Grass silage contaminated with *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*): a possible source of paratuberculosis infection in ruminants?

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ABSTRACT: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis (Johne's disease) in ruminants. Paratuberculosis can cause severe economic losses and is acknowledged as one of the most important diseases of ruminants today. High amounts of *MAP* can be shed in the faeces of infected individuals and can survive for a long period in the environment. In the presented trial, baled grass silage was inoculated with a *MAP*-suspension, and the viability of *MAP* was studied over time. Samples from the bales were taken at increasing intervals and subsequently tested for the presence of *MAP* by solid culture on Herrold's Egg Yolk Media (HEYM), liquid culture and real time Polymerase Chain Reaction (PCR) for the IS*900* and F*57* fragments. No growth of *MAP* was observed at any time on solid or in liquid cultures, except at the time of inoculation; PCR detections were positive in the majority of the bales. From the results of the presented study, baled grass silage can be classed as a minor risk for the transmission of *MAP*.

Keywords: cattle; disease control; Johne's disease; feed contamination; *Mycobacterium avium* subsp. *paratuberculosis*; paratuberculosis; risk of infection

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is the causative agent of paratuberculosis (Johne's disease) in ruminants. *MAP* infects many species of domestic and wild ruminants (Hirsh and Biberstein, 2004; Kopecna et al., 2008), in which its distribution is worldwide (Ayele et al., 2001). *MAP* can be shed in high amounts in the faeces of infected individuals and is also found in colostrum and milk. Faecal-oral transmission is the most important route of infection; young animals are most susceptible, but adult ruminants can also become infected (Whitlock, 1996). Due to its long incubation period, *MAP* spreads slowly, and years can pass before a herd is recognized as infected. Young infected cattle do not show clinical signs, but can still shed *MAP* into the farm environment (Bolton et al., 2005). The first clinical signs can usually be seen at the ages of three to six years as chronic or intermittent diarrhoea with periods of normal faecal consistency. Cattle with advanced clinical disease become weak and emaciated. Pipestream diarrhoea and cachexia characterize the terminal stage of the disease for which the prognosis is poor. Paratuberculosis is untreatable and ends with the death of the diseased animal (Hirsh and Biberstein, 2004).

Due to severe economic losses in affected herds (Hasonova and Pavlik, 2006), paratuberculosis is acknowledged as one of the most important disease of ruminants in developed countries today (Ayele et al., 2001). The prevalence of the disease varies and reaches up to an 84.7% *MAP* positivity in dairy herds in some parts of Germany (Hacker et al., 2004). Van Leeuwen et al. (2001) reported 43.0% positive dairy herds in Canada and 68.1% of the US dairy herds were found to be positive for *MAP*

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in 2007 (Anonymous, 2008). In Austria, 19.0% of cattle herds were found to be serologically positive from 2002 to 2003 (Baumgartner et al., 2005).

Bacterial culture from faecal or tissue samples serves as the gold standard for the detection of *MAP*. The specificity of faecal and tissue samples is high, but the sensitivity is low due to the late onset of faecal shedding, intermittent shedding and the late occurrence of *MAP* in regional lymph nodes. Polymerase Chain Reaction (PCR) can be used to confirm the results of bacteriological culture as well as for the detection of *MAP* in faeces, tissue samples, blood and milk. The advantages of PCR are a high specificity (90%) and the possibility of obtaining results in less than 24 hours. The handicap of this technique is that there is no way of distinguishing between viable and non-viable *MAP* cells (Nielsen et al., 2001).

MAP is known to have high tenacity and can survive for a long period in the environment. A survival time of 55 weeks in the environment and up to 120 weeks in lakes has been reported by Rowe and Grant (2006). Another study showed that this bacterium can survive for 48 weeks in shaded water sediment and 12 weeks in the ground (Whittington et al., 2005), and if *MAP* was mixed with manure and stored in a shaded place it could even be detected by culture after a period of 55 weeks (Whittington et al., 2004). High temperature, ultraviolet radiation, as well as low pH and humidity are known to reduce the survival time of *MAP* in the environment (Sung and Collins, 2003; Whittington, et al., 2004; Grewal et al., 2006).

