

Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples by faecal culture and *real-time* PCR in relation to apparent within-herd prevalence as determined by individual faecal culture

K. DONAT¹*, J. KUBE¹, J. DRESSEL¹, E. EINAX¹, M. PFEFFER² AND K. FAILING³

¹Animal Health Service, Thuringian Animal Diseases Fund, Jena, Thuringia, Germany

²Institute of Animal Hygiene and Veterinary Public Health, Centre of Veterinary Public Health, University of Leipzig, Leipzig, Saxony, Germany

³Unit for Biomathematics and Data Processing, Veterinary Faculty, Justus-Liebig-University, Gießen, Hesse, Germany

Received 18 April 2014; Final revision 24 August 2014; Accepted 30 August 2014;
first published online 2 October 2014

SUMMARY

Environmental samples are a cost-saving and easy-to-use approach to diagnose paratuberculosis at the herd level. Detailed knowledge concerning its uncertainties in herds with a low prevalence of *Mycobacterium avium* spp. *paratuberculosis* (MAP) is required to design sampling strategies in control programmes. This study aimed to calculate a threshold level of the apparent within-herd prevalence (WHP_{app}) as determined by individual mycobacterial cultivation (faecal culture; FC) of all cows thus allowing the detection of a herd as MAP-positive at a certain probability level (P_d). Out of 200 environmental samples taken twice from five predefined locations in a barn, 25 were positive by FC and 60 were positive by a quantitative real-time PCR method (qPCR). A logistic regression model was used to calculate the WHP_{app} threshold of detection. For 50% P_d , a WHP_{app} threshold of 2.9% was calculated for the combination of three samples (milking area, main cow alleyways, holding pen) tested simultaneously both by FC and qPCR. The threshold increased to 6.2% for 90% P_d . Repeated environmental sampling did not reduce the WHP_{app} threshold. Depending on the particular needs for prevalence estimation or in control programmes (single or repeated sampling) the provided WHP_{app} thresholds at different P_d will enable decisions to be made about various sampling strategies.

Key words: Faecal culture, infectious disease control, *Mycobacterium (avium paratuberculosis)*, veterinary epidemiology and bacteriology, zoonoses.

INTRODUCTION

Paratuberculosis, or Johne's disease, is a chronic granulomatous enteric disease, predominantly of ruminants, which is caused by *Mycobacterium avium* subspecies (spp.) *paratuberculosis* (MAP). It is characterized by a

long incubation period of up to 10 years, resulting in a latent, a subclinical, and a clinical stage of disease. Therapy-resistant aqueous diarrhoea and formation of oedema due to hypoproteinaemia are cardinal symptoms of late-stage clinical paratuberculosis [1]. Economic losses are caused by reduced slaughter weight [2], increased susceptibility to other diseases [3], premature culling [4] and, in the dairy industry, decreased milk production even in cows without clinical symptoms [5–8]. Furthermore, there may be a role

* Author for correspondence: Dr. K. Donat, Thüringer Tierseuchenkasse, Victor-Goerttler-Strasse 4, D-07745 Jena, Germany.
(Email: kdonat@thueringertierseuchenkasse.de)

of MAP as a potential zoonotic pathogen. A link between MAP and Crohn's disease, a human inflammatory bowel disease, is discussed [9, 10]. MAP survives current pasteurization treatments and, therefore, is a potential human foodborne pathogen [11].

With respect to the economic impact of Johne's disease and the potential risk for human health, effective measures to control the disease should be implemented in MAP-positive herds to limit the spread of the disease, and to reduce the shedding of MAP into the environment, and the carry-over into the food chain. Currently, identification of MAP-positive herds by testing individual animals is hampered by the lack of affordable, specific and sufficiently sensitive approaches for a diagnosis at the herd level. First, bacterial culture of faecal samples on solid or liquid media (faecal culture; FC) is still considered the 'gold standard' for MAP identification [9, 12], but it is time-consuming and costly. Further, it may render false-negative results when applied at a sampling time point without MAP shedding. Bacterial culture of pooled faecal samples can reduce the testing costs per animal by 43% up to 73%, but it decreases test sensitivity at the individual animal level to 60% [13]. Second, ELISA tests, which are commercially available and commonly used, perform with a lack in sensitivity and specificity, and do not allow the categorization of a herd as MAP-positive or MAP-negative in specific cases [1, 14]. Therefore, a cost-efficient and manageable screening method to categorize herds with regard to their MAP status would be valuable. Bacterial culture of environmental faecal samples has been shown to detect dairy herds with an estimated moderate to high within-herd prevalence (WHP) as MAP-positive [15–17]. This approach utilizes the long-term survival of MAP under various environmental conditions. In liquid manure MAP can survive at 5 °C for more than 9 months and at 15 °C for at least 3.5 months [18, 19]. In the absence of essential nutrients MAP may enter a 'state of dormancy' and return to a viable state under better conditions [20]. In freestall herds, targeted sampling in alleyways, waiting yards, manure storage areas and holding pens showed the best outcome of detection [15–17, 21, 22].

The number of positive environmental samples and the amount of MAP in those samples are positively correlated with WHP [15, 16]. The sensitivity at the herd level as determined by composite environmental sampling varies in a wide range from 33.3% to 89.7% depending on the number of samples and WHP [23].

Recently, sensitivity of a set of six environmental samples collected from different locations within a barn and tested by FC was found to be 71% (95% confidence interval 49–86) compared to a herd classification based on pooled FC samples, with samples from five cows in each pool [24].

