Epidemiol. Infect. (2013), **141**, 1536–1544. © Cambridge University Press 2012
doi:10.1017/S0950268812002452

Comparison of prevalence estimation of *Mycobacterium avium* subsp. *paratuberculosis* infection by sampling slaughtered cattle with macroscopic lesions *vs*. systematic sampling

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Received 17 October 2011; Final revision 21 September 2012; Accepted 10 October 2012; first published online 13 November 2012

SUMMARY

The objective of this study was to identify the most reliable approach for prevalence estimation of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) infection in clinically healthy slaughtered cattle. Sampling of macroscopically suspect tissue was compared to systematic sampling. Specimens of ileum, jejunum, mesenteric and caecal lymph nodes were examined for MAP infection using bacterial microscopy, culture, histopathology and immunohistochemistry. MAP was found most frequently in caecal lymph nodes, but sampling more tissues optimized the detection rate. Examination by culture was most efficient while combination with histopathology increased the detection rate slightly. MAP was detected in 49/50 animals with macroscopic lesions representing 1·35% of the slaughtered cattle examined. Of 150 systematically sampled macroscopically non-suspect cows, 28·7% were infected with MAP. This indicates that the majority of MAP-positive cattle are slaughtered without evidence of macroscopic lesions and before clinical signs occur. For reliable prevalence estimation of MAP infection in slaughtered cattle, systematic random sampling is essential.

Key words: Cattle, *Mycobacterium avium* ssp. *paratuberculosis*, prevalence estimation, sampling, slaughterhouse.

INTRODUCTION

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis, a chronic granulomatous enteritis affecting mainly ruminants. Although paratuberculosis is present worldwide, regional prevalences at the individual animal level are not well known. This is due to the difficulty of accurately detecting infected animals by ante-mortem test methods. Prevalence estimations in Germany range from 4·38% to 33·0% [1–4].

The different test methods used hamper comparison and interpretation of the results of prevalence studies. Serological tests are unsuitable especially for detection of animals in the latent stages of the disease [5]. Use of serological tests alone allows the identification of just a small proportion of infected animals [6]. Faecal culture is the most reliable method for ante-mortem diagnosis with an overall sensitivity ranging from 38·0% to 85·6%. The specificity is considered 100% when culture results are confirmed by polymerase chain reaction (PCR) and/or subcultivation [7–9]. However, as for serology, the sensitivity of faecal culture depends on the stage of disease

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in the animals tested. Infected animals shed MAP intermittently, particularly in the early stages of disease. [10]. Furthermore, MAP-infected cattle excrete MAP organisms to a different extent [8]. False-negative results can be caused by shedding below the detection limit and by heterogeneous distribution of the bacteria in faecal samples. Cultural and histological examination of tissue samples are considered the methods with the highest sensitivity and specificity for detection of infected animals [11, 12]. These methods can be useful in confirming the true prevalence of MAP-infected cattle, because of the higher probability of detecting animals in the early stages of disease [10]. The distal part of the jejunum, ileum, ileocaecal valve and associated lymph nodes have been recommended as sampling sites [13]. Gross pathological findings associated with MAP infection are segmental thickening of the intestinal wall and thick rugose mucosa. Thickening of mesenteric lymphatic vessels, granular appearance of the intestinal mucosa and enlargement of mesenteric lymph nodes have also been reported [14, 15].

The pathological alterations of the intestinal wall cause malabsorption, followed by diarrhoea, hypoproteinaemia and weight loss [10]. This results in reduced slaughter value, decreased milk yield, poor fertility and eventually a loss of diseased animals with detrimental economic consequences worldwide [16, 17].

Furthermore, as long as the association of MAP with Crohn's disease in humans is not finally excluded [18, 19], loss of consumer confidence in ruminant products has to be expected. Therefore, control of the disease is desirable. The true prevalence of paratuberculosis needs to be known in order to support decisions about future national control strategies in Germany. Because of the limitations of methods applicable to live animals, testing of slaughtered cattle is assumed to be more suitable for prevalence estimation.

The objective of this study was to identify the most reliable approach for prevalence estimation of MAP infection at the individual animal level in slaughtered cattle. Testing of macroscopically suspect tissue and systematic sampling were compared, thus determining whether macroscopic pre-selection of specimens is necessary. Bacteriological, histological and immunohistochemical detection methods were used and evaluated for their suitability. The prevalence of MAP infection in cattle with and without macroscopic intestinal lesions was determined in the study population.