Besides its lipid rich cell wall (Rowe and Grant, 2006), two additional survival tactics of *MAP*, uptake by microorganisms and dormancy, have recently been described. The interaction of *MAP* with, and uptake by ubiquitous environmental protozoa and microbes has been reported (Whittington et al., 2005; Whan et al., 2006). This uptake leads to a higher resistance against chlorine and enables the bacterium to survive for a long time, and even reproduce inside microorganisms (Rowe and Grant, 2006; Whan et al., 2006).

The entering of *MAP* into an inactive phase, which is called dormancy, was identified recently as another possible survival tactic of *MAP* in the environment (Whittington et al., 2004, 2005; Rowe and Grant, 2006). Genes, which are similar to those that are responsible for dormancy in *M. smegmatis* have been found in *MAP*. The reasons for entering dormancy as well as for the return into the vegetative lifeform have not yet been identified (Whittington et al., 2004).

The survival of *MAP* on pastures, in manure, water and water sediments has been extensively studied but only one study concerning the resistance of the bacterium after ensiling has been reported. In a laboratory experiment, Katayama et al. (2001) showed that the survival of *MAP* was related to silage quality. *MAP* could be detected in poorly preserved silage and in well preserved silage with low pH and a high concentration of lactic acid.

The aim of the present study was to determine the viability of *MAP* in baled grass silage under field conditions over time.

MATERIAL AND METHODS

Silage production, storage and disposal

For the trial, 40 bales of grass silage were produced in the summer of 2006 at a farm of the University for Veterinary, Vienna, Austria. After harvesting, the grass was pressed into bales of approximately 40 kg each, sealed with a plastic wrap and stored in the open air. No additives to improve the silage process were added to the bales. Apart from bale size, which is usually larger, this represents the normal way of silage production in Austria and many other countries. Three months after production, the bales were transported to an unheated room at the University of Veterinary Medicine, Vienna, where they were stored. *MAP* is listed as a "pathogen that could cause disease in humans" according to Austrian law and, therefore, specific safety measures have to be taken. The storage room had to be locked at all times with a sign reading "biohazard" attached to the door. Due to the fact that the bales were entirely covered with a plastic wrap contamination of the environment with *MAP* after inoculation was considered as very unlikely. In any case, an additional plastic wrap was placed underneath the bales, and tuberculocide disinfectant along with an emergency protocol was provided at the entrance of the room. All of the bales were disposed of as medical hazardous waste during and after the trial in order to provide maximal security for humans and the environment.

A digital thermometer with a measuring range of –10 to +60°C and a resolution of 0.1°C was used for temperature recording in the centre of one silage bale in the storage room and throughout the entire

trial. One probe of the thermometer was placed in the middle of the bale and the other one between the bales. The actual temperature as well as the minimum and maximum of the last 24 hours were recorded every day.

Inoculation of bales

A total of 35 bales were inoculated with 300 ml of a *MAP*-suspension (bovine reference strain CAPM 6381 from Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Brno, Czech Republic) in a concentration of 10^8 Colony Forming Units (CFU)/ml using a trocar and tube. Inoculation was performed under sterile conditions. Protective clothing, gloves, breathing masks and safety glasses had to be worn by all the people involved. The trocar was placed with the tip in the centre of the bale, a sterile tube was placed in the trocar and the *MAP*-suspension was injected. After injection, the trocar with the tube was removed and the bales were sealed with adhesive tape. Each bale was rolled for several minutes after inoculation to achieve a better distribution of the suspension. Before the inoculation of the next bale, the trocar was cleaned with alcohol and a new sterile tube was used for the next bale. The bales were stored with the inoculation site placed up at all times. Five bales remained uninoculated and served as negative controls.