Due to the higher test sensitivity and test specificity, the studies that determined individual Johne's disease status by identification of the organism [21, 22, 25], are less susceptible to misclassification bias than those using identification based on percentage of antibodies against MAP [26], which is particularly important in herds with low prevalence. These studies indicated that environmental samples tested by FC do not identify MAP-positive herds with a WHP <2% and showed inconsistent results when apparent WHP (WHP_{app}) ranged between 2% and 10%.

For the last decades attempts have been made to apply polymerase chain reaction (PCR) techniques directly on faecal samples, which is able to provide much faster results than FC [1]. A variety of primers against different targets in the MAP genome has been established, i.e. IS900, IS Mav3, F57 and locus 255. Most of the single-step methods are reliable, while nested PCR methods are at a higher risk of being disturbed by contaminations [27]. Sensitivity of PCR from faecal samples depends on the DNA extraction procedures, which should ensure an effective removal of PCR inhibitors like phytic acid, polysaccharides, phenolics or bile salts [28]. Therefore, most PCR methods are less sensitive than FC [29]. Recently, real-time PCR for the detection of MAP combined with a high-efficiency DNA extraction was developed which was reported to have an equal or higher sensitivity compared to FC on Herrold's egg yolk medium (HEYM) [30–32].

Therefore, our study aimed to calculate a threshold of WHP that allows the identification of low-prevalence herds as MAP-positive with an acceptable probability using environmental samples tested by FC and quantitative real-time PCR (qPCR).

MATERIAL AND METHODS

Study population

Twenty Holstein dairy herds enrolled in the 'Paratuberculosis Control Programme in Thuringian Cattle Herds' and housed in freestalls, with an average herd size of 253 cows (40–538) were selected for this study. The programme included, among other

measures, annual screenings of all cows for MAP by FC. If a cow tested positive by FC (FC+), it was not retested the next year. The Thuringian MAP control programme followed the recommendations of Sweeney *et al.* [9], who suggested immediate culling of heavy shedders and the elimination of low shedders at the end of lactation or in case of other problems.

According to the aim of the present study, 14 herds with a WHP_{app} (see below) less than 10% were selected based on the results of herd screenings for the years 2008–2010, and three herds had a WHP_{app} larger than 10%. Additionally, three herds which never had a FC+ test result in the annual herd screenings during the previous three years were included (Table 1).

Estimation of WHP_{app}

All cows in the 20 herds without a FC+ test result, i.e. first lactation cows and cows tested FC-negative (FC-) in previous years were tested once by individual FC during January and June 2011. Samples were taken by a veterinarian of the Thuringian Animal Health Service using a new glove for each cow and a sterile 125 ml plastic cup with a screw cap and bar code for sample identification. The numbers of tested cows, cows with MAP-positive test results from previous years, and number of cows in each herd are given in Table 1. We calculated WHP_{app} using the FC result of each cow in the herd (Table 1) and therefore, WHP_{app} was estimated with a high accuracy level and not biased by pooling or selection of sampled individuals.

Environmental sampling

Faecal samples from the barn environment of the cattle were collected by a veterinarian of the Animal Health Service. In a previous study [22] a total of five sampling sites within the barns were identified and proven to be suitable for environmental sampling: milking area (waiting pen), main alleyway, lactating cow floor (fresh cow pen), maternity (calving) pen, and crossover to calf area. For each of these five areas a composite sample consisting of ten randomly collected subsamples was taken at different sites within the sampling location using a clean and disinfected scraper. The subsamples were placed together in a sterile 125 ml plastic cup with a screw cap and transported in a cooler to the laboratory within 2 h. Environmental samples were taken twice for each herd within 4 months with a median interval of 132

days (minimum 118, maximum 160) in different seasons. In the cool season sampling was performed during March and April 2011 and in the warm season samples were taken during July and August 2011.

Bacterial culture

After transportation to the laboratory all faecal samples were stored at -20°C until cultivation to avoid undesired bacterial and fungal growth and to ensure consistent sample handling. FC of individual samples and environmental samples was performed according to the official manual of diagnostic procedures published by the FLI [33]. Differentiation of characteristic colonies was done by Ziehl-Neelsen staining and an IS900 PCR [29].

DNA isolation from faeces

The DNA was extracted from faeces using the MagMax™ Total Nucleic Acid isolation kit (Life Technologies GmbH, Germany) according to the manufacturer's instructions. The samples were thawed, homogenized and 0.3 g was transferred into 1 ml phosphate buffered solution. The MagMax™ Express 96 instrument (Life Technologies GmbH) was applied for nucleic acid purification and DNA was eluted in a final volume of 200 μl buffer solution.

DNA amplification and real-time PCR

For the detection of MAP DNA, the *TaqMan*® MAP (Johne's) Reagents kit (Life Technologies GmbH), performed on a 7500 fast real-time PCR cycler (Life Technologies GmbH), was used according to the manufacturer's instructions.

Statistical data analysis

Results of bacterial culture and qPCR were recorded and descriptive statistics were generated using a Microsoft Excel calculation spreadsheet (Microsoft Corporation, USA). All other statistical analyses were done using the statistical software package BMDP/Dynamic (release 8.1; W. J. Dixon, Statistical Solutions Ltd, Ireland). At each step a statistical significance level of $\alpha = 0.05$ was used.

