Individual faecal and boot swab sampling to determine John's disease status in small cattle herds

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Summary
Individual faecal samples were collected from adult animals in 275 cattle farms previously positive for *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In addition, boot swab samples were collected in 30 randomly chosen farms. Faecal samples were tested for MAP by a combination of bacterial culture and PCR. A logistic regression and the Pearson Correlation were used to calculate the relation between the number of MAP-positive cows and boot swab results. In 66.9% of all previously tested herds, no positive individual faecal sample was detected, indicating possible fadeout of the infection. In 9 (30.0%) of the 30 selected farms, at least one MAP-shedding animal was detected in faecal samples individually collected, while only 5 (16.7%) of these farms were found positive when the boot sampling method was used. The sensitivity of the boot swab sampling increased up to 92% (95% CI: 41%-99%), if at least 12 animals were faecal MAP-shedders in a herd. The current study shows possible fadeout of JD in a substantial percentage of previously infected herds. Furthermore, in small herds, a relatively high within-herd prevalence of MAP-shedding animals is needed to assure reliable positive boot swab results.

Introduction

Johne's disease (JD), which is also called paratuberculosis, is caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP; Sweeney 1996). JD is a chronic infection in cattle, usually transmitted via the faecal-oral route within the first months of life. A period of at least two years of latency usually follows the early exposure (Sweeney 2011). In this early stage of the infection, neither faecal shedding of MAP nor specific antibodies (Ab) occurs, thereby hampering the detection of infected individuals (Sweeney 1996). As the infection progresses, the faecal shedding of MAP and the production of specific Ab commences, but clinical signs of JD are still absent at this time (Sweeney 2011). Even though the onset of characteristic clinical signs of JD, such as watery diarrhoea and weight loss despite normal appetite, is quite variable, they usually do not become evident in cattle younger than 2 years of age (Sweeney 1996). The disease is incurable (Fecteau and Whitlock 2011). Vaccines, which are available in some countries, contribute to reduce production losses.

MAP infections in cattle are emerging in most parts of the world. In Europe, up to 68% of cattle herds were found MAP positive, based on the available data (Nielsen and Toft 2009). In Austria, the herd prevalence was 19.1% (Baumgartner *et al.* 2005).

The ELISA is the most commonly indirect detection method used for JD today (Gilardoni *et al.* 2012). Considering its low sensitivity, this method is not appropriate for either detecting the early stages of

MAP infection in individual animals (Diéguez *et al.* 2009) or defining a MAP herd status on cattle farms (Donat *et al.* 2012).

Bacterial culture and identification is considered the "gold standard" in MAP diagnostics. It requires from 8 to 20 weeks and can be applied to faecal and tissue samples (Gilardoni *et al.* 2012, Whittington 2010).

Because of the long incubation time of bacterial culture, PCR is increasingly used for MAP-detection in faecal samples with comparable results concerning sensitivity and specificity. The PCR sensitivity is even improved if applied on faecal samples following enrichment culture (Fawzy *et al.* 2015). Sample pooling with pool size of 10 samples collected either from the animals or from the areas of the stable with high animal density may reduce the costs of MAP-diagnosis. The sensitivity of pooled samples were reported to range from 48% to 69% (van Shaik *et al.* 2007, Tavorpanich *et al.* 2004). A more recent study stated that pool sizes of five or ten faecal samples may reduce costs with an acceptable reduction of the sensitivity (McKenna *et al.* 2018). Environmental sampling was able to correctly identify 70% of MAP positive herds, if six samples were collected from different places throughout the farm (Wolf *et al.* 2017). In 2013, boot swab sampling was suggested for the first time as a technique to establish the MAP-herd status in cattle, with 90.6% of MAP-infected herds being detected (Eisenberg *et al.* 2013). Disposable cover boots, equipped with an absorbent material on the sole and worn while walking around the animals inside the barn and the milking parlour are just what this sampling method required (Eisenberg *et al.* 2013). After walking through the herd, the absorbent material soaked with manure is removed and used for MAP detection by PCR or culture (Eisenberg *et al.* 2013). In a recent study, the sensitivity of boot swabs for MAP-detection was calculated to be only 43.5% (Wolf *et al.* 2016). Another paper showed that the probability for the detection of MAP-positive farms by boot swabs ranged between 50% and 90%, depending on the intra-herd-prevalence of MAP (Donat *et al.* 2016).

