THREE-YEAR MONITORING OF PARATUBERCULOSIS IN DAIRY CATTLE BY POOLED FAECAL CULTURE AND INDIVIDUAL PREVALENCE ESTIMATION*

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Abstract

The objective of this study was to assess the implementation of a three-year *Mycobacterium avium* subsp. *paratuberculosis* monitoring programme using pooled faecal culture in small and medium-sized dairy herds to classify them as infected or non-infected and apply proper hygiene and biosecurity measures. Over a three-year period, 35 dairy herds were analysed annually by faecal culture of ten pooled samples. In addition, proper hygiene and biosecurity protocols were implemented in the farms after the first testing round. Considering a herd as infected with at least one culture positive in any of the three years, the accumulated percentage of infected herds was 25.7%, 40% and 45.7%, for each year respectively. Assuming that all infected herds had been detected at the end of the study, the percentage of infected herds detected each year was 56.25% and 87.5% for the first and second year, respectively. Using frequentist and Bayesian approaches, the estimated individual prevalence revealed a downward trend from 3.30–3.65% in the first year to 1.66–1.86% in the third year. The results of this study indicate that pooled faecal culture allowed for proper classification of the herds and can be a useful tool for monitoring dairy herds against paratuberculosis. In addition, statistical analysis of pooled faecal culture results can be used to evaluate the evolution of individual prevalence in the population and therefore the function of the implemented control programmes.

Key words: Bayesian estimation, cattle, monitoring, paratuberculosis, pooled faecal culture

* Work financed from: Agris SCG and Xunta de Galicia, Spain, project n° 08MRU023E.

Bovine paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a globally-distributed chronic intestinal disease that causes significant direct and indirect economic losses in livestock production (Beaudeau et al., 2007; Raizman et al., 2009). In this regard, implementation of control programmes is essential in order to minimize the spread of MAP in infected farms and to avoid the infection of disease-free herds.

Control plans against paratuberculosis are usually supported on annual serological analysis of a representative number of animals, confirmatory testing and classification of herds according to the results (Geraghty et al., 2014). Nevertheless, there are no tests available that allow for the identification of all infected animals in a population (Smith, 2009). ELISA tests used for screening herds have low sensitivity and specificity problems (Osterstock et al., 2007). As a result, misclassification of farms is frequent, especially in areas or countries with small average herd sizes (Greiner and Dekker, 2005). Individual faecal culture (IFC) shows greater sensitivity and specificity than serological techniques; however, it is a costly, laborious and time-consuming method and thus usually only employed as a confirmatory test (Sweeney et al., 2012). These limitations are evidence of the great difficulty of implementing paratuberculosis eradication programmes; although all test-positive cattle are culled, undetected infected animals remain in the herd allowing the maintenance of the infection.

In this context, one of the most effective strategies for reducing the prevalence of MAP in the herd is the establishment of hygiene management measures (Collins et al., 2010). Considering the diagnostic difficulties at the individual level, it has been suggested that the most cost-effective model is to firstly assess the presence or absence of paratuberculosis on farms, and then to implement proper hygiene management measures in the infected herds and rigorous biosecurity protocols in those disease-free farms (Sergeant et al., 2008). The use of pooled faecal cultures (PFC) has been proposed as an alternative to the current serology-based surveillance programmes (Kalis et al., 2004; van Schaik et al., 2007); this method has been proven to reduce testing costs and allow for more accurate classification than serological methods (Tavornpanich et al., 2004). Moreover, several statistical methods have been developed in order to estimate individual prevalence using results from pooled samples (Cowling et al., 1999; Messam et al., 2008) and have been used in some studies concerning paratuberculosis diagnosis (Toribio and Sergeant, 2007; Raizman et al., 2011). Consequently, these statistical approaches could theoretically be applied to the evaluation of control programme effectiveness after the implementation of both hygiene and on-farm biosecurity measures.

The aim of this study was to assess the efficacy of PFC for achieving a correct health classification of paratuberculosis infection of dairy cattle herds after the implementation of a control programme based on hygiene management and biosecurity measures, by means of a PFC monitoring over a three-year period with comparison to traditional serological classification. In addition, the evolution of the annual individual prevalence was inferred statistically from PFC using frequentist and Bayesian approaches as a means to evaluate the effectiveness of the control programme.

