ELISA is the least expensive way to determine the status of individual cows but has a lower Se and Sp than individual culture.

Keywords: *Paratuberculosis*; Fecal culture; Pooling; ELISA; Validity; Costs

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1. Introduction

Paratuberculosis (Johne's disease) in cattle is a chronic enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). The disease is widespread in cattle populations in almost all countries with a dairy industry. In several countries, disease-control programs are developed to reduce *Map* prevalence in the participating dairy farms (Shin, 1989; Benedictus et al., 2000; Wells et al., 2002). The programs aim at control of the disease by both management and testing strategies. Various laboratory tests are used to identify infected animals, for which the necessary measures can be taken (e.g. colostrum management, segregation or culling). In general, two tests are used on a regular basis: ELISA and fecal culture. An ELISA is a fast and low-cost serology test; however, it is less sensitive and specific than fecal culture (Whitlock et al., 2000). Fecal culture is considered a better ante mortem diagnostic test for *Map* infection because the specificity is considered to be 100% and it detects cows in an earlier stage of infection (Van Schaik et al., 2003b); however, it is costly and, on solid media, requires 3–4 months for results. One way to overcome the high costs of fecal culture is to pool fecal samples of individual animals, which can be a cost-effective and a sensitive way to test a herd for a disease (Benedictus et al., 2000; Kalis et al., 2000; Van Schaik et al., 2003a; Weber et al., 2004). However, validation in the field in which ELISA, fecal culture of individual cows and culture of pools are carried out in parallel (i.e. at the same time) need to confirm the results from those studies.

In Chile, the *Map* prevalence is likely to be similar to the prevalence in dairy herds in other countries; a considerable proportion of the herds are infected with a low cow-level prevalence. Several published and unpublished reports indicated a herd prevalence of 37–71% and a cow-level prevalence of 2.8–16% in Chile (Soto et al., 2002a,b). For future control efforts, it is essential to have valid and economical diagnostic tools to lower the animal level prevalence or eradicate *Map* in affected herds. Independent whether farmers want to reduce the economic damage of *Map* or to reduce the possible risk for humans, the benefits have to outweigh the costs of the efforts. Otherwise, farmers will not be motivated to control *Map*.

The objective of the study was to investigate the diagnostic validity and costs of pooling of fecal samples in dairy farms relative to culture of individual samples or individual ELISA to determine the cow- or herd-status for *Map*.

2. Materials and methods

2.1. Sampling and data collection

The study was carried out in 12 commercial dairy herds in southern Chile between September 2003 and March 2004. Herds were selected based on the willingness of the farmers to pay for fecal culture of at least 50 of their cows to assure a sufficiently large sample from the herd. Blood and fresh fecal samples were collected simultaneously from cows in their second or later lactation but older cows and cows that showed clinical signs were favored over younger cows to increase the chance of including cows that shed *Map* in the study. Blood samples were processed and tested with the ELISA test for *Map* antibodies following the recommendations of the manufacturer (IDEXX Laboratories, Inc., Westbrook, ME, USA). Individual fecal samples were collected from the rectum and transferred to a vial that was closed with a lid. Fecal samples were not cooled and transported to the laboratory and processed for culture within 24 h of collection. In the laboratory, the individual samples were cultured and pools of 5 and 10 individual samples (the latter from the same 10 cows that constituted the two pools of 5) were formed by age

(i.e. oldest cows in one pool). The samples were pooled by thoroughly mixing 2 g of faeces from the homogenized individual samples and 2 g of faeces were taken from the pool to be decontaminated and cultured with the same procedure as for the individual samples. Culture was carried out on Modified Herrold's Egg Yolk Medium (HEYM) with (three tubes) and without (one tube) mycobactin J according to the recommendations of the Diagnostic Laboratory of Cornell University, USA (Shin, 1989). Decontamination prior to culture was carried out following the procedure recommended by the National Animal Disease Centre, USA as described by Soto et al. (2002b). Tubes were incubated at 37 °C for 16 weeks. Colonies resembling *Map* and showing mycobactin-dependence were tested by IS*900* PCR. In a recent validation study on individual samples, the ELISA had a Se of 26% and a Sp of 98.5% and fecal culture a Se of 54% and a Sp of 100% (Van Schaik et al., 2007). The pool Se (PSe) was the probability to detect a pool that contained at least one shedding cow.

2.2. Analysis

The sensitivity of pooling was estimated given the pool-size, amount of shedding in the pool and the prevalence in the herd. Herds were classified according to their prevalence in low ($\leq$4%), medium (4–12%) and high (>12%) prevalence herds. Logistic regression to determine whether the sensitivity was significantly different between the categories was not feasible because of missing data in some categories (e.g. zero positive pools of 5 in the low prevalence category and 4 out of 4 positive pools for pools of 10). Therefore, the two-sample proportion test in STATISTIX 8 (Analytical Software, Tallahassee, FL, USA) was used to determine the difference between the sensitivities in the categories by Fisher's exact test at $P \leq 0.05$. The null hypothesis for the two-sample test is that the two proportions are equal. This procedure did not allow correction for a herd-effect (more pools from the same herd). The total costs were estimated for an average herd in the study (300-cows, 5% fecal shedders). The costs of an individual fecal culture amounted to US$24, while the costs for culturing a pool were estimated at US$27. The costs for an ELISA were US$6.