Sampling scheme

One bale was opened for sampling immediately after inoculation. The next two bales were opened four days later, followed by the sampling of three bales at a time in increasing intervals until Day 107. From Day 30 of the trial, one control bale was opened at each sampling date (Table 1). Because of negative results in bacterial culture, the trial was stopped after 107 days and the remaining bales were disposed of as described.

Culture for *MAP* and feed analysis were performed from each opened bale (no feed analysis was performed from bale nos. one and two). In addition, a real-time PCR was run on samples from all the inoculated bales starting at Day 15 (Table 1).

Samples were taken from the surface, centre and middle parts of each bale and put together for culture. PCR was performed from the sample taken in the centre only. For feed analysis, a representative sample from each bale (excluding the centre) of approximately 2 kg were sent to the Laboratory for Feed Analysis, Rosenau (Austria).

Sample decontamination for bacterial culture and real-time qPCR

Silage samples were collected in sterile plastic bags and decontaminated for further analysis at the day of sampling, following a modified protocol from Pavlik et al. (2000). For decontamination, approximately 2 g of silage were taken from the bags, transferred to a jar containing 60 ml of distilled water and agitated in a horizontal shaker for 30 min at room temperature. After shaking large particles and debris were allowed to settle for an additional 30 min. After sedimentation, 1 ml from the surface was transferred to a micro tube (1.5 ml, Sarstedt, Nümbrecht, Germany) and frozen at –70°C for real-time qPCR analysis. Five ml from the same jar were transferred into a fresh glass containing 25 ml 0.9% HPC (Hexadecyl Pyridinium Chloride: N-cetylpyridinium chloride monohydrate, No. 102340 Merck, USA) and shaken for 30 min. After decontamination for three days the samples were inoculated onto solid and into liquid culture media (see below).

Inoculation for bacterial culture and colony reading

After decontamination, 200 µl of the sediment were placed on each of the three vials of Herrold's Egg Yolk Media (HEYM). The same amount was inoculated into three Mycobacteria Growth Indicator Tubes (MGIT, Becton Dickinson, USA) containing an MGIT growth supplement (Becton Dickinson, USA) and PANTA antibiotic suspension (Becton Dickinson, USA) and cultured at 37°C. Solid and liquid media cultures were observed at two weeks after inoculation to rule out fast growing mycobacteria and early contamination. Further readings were performed monthly up to the 16^{th} week. If any signs of possible *MAP* growth were present, smears were prepared, stained according to Ziehl-Neelsen and observed by microscopy. In liquid media, visible growth was measured by a fluorescence detection signal and the presence of purple colour, which is caused by a neotetrazolium chloride dye that is added to the media.

Table 1. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in baled grass silage

Day	Bale number	HEYM[1]	MGIT[2]	IS*900*[3]	F*57*[3]
0	PC0[4]	+ (6, 18)	+	4.2×10^7	3.8×10^7
4	1	–	–	Nt	Nt
	2	–	–	Nt	Nt
15	3	–	–	3.4×10^3	0
	4	–	–	0	0
	5	–	–	0	0
30	6	–	–	1.0×10^7	1.3×10^7
	7	–	–	2.2×10^4	4.8×10^4
	8	–	–	2.8×10^5	2.3×10^5
	C1[5]	–	–	0	0
44	9	–	–	0	0
	10	–	–	2.1×10^7	2.9×10^7
	11	–	–	2.1×10^7	5.8×10^7
	C2[5]	–	–	0	0
75	12	–	–	1.6×10^4	1.5×10^4
	13	–	–	0	0
	14	–	–	3.7×10^5	5.5×10^5
	C3[5]	–	–	0	0
107	15	–	–	2.3×10^7	1.1×10^7
	16	–	–	0	0
	17	–	–	8.7×10^5	1.3×10^6
	C4[5]	–	–	0	0