METHODS

Study design and sampling

Clinically healthy cattle were sampled in two German slaughterhouses (slaughterhouses A and B) at the evisceration table. The survey was subdivided into two substudies.

In substudy 1, intestines of 3630 female cattle were examined visually and by palpation by one assessor. Only cows were inspected in slaughterhouse A while in slaughterhouse B the study population consisted of 73·1% cows, 21·1% heifers and 5·8% calves.

The following gross pathological findings on the closed and opened intestine were used as selection criteria:

- thickening of the intestinal wall,
- thick rugose mucosa,
- granular appearance of the intestinal mucosa,
- thickened mesenteric lymphatic vessels.

Fifty cattle were sampled (33 in slaughterhouse A, 17 in slaughterhouse B) that fulfilled at least one of the criteria listed above without consideration of age or breed.

In substudy 2, systematic sampling was done on 150 cattle without macroscopic intestinal lesions according to a pre-defined sampling plan (slaughterhouse A: 100 cattle, sampling of every tenth animal; slaughterhouse B: 50 cattle, sampling of every fifth animal). The percentage distribution of the 150 animals sampled in slaughterhouses A and B equates to the percentage distribution of the sampled animals in slaughterhouses A and B in substudy 1. Slaughterhouse staff were the only individuals with control over the order of animals. Sampling started at random. The number of cattle sampled from any one herd was limited to four per day to prevent an accumulation of animals from single herds. Because of the results of substudy 1, sampling in substudy 2 was limited to female cattle aged >2 years. If a tenth (slaughterhouse A) or a fifth (slaughterhouse B) animal had macroscopically suspect lesions of paratuberculosis, the next female animal without such lesions aged $\geqslant 24$ months was selected and the sampling frequency was then resumed.

Samples consisted of ileum, jejunum, mesenteric and caecal lymph nodes. They were collected with sterile scissors and forceps. Tissue for microbiological examination was placed in individual sterile containers and transported fresh. Samples for

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Table 1. *Breed of cattle sampled in substudies 1 and 2*

Breed	Substudy 1 (*n*)	Substudy 2 (*n*)
Holstein-Friesian	43	119
Jersey	1	—
Crossbred dairy cattle	1	5
Charolais	1	1
Limousin	—	1
Hereford	—	1
Highland	—	1
Fleckvieh cattle	—	4
Crossbred beef cattle	2	10
Cross beef × dairy cattle	—	4
Other breeds	2	4
Total	50	150

histopathological and immunohistochemical examinations were fixed in 3·5% neutral buffered formalin.

Samples

In substudy 1, heifers and cows were sampled, with 90% belonging to dairy breeds (Table 1). The average age was 1680 days (4·6 years), with the youngest aged 794 days (2·2 years). The cows originated from 38 farms with the maximum number of four cows from one herd. Forty-eight cows were from Germany, one from The Netherlands and one from Denmark.

In substudy 2, samples were limited to female cattle, with 82·6% being dairy cows (Table 1). The minimum age was set at 2 years because of the results of substudy 1. The oldest animal was aged 4793 days (13·1 years) and the average age was 1842 days (5 years). The cows came from 113 farms in Germany. The maximum number of cows from one herd was six and the maximum number of cows from one herd sampled on the same day was two.

Bacteriological examination

To examine the tissue samples by bacterial microscopy, smears were prepared on glass slides and stained with acridine orange (Riedel-de-Haën, Germany). The stained smears were scanned for clumps of acid-fast bacteria (AFB) by fluorescence microscopy under oil immersion. The number of AFB was estimated in about 100 fields and recorded semi-quantitatively.

For cultural examination, the intestinal mucosa was rinsed with sterile phosphate-buffered saline (PBS) to eliminate intestinal contents. Mesenteric fat and connective tissue were removed from lymph nodes. Samples of 1 g were prepared using sterile scissors and forceps, and further disrupted using a stomacher, decontaminated with 0·9% hexadecylpyridinium chloride (HPC, Merck, Germany) for 24 h as described previously [6], inoculated on four slants of Herrold's egg yolk medium with mycobactin J (HEYM, Becton Dickinson, USA) and incubated at 37 °C for 16 weeks. The slants were examined every 2 weeks for colony growth. The number of colonies was recorded semi-quantitatively. Colonies were stained by the Ziehl–Neelsen technique and checked microscopically for the presence of AFB. IS*900* PCR [20] and subcultivation for testing mycobactin dependence were performed to confirm the presence of MAP. Growth of *M. avium* ssp. *avium* and *M. avium* ssp. *hominissuis* was excluded by PCR targeting IS*901* and IS*1245* [21, 22].