3. Results

Results for the 12 herds are presented in Table 1. *Map* was cultured in 83% of the herds and 7% of the cows. The individual prevalence based on fecal culture in infected herds varied from 2 to 20% of the cows. The distribution of shedders varied considerably by herd but was on average 71% low, 19% moderate, and 10% heavy shedders in the actual data. About 60% of the tested cows were in $\geq$4th lactation. There was at least one positive pool (irrespective of pool-size) in 5 of the 10 infected herds.

Sixteen percent of the pools of 5 cows and 22% of the pools with 10 cows were positive. Three pools of five were positive while none of the individual cultures were positive, but these pools were considered true positives. The probability to detect a positive herd by pooled culture, relative to individual culture, was 19 (16 + 3 positive pools) over 35 (32 culture positives in both individual as pooled samples +3 positive pooled samples), 54.3% (95% CI 36.3–72.2%) and 13 over 28, 46.4% (26.2–66.7%) for pools of 5 and 10 cows, respectively (Table 2).

Table 3 shows that culture of pooled fecal samples had a lower sensitivity in pools with low shedders only (26% and 24% for pools of 5 and 10, respectively) than pools with moderate or heavy shedders (>71% sensitivity). The PSe for pools with at least one low, moderate or heavy shedder was not significantly different between pools of 5 or 10 cows.

Table 1
Culture results of *Mycobacterium avium* subsp. *paratuberculosis* of individual culture and pools of 5 ($n = 120$ pools) and 10 cows ($n = 60$ pools) in 12 Chilean dairy herds

Herd	Individual culture			Pools of five cows		Pools of 10 cows	
	N	# pos.	% pos.	# pools with ≥1 pos. cow	% pos.	# pools with ≥1 pos. cow	% pos.
1	50	5	10	5	100[a]	4	100
2	50	10	20	8	75[a]	5	100
3	50	2	4	2	0	2	50
4	50	1	2	1	0	1	0
5	50	1	2	1	0	1	0
6	50	2	4	2	0	2	0
7	50	6	12	4	75	4	25
8	50	4	8	3	100[a]	3	0
9	50	10	20	8	13	5	40
10	50	1	2	1	0	1	0
11	48	0	0	0	–	0	–
12	50	0	0	0	–	0	–

[a] One pool was culture positive although none of the individuals in the pool was culture positive.

In two high prevalence herds (both 20% prevalence), two pools in which all individual culture results were negative had a positive culture for the pool as well as one pool in a herd with a moderate prevalence of 8% (Table 1). Table 4 shows that the sensitivity of the pools depended on the prevalence in the herd. For pools of 10 cows, the sensitivity of the pools increased with higher prevalence based on individual culture. For pools of five this association was less clear.

Table 5 shows the costs for whole-herd testing using individual fecal culture, individual ELISA and pooled fecal culture with pools of 5 and 10 cows with the costs for testing individual samples of cows in positive pools included in the total costs for pooling. Between brackets are the costs for using the pools solely as a herd test, without follow-up of individuals. Testing with pools of 10 cows is the least expensive option when a high cow-level Sp is required and/or when the herd-status needs to be determined more accurately. A whole-herd ELISA is the second cheapest option when the individual cow-status is required, but it is also the test with the lowest Sp and an especially low-herd specificity (HSp).

Table 2
The sensitivity of pools of 5 cows or 10 cows relative to the individual culture results of *M. avium* subsp. *paratuberculosis* in Chilean dairy cattle

	≥1 positive cows for individual culture	All negative cows for individual culture	Total
Pools of five cows			
Positive	16	3	19
Negative	19	82	101
Total	35	85	120
Pools of 10 cows			
Positive	13	0	13
Negative	15	32	47
Total	28	32	60

Table 3
Pool sensitivity (PSe) for pools with low, moderate or heavy shedders of *M. avium* subsp. *paratuberculosis* in Chilean dairy cattle

Pool with ≥1	Pools of five cows			Pools of 10 cows		
	Total no. of pools	No. of positive pools	PSe	Total no. of pools	No. of positive pools	PSe
Low shedders (<10 cfu/g)	23	6	26.1 a	17	4	23.5 b
Moderate shedders (10–300 cfu/g)	8	7	87.5 a	7	5	71.4
Heavy shedders (>300 cfu/g)	4	3	75.0	4	4	100.0 b

Figures with the same letters (a, b) are significantly different ($P < 0.05$).