[1]examined by solid culture on Herrold's Egg Yolk Media (HEYM)
[2]examined by liquid culture in Mycobacteria Growth Indicator Tubes (MGIT™)
[3]examined by quantitative real-time PCR. The values in the columns represent the rounded amount of the target molecules per doublets of 1 g of the silage samples: IS*900* quantitative real-time PCR for the IS*900* target gene, F*57* quantitative real-time PCR for the F*57* target gene
[4]PC = positive control (*MAP*-contaminated) bale of silage at time 0
[5]C = negative controls (not *MAP*-contaminated).
+ = positive results (number of CFU on two HEYM); – = negative results; Nt = not tested

Detection of *MAP* by real-time qPCR

MAP cells eluted from the silage to water were defrosted and centrifuged at 18 000 G for 15 min. The supernatant was discarded and 350 mg of zirconia-silica beads (Biospec, Ohio, USA) and 300 µl of TE buffer (Serva, Heidelberg, Germany) were added to the pelleted cells. The *MAP* cells were mechanically disrupted by centrifugation of the pellet at 6400 rpm for 60 s (MagNA Lyser, Roche, Grenzach, Germany), followed by another centrifugation at 18 000g for 10 min. The supernatant served as the DNA template for the subsequent real-time PCR.

Two independent real-time qPCR assays for the *MAP* specific multiple copy insertion sequence IS*900* and the single copy fragment F*57* were performed (Slana et al., 2008). The total amount of *MAP* cells was recalculated to 1 g of silage. It was assumed that IS*900* is present in the *MAP* genome in 15 copies on average, and this parameter was taken into account in the assessment of the absolute amount of *MAP* in the sample.

RESULTS

Temperature recording

The room temperature rose from approximately 10°C at the beginning of the trial to a temperature of around 20°C at the end, with fluctuations according to the outside temperature. The temperature recorded inside the bale was slightly higher than the room temperature but there were no major differences between the two temperature curves.

Silage quality

The dry matter of the silage bales varied between 469 and 774 g/kg. The total nitrate did not exceed 19.7 g/kg and showed a mean value of 14.8 g/kg. The ammonia-nitrate was at maximum 4.2% of the total nitrate (mean value 2.6%). The pH value of the silage bales varied between 4.9 and 6.4 with acetic acid as the predominant acid in most of the bales (Table 2).

Detection of *MAP* by solid and liquid culture

No growth of *MAP* was observed at any time during the experiment on a solid or in liquid culture except for at the time of inoculation (Table 1).

Detection of *MAP* by real-time qPCR

Real-time qPCR for the detection of the IS*900* was positive in 10 (66.7%) of the tested bales. At least one bale was positive at each sampling date, and the amount of the target molecules varied between 3.4×10^3 and 4.2×10^7 *MAP* cells per 1 g of silage. Real-time qPCR for F*57* was positive in nine (60.0%) of the bales tested. At Day 15 only was no bale found to be positive for F*57*; at every other sampling date two or three bales gave positive results, corresponding to the results of the real-time qPCR for IS*900*. The levels of the target gene per ml translated into total *MAP* cells of between 1.5×10^4