Material and methods

Herds

Thirty-five dairy herds, randomly selected from all farms belonging to a dairy cooperative in Galicia (NW Spain), were studied during 2009–2011. All herds were 100% Holstein under an intensive management system, with 28.5% of farms (10/35) closed to live animal introductions for at least the previous five years. Average herd size was 56 cows above two years of age (range 26–236). All farms were confirmed as free of tuberculosis according to the Spanish Programme for Tuberculosis Eradication, and none had previously performed any diagnostic test to evaluate the presence of paratuberculosis.

Study design and scheme

The sampling scheme and chronology for this study is summarized in Figure 1. All herds were sampled once per year from 2009 to 2011. At each sampling, faecal samples were taken from all animals above two years of age, totalling 5828 faecal samples across the study period. Samples were ordered according to the age of the animals for each herd and pooled in groups of ten samples, resulting in 595 faecal pools. Each pooled sample was subsequently cultured, and farms with at least one positive PFC in any of the three samplings were considered as positive. Herd status (positive or negative) was recorded yearly, and a final PFC herd classification was determined after three years. Additionally, blood samples were taken from all sampled animals ($n = 1942$) in the first annual sampling in order to obtain a serological herd classification by ELISA and to also determine the within-herd seroprevalence.

Figure 1. Sampling scheme and chronology of the annual and follow-up testing rounds of pooled and individual faecal culture of 35 mid-size dairy farms in Spain (PFC = pooled faecal culture; IFC = individual faecal culture)

The occurrence of misclassified herds by ELISA was evaluated according to both ELISA and PFC results from the first annual sampling with a follow-up study of the five PFC-negative farms with the highest within-herd seroprevalence. In addition to the annual samplings, these herds were analysed two subsequent times (follow-up samplings) by IFC and PFC six months after the second and third annual samplings (Figure 1). However, the second follow-up sampling round was only performed in PFC-negative herds. In the first follow-up sampling, 182 faecal samples were collected for PFC and IFC of the five selected herds. In the second follow-up sampling, only 39 faecal samples from one herd were analysed by both methods, since three farms tested positive in the second annual sampling and were therefore removed from follow-up sampling. The remaining farm abandoned the dairy business before this follow-up sampling.

Sampling procedure and management data collection

Faecal samples were collected directly from the rectum and blood samples were taken from the coccygeal vein. Both faecal and blood samples were kept refrigerated until processing and faecal cultures were performed within the first 24 h of collection. In addition, biosecurity and hygiene management critical points were carefully revised following previously described checklists (Weber, 2006). The same member of the research team, registering which practices should be modified or implemented, assessed each case *in situ*.

Serological analysis

Two commercial indirect ELISA tests were used for seroanalysis, following the manufacturer's instructions: ELISA A (ID Screen Paratuberculosis Indirect ELISA kit-screening, ID Vet, Montpellier, France) and ELISA B (Pourquier ELISA Paratuberculosis Screening/Paratub. Serum-S, Institut Pourquier, Montpellier, France). Both tests utilize a pre-dilution step with *Mycobacterium phlei* antigen to reduce cross-reactions (Yokomizo et al., 1985). Herds were considered positive if any animal tested positive with at least one of the ELISA tests.

Pooling procedure

Individual faecal samples were homogenized and sorted by age in groups of ten samples. In cases where an exact number of pools of ten samples was not possible, a smaller pool was formed with the remaining samples if they represented more than 10% of the herd census; otherwise, samples from the youngest animals were discarded. For example, in a herd of 56 cows, six pools were formed (five pools of ten samples and one pool of six samples), and in a herd of 55 cows, five pools of ten samples were formed, and the five remaining samples were discarded (those from the five youngest animals). Faecal pools were formed by placing 2 g from each individual faecal sample into a 14 cm sterile petri dish, and then mixing thoroughly with a wooden tongue depressor. Finally, an aliquot of 2 g from each faecal pool was transferred into a falcon tube.