To analyse the relationship between the WHP_{app} and the colony growth score (FC) or the cycle threshold (C_t) value (qPCR) of environmental samples,

Table 1. Determination of apparent within-herd prevalence (WHP_{app})

Herd	Cows (n)	Known FC positive cows ^a (n)	Tested cows (n)	FC pos. ^b (n)	WHP_{app} (%)
1	253		253	0	0
2	417		417	0	0
3	204		204	1	0.49
4	211		211	0	0
5	224		224	0	0
6	198		198	7	3.54
7	301	4	297	9	4.32
8	465		465	32	6.88
9	380	2	378	14	4.21
10	182		182	13	7.14
11	322	4	318	15	5.90
12	259	4	255	9	5.02
13	538	9	529	17	4.83
14	40		40	1	2.50
15	150		150	9	6.00
16	168		168	14	8.33
17	236		236	10	4.24
18	185		185	22	11.89
19	195		195	66	33.85
20	135	25	110	31	41.48
Total	5063	48	5015	270	

FC, Faecal culture; MAP, *Mycobacterium avium* spp. *paratuberculosis*.

^a MAP-positive cows tested by FC in previous years and still kept in the herd.

^b FC MAP-positive cows.

respectively, Spearman's rank correlation coefficients were calculated for each location and test method.

As environmental samples were collected twice, and WHP_{app} could be calculated only once for each herd, additionally to the observation in spring, the FC and qPCR results were aggregated using the highest colony growth score for FC and the lowest C_t value for qPCR observed in spring or summer for each location.

Concerning WHP_{app} of a certain herd as an influencing factor of interest ($n = 20$), its relationship to the dichotomized MAP findings (negative or positive) in the environmental samples was assessed using a logistic regression model analysed with the asymptotic logistic regression procedure BMDPLR [34].

Due to significant relationships for WHP_{app} in the logistic regression model, two, three or five locations were selected in order to combine their findings into a single binary outcome, being positive if at least one of these locations showed a positive result, and negative if all locations were negatively tested.

Inversion of the logistic function yields a WHP_{app} estimate associated with a given probability of detection (P_d) of a MAP-positive herd. For practical reasons in a control programme or for prevalence estimation, a high P_d is desirable. A value of $P_d = 0.9$ was selected because on the one hand it is commonly used in epidemiological problems and on the other, the slope of the logistic function is still rising steeply enough to limit the uncertainty of WHP_{app} estimation. Furthermore, $P_d = 0.5$ was selected because this is the inflexion point of the sigmoid logistic function, and this is the point of maximal slope which minimizes the uncertainty of WHP_{app} estimation. By means of the program BMDPLE [34], which uses maximum likelihood techniques, estimates as well as asymptotic standard errors for these detection thresholds were found. From this, approximate 95% confidence intervals for the thresholds of interest were computed.

The formula for the detection threshold is:

$$WHP_{app}(P_d) = (\ln(P_d/(1 - P_d)) - a)/b,$$

where P_d = desired probability of detection; $WHP_{app}(P)$ = apparent within-herd prevalence with detection probability P ; a = constant parameter in the logistic function; b = model coefficient relating to WHP_{app} in the logistic regression; and \ln = natural logarithm.

In order to analyse the accordance between the FC and qPCR results, Cohen's kappa coefficient was calculated. Additionally, McNemar's procedure was used to test for deviation of symmetry of differing results in the fourfold table of the dichotomized FC and qPCR findings.

RESULTS

WHP_{app}

Out of a total of 5015 individual faecal samples, 270 were MAP-positive and 4710 MAP-negative by FC (Table 1). The 20 study herds had a median WHP_{app} of 4.6% (first quartile 2.0%, third quartile 6.9%). In four herds none of the cows was positively tested.

Environmental samples

Regarding the first sampling (cool season), FC resulted in 18 FC+ and 76 FC- environmental samples. When qPCR was applied, 31 samples showed a positive (qPCR+) and 69 a negative (qPCR-) result.

Table 2. Environmental samples tested by FC and qPCR for MAP grouped by location of sampling

Location of sampling	Method of MAP detection	FC			
		Pos. ^a	Neg. ^b	n.a. ^c	
Milking area	qPCR	Pos.	7	4	2
		Neg.	2	17	8
Maternity pen	qPCR	Pos.	0	12	2
		Neg.	1	23	2
Crossing to calf area	qPCR	Pos.	1	7	1
		Neg.	1	23	7
Main alleyway	qPCR	Pos.	6	9	1
		Neg.	1	20	3
Holding pen	qPCR	Pos.	6	8	0
		Neg.	0	24	2
Total	qPCR	Pos.	20	40	6
		Neg.	5	107	22
		Total	25	147	28

FC, Faecal culture; qPCR, quantitative real-time polymerase chain reaction; MAP, *Mycobacterium avium* spp. *paratuberculosis*.

^a MAP-positive.

^b MAP-negative.

^c Not assessable FC due to contamination.

Due to fungal or bacterial contamination six samples were not assessable during the cool season and 22 samples during the warm season, where 35 samples were qPCR+ and 65 qPCR-. Taken together, 33% of the environmental samples were qPCR+ and 12.5% FC+ (Table 2). Based on the results of 172 environmental samples with valid results by FC and qPCR, Cohen's kappa was 0.334 indicating a moderate but significant ($P < 0.001$) association of FC and qPCR in environmental samples. McNemar's test showed a significant asymmetry of the differing test results by FC and qPCR ($P < 0.0001$), where PCR resulted in a higher number of positive tests.

Association between WHP_{app} and FC or qPCR results

Spearman's rank correlation coefficients (r_s) for the relationship between WHP_{app} and colony growth score (FC) of environmental samples or the C_t values of qPCR for each location are given in Table 3. Additionally, we calculated r_s for combinations of two or three locations which showed a significant association with WHP_{app}, and for a combination of all locations. All combinations showed a significant correlation with WHP_{app} gaining best results for the FC colony score using a combination of three samples (milking area, main alleyway, holding pen) or all samples.