The aim of the present study was to evaluate the development of the MAP-herd status determined by boot swab samples in small structured cattle herds. Furthermore, the association of the boot swab results with the number of animals shedding MAP with faeces within a herd should be investigated. Therefore, individual faecal samples were collected in previously MAP-boot swab positive cattle farms. In some of these farms, boot swabs were taken for a second time simultaneously in order to reassess the MAP-herd status.

Materials and methods

Background

Between 2013 and 2014, the Veterinary Animal Health Service Tyrol and the Regional Veterinary Office conducted an investigation to evaluate the MAP herd status of cattle farms in the Austrian province of Tyrol. The purpose of this investigation was to establish a program for the control and eradication of JD in cattle. In the course of this voluntary survey, boot swab samples were collected as previously described (Donat *et al.* 2016) on 4,679 farms and tested for the presence of MAP by bacterial culture and PCR. MAP was detected in 349 (7.5%) boot swab samples (Köchler *et al.* 2017).

Study population

Of the MAP-positive farms detected in the previous survey (2013-2014), 275 farms, voluntarily joined the individual animal testing-program launched by the Veterinary Health Service in autumn 2015, were enrolled in the present study. Resampling, as part of the present study, was performed approximately 18 months (min. 13, max. 26 months) after the first boot swab collection. On these farms, individual faecal samples were taken from all animals with a minimum age of 2 years. Furthermore, another boot swab sample was collected at the same time from 30 randomly selected farms. These farms were chosen by simple randomisation from the 275 positive farms, but selection of farms had to be modified due to willingness of the owners to participate in the study. This resulted in a total of 3,758 individual faecal samples from 275 farms. An overview of the study population and its origin is given in Figure 1.

The size of the farms enrolled in the study ranged from one to 82 tested individuals with a minimum age of 2 years. The 275 farms are referred to as "all farms" and had a mean of 14 individuals tested per farm (median 11). The 30 randomly selected herds, called "selected farms", held a mean of 24 (median 19.5) animals with a minimum age of 2 years. Animals tested throughout the study were kept in tight stalls or loose housing systems and pastured throughout the summer. Details for the 30 selected farms are given in Table II.

Collection and testing of boot swab samples

Boot swab samples, for determining the MAP-herd status, were collected as previously described (Donat *et al.* 2016). Samples for the 2013-2014 study were collected by the local veterinarians, except for the re-evaluation of the 30 randomly chosen farms,