Histopathological examination

Slices of formalin-fixed tissues were embedded in paraffin. Paraffin sections were stained with haematoxylin and eosin (H&E). Lesions associated with paratuberculosis were characterized by an infiltration with epithelioid cells, multinucleated giant cells and lymphocytes. Focal lesions with 1–5 distinct granulomatous infiltrates, multifocal lesions with >5 such infiltrates, and diffuse lesions with epithelioid cells and/or multinucleated giant cells throughout the section were distinguished [11].

Immunohistochemical examination

Immunohistochemistry was performed using the indirect immunoperoxidase method on paraffin sections. Endogenous peroxidase activity was blocked with methanol and 0·5% hydrogen peroxide. After trypsin treatment, slides were incubated with heat-inactivated sheep serum. A polyclonal antiserum against MAP (dilution 1:6000, DakoCytomation, Denmark) was used as primary antiserum. Slides were then incubated with peroxidase-labelled goat anti-rabbit IgG antiserum (Dianova, Germany). Peroxidase activity was detected by incubation in a solution of 0·05% 3,3′–diaminobenzidine tetrahydrochloride and 0·03% hydrogen peroxide in PBS (pH 7·4) and intensified with 0·001% osmium

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Table 2. *Results of substudy 1: prevalence of MAP-infected slaughtered cattle with macroscopic lesions of granulomatous enteritis*

	Total number of cattle examined	Macroscopically suspicious cattle		Verification of MAP infection	
		n	Prevalence (95% CI)	*n*	Prevalence (95% CI)
Slaughterhouse A	2821	33	1·17 (0·81–1·64)	32	1·13 (0·78–1·60)
Slaughterhouse B	809	17	2·10 (1·23–3·34)	17	2·10 (1·23–3·34)
Total	3630	50	1·38 (1·02–1·81)	49	1·35 (1·00–1·78)

MAP, *Mycobacterium avium* ssp. *paratuberculosis*; CI, Confidence interval.

Table 3. *Results of substudy 2: prevalence of MAP-infected slaughtered cows without macroscopic intestinal lesions*

	Total number of cattle examined	Verification of MAP infection	
		n	Prevalence (95% CI)
Slaughterhouse A	100	24	24·0 (16·0–33·6)
Slaughterhouse B	50	19	38·0 (24·7–52·8)
Total	150	43	28·7 (21·6–36·6)

MAP, *Mycobacterium avium* ssp. *paratuberculosis*; CI, Confidence interval.

tetroxide. Slides were counterstained in a 2% aqueous solution of methylene green. Control sections of MAP–positive and MAP–negative tissue were prepared in the same manner. Slides were examined for MAP under oil immersion and assessed semi-quantitatively.

Statistical analysis

Animals were classified as MAP infected when cultural examination was positive and/or when histological lesions of paratuberculosis were evident in one of the tissues examined. All data analyses were performed using SPSS v. 15.0 (SPSS GmbH, Germany). In substudy 1, prevalence was calculated as the quotient between animals demonstrated to be MAP infected and the total number of animals whose intestines were examined visually and by palpation. In substudy 2, prevalence in cattle without macroscopic findings was calculated as the quotient between animals shown to be MAP infected and the number of selected cattle ($n=150$). Prevalence estimations were characterized by their 95% confidence intervals (CI). Statistical significance of the differences in the prevalence between substudies 1 and 2 was estimated using Fisher's exact test. Detection rates of combinations of tissues sampled and diagnostic methods were analysed using data from substudy 2.

RESULTS

Prevalence

In substudy 1, 49/50 animals with macroscopic lesions of granulomatous enteritis were shown to be infected with MAP by laboratory methods. One cow had macroscopic suspect lesions, but MAP infection was not confirmed. Histological examination of the jejunum and the mesenteric lymph node revealed a moderate purulent to necrotizing enteritis and lymphadenitis in this animal. Based on the data of this substudy, it can be estimated that a minimum of 91% (lower limit of 95% CI) of the animals would be MAP positive when selected by sampling macroscopically suspect tissue as described above. The proportion of slaughtered cattle with macroscopic lesions and confirmed as MAP infected was 1·35% of all animals examined (Table 2).