Association between WHP_{app} and herds' MAP status as determined by FC or qPCR

When the single sampling strategy was implemented, the main alleyway was the only location where both techniques (FC and qPCR) and the combination of both were significantly associated with WHP_{app}. For the holding pen this was the case only when qPCR was applied, and for the combination of both methods. Double sampling resulted in a significant association with WHP_{app} for the main alleyway tested by both methods and the combination of both. Additional significance was found for the maternity area tested by qPCR and the combination of qPCR and FC. Despite two combinations in qPCR testing in the single sampling, all combinations of locations resulted mostly in significant associations with WHP_{app} (Table 4).

Estimates of WHP_{app} associated with a given probability of detection

For a combination of two samples, the estimate of WHP_{app} (\pm standard error) associated with 90% probability of detection of a MAP-positive herd yielding from the inversion of the logistic function was $10.2 \pm 2.6\%$ for FC and $7.9 \pm 1.5\%$ for qPCR without relevant differences between single and double sampling (Table 5).

Using a combination of three samples once, the estimated WHP_{app} associated with 90% probability of detection decreased to $6.4 \pm 0.9\%$ for FC and increased to $10.9 \pm 4.0\%$ for qPCR. When reducing the probability of detection to 50%, the estimate decreased to $5.1 \pm 0.5\%$ for FC and $4.9 \pm 1.5\%$ for qPCR. For double sampling, the latter decreased to $3.5 \pm 1.1\%$. Applying both tests and a combination of three or all samples resulted in an estimate of $6.2 \pm 4.0\%$ for 90% and $2.9 \pm 1.0\%$ for 50% probability of detection for both single and double sampling (Table 5).

DISCUSSION

Control of paratuberculosis, or Johne's disease, in cattle herds requires the identification of MAP-positive herds in order to implement control measures. Testing individual cow samples is time-consuming and expensive and may be hampered by the lack of sensitivity or specificity if only one sample per individual is tested. Consequently, environmental sampling

Table 3. Spearman's rank correlation coefficient (r_s) for the correlation between within-herd prevalence and colony growth score of the FC or C_t value of the qPCR for single and double sampling and for each location sampled for the combination of two, three or five samples

Location	Single sampling						Double sampling					
	Colony score ^a			C_t value ^b			Colony score			C_t value		
	n^c	r_s^d	P value	n	r_s	P value	n	r_s	P value	n	r_s	P value
Milking area	18	0.597	0.007	20	-0.581	0.007	18	0.597	0.009	20	-0.627	0.003
Maternity	19	0.0	n.s.	20	-0.396	n.s.	20	0.260	n.s.	20	-0.730	<0.001
Crossing to calf area	18	0.146	n.s.	20	-0.329	n.s.	20	0.181	n.s.	20	-0.547	0.012
Main alleyway	19	0.695	<0.001	20	-0.704	<0.001	19	0.695	<0.001	20	-0.694	<0.001
Holding pen	20	0.576	<0.001	20	-0.780	<0.001	20	0.579	0.007	20	-0.752	<0.001
2 locations combined ^e	20	0.691	<0.001	20	-0.747	<0.001	20	0.700	<0.001	20	-0.767	<0.001
3 locations combined ^f	20	0.848	<0.001	20	-0.712	<0.001	20	0.858	<0.001	20	-0.780	<0.001
5 locations combined ^g	20	0.846	<0.001	20	-0.688	<0.001	20	0.860	<0.001	20	-0.758	<0.001

FC, Faecal culture; C_t , cycle threshold; qPCR, quantitative real-time polymerase chain reaction; n.s., not significant.

^a Colony score of environmental samples' FC test (c.f.u.): +, 1–10; ++, 11–50; +++, 51–100; +++++, >100.

^b C_t value of environmental samples' qPCR.

^c Number of herds with valid test results of environmental samples tested by either qPCR or FC.

^d Spearman's rank correlation coefficient.

^e Combination of two locations (main alleyway, holding pen).

^f Combination of three locations (milking area, main alleyway, holding pen).

^g Combination of all five locations.

has been described as an easy and cost-effective herd-level screening method [14–17]. Although there is controversy as to whether this approach is appropriate for herds with low MAP prevalence [21, 24, 25], herd-level sensitivity and specificity values have been published [24] and, for a given WHP_{app} to be detected, found to be comparable or even better than serological testing [14]. Therefore, environmental samples tested by FC can be considered an alternative method for herd diagnosis in low-prevalence herds [24]. Using two methods of MAP detection simultaneously, FC and qPCR, this prospective study was performed in well-characterized dairy herds with a low-level WHP_{app} known from individual FC testing in previous years. Thus, we were able to fill a gap in the knowledge regarding the benefit of using qPCR instead of, or simultaneously with, FC.

Compared to previous studies [14–17], the strength of our study design is that we used the gold standard for the determination of WHP_{app} , i.e. the individual testing of all cows in the herd. Thus, the WHP_{app} we used in the model was not influenced by any bias of pooling or selection of sampled individuals [9, 12, 35] resulting in a WHP_{app} estimation with a high accuracy level. Nonetheless, a certain level of uncertainty remains in the estimation of WHP_{app} that results

from the use of an imperfect test. FC is highly specific (>99%) but has a sensitivity that is estimated to be approximately 60% relative to necropsy [9]. Because necropsy is not a realistic option for field studies and for diagnosis in the framework of control programmes, we decided to refer to the FC-based WHP_{app} . The estimation of WHP_{app} was animal based with numerous animals per herd, and we cannot exclude that variability may be caused by fluctuating shedding, which only becomes apparent by repeated sampling. Because of the high cost of our approach based on individual culture of each sample, the individual FC test was not repeated. Hence, this uncertainty cannot be excluded from the estimation of the WHP_{app} threshold.