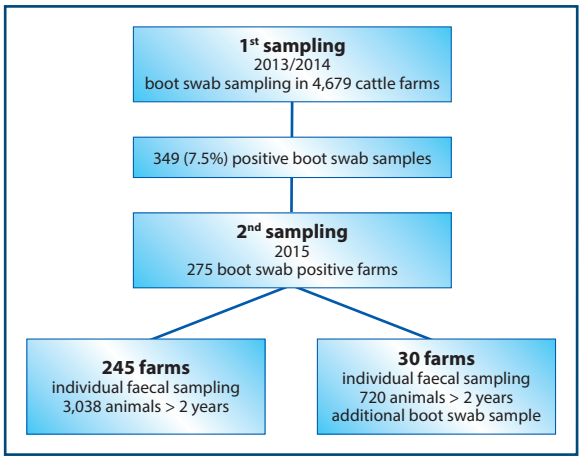

Figure 1. *Study population and sampling scheme.*

wherein the boot swab samples were collected by the authors of this paper (Gschaider and Köchler). To avoid contamination, sample takers had to put on single use plastic overshoes first. Then, the actual boot swabs (Sodibox, Névez, France) made out of knitted jersey, were pulled over the bottom of the overshoes. In tie-stalls with cows tethered in rows next to each other, sample takers were instructed to walk through the manure channel while, in free stall barns, samples were collected by walking along the alleyways, including the spaces around feeding or watering devices and the waiting area in front of the milking parlour (Eisenberg *et al.* 2013). To collect a sufficient amount of sampling material, the boot swabs had to be soaked with at least 50 g of manure. After collection, the boot swabs were put into sterile plastic twirl type bags, stored cooled and sent to the Institute of Microbiology at the University for Veterinary Medicine in Vienna for MAP-detection.

For the detection of MAP, the boot swab samples were transferred to Stomacher® bags (Seward Ltd., Worthing, UK) and then homogenized in a LB 400 circulator (VWR International LLC, Vienna, Austria) for 60 seconds after adding 50 ml of phosphate buffered saline (PBS). Following centrifugation at 3,000 x g for 15 minutes, the supernatant was discharged and 3 g of the remaining manure were resuspended with 30 ml of 0.75% hexadecylpyridinium chloride (HPC, Sigma Aldrich Handels GmbH, Vienna, Austria). Thereafter, samples were shaken for 60 minutes and left for 5 minutes for the sedimentation of the large particles afterwards. Following sedimentation, 15 ml of the supernatant were filled into a sterile tube and incubated in the dark for 48 hours at room temperature. Subsequently, the samples were centrifuged again at 3,000 x g for 15 minutes, the supernatant was discharged and the pellets remixed with 1 ml of 0.75% HPC. Four tubes of Herrolds egg yolk medium (HEYM), prepared in house and containing 2 mg of Mycobactin J (IDEXX GmbH, Ludwigsburg, Germany) per litre, were inoculated with each sample. All HEYM media used for the study were prepared as single batch and underwent quality control including control for sterility and prove of MAP growth by inoculation of 10 media with 100 cfu/ml of MAP at 37 °C. The HEYM tubes were then incubated at 37 °C and checked for growth once a week. After 4 weeks of incubation, one of the 4 tubes per sample was rinsed with 200 µl PBS and the fluid was used for the subsequent MAP detection by real-time PCR. The QIAmp DNA Stool Mini Kit (QIAGEN N.V., Venlo, Netherlands) was used for the extraction of the DNA following the user's manual. Subsequently, the MAP specific sequence element was amplified using the VetMAXTM MAP Real-Time PCR Screening Kit (Fisher Scientific - Austria GmbH; Vienna, Austria), again following the manufacturer's instructions using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories GmbH, Vienna, Austria) for the amplification.

The 3 remaining tubes were incubated at 37 °C for another 8 weeks and checked for growth of MAP weekly. If growth of MAP occurred colonies were sampled by PCR as described above for confirmation and the culture rated as MAP-positive. Culture tubes not showing any MAP colonies were rated as negative after 12 weeks of incubation. More samples were positive in the combination of culture and PCR than in the culture alone, which is in accordance with the study of Köchler and colleagues (Köchler *et al.* 2017). Therefore, results of the described combination of culture and PCR are presented here and were used for statistical evaluation only.

Collection and testing of individual faecal samples

Individual faecal samples were taken directly from the rectum, using a new single use plastic glove for each animal. The faeces were put into a sterile plastic container, stored cooled, and sent to the Institute of Microbiology at the University for Veterinary Medicine in Vienna. For the detection of MAP, bacterial culture on HEYM and real-time PCR were performed following the procedure previously used for boot swab samples.