In substudy 2, 43 (28·7%) of 150 systematically selected cows with no macroscopic lesions were demonstrated to be MAP positive (Table 3). From these data it can be estimated that a maximum of 34% (upper limit of 95% CI) of the animals would be MAP positive when systematic sampling is done

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Table 4*a*. *Results of the diagnostic methods used for the detection of MAP infection in tissue samples* (*number of positive animals*)

		Number of positive animals			
Substudy	*n**	Bacterial microscopy	Isolation	Histology	Immuno-histochemistry†
1	50	46	49	49	47
2	150	2 (1‡)	42	17	0

MAP, *Mycobacterium avium* ssp. *paratuberculosis*.
* Number of animals examined.
† Immunohistochemical labelling of *Mycobacterium* spp.
‡ MAP infection could not be confirmed with other methods.

on macroscopically non-suspect animals. The proportions of MAP-positive cows differed by a ratio of nearly 1:2 between slaughterhouses A and B (Tables 2, 3).

The prevalence of animals with confirmed MAP infection differed significantly between substudies 1 and 2 ($P<0{\cdot}00001$). The difference in estimated prevalence between the two substudies was much greater than the difference in prevalence between the two slaughterhouses.

Detection rate of the diagnostic methods used

Substudy 1: sampling macroscopically suspect tissue

For tissue with macroscopic lesions the detection rates of all applied methods were high, culture and histopathology being most sensitive (Table 4*a*). By bacterial microscopy, AFB were present in 174 tissue samples representing 46 animals. In terms of the abundance of AFB, 35·0% of all 200 samples showed numerous AFB. After culture on HEYM, colony growth occurred in 193 tissue samples from 49 animals. Almost half (46·0%) of the 200 samples developed a dense layer of bacterial growth. By histopathology, 190 samples of intestine and lymph nodes representing 49 animals had lesions of granulomatous inflammation characterized by a focal to diffuse infiltration of epithelioid cells, multinucleated giant cells and lymphocytes. Fifty-one percent of the sampled organs showed a diffuse, 34·5% a multifocal and 9·5% a focal distribution of lesions. In some animals the alterations involved all layers of the intestinal wall. The entire paracortex and, less frequently, lymphoid follicles were infiltrated in the lymph nodes.

Using immunohistochemistry, 178 samples originating from 47 animals were positive for *Mycobacterium* spp. Overall, numerous *Mycobacterium* spp. could be found in 54·0% of the slides (data not shown).

Substudy 2: systematic sampling

Culture was the most efficient method for detecting MAP infection in macroscopically non-suspect tissue (Table 4*a*). By bacterial microscopy, AFB were found in smears of two animals, each with one positive organ. MAP infection could be confirmed by other detection methods only for one of the two animals. This animal showed scattered AFB in the mesenteric lymph node sample. Cultural isolation of MAP was positive in 80 samples representing 42 animals. Most (77·5%) of the positive slants showed only sparse colony growth. From the 150 animals examined in substudy 2, 22 samples originating from 17 animals had histopathological lesions of granulomatous inflammation. Fifteen (68·2%) of these 22 samples had a focal and seven (31·8%) had a multifocal distribution of lesions. *Mycobacterium* spp. were not found in any of the samples by immunohistochemical labelling (Table 4*a*, *b*).

Since bacterial microscopy and immunohistochemistry did not reveal any additional positive sample in comparison to culture, only the detection rates of culture alone and of culture plus histopathology were analysed for combinations of the different tissue samples. By culture, MAP was found most frequently in caecal lymph nodes. Most positive animals were identified when all four tissues were examined. Reduction to three specimens was very sensitive using the caecal lymph node in combination with two other tissues. Testing only two samples resulted in detection rates <70% except for a combination of mesenteric and caecal lymph nodes. Performing culture and histopathology in parallel

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Table 4*b*. *Results of the diagnostic methods used for the detection of MAP infection in tissue samples* (*number of positive tissue samples*)