Our results demonstrate the advantages and limitations of using a qPCR to detect MAP in environmental samples. Contrary to the time-consuming FC method, qPCR allows MAP detection within a few days and detected a significantly higher number of environmental samples as MAP-positive without specificity problems [36]. Environmental samples that tested MAP-positive originated from herds known to be MAP-positive, except for five samples from herd 2 that was known to be MAP-negative for at least 5 years. The herd was retested by individual FC of

Table 4. Results of the logistic regression to analyse the association between WHP_{app} and MAP status of herds ($n = 20$) determined by FC, qPCR or the combination of both for different combinations of locations

Location	Single sampling						Double sampling					
	FC ^a		qPCR ^b		FC and qPCR ^c		FC		qPCR		FC and qPCR	
	OR ^d	<i>P</i> ^e	OR	<i>P</i>	OR	<i>P</i>	OR	<i>P</i>	OR	<i>P</i>	OR	<i>P</i>
2 locations combined ^f	2.07	0.044	2.73	0.030	2.48	0.030	2.07	0.044	1.74	0.041	1.74	0.041
3 locations combined ^g	5.76	0.016	1.44	0.094	1.96	0.026	5.76	0.016	1.74	0.041	1.96	0.026
5 locations combined ^h	5.76	0.016	1.48	0.078	1.96	0.026	5.76	0.016	1.96	0.026	1.96	0.026

WHP_{app} , apparent within-herd prevalence; MAP, *Mycobacterium avium* spp. *paratuberculosis*; FC, faecal culture; qPCR, quantitative real-time polymerase chain reaction; OR, odds ratio.

^a Herds classified as MAP-positive by testing the sample by FC.

^b Herds classified as MAP-positive by testing the sample by qPCR.

^c Herds classified as MAP-positive by testing the sample by FC or qPCR.

^d Odds ratio for the description of the association between WHP_{app} and MAP status.

^e *P* value for odds ratio.

^f Combination of two locations (main alleyway, holding pen).

^g Combination of three locations (milking area, main alleyway, holding pen).

^h Combination of all five locations.

all cows the following year and 3 years after the study without any positive results. The samples were retested using another PCR protocol targeting the F57 locus with negative results. Although we suppose that the initial qPCR results of these samples might be false positive, another reason for the positive results could be a MAP shedder with low or intermittent shedding resulting in very low WHP. We did not exclude these samples from the model because in the practical use of qPCR-tested environmental samples, these samples are regarded as positive which should be represented by our model.

With respect to the locations of sampling, the qPCR performed much better for samples from the maternity pen and the crossing to calf area with 20 samples qPCR MAP-positive compared to only four samples with positive results by FC. Because all study herds are involved in the control programme, this may result from the activities of the herd managers to ensure a high hygienic standard by frequent disinfection in areas of critical importance (maternity pen, crossing to calf area), and consequently, MAP was not cultivable but detectable by PCR. Regarding the correlation of the C_t value with WHP_{app} , we observed a significant Spearman's rank correlation coefficient of -0.730 for the maternity pen and -0.547 for the crossing to calf area for double sampling (Table 3). In samples from the main alleyways, the holding pens of lactating cows

and the milking area, qPCR nearly doubles the number of MAP-positive samples compared to FC; the correlation with WHP_{app} was significant for the C_t values and the FC colony score as well indicating that these three locations are appropriate for environmental sampling and testing by FC as well as by qPCR. The best correlation with WHP_{app} ($r_s = 0.78$) was achieved when samples from the holding pen were tested by qPCR. This corresponds to the correlation between C_t values and colony-forming units in a previous study with a non-standardized sampling protocol [32]. The combination of three samples (holding pen, main alleyway, milking area) enhanced correlation markedly for colony scores, but not for C_t values. The combination of all five sample locations did reduce and double sampling did not improve the correlation compared to single sampling. When focusing on cow concentration areas (lactating cow floor, cows' alleyway), our results are in line with the results of previous studies [15, 21]. Although manure storage areas were shown to be suitable locations for MAP detection by environmental sampling in previous studies [15–17, 21] we excluded them from our study on health and safety grounds. As we intend to establish environmental sampling as a sufficiently sensitive, cost-saving and easy-to-use approach to identify MAP-positive dairy herds, we aim to avoid a hazardous situation for the person sampling.

Table 5. Estimates of WHP_{app} threshold value \pm asymptotic s.e. and approximate 95% confidence intervals for the detection of a MAP-positive herd using a combination of two, three or five environmental locations tested by FC, qPCR or the combination of both, respectively, at different probabilities of detection