Table 4
Pool sensitivity (PSe) for pooled fecal culture of *M. avium* subsp. *paratuberculosis* in Chilean dairy herds that differ in prevalence

Prevalence (%)	No. of herds	Pools of five cows			Pools of 10 cows		
		Total no. of pools	No. of pools with ≥1 shedder	PSe	Total no. of pools	No. of pools with ≥1 shedder	PSe
≤4	7	70	7	0.0 a	35	7	14.3 b
>4 ≤ 12	3	30	12	100.0 a, b	15	11	45.5 a
>12	2	20	16	43.8 b	10	10	70.0 a

Figures with the same letters (a, b) are significantly different ($P < 0.05$).

4. Discussion

In the study we found only a slight but non-significant difference in sensitivity for pools of 5 or 10 animals. Thus, the dilution effect of pooling a positive with negative samples may be less than expected or may be compensated by an increased chance to include more than one shedder and/or a moderate to heavy shedder in a pool of 10. The results in Tables 3 and 4 show that the PSe did increase for pools with moderate to heavy shedders and in herds with a higher prevalence.

As reported by Tavornpanich et al. (2004), we also found positive pools in which all individual samples were culture negative. This may be a result of the homogenization of fecal samples and

Table 5
Costs, sensitivity (Se), specificity (Sp) and herd-level sensitivity (HSe) and specificity (HSp) for individual culture of fecal samples, for individual ELISA and for pools of 5 and 10 cows to detect *M. avium* subsp. *paratuberculosis* in for a 300-cow Chilean dairy herd with a shedding prevalence of 5% in which all cows are sampled

Testing scheme	Costs (in US$)	Se	Sp	HSe[a]	HSp[b]
Individual culture	7200	54	100.0	100.0	100.00
Individual ELISA	1800	26	98.5	99.9	0.01
Pools of five cows	1860 (1620)[c]	29[d]	100.0	98.7	100.00
Pools of 10 cows	1050 (810)[c]	25[d]	100.0	97.7	100.00

[a] $HSe = 1 - (1 - AP)^{300}$.
[b] $HSp = Sp^{300}$.
[c] Costs between brackets are without follow-up to detect individuals in positive pools.
[d] Assuming independence between the PSe and Se of the individual culture to detect the infected cows in a positive pool the cow-level Se is $PSe \times Se_{FC}$.

the uneven distribution of the bacteria in the fecal sample. Although, the samples were well mixed there was a chance that a 2 g aliquot did not contain a bacterium.

The study contained older cows, which seemed to have slightly increased the prevalence but not the proportion of moderate to heavy shedders compared to other studies (Wells et al., 2002; Van Schaik et al., 2003a, 2007). The distribution of shedders varied considerably by herd but was on average 71% low, 19% moderate, and 10% heavy shedders in the actual data. In five herds no positive pools of 5 or 10 were detected even though those pools contained at least one shedder. However, all herds had a prevalence <4% and only 50 cows were sampled and thus only one or two pools included shedders. The probability not to detect these pools as positive was fairly large (30–55%).

Whether pooling is a suitable alternative for whole-herd ELISA testing or individual culture depends on several issues. The objective of the farmer for testing the herd has to be clear: e.g. whether testing is needed to provide knowledge about the infection status of the herd or of the individual cows. Next, timeliness is an issue, ELISA results are available in 24–36 h while fecal culture takes at least 3 months and if individuals from positive pools are cultured subsequently (frozen individual samples or resampled at the farm) it will take at least 6 months. Pools of 5 or 10 cows are not a cost effective option when a farmer wants to determine the infections status of the individual cows for eradication or control purposes. When a farmer is willing to pay, whole-herd culture is the most efficient way to obtain the status of the cows and take measures for the test positive cows such as culling. In addition, the fecal culture positive cows are infected with a 100% certainty. A whole-herd ELISA is the quickest and least expensive screening tool. However, the true infection status of the cows and the herd is more uncertain as a result of the lower sensitivity and specificity of the test. Test reactors should only be culled when the specificity of the ELISA is high or when the infection status is confirmed with fecal culture (which would undo the advantage of timeliness). Nonetheless, repeated whole-herd testing with a highly specific (>99%) ELISA will be a cost effective way to control a *Map* infection in a herd (Weber et al., 2004).

Recently, culture of environmental samples was investigated as a predictor for the herd infection status in large Californian dairy herds (Berghaus et al., 2006). However, the applicability of environmental pooling in different housing systems is not yet determined. In a system such as that in southern Chile where cows are not stabled it may be more difficult to obtain representative environmental samples to determine the herd status. In our study, pools of 10 cows were the preferred option to determine the herd-status with the highest sensitivity and lowest costs. The sensitivity of pools is strongly related to the prevalence in a herd and the infection status of the cows in the pools. In our study we found no difference in sensitivity between pools of five or ten cows. Nevertheless, there is a trend that the sensitivity of pooling is higher for the more advanced stages of infection and in herds with a higher prevalence. We think that these results can be generalized because the shedders in these herds and the prevalence were representative for shedders and the range in prevalence found in any infected herd.

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