Substudy	Tissue	*n**	Number of positive samples			
			Bacterial microscopy	Isolation	Histology	Immuno-histochemistry†
1	Ileum	50	45	48	48	46
	Jejunum	50	42	48	47	43
	Mesenteric Ln	50	44	49	48	46
	Caecal Ln	50	43	48	47	43
2	Ileum	150	0	19	5	0
	Jejunum	150	0	16	3	0
	Mesenteric Ln	150	1	21	10	0
	Caecal Ln	150	1‡	24	4	0

MAP, *Mycobacterium avium* ssp. *paratuberculosis*; Ln, lymph node.
* Number of tissues examined.
† Immunohistochemical labelling of *Mycobacterium* spp.
‡ MAP infection could not be confirmed with other methods.

led to an increase in the proportion of animals characterized as positive (Table 5).

DISCUSSION

Paratuberculosis is a globally occurring disease with a high economic impact [16]. Control of the disease is desirable. Knowledge about the prevalence of MAP infection at the herd level, and most importantly, at the individual animal level is essential for decisions about the most appropriate control measures and for verification of their success. None of the diagnostic methods applied to live animals gives reliable information about the prevalence at the individual animal level [23].

Only a few cow-level prevalence estimates have been performed on slaughtered cattle applying different sampling schemes, diagnostic methods and sampled tissues [23–26]. Slaughtered cattle are generally a pre-selected group containing higher numbers of animals that are older or have low performance or high veterinary costs. For this reason the possibility of finding infected cattle seems to be generally higher by sampling slaughtered animals [27].

Prior knowledge about the efficiency of the testing strategy is necessary for proper interpretation of the results. Therefore, it was the objective of this study to compare different sampling strategies and diagnostic methods for prevalence estimation on slaughtered cattle and to give recommendations for the most reliable sampling scheme.

Two completely different approaches were compared, sampling of slaughtered cattle based on evidence of macroscopic lesions (substudy 1) and systematic sampling according to a predefined sampling scheme (substudy 2). In substudy 1, characteristic macroscopic lesions were seen in 1·38% (95% CI 1·02–1·81) of all examined cattle, and 98% of the cattle with lesions were shown to be infected with MAP. The criteria used in substudy 1 for selection on the basis of macroscopic evidence were very stringent and specific, because in contrast to our results, a Canadian study identified physical attributes of MAP infection (intestinal thickening, loose faeces, enlarged mesenteric lymph nodes) in 134/984 cows, but only 20·1% could be confirmed by culture [23]. Furthermore, the efficiency of diagnosis by gross pathology is highly dependent on the experience of the assessor. A study performed in Australia revealed that under normal meat processing conditions, the sensitivity of abattoir inspection for ovine paratuberculosis relative to histology varied from 53% to 87% between inspectors; specificity varied from 97% to 100% [28]. Data from the USA point to the fact that prevalence of MAP infection in cattle might be underestimated by sampling only macroscopically suspect animals, because 37% of culled dairy cows with disseminated culturally confirmed MAP infection had no or minimal gross pathological evidence of infection [29]. The results of substudy 2 support this view. By systematic sampling of animals without macroscopic intestinal lesions, a prevalence of 28·7%

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Table 5. *Detection rate (%) by culture alone or a combination of culture and histopathology when individual tissues and combinations of tissues were examined (substudy 2)*

Tissues sampled	Culture	Culture and histopathology
Caecal Ln	55·8	55·8
Ileum	44·2	46·5
Jejunum	37·2	39·5
Mesenteric Ln	48·8	58·1
Caecal Ln, ileum	67·4	69·8
Caecal Ln, jejunum	74·4	74·4
Caecal Ln, mesenteric Ln	72·1	83·7
Jejunum, ileum	62·8	62·8
Jejunum, mesenteric Ln	65·1	72·1
Mesenteric Ln, ileum	67·4	72·1
Caecal Ln, ileum, jejunum	83·7	83·7
Caecal Ln, ileum, mesenteric Ln	86·0	90·7
Caecal Ln, jejunum, mesenteric Ln	86·0	93·0
Jejunum, mesenteric Ln, ileum	79·1	83·7
Caecal Ln, ileum, jejunum, mesenteric Ln	97·7	100·0*