	Single sampling						Double sampling					
	FC ^a		qPCR ^b		FC and qPCR ^c		FC		qPCR		FC and qPCR	
P_d^d (%)	$WHP_{app} \pm s.e.^e$	95% CI_{app}^f	$WHP_{app} \pm s.e.$	95% CI_{app}	$WHP_{app} \pm s.e.$	95% CI_{app}	$WHP_{app} \pm s.e.$	95% CI_{app}	$WHP_{app} \pm s.e.$	95% CI_{app}	$WHP_{app} \pm s.e.$	95% CI_{app}
Combination of two environmental samples (main alleyway, holding pen)												
50	7.2 \pm 1.2	4.7–9.6	5.7 \pm 0.7	4.2–7.2	5.2 \pm 0.8	3.7–6.7	7.2 \pm 1.2	4.7–9.6	3.5 \pm 1.1	1.3–5.7	3.5 \pm 1.1	1.3–5.7
90	10.2 \pm 2.6	4.9–15.4	7.9 \pm 1.5	4.8–11.0	7.7 \pm 1.6	4.5–10.8	10.2 \pm 2.6	4.9–15.4	7.5 \pm 2.0	3.4–11.5	7.5 \pm 2.0	3.4–11.5
Combination of three environmental samples (milking area, main alleyway, holding pen)												
50	5.1 \pm 0.5	4.2–6.1	4.9 \pm 1.5	1.9–7.9	2.9 \pm 1.0	1.0–4.9	5.1 \pm 0.5	4.2–6.1	3.5 \pm 1.1	1.3–5.7	2.9 \pm 1.0	1.0–4.9
90	6.4 \pm 0.9	4.6–8.2	10.9 \pm 4.0	3.0–18.9	6.2 \pm 1.6	3.0–9.4	6.4 \pm 0.9	4.6–8.2	7.5 \pm 2.0	3.4–11.6	6.2 \pm 1.6	3.0–9.4
Combination of five environmental samples (milking area, maternity, crossing to calf area, main alleyway, holding pen)												
50	5.1 \pm 0.5	4.2–6.1	4.1 \pm 1.4	1.3–6.9	2.9 \pm 1.0	1.0–4.9	5.1 \pm 0.5	4.2–6.1	2.9 \pm 1.0	1.0–4.9	2.9 \pm 1.0	1.0–4.9
90	6.4 \pm 0.9	4.6–8.2	9.9 \pm 3.3	3.3–16.4	6.2 \pm 1.6	3.0–9.4	6.4 \pm 0.9	4.6–8.2	6.2 \pm 1.6	3.0–9.4	6.2 \pm 1.6	3.0–9.4

WHP_{app} , Apparent within-herd prevalence; s.e., standard error; MAP, *Mycobacterium avium* spp. *paratuberculosis*; FC, faecal culture; qPCR, quantitative real-time polymerase chain reaction; CI, confidence interval.

^a Herds classified as MAP-positive by testing environmental samples by FC.

^b Herds classified as MAP-positive by testing environmental samples by qPCR.

^c Herds classified as MAP-positive by testing environmental samples by using both qPCR and FC.

^d Probability of detection MAP-positive herds as positive.

^e Estimated threshold value of the $WHP_{app} \pm$ approximate s.e. for the detection of a MAP-positive herd.

^f Approximate 95% CI of the estimated threshold value of the WHP_{app} for the detection of a MAP-positive herd.

The estimation of the WHP_{app} threshold value for detection of a MAP-positive herd using different combinations of sampling and methods of testing showed the lowest WHP_{app} percentage as 90% P_d for a combination of three and five samples tested by FC and qPCR simultaneously. Relaxing P_d to 50% resulted in a lowering of this estimate from $6.2 \pm 1.6\%$ to $2.9 \pm 1.0\%$ WHP_{app} . Our results are in line with previous studies [24] using a set of six samples with a sensitivity approaching 100% at moderate WHP_{app} levels of 8%, and 90% sensitivity at a WHP_{app} of $\sim 5\%$. This may encourage the use of a set of two or three environmental samples instead of six samples which is demanded by the Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program published by the USAD [37]. Furthermore, in contrast to our study, Lavers *et al.* [24] estimated the WHP_{app} of the study herds using initially a pooled FC consisting of five samples followed by individual culture of samples from positive pools. This less elaborate approach lowers the sensitivity of the FC method [13, 38] because a sample from a single cow containing a low amount of MAP would not be detected by the pool FC due to the dilution effect. Consequently, WHP_{app} could be underestimated, as the individual FC was done only from MAP-positive pools. To a certain degree, our results confirm the limitations of detecting low-prevalence MAP-positive herds using only environmental samples, that have been postulated by other studies regardless of the detection methods used [21, 22, 25]. The WHP_{app} thresholds resulting from our model refer to WHP_{app} calculated from individual FC results. Although FC is currently the most sensitive method to diagnose paratuberculosis in living animals, its sensitivity is estimated to be about 60–70% relative to necropsy, with a specificity $\geq 99\%$ [9, 39]. If we had used the true prevalence in our model, the estimated threshold value would be even higher. We waived this option because necropsy is not a realistic option. Nonetheless, for practical use in control programmes, the 90% P_d WHP_{app} threshold is an orientation value of what can be expected from the use of a single environmental sampling. In voluntary control programmes it is important to identify herds with a high WHP_{app} because these herds account for the highest risk of MAP shedding into the environment and the food chain. These farms should be identified and given advice as a priority. Applying two test methods on a set of three samples resulted in a WHP_{app} threshold of 6.2% and, therefore, meets the requirement necessary to identify these herds.