Faecal culture procedure

Individual and pooled faecal samples were decontaminated and cultured by the National Animal Disease Center method (Stabel, 1997). Culturing was carried out on Herrold's Egg Yolk Medium (HEYM, Becton Dickinson, New Jersey, USA) with (three tubes) and without (one tube) mycobactin J. Tubes were incubated at 37°C for 16 weeks and growth checked once per week. MAP-compatible colonies based on slow growth rate, morphological characteristics (small, smooth to slightly rough, from white to slightly yellow) and no growth in HEYM without mycobactin, were first confirmed by Ziehl-Neelsen staining as acid-alcohol resistant bacilli. Subsequently, positive isolates from Ziehl-Neelsen staining were confirmed as MAP by F57-qPCR (Paratub Kuanti-VK, Vacunek, Derio, Spain). A sample was considered positive if isolates from one or more HEYM tubes containing mycobactin J were confirmed as described.

Communication of results and establishment of hygiene management and biosecurity measures

Upon completion of the first testing round, farms were visited again in order to discuss the obtained results (individual ELISA and PFC) with each farmer. Elimination of animals based on ELISA results was strongly discouraged, unless compatible clinical signs were detected. A detailed list of critical management points was given to each farmer and all were advised about the specific practices to be implemented or modified to prevent the entrance or dissemination of the disease. In general, these recommended measures included, among others:

- set up a separated calving area, always keep it clean and dry and disinfect it between parturitions,
- separate calves from their dams immediately before parturition and house them individually until weaning,
- feed calves with colostrum from their own dam only and subsequently with artificial milk replacer instead of cows' milk,
- clean and disinfect all feeding buckets before using, or provide own bucket to each calf,
- house post-weaned calves completely separated from adult cows,
- use different working footwear for each animal area (calving area, calf area and adult cows),
- use disposable plastic boot covers for any visitor (practitioners, technicians, etc.),
- avoid purchasing animals (calves or cows), independently of any type of previous negative test.

The compliance with these management measures was evaluated by the research team in the subsequent samplings, and farmers were warned about the non-modified routines. In contrast, results from the follow-up samplings of the five selected herds were not communicated to producers in order to avoid differences in the application of the recommended management protocols.

Statistical analysis

Kappa statistic and Mc Nemar's test were used to evaluate the existing agreement between the herd classifications obtained by both ELISA tests. For these analyses,

the statistical software R (R v.2.12.2; R Development Core Team, 2011) was employed, using the functions `kap()` from `epicalc` package (Chongsuvivatwong, 2012) and `mcnemar.test()`.

Annual individual prevalence was estimated from PFC results by frequentist and Bayesian approaches (Cowling et al., 1999). Both estimations were calculated using the web tool “Epitools Epidemiological Calculators” (Sergeant, 2015). For the frequentist approach, a method assuming a fixed size pool and imperfect test sensitivity and specificity was chosen (Method 6; Cowling et al., 1999). This procedure assumes that the number of test-positive groups is binomially distributed and uses maximum likelihood methods to estimate the true prevalence at the individual level. The variance of the point estimate is adjusted for additional uncertainty associated with the estimates of sensitivity and specificity. The Bayesian approach, however, requires inputs for prior estimates of true prevalence and test sensitivity and specificity based on expert knowledge or previous data (Method 7; Cowling et al., 1999). These prior estimates are specified as Beta probability distributions, with parameters alpha and beta (α ; β), and are used to calculate posterior probability distributions of prevalence, test sensitivity and specificity. In this study, prior beta distributions for test sensitivity (25; 27) and specificity (10001; 1) were calculated using previously published data (van Schaik et al., 2007). In contrast, an uninformed prior beta distribution (1; 1) was used for true prevalence due to the lack of a reliable previous knowledge about the disease prevalence in the study area. Results from both approaches were used to assess possible fluctuations in individual prevalence after the implementation of suggested biosecurity and hygiene management practices.

Results

Herd classification

Of the herds tested, 58.3% and 66.7% of the herds were classified as positive by ELISA A and B, respectively. Table 1 shows the within-herd prevalence for all studied farms. Five herds (14.3%) presented a different status depending on the ELISA used. Agreement between both ELISA tests for herd classification showed a Kappa value of 0.69 (CI95% = 0.44–0.94); Mc Nemar’s test result showed no significant differences between both herd classifications ($p=0.371$).

The number of confirmed isolates from PFC was 62 of 595. Results per herd and year are summarized in Table 2. The percentage of MAP-positive herds was 25.7% in the first year (9/35) and 28.6% in the second and third years (10/35 per year). A herd was considered positive when at least one positive PFC was detected in any of the three samplings. Accordingly, the accumulated percentages for each year were 25.7% (9/35), 40% (14/35) and 45.7% (16/35), respectively.