Statistical analysis

Statistical analysis of the data was performed using the IBM SPSS Statistics v19 software (IBM Österreich Internationale Büromaschinen GesmbH, Vienna, Austria). A logistic regression to calculate the relation of the number and percentage of MAP-positive cows to a negative or positive boot swab result in the 30 randomly selected farms was performed. The boot swab result (positive/negative) was used as dependent and the amount of animals

shedding MAP with faeces as independent variable (probability of entry 0.5, removal of 0.10, iterate 20 and cut 0.5). Because of the small sample size, leading to a possible bias when referring to the quantity and the percentage of samples, the probability related to the total number of positive individuals was calculated, combining the quantity of MAP-positive animals with the predicted probability for a positive boot swab result from the logistic regression.

Additionally, the Pearson Correlation was calculated to define the relation between the number of MAP-shedders and the herd size.

Results

Detection of MAP in individual faecal samples

No MAP-positive individual faecal sample was detected in 184 (66.9%) of the previously boot swab positive farms, together holding 3,417 animals with a minimum age of 2 years (Table I).

Altogether, 248 animals (6.6%) originating from 83 (30.2%) different farms were tested positive for MAP in the individual faecal samples (Table I). Relating to MAP-positive cattle premises only, 17.3% (248 out of 1,430) of cows or heifers tested were shedding MAP with their faces. The mean intra-herd-prevalence of cattle shedding the bacterium with their faces in farms with at least one positive animal turned out to be 21.2% (min. 1.2%, max. 75.0%), with a mean samples size of 17 (median 14) tested animals in farms with at least one positive result. The intra-herd-prevalence of MAP-shedding animals in relation to the herd size is shown in Figure 2. Four cattle premises turned out to have 50% or more animals shedding MAP with faeces, but the size of those 4 farms was small with a maximum of 12 animals sampled (Figure 2). The highest intra-herd-prevalence of MAP-shedders found was 75%, but this farm was holding 4 animals above 2 years of age only. Altogether, MAP was detected in individual faecal samples of less than 20% of the individuals tested in more than half of the MAP-positive cattle premises. The Pearson correlation showed a weak negative correlation between the herd size and amount of MAP-shedding individuals with a correlation coefficient of - 0.36.

Relation of MAP-shedding individuals and boot swab result

In 9 (30.0%) of the 30 selected farms, in which individual faecal testing as well as boot swab sampling was performed simultaneously, at least one MAP-shedding individual was found (Table I). In total, 36 (5.0%) animals turned out to be MAP-shedders, and boot swab samples were

Table I. *Detection of* Mycobacterium avium *subsp.* paratuberculosis *(MAP) in boot swab samples and individual faecal samples, absolute numbers with the percentage in brackets.*

				Positive	Negative	Missing	Total
Farm	**Breed**	**Housing system**	**Animals tested[1]**	**MAP positive[2]**	**MAP Boot swab**		
16	RF[3]	loose housing	17	2 (11.8%)	negative		
17	AS[4]	tight stall	2	1 (50.0%)	negative		
18	BS[5]	tight stall	4	0 (0.0%)	negative		
24	TG[6]	loose housing	15	0 (0.0%)	negative		
53	AS	tight stall	14	3 (21.4%)	positive		
55	AS	loose housing	20	4 (20.0%)	positive		
56	AS	tight stall	18	6 (33.3%)	positive		
57	BS	tight stall	17	3 (17.6%)	negative		
58	BS	loose housing	34	12 (35.3%)	positive		
88	FV	loose housing	29	4 (13.8%)	positive		
89	FV	tight stall	19	0 (0.0%)	negative		
109	AS	tight stall	14	0 (0.0%)	negative		
All farms		Farms		83 (30.2)	184 (66.9)	8 (2.9)	275 (100)
		Individuals		248 (6.6)	3,417 (90.9)	93 (2.5)	3,758 (100)
Selected farms		Farms		9 (30.0)	21 (70.0)	0 (0)	30 (100)
		Individuals		36 (5.0)	684 (95.0)	0 (0)	720 (100)
		Boot swab samples		5 (16.7)	25 (83.3)	0(0)	30 (100)

[1]Number of cattle with a minimum age of 2 years, included in the study; [2]Number of animals with *Mycobacterium avium* subsp. *paratuberculosis*-positive faecal samples with percentage in brackets; [3]Red Frisian; [4]Austrian Simmental; [5]Brown Swiss; [6]Tyrolean Grey Cattle.