In our dataset double sampling did not reduce threshold value compared to single sampling by FC or the combination of FC and qPCR. This confirms the results of Lavers *et al.* [24] who stated only minimal improvement of sensitivity with a second sampling. This may disprove the assumption that repeated sampling, which is frequently used in control programmes, will reduce the WHP_{app} threshold that allows the detection of a herd as MAP positive. By contrast, when only qPCR was applied, double sampling lowered the estimated threshold calculated for single sampling. This may be due to the perceptible influence of the positive qPCR results in herd 2. That would, at least in part, explain the worse performance of qPCR in the logistic regression model, particularly in the single sampling approach. Because our model is based on only 20 herds, it presumably reacts rather sensitively to misclassification causing a bias in threshold estimation. Although repeated sampling did not reduce the WHP_{app} threshold it enhances P_d at certain WHP_{app} levels. For example, using the calculated WHP_{app} threshold of 2.9% at $P_d = 0.5$ for a set of three samples tested by both FC and qPCR and assuming five statistical independent observations, the resulting P_d would be 0.969, which is an acceptable value even for a monitoring programme that aims to detect herds with a $WHP_{app} > 3\%$ with a probability of > 0.95 . Obviously, limitations remain regarding the use of environmental samples for monitoring herds not suspected of paratuberculosis in certification programmes.

In our study the high number of unanalysable FCs from samples collected during the warm season (Table 2) might have limited the improvement which would be expected from repeated sampling. As in most cases fungal contamination hampered the identification of MAP cultures and we suspect that the higher air temperature and humidity during the summer facilitated the growth of mould in the faeces which accumulated in the barn. Therefore, the decontamination procedure was not able to inactivate the mould spores completely, and consequently, more culture tubes were affected by fungal overgrowth.

Taken together, our results favour a combination of three environmental samples taken once per herd and tested simultaneously by FC and qPCR to be an option that is less laborious but similar in sensitivity compared to a set of five environmental samples. Repeated sampling as well as the inclusion of more samples did not improve the probability of identifying a herd as MAP-positive. The set of three

environmental samples showed a similar probability of detecting a MAP-positive herd compared to a set of five samples.

If this easy-to-use approach were to be applied as a first diagnostic step in a control programme, it would allow the detection of those herds that need to implement control measures as soon as possible because they are likely to have a relevant number of MAP shedders. This may advance the implementation of control measures and the entry into a paratuberculosis control programme to reduce the spread of the disease within the herd. On the other hand, our results show that environmental sampling is not an adequate diagnostic approach to detect MAP-positive dairy herds with a WHP_{app} of less than $\sim 3\%$. Individual testing is still needed for the detection of herds with a very low WHP_{app} as well as for control measures within a herd (e.g. specific hygienic measures, separation and culling of shedders). Further research is required for the use of repeated environmental sampling to monitor certified herds in order to detect a re-introduction of the infectious agent.

ACKNOWLEDGEMENTS

The present study was a part of the ‘Paratuberculosis control programme in Thuringian dairy herds’ of the Thuringian Ministry of Social Welfare and Health, which is financed by the Thuringian Animal Disease Compensation Fund. The work was supported by ‘Landesvereinigung Thüringer Milch e.V.’. The authors thank Ute Schau and her team for performing the faecal culture and Felix Häslér, Life Technologies GmbH, for the technical support concerning qPCR.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Tiwari A, et al.** Johne’s disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *Canadian Veterinary Journal* 2006; **47**: 874–882.
2. **Whitlock RH, Hutchinson LT, Merkal RS.** Prevalence and economic consideration of Johne’s disease in the northeastern U.S. *Proceedings of the United States Animal Health Association* 1985; **89**: 484–490.
3. **Raizman EA, et al.** The association between culling due to clinical Johne’s disease or the detection of *Mycobacterium avium* subsp. *paratuberculosis* faecal shedding and the diagnosis of clinical or subclinical diseases in two dairy herds in Minnesota, USA. *Preventive Veterinary Medicine* 2007; **80**: 166–178.
4. **Smith RL, et al.** Effect of Johne’s disease status on reproduction and culling in dairy cattle. *Journal of Dairy Science* 2010; **93**: 3513–3524.
5. **Raizman EA, et al.** The association between *Mycobacterium avium* subsp. *paratuberculosis* faecal shedding or clinical Johne’s disease and lactation performance on two Minnesota, USA dairy farms. *Preventive Veterinary Medicine* 2007; **78**: 179–195.
6. **Beauveau F, et al.** Reduction in milk yield associated with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in dairy cows. *Veterinary Research* 2008; **38**: 625–634.
7. **Raizman EA, Fetrow JP, Wells SJ.** Loss of income from cows shedding *Mycobacterium avium* subspecies *paratuberculosis* prior to calving compared with cows not shedding the organism on two Minnesota dairy farms. *Journal of Dairy Science* 2009; **92**: 4929–4936.
8. **Smith RL, et al.** A longitudinal study on the impact of Johne’s disease status on milk production in individual cows. *Journal of Dairy Science* 2009; **92**: 2653–2661.
9. **Sweeney RW, et al.** Paratuberculosis (Johne’s disease) in cattle and other susceptible species. *Journal of Veterinary Internal Medicine* 2012; **26**: 1239–1250.
10. **Over K, et al.** Current perspectives on *Mycobacterium avium* subsp. *paratuberculosis*, Johne’s disease, and Crohn’s disease: a review. *Critical Reviews in Microbiology* 2011; **37**: 141–156.
11. **Antognoli MC, et al.** Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with ante-mortem test results. *Veterinary Microbiology* 2008; **127**: 300–308.
12. **Ayele WY, Machackova M, Pavlik I.** The transmission and impact of paratuberculosis infection in domestic and wild ruminants. *Veterinary Medicine Czech* 2001; **46**: 205–224.
13. **Vialard J, et al.** Detection of bovine paratuberculosis by culture of pooled faeces [in French]. *Revue de Médecine Vétérinaire* 1993; **144**: 527–533.
14. **Donat K, et al.** Herd prevalence studies of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in cattle using serological tests: opportunities, limitations and costs [in German]. *Berliner und Münchner Tierärztliche Wochenschrift* 2012; **125**: 10–19.
15. **Raizman EA, et al.** The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. *Journal of Dairy Science* 2004; **87**: 2959–2966.
16. **Berghaus RD, et al.** Use of age and milk production data to improve the ability of enzyme-linked immunosorbent assay test results to predict *Mycobacterium avium* ssp. *paratuberculosis* faecal culture status. *Journal of Dairy Science* 2006; **89**: 963–970.
17. **Lombard JE, et al.** Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd

- infection status on US dairy operations. *Journal of Dairy Science* 2006; **89**: 4163–4171.
18. **Lovel R, Levi M, Francis J.** Studies on the survival of Johne's bacilli. *Journal of Comparative Pathology* 1944; **54**: 120–129.
 19. **Jørgensen JB.** Survival of *Mycobacterium paratuberculosis* in slurry. *Nordisk veterinærmedicin* 1977, **29**: 267–270.
 20. **Gumber S, Whittington RJ.** Analysis of the growth pattern, survival and proteome of *Mycobacterium avium* subsp. *paratuberculosis* following exposure to heat. *Veterinary Microbiology* 2009; **136**: 82–90.
 21. **Pillars RB, et al.** Prevalence of Michigan dairy herds infected with *Mycobacterium avium* subspecies *paratuberculosis* as determined by environmental sampling. *Canadian Veterinary Journal* 2009; **50**: 1039–1046.
 22. **Donat K, Schau U, Soschinka A.** Identification of *Mycobacterium avium* ssp. *paratuberculosis* infected dairy herds by environmental sampling [in German]. *Berliner und Münchner Tierärztliche Wochenschrift* 2011; **124**: 10–17.
 23. **Lombard JE, et al.** Herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in United States dairy herds in 2007. *Preventive Veterinary Medicine* 2013; **108**: 234–238.
 24. **Lavers CJ, et al.** Evaluation of environmental faecal culture for *Mycobacterium avium* subspecies *paratuberculosis* detection in dairy herds and association with apparent within-herd prevalence. *Canadian Veterinary Journal* 2013; **54**: 1053–1060.
 25. **Smith RL, et al.** Environmental contamination with *Mycobacterium avium* subsp. *paratuberculosis* in endemically infected dairy herds. *Preventive Veterinary Medicine* 2011; **102**: 1–9.
 26. **McKenna SL, et al.** Johne's disease in Canada part II: disease impact, risk factors, and control programs for dairy producers. *Canadian Veterinary Journal* 2006; **47**: 1089–1099.
 27. **Möbius P, et al.** Comparison of 13 single-round and nested PCR assays targeting IS900, ISMav2, f57 and locus 255 for detection of *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology* 2008; **126**: 324–33.
 28. **Pozzato N, et al.** Evaluation of a rapid and inexpensive liquid culture system for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine faeces. *Journal of Microbiological Methods* 2011; **84**: 413–417.
 29. **Englund S, et al.** Single PCR and nested PCR with a mimic molecule for detection of *Mycobacterium avium* subsp. *paratuberculosis*. *Diagnostic Microbiology and Infectious Disease* 1999; **33**: 163–171.
 30. **Logar K, et al.** Evaluation of combined high-efficiency DNA extraction and real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in sub-clinically infected dairy cattle: comparison with faecal culture, milk real-time PCR and milk ELISA. *BMC Veterinary Research* 2012; **8**: 49.
 31. **Rommel A.** New diagnostic tool for detection of Johne's disease [in German]. *Tierärztliche Umschau* 2012; **67**: 257–259.
 32. **Aly SS, et al.** Correlation between Herrold egg yolk medium culture and real-time quantitative polymerase chain reaction results for *Mycobacterium avium* subspecies *paratuberculosis* in pooled faecal and environmental samples. *Journal of Veterinary Diagnostic Investigation* 2010; **22**: 677–683.
 33. **Friedrich-Loeffler-Institut (FLI) Federal Research Institute for Animal Health.** Amtliche Methodensammlung Stand: April 2012 (http://www.fli.bund.de/fileadmin/dam_uploads/Publikationen/Amtliche_Methodensammlung/Methodensammlung_201204.pdf). Assessed 15 August 2013.
 34. **Dixon WJ (ed.).** BMDP Statistical Software Manual, *Volumes I and II*. Berkeley, Los Angeles, London: University of California Press, 1993, pp. 775–794 and 1105–1144.
 35. **Whittington RJ, Sergeant ES.** Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. *Australian Veterinary Journal* 2001; **79**: 267–278.
 36. **Dressel J.** Direct detection of *Mycobacterium avium* spp. *paratuberculosis* in bovine faeces – a comparative study (diploma thesis). Zwickau, Germany: Hochschule Fresenius University of Applied Sciences, 2012, 71 pp.
 37. **United States Department of Agriculture (USDA).** Animal and Plant Health Inspection Service, Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program, effective 1 September 2010 (http://www.aphis.usda.gov/animal_health/animal_diseases/johnes/downloads/johnes-ups.pdf). Assessed 27 February 2014.
 38. **Raizman EA, et al.** Estimated within-herd prevalence (WHP) of *Mycobacterium avium* subsp. *paratuberculosis* in a sample of Minnesota dairy herds using bacterial culture of pooled faecal samples. *Canadian Journal of Veterinary Research* 2011; **75**: 112–116.
 39. **Köhler H, et al.** Evaluation of five ELISA test kits for the measurement of antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in bovine serum. *Berliner und Münchner Tierärztliche Wochenschrift* 2008; **121**: 203–210.