Comparison between ELISA herd classifications and the final PFC herd classification showed that in the first case (ELISA A versus PFC), seven herds presented a different status, with six herds positive by serology but not by PFC (17%). In the second case (ELISA B versus PFC), there were twelve farms with differing results depending on the method used, with ten ELISA-positive, but PFC-negative farms (28%).

Table 1. Within-herd seroprevalence for both ELISA tests in the 35 studied farms

Farm	No. of animals	ELISA A % seroprevalence	ELISA B % seroprevalence
1	45	6.67	8.89
2	31	3.23	3.23
3	34	2.94	8.82
4	42	0	0
5	70	0	2.86
6	44	2.27	4.55
7	39	12.82	12.82
8	40	5	5
9	72	1.39	2.78
10	53	9.43	18.87
11	34	0	0
12	30	0	0
13	77	1.3	0
14	35	11.43	11.43
15	53	0	0
16	201	0	0
17	59	1.69	1.69
18	52	3.85	3.85
19	51	3.92	5.88
20	68	1.47	1.47
21	31	0	0
22	43	2.33	6.98
23	236	2.12	4.66
24	30	0	3.33
25	26	0	3.85
26	56	0	3.57
27	25	0	0
28	33	3.03	6.06
29	49	0	0
30	32	0	0
31	37	8.11	5.41
32	58	15.52	20.69
33	53	3.77	3.77
34	60	1.67	3.33
35	43	0	0

Table 2. Proportion and percentage of positive pooled faecal cultures by herd and year and number of positive testing rounds for each farm

Farm	Proportion of positive pooled faecal cultures (%) by herd			No. of positive testing rounds
	May–June 2009	May–June 2010	May–June 2011	
1	5/5 (100)	3/5 (60)	1/5 (20)	3
2	1/3 (33.33)	0/4 (0)	0/4 (0)	1
3	0/4 (0)	0/4 (0)	0/4 (0)	0
4	0/4 (0)	0/4 (0)	0/4 (0)	0
5	0/7 (0)	0/7 (0)	0/7 (0)	0
6	1/4 (25)	0/4 (0)	0/5 (0)	1
7	3/4 (75)	0/4 (0)	0/4 (0)	1
8	0/4 (0)	0/5 (0)	0/6 (0)	0
9	0/7 (0)	4/7 (57.14)	2/7 (28.57)	2
10	0/5 (0)	1/5 (20)	0/5 (0)	1
11	0/3 (0)	0/4 (0)	0/4 (0)	0
12	0/3 (0)	0/3 (0)	0/3 (0)	0
13	0/7 (0)	0/7 (0)	1/7 (14.29)	1
14	0/3 (0)	1/3 (33.33)	0/3 (0)	1
15	0/5 (0)	0/5 (0)	0/4 (0)	0
16	1/20 (5)	2/22 (9.09)	1/22 (4.55)	3
17	0/6 (0)	0/6 (0)	0/6 (0)	0
18	3/5 (60)	0/5 (0)	1/6 (16.67)	2
19	3/5 (60)	2/5 (40)	1/5 (20)	3
20	0/7 (0)	0/8 (0)	0/8 (0)	0
21	0/3 (0)	0/3 (0)	0/3 (0)	0
22	0/4 (0)	0/5 (0)	0/4 (0)	0
23	6/22 (27.27)	3/24 (12.50)	2/24 (8.33)	3
24	0/3 (0)	0/3 (0)	0/3 (0)	0
25	0/3 (0)	0/3 (0)	0/3 (0)	0
26	0/5 (0)	0/5 (0)	0/6 (0)	0
27	0/3 (0)	0/3 (0)	0/3 (0)	0
28	0/3 (0)	0/4 (0)	0/3 (0)	0
29	0/5 (0)	0/5 (0)	0/4 (0)	0
30	0/3 (0)	0/4 (0)	0/5 (0)	0
31	0/4 (0)	1/4 (25)	2/4 (50)	2
32	3/6 (50)	3/7 (42.86)	3/6 (50)	3
33	0/5 (0)	0/5 (0)	1/6 (16.67)	1
34	0/6 (0)	1/6 (16.67)	0/6 (0)	1
35	0/4 (0)	0/4 (0)	0/4 (0)	0
TOTAL	26/190 (13.7)	21/202 (10.4)	15/203 (7.4)	

Individual prevalence estimation from PFC results

Individual prevalence estimations from annual PFC results according to the frequentist method were 3.30%, 2.41% and 1.66% for 2009, 2010 and 2011, respectively. By means of Bayesian approach, the calculated estimations were 3.65%, 2.66% and 1.86% for each year respectively. Figure 2 shows both estimations with 95% intervals (95% confidence interval for frequentist approach and 95% probability interval for Bayesian approach).