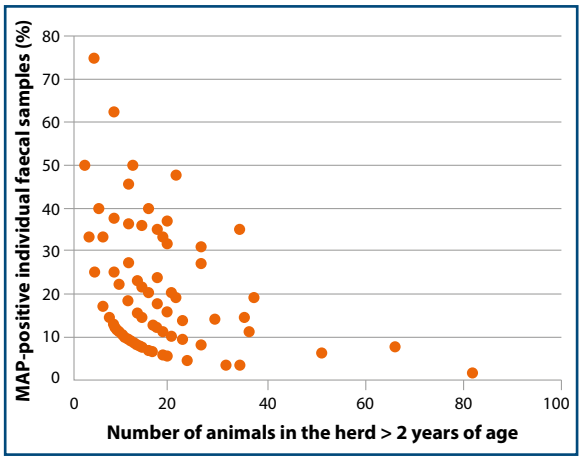

Figure 2. *Relation between the within-herd prevalence of MAP-shedding individuals and the herd size.*

positive in 5 (16.7%) of these farms (Table II). There was no farm without a MAP shedding animal giving a positive boot swab result, but 4 premises, with at least one individual animal shedding the bacterium, showed a MAP-negative boot swab sample. The detailed results of the selected farms are shown in Table II.

The calculation of the logistic regression revealed that the probability of obtaining a MAP-positive result in the boot swab sample depends on the within-herd prevalence of animals shedding MAP with their faeces (Figure 3a). As sample size, which was small in this study, can produce a bias referring to the quantity and the percentage of positive samples, the probability related to the total number of positive individuals is shown in Figure 3b. When the number of positive animals was combined with the predicted probability for a boot swab to be positive, (Figure 3a), the sensitivity of the MAP-detection methods was 48% if there are 6 MAP shedding animals in a herd and 92% if the MAP shedding animals are 12 (Figure 3b). As seen in Figure 3b, the probability of a positive boot swab sample drops to 28% (95% CI: 4%-79%) if there are 4 MAP-shedding animals in a herd.

Table II. *Results of* Mycobacterium avium *subsp.* paratuberculosis *(MAP) detection in individual and boot swab samples in selected farms.*

Farm	Breed	Housing system	Animals tested[5]	MAP positive[6]	MAP Boot swab[7]
16	RF[1]	loose housing	17	2 (11.8%)	negative
17	AS[2]	tight stall	2	1 (50.0%)	negative
18	BS[3]	tight stall	4	0 (0.0%)	negative
24	TG[4]	loose housing	15	0 (0.0%)	negative
53	AS	tight stall	14	3 (21.4%)	positive
55	AS	loose housing	20	4 (20.0%)	positive
56	AS	tight stall	18	6 (33.3%)	positive
57	BS	tight stall	17	3 (17.6%)	negative
58	BS	loose housing	34	12 (35.3%)	positive
88	FV	loose housing	29	4 (13.8%)	positive
89	FV	tight stall	19	0 (0.0%)	negative
109	AS	tight stall	14	0 (0.0%)	negative
111	AS	loose housing	29	0 (0.0%)	negative
112	AS	tight stall	30	0 (0.0%)	negative
114	AS	loose housing	31	0 (0.0%)	negative
115	AS	loose housing	82	1 (1.2%)	negative
123	AS	tight stall	27	0 (0.0%)	negative
126	AS	tight stall	23	0 (0.0%)	negative
130	AS	tight stall	32	0 (0.0%)	negative
131	AS	tight stall	37	0 (0.0%)	negative
137	BS	loose housing	17	0 (0.0%)	negative
138	BS	loose housing	30	0 (0.0%)	negative
163	BS	tight stall	15	0 (0.0%)	negative
165	TG	tight stall	7	0 (0.0%)	negative
166	TG	tight stall	4	0 (0.0%)	negative
167	AS	tight stall	3	0 (0.0%)	negative
168	TG	tight stall	3	0 (0.0%)	negative
170	BS	loose housing	22	0 (0.0%)	negative
207	AS	loose housing	44	0 (0.0%)	negative
239	RFxAS	loose housing	81	0 (0.0%)	negative