MAP, *Mycobacterium avium* ssp. *paratuberculosis*; Ln, lymph node.
Data represent the proportion of evidently MAP-infected animals that had a positive result in any of the tissues sampled when examined by culture with or without histopathology.
* This value is necessarily 100 %

(95 % CI 21·6–36·6) of MAP-infected slaughtered cattle was determined. This proportion is comparable to other recently published data, but much higher than the results of earlier studies. In a Canadian study from 2004, systematic random sampling of 984 macroscopically suspect and non-suspect cattle yielded an overall prevalence of 16·1 % (95 % CI 13·8–18·3) culturally positive animals. Seasonal variation was discovered and the monthly proportion of MAP-positive cows varied from 2·4 % to 42·5 % [23]. More than 15 years earlier, only 5·5 % of 400 culled cows sampled in Canada between 1986 and 1989 were MAP positive by culture [25]. These data most likely reflect the spread of MAP infection in the cattle population over time.

In both substudies, a marked difference in the prevalence of MAP-infected cattle between the two slaughterhouses was found. It could not be attributed to differences in the breeds and production systems, because dairy cows constituted the majority of the slaughtered cows in both facilities (slaughterhouse A: 85–87·5 %; slaughterhouse B: 78–100 %). Generally, it is assumed that the prevalence of paratuberculosis is much lower in beef than in dairy cattle. Risk-based sampling of thin market cows in the USA ($n = 539$) resulted in MAP isolation from 34 % of sampled dairy cows and 3 % of sampled beef cows [27]. Regarding our data, we suspect a higher prevalence of MAP infection in the farms delivering animals to slaughterhouse B.

When the different diagnostic methods were compared, bacterial microscopy and immunohistochemistry were much less sensitive than bacterial culture in detecting positive tissue; thus confirming the results of previous studies [12]. Both cultural and histological examination revealed early stages of paratuberculosis [10]. Agreement in the detection rates of cultural isolation and histopathology was different in the two substudies. We assumed that this is due to the types of lesions predominating in the two sets of samples. More than half (51·0 %) of the samples in substudy 1 were characterized by severe diffuse granulomatous lesions and often a heavy bacterial load. This represents more advanced stages of the disease [11]. With these samples cultural and histological examination showed similar detection rates.

In substudy 2, which included mainly animals in early stages of the disease, cultural examination was most sensitive. Although the combination of culture and histopathology resulted in a further increase of

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the detection rate, it is questionable whether the additional effort justifies the benefit. Conflicting data regarding the usefulness of culture or histopathology can be found in the literature. In sheep, histopathology was considered a better indicator of paratuberculosis infection than bacteriology [30]. On the other hand, 32% of intestinal tissues of sheep without histological evidence of Johne's disease from infected flocks were culture positive [31], indicating a higher sensitivity of culture. Similarly, for detecting infected cattle, histological testing was far less sensitive than bacteriological methods [23]. Taken together, cultivation is the method recommended for prevalence estimation using tissue samples from randomly selected slaughter cattle.

The tissues collected in epidemiological slaughterhouse studies were mesenteric lymph nodes alone or in combination with ileum [23, 25, 26]. Either mesenteric lymph nodes [23] or ileum [32] yielded the highest proportion of positive culture results. In our study, caecal lymph nodes were most frequently culture positive. However, only combined testing of more than two tissues including caecal lymph nodes resulted in high detection rates.

In conclusion, estimation of the prevalence of MAP infection at the individual animal level is possible by sampling slaughtered cattle. The results of the two substudies stress the fact that it is necessary to examine both macroscopically suspect and non-suspect animals. The MAP-positive cattle found in this survey were in the subclinical stages of the disease. Animals in substudy 2, in contrast to substudy 1, had predominantly focal lesions and only a low bacterial load attributable to very early stages of the disease [33]. This indicates that the majority of MAP–infected cattle are slaughtered even before macroscopic lesions become detectable. Therefore, reliable prevalence estimation only seems possible when both macroscopically suspect and non-suspect animals are sampled by a systematic random sampling strategy. Sampling of animals with macroscopic intestinal lesions only will result in an underestimation of the real prevalence of MAP infection.

ACKNOWLEDGEMENTS

The authors thank Uta Brommer, Monica Godat, Sabine Lied and Danny Michel for their excellent technical assistance. The support of the state veterinarians and staff of two German slaughterhouses is kindly acknowledged.

DECLARATION OF INTEREST

None.

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