Figure 2. Annual individual prevalence estimation (2009–2011) calculated by frequentist and Bayesian inference with their respective 95% confidence and 95% probability intervals

Table 3. Results of follow-up testing by pooled faecal culture (PFC) and individual faecal culture (IFC) of the five selected farms, and final herd classification after 3-year PFC monitoring

Farm No.	% Seroprevalence (ELISA A – ELISA B)	Follow-up PFC Positive/Total	Follow-up IFC Positive/Total	Final PFC herd classification
December 2010				
3	2.94–8.82	0/4	0/36	Negative
10	9.43–18.87	0/4	0/40	Positive
14	11.43–11.43	2/3	2/27	Positive
22	2.33–6.98	0/4	0/40	Negative
31	8.11–5.41	1/4	2/39	Positive
December 2011				
3	2.94–8.82	0/4	0/39	Negative
10	9.43–18.87	Farm removed from follow-up (positive in annual PFC in 2010)		Positive
14	11.43–11.43	Farm removed from follow-up (positive in annual PFC in 2010)		Positive
22	2.33–6.98	Not performed ^a		Negative
31	8.11–5.41	Farm removed from follow-up (positive in annual PFC in 2010)		Positive

^afollow-up analysis was not performed due to the closure of this farm in 2011.

Results from follow-up study

The five selected herds showed a within-herd prevalence of 2.33–11.43% for ELISA A and 5.41–18.87% for ELISA B. Table 3 shows the IFC and PFC results for each follow-up testing in the selected herds. It must be pointed out that the three positive herds identified by follow-up testing were also previously identified in the annual samplings (Table 2).

Discussion

The scientific community currently suggests that paratuberculosis control programmes should not be based on test-and-cull schemes alone, but also include the implementation of proper biosecurity and hygiene measures (Behr and Collins, 2010). Therefore, the first step in a paratuberculosis control programme must be to achieve a precise herd classification.

Our results showed discrepancies between the ELISA herd classifications and the final PFC herd classification, most of which related to ELISA-positive, but PFC-negative herds (17% of herds for ELISA A and 28% for ELISA B). In addition, the concordance between both ELISAs was not perfect and up to five farms (14% approximately) presented conflicting results depending on the ELISA used (Table 2). The farms included in this study were monitored by PFC for three years; it is therefore possible that a major proportion of these ELISA-positive herds were actually uninfected herds showing false positive serological reactions. Additionally, results from the follow-up testing performed in the five selected farms in the first year demonstrate this probable misclassification, as two tested negative either by PFC or by IFC in the subsequent sampling rounds (Table 3). It is worth mentioning that one limitation of this comparison between classifications lies in the fact that closed herd management is not a common practice in the analysed farms. It is therefore possible that truly negative farms purchased infected animals after the serological testing in the first sampling round. Nevertheless, this fact does not affect the ELISA-misclassification of negative farms in the first year.

Our observations regarding the ELISA herd specificity are consistent with those reported by Tavoranpanich *et al.* (2008), who affirmed that diagnostic strategies based exclusively on ELISA have good herd sensitivity at low cost despite false positive results. The implementation of this strategy in non-infected farms will lead to the misclassification of many due to the previously-cited low herd specificity. Another study assessing serial testing strategies (ELISA + IFC) concludes that these approaches will be unable to detect a large proportion of farms with low within-herd prevalence due to reduced herd sensitivity (Wells *et al.*, 2002 a). The same authors reported greater detection frequency of infected farms by ELISA when confirmation by IFC was not subsequently performed; consequently, a considerable proportion of non-infected herds would be misclassified. Therefore, the imperfect sensitivity and specificity of ELISA techniques frequently produces a misclassification of herds and false-positive farms can be affected by restrictions arising from the pertinent control