[1]Red Friesian; [2]Austrian Simmental; [3]Brown Swiss; [4]Tyrolese Gray Cattle; [5]Number of cattle with a minimum age of 2 years, included in the study; [6]Number of animals with *Mycobacterium avium* subsp. *paratuberculosis*-positive faecal samples with percentage in brackets; [7]Result of boot swab testing for *Mycobacterium avium* subsp. *paratuberculosis*.

Discussion

All of the farms included in the present study had shown a positive boot swab result for MAP before (2013 or 2014). About 18 months later, MAP could not be detected in individual faecal samples of any of the adult cattle in two third of these farms.

Although it has to be considered that MAP is not shed continuously in the faeces of animals with subclinical JD (Mitchell *et al.* 2015), this result may point to a possible change in the MAP-status of these farms. Marcé and colleagues (Marcé *et al.* 2011) observed spontaneous fadeout of JD in 43% of cattle herds within 2 years after MAP introduction. Altogether, the infection was not detected in 66% of herds within several years in this model. In this study, it was furthermore shown, that more farms remained infected, if animals with clinical JD stayed in the herd for a prolonged time and MAP removal from the environment was reduced (Marcé *et al.* 2011).

Most of the Tyrolean cattle herds spend at least two months on alpine pastures during the summer. Cows in weak condition might be slaughtered before

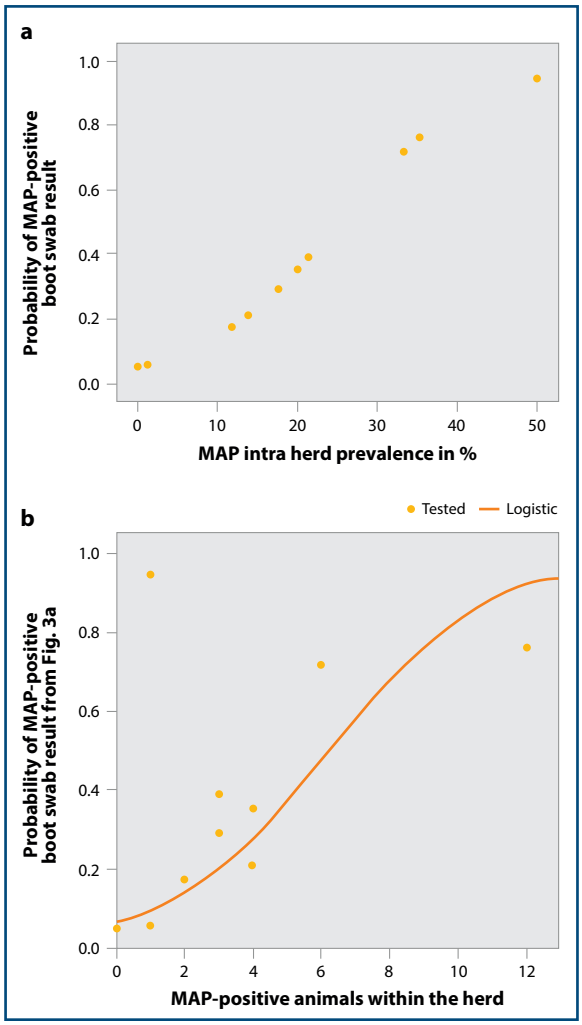

Figure 3. *Logistic regressions for* Mycobacterium avium *subsp.* paratuberculosis *(MAP) positive boot swab results depending on the within-herd prevalence of MAP-shedding individuals.* **a.** Probability of obtaining a MAP-positive result in the boot swab sample depending on the within-herd prevalence. **b.** Probability of obtaining a MAP-positive boot swab sample as calculated in Figure 3a, related to the total number of MAP-positive animals (test results and logistic regression).

