IS*900* RFLP types of *Mycobacterium avium* subsp. *paratuberculosis* in faeces and environmental samples on four dairy cattle farms

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ABSTRACT: *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is the causative agent of paratuberculosis, an economically serious, chronic and progressive intestinal disease of ruminants. *MAP* is shed into the environment particularly in the faeces of ruminants. The purpose of the present study was to monitor *MAP* occurrence in animal faeces and different components of the environment in four herds of infected cattle and to investigate the distribution of different restriction fragment length polymorphism (RFLP) types. Faecal and environmental samples were tested by cultivation and identified using a Mycobactin J dependence test and IS*900* polymerase chain reaction. Randomly selected *MAP* isolates were typed using a standardised IS*900* RFLP method. A total of 2 410 samples were examined by culture. *MAP* isolates were detected in 111 (6.4%) faecal samples, 25 (3.9%) environmental samples and two (5.1%) small terrestrial mammals