programme, such as limitations regarding animal movement. These reasons suggest that ELISA techniques alone may not be suitable for herd classification. In addition, serial testing strategies raise the cost of the final herd classification, since the control programme must assume the expenses of confirmatory tests and indirect costs (visits to farms, samplings, etc.). However, in contrast to ELISA tests, the PFC technique does not produce false positive results if isolates are further confirmed by molecular methods. Although PFC sensitivity is not perfect, carrying out regular controls for several years can compensate and lead to a more precise and cost-effective herd classification. Furthermore, due to the absence of a “perfect” diagnostic test for paratuberculosis and low individual prevalence, this improvement in herd classification is especially interesting for small herds, which occur in the main European dairy producer countries, such as Spain, France, Netherlands or Germany (average herd size of 40–50 animals) (Eurostat, 2013). In addition, herd sensitivity in small farms cannot be compensated by more extensive sampling (Greiner and Dekker, 2005). Thus, herd sensitivity can only be improved by continuous surveillance (regular testing) of at-risk herds.

Several studies comparing PFC with IFC have been conducted, but mainly focussed on some aspects such as sensitivity based on pool size and detection limits (Wells et al., 2002 b, 2003; Tavornpanich et al., 2004; van Schaik et al., 2007). Nevertheless, studies regarding herd monitoring over several years are still limited. Thus, results from the present study can be helpful to evaluate the performance of PFC for the classification and monitoring of herds against paratuberculosis, as well as the necessity of carrying out long-term periodic controls. A similar study (Kalis et al., 2004) tested 90 Danish dairy herds with closed management and without previous history of paratuberculosis using biannual PFC. In this four-year study, assuming that all infected farms had been detected at the end of the study period, an increase in the cumulative annual detection percentage of positive herds (51%, 70%, 90% and 97% yearly) was observed; the total herd-prevalence at the end of the study was 61%. Our data are consistent with Kalis et al. (2004), showing that the cumulative percentage of positive farms detected increased with each year of monitoring. Thus, the proportion of infected but undetected herds was lower after each testing round, even when considering that the farms included in this work did not practice closed management. One difference between both studies is the pool size (5 samples/pool versus 10 samples/pool). In regard to this issue, several authors have reported slightly higher sensitivity for five-sample pools when compared to ten-sample pools, although the economic saving of employing pools of 10 samples can compensate for the difference (Wells et al., 2002 b; van Schaik et al., 2007), particularly for continuous monitoring. Another difference is the final purpose of the study, since the Danish study used their results for a certification programme of disease-free farms and our research was principally focused on the use of PFC for monitoring the fluctuation of prevalence after implementation of biosecurity and hygiene measures.

As has been mentioned, the establishment of proper hygiene management and biosecurity protocols is essential to achieve a significant reduction in the prevalence of paratuberculosis in infected herds. Consequently, the evaluation of individual prevalence can be a helpful tool for assessing the effectiveness of implemented recom-

mentations. Our results (using two different statistical approaches) show a decreasing yearly trend in individual prevalence after the implementation of recommended measures within a control programme based on annual PFC monitoring (Figure 2). Therefore, results from the PFC method are suitable for calculating the individual prevalence and could therefore be used for evaluating the effectiveness of control programmes based on the application of management and biosecurity standards. Both statistical methods (frequentist and Bayesian) yielded similar estimations, but the Bayesian approach showed a slightly higher prevalence for each year. This observation is in agreement with Raizman et al. (2011), who calculated the within-herd prevalence of MAP in dairy farms by these two methods, but in a single sampling. Similarly, Toribio and Sergeant (2007) analysed the individual MAP prevalence in ovine flocks by seven different statistical methods, obtaining more variable prevalence estimations and narrower credible intervals with the Bayesian, rather than the frequentist approach. These differences could be related to an inadequate selection of the prior distribution for prevalence. The utilization of an uninformed prior distribution (a distribution where all values between 0 and 1 are equally likely) may be more appropriate for prevalence estimation, since our data showed less variability between both Bayesian and frequentist methods.

The current study confirms annual PFC monitoring as a suitable tool for detecting MAP in dairy herds, allowing a more precise herd classification than serological methods, particularly in areas with small farm sizes. In addition, results from PFC testing can be evaluated using Bayesian or frequentist approaches in order to assess the population prevalence after the implementation of hygiene management and biosecurity measures. Thus, this information will be very useful for studying the effectiveness of paratuberculosis control programmes.

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Received: 27 IV 2017

Accepted: 18 IX 2017