pasturing, and MAP-positive animals likely removed from the herd. During this time, barns are also usually thoroughly cleaned with a high pressure cleaner and left empty until the animals return in autumn. This management practice may lead to a significant reduction of MAP in the stable and could, therefore, contribute to possible fadeout of the infection, as evidenced in the present study. However, MAP-DNA was detected in the environmental samples after the complete destocking of a herd with a known history of clinical paratuberculosis as well as 24 months after cleaning and disinfection (Moravkova *et al.* 2012). Clinical JD is a notifiable disease in Austria and affected cattle have to be culled within 3 days after confirmation of a MAP-infection (Khol *et al.* 2007). This timely removal of clinically ill animals contributes to the reduction of MAP contamination of the environment and, thereby, to the fadeout of the infection. Further studies, including a follow up of the farms enrolled in the present study, are needed to elucidate possible spontaneous fadeout of MAP in cattle herds.

A negative correlation exists between the herd size and the number of MAP-shedding individuals. The mean within herd prevalence of MAP-shedding animals in the present study turned out to be 21.2%. Farms with a maximum of 15 animals showed a mean within herd prevalence of MAP-shedding of 24.6%, whereas it was 16.8% in herds with 16 to 82 animals only. This finding is in contradiction to the literature, where an increasing herd size has been reported to correlate with a higher MAP within-herd prevalence in infected cattle farms (Hirst *et al.* 2004, Muskens *et al.* 2003). One possible explanation for this could be the close relationships of the individuals in small structured cattle herds. The farmers often keep the female offspring of a cow over several years for breeding, if it is believed to be of high genetic value. Therefore, if this cow is MAP positive, there is an increased chance that its offspring is infected with MAP as well, as MAP infection can occur in utero, via colostrum or via manure-contaminated teats (Sweeney 1996).

The size of the cattle herds in the part of Austria where the study was performed is quite small compared to dairy cow premises worldwide. The mean herd size in our study was 14 sampled animals with a minimum age of 2 years per herd only. In the German federal states Thuringia, Hesse, and Saxony, boot swab sampling was performed in 77 cattle herds with a known JD status and an average sample size of 272 animals per herd (Donat *et al.* 2016). In that study, it was shown that the MAP within-herd prevalence had to be at least 2.39% to obtain a positive boot swab result with a probability of 50% (Donat *et al.* 2016). For the probability of positive boot swab results to be raised to 90%, the within-herd prevalence of animals shedding MAP with their faeces had to be at least 5.85%, when the samples were tested by faecal culture and PCR simultaneously (Donat *et al.* 2016). Our study on the Tyrolean cattle premises showed that the within-herd prevalence of animals shedding MAP had to exceed 25% to obtain a probability of at least 50% for a positive boot swab result. This is a markedly higher percentage than found in the aforementioned study (Donat *et al.* 2016), but interpretation of the results of the present study is hampered by the small sample size, questioning the relevance of the results. Importantly, only 25 of the 83 positive farms held more than 25% of the animals shedding MAP with their faeces in our study population. In relation to the total number of MAP positive individuals in a herd, statistical calculations in our study revealed

that the boot swab sample will be MAP positive with a probability near 90% when there are at least 12 MAP-shedding animals in a herd, irrespective of the herd size. If there are at least 6 MAP-shedding individuals, the calculated probability of a positive boot swab result drops to 50%. Because of the small number of positive farms detected in the present study 95% confidence intervals are rather wide, which has to be considered when interpreting the results. Nevertheless, the calculated within-herd prevalence of cattle shedding MAP in their faeces must be 10 times higher in our study than in the aforementioned one (Donat *et al.* 2016) in order to achieve a positive boot swab sample with a probability of 50%. The reason for this marked difference remains unknown, but the small size of the herds included in our study may contribute to this finding. The housing conditions could possibly also influence the results of the boot swab samples. MAP-shedding cows in tethered stalls may lead to a smaller contamination of the environment than animals in a free stall which are able to move around. Because of the relatively low number of boot swab positive farms in the present study, a comparison of the two housing systems was not performed and warrants further investigations. Furthermore, it has been shown, that bedding material, such a straw as well as low outside temperatures are able to hamper the detection of MAP in environmental samples (Wolf *et al.* 2017).