Table 2. Silage quality analysis results

Bale No.	Dry matter (g/kg)	Nitrate (g/kg)	Ammonia-nitrate (% of total nitrate)	pH	Lactic acid	Acetic acid	Butyric acid
					% of total acid (g/kg dry matter)		
0	560	14.6	3.8	6.2	18.3 (2.3)	76.1 (9.6)	5.6 (0.7)
3	585	19.7	3.3	6.0	0 (0)	82.4 (2.4)	17.6 (0.5)
4	623	16.7	2.1	5.6	50 (1.9)	50 (1.9)	0 (0)
5	630	16.2	2.5	5.6	61.1 (3.5)	38.9 (2.2)	0 (0)
6	728	14.0	2.0	5.7	0 (0)	100 (2.2)	0 (0)
7	664	14.0	3.6	5.7	0 (0)	100 (2.6)	0 (0)
8	639	12.4	2.7	5.6	48.4 (2.3)	51.6 (2.5)	0 (0)
9	572	12.6	2.9	5.6	29.4 (0.9)	70.6 (2.1)	0 (0)
10	469	12.4	2.5	5.5	0 (0)	100 (1.7)	0 (0)
11	511	13.2	2.3	4.9	73.3 (16.9)	25.0 (5.7)	1.7 (0.4)
12	561	12.5	3.5	5.5	46.3 (3.4)	48.8 (3.6)	4.9 (0.4)
13	766	15.9	2.2	5.5	24.2 (1.0)	66.7 (2.9)	9.1 (0.4)
14	679	19.1	2.5	5.4	0 (0)	100 (1.8)	0 (0)
15	596	15.8	2.6	5.1	63.5 (5.5)	34.6 (3.0)	1.9 (0.2)
16	690	14.6	1.6	5.7	0 (0)	100 (0.9)	0 (0)
17	478	10.5	2.4	5.7	0 (0)	100 (2.5)	0 (0)
C1	742	13.9	2.8	5.6	0 (0)	100 (3.2)	0 (0)
C2	729	16.7	1.6	5.8	0 (0)	100 (2.3)	0 (0)
C3	774	15.9	1.4	5.7	0 (0)	100 (1.0)	0 (0)
C4	694	14.5	4.2	6.4	0 (0)	100 (1.4)	0 (0)

and 5.8×10^7 per 1 g of silage in real-time qPCR for F*57* (Table 1). The total amount of *MAP* cells as determined by IS*900* and F*57* real-time qPCR showed a good correlation and, therefore, it can be considered that the quantification of *MAP* in the sample was reliable.

DISCUSSION AND CONCLUSIONS

Silage temperature and quality

The core temperature of the silage measured in one bale increased according to the season and outside temperature. Due to the time span between the bale production and start of the trial as well as the temperatures measured within the bale, it can be presumed that no fermentation processes were going on in the silage during the trial.

Analyses showed that the silage bales that were used in the trial can be rated as representative for fair ensiled grass silage. The silage quality was rated as fair for most of the bales, good for bale 14 and 15, very good for bale 11 and poor for bale zero and the control bale four, according to the rules of the German Agricultural Society (Anonymous, 2006). The main reasons for fair to poor quality were the high pH values and high amounts of acetic acid. Both were most probably due to the high amount of dry matter seen in the bales, leading to a reduced production of lactic acid, higher pH within the bales and an increased production of acetic acid (Kamphues et al., 2004).

Detection of *MAP* culture and real-time qPCR

Viable *MAP* cells were recovered from the silage sample contaminated at day zero. On consecutive days after the inoculation, the silage samples were negative in the solid and liquid cultures. There are several possible ways to explain these rather surprising results: the collected samples contained a low amount of the inoculated *MAP* (the bales after the 15th days after inoculation) and the handling or decontamination procedure of the samples could be considered as unsuitable or, which is more likely, the *MAP* cells were inactivated in the grass silage after a very short period (one day). *MAP* then might have entered into an inactive phase, which is known as dormancy.

Because *MAP* could be detected by real-time qPCR in most of the samples it could be shown that the procedures that were used were suitable for the recovery of the inoculated bacterium from spiked silage bales during this trial. Although real-time qPCR was not performed in the bales opened prior to Day 15 after inoculation, *MAP* could most probably also be retrieved in the first bales because the sampling procedures and silage quality were not changed throughout the trial. Unfortunately, the PCR technique is not able to differentiate between viable and not viable bacterial DNA and, therefore, no information about the survival of the bacterium in the grass silage can be gained from these results. Further studies are needed to elucidate whether the *MAP* is truly inactivated under these conditions or if it just enters a dormancy phase from which it can be reactivated and be of possible risk when fed to susceptible livestock.

Decontamination and preparation of the samples are crucial steps in the cultivation of *MAP*. To avoid overgrowing the cultures, the samples were decontaminated for a prolonged time using 0.75% HPC. This decontamination could have led to a reduction of *MAP* in the samples, but previous studies show that HPC is tolerated by the bacterium for at least five days (Pavlik et al., 2000). Even if the decontamination protocol could have caused the underestimation of *MAP* in culture, it is very unlikely that it is the reason as to why the bacterium could not be cultivated in any solid or liquid culture.

The viability of *MAP* in the environment depends on temperature, humidity, pH (Grewal et al., 2006) and ultraviolet radiation (Whittington et al., 2004). While temperature and UV light can be considered to be of negligible or no influence at all for the survival of *MAP* in silage bales, humidity and pH as well as the concentration of ammonia could be responsible for the short life span of the bacteria within the milieu of the bales.

No influence of humidity on the survival of *MAP* was found on pastures (Whittington et al., 2004), but in a laboratory trial it was shown that the bacterium could be recovered from silage with a moisture of 60% or more, but not from those with a moisture of 45% or below (Katayama et al., 2001). The silage bales that were used in the present study had a high amount of dry matter of more than 55% in most bales, in turn resulting in low moisture and unsuitable conditions for the survival of *MAP*. Even in this silage, the survival of *MAP* was undetectable wherein in well preserved silage with low pH and a

high content of lactic acid, the likelihood of survival is even lower (Katayama et al., 2000).

Low pH seems to be one of the most important reasons for the inactivation of *MAP* in the environment. Wu et al. (2007) showed that a pH of 5.0 causes the highest stress to *MAP* and results in altered gene regulation in the bacterium. In two laboratory studies, *MAP* could not be recovered after 14 days from grass silage with a low pH, but was detectable with culture in silage with a pH of above 4.8 (Katayama et al., 2000, 2001). In contrast, Sung and Collins (2003) showed that *MAP* can survive a pH of 4.0 for 112 days under laboratory conditions. This difference can be explained by more complex stress conditions in silage than in a simple laboratory trial. The silage pH in the presented study varied between 4.9 and 6.4, with only three bales showing a pH of less than 5.5. According to the literature these rather high pH values are unlikely to represent the major cause of the negative culture samples in this trial.

It should also be taken in account that the environmental condition within the bales could have caused the entering of *MAP* into an inactive dormancy phase. It has been suggested that *MAP* can survive for a long time in such an inactive stage under unsuitable environmental conditions (Whittington et al., 2004, 2005; Beran et al., 2006; Rowe et al., 2006). Incentives for the bacterium to enter dormancy have not yet been identified with the absence of nutrients and a lack of an appropriate host being the most discussed. The reasons for reentry into the vegetative phase are also unknown, but the incubation of *MAP* for bacterial culture does not re-activate the growth of the bacterium in some cases corresponding to the results that were obtained in this study (Whittington et al., 2004).

The main sources of paratuberculosis infections in cattle are from sub-clinically or clinically ill animals shedding a high amount of *MAP* into their environment leading to direct faecal-oral transmission of the disease. Additional, but by far less important, sources for the transmission of paratuberculosis such as free ranging wild ruminants, contaminated pastures and feed have long been discussed. In the present study *MAP* was injected directly into the silage bales within broth. This might have had an influence on the viability of the bacterium compared with *MAP* shed in the excrement of infected animals and should be considered. Clusters of *MAP* protected from the environmental conditions by faecal matter might have a longer survival span than shown in this study.

In the present study, it was shown that baled grass silage contaminated with *MAP* can be rated as a minor risk for the transmission of paratuberculosis infections. Unsuitable environmental conditions such as low pH, high amounts of organic acids and storage time might be the main reasons for the decrease of viable *MAP* cells in well preserved grass silage. No viable *MAP* organisms could be found in this trial in baled grass silage. Further studies are needed to elucidate whether the organism is really inactivated under these conditions or if it just enters a dormancy phase from which it might be reactivated and be of possible risk when fed to susceptible livestock.

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