Overall, due to the small sample size and corresponding to the fact that only 16.7% of the farms turned out to be MAP positive by boot swab sampling, the conclusions of the present study must be interpreted with caution.

A prior study reported a rather low sensitivity of boot swap samples to detect MAP-positive cattle herds of 43.5% only (Wolf *et al.* 2016), thus questioning their application for assessment of the MAP-herd status. Possible methodological deficiencies of the sampling procedure applied in our study, related to either the procedure itself or the sampling person should be considered. Comparison of the results of the present study to the literature also is hampered by the different detection methods applied. A test protocol combining solid culture and PCR was used in our study, while MAP was detected by 3 different PCR protocols by Donat and colleagues (Donat *et al.* 2016) and by liquid culture in the investigation of Wolf and colleagues (Wolf *et al.* 2016). It has been shown, that the combination of bacterial culture for the enrichment of MAP, followed by PCR has a high sensitivity for the detection of MAP (Fawzy *et al.* 2015). Then again previous studies showed that the method of DNA extraction is crucial for the success of MAP detection in faecal samples and differs between different protocols (Fernando *et al.* 2013, Sting *et al.* 2014). Unfortunately, no data concerning the overall test performances for the combination of protocols for DNA extraction and PCR used in the present study are available. Although this should be considered when interpreting the results, it can be assumed to be of minor importance, as in this and in the 2013-2014 surveys, samples were tested in the same laboratory using the same protocols.

The results of the present study indicate that examination of individual faecal samples is more sensitive than boot swabs for detecting MAP positive herds, as 4 out of 9 farms showed positive individual faecal results and were boot swab negative (Table II). Individual faecal sampling is not an option to establish the MAP-herd status in large herds because of financial and management issues, although it has been shown that may reduce costs with an acceptable reduction of sensitivity (McKenna *et al.* 2018). Nevertheless, in small premises, holding a few adult cattle only, individual faecal samplings might give better results compared to boot swab sampling because of a possible low sensitivity of the latter, with a reasonable increase of costs and workload.

Boot swabs are a suitable sampling method for defining the MAP-herd status in cattle, as they are quick and easy to perform as well as cost effective (Eisenberg *et al.* 2013, Wolf *et al.* 2016). The sensitivity of boot swab samples for MAP assessement depends on the within-herd prevalence (Donat *et al.* 2016) and the total amount of animals shedding MAP with their faeces. Nevertheless, results from the present study indicate that boot swab sampling have to be used with caution in smaller herds, as a relatively high within-herd prevalence of MAP-shedding animals is needed to assure reliable results. To avoid false negative results, repeated sampling should be applied. Due to the long incubation period and chronic nature of JD in cattle with a late onset of faecal MAP-shedding, as well as intermittent shedding of the bacteria, repeated faecal sampling increases the probability to detect infected individuals (Gilardoni *et al.* 2012). Consequently, repeated sampling also increases the chance to detect MAP-positive herds, by both environmental and boot swab sampling (Eisenberg *et al.* 2013, Khol *et al.* 2009). Furthermore, combining boot swab with the slurry tank sampling for MAP as suggested before (Donat *et al.* 2016) might increase the sensitivity. According to the results of this study, on very small premises, holding a few adult cattle only, individual faecal samplings should be considered instead of boot swab sampling. Further studies, including larger sample sizes and long-term investigations, are needed to elucidate the use of boot swab samples for MAP-detection in small cattle herds and possible spontaneous fadeout of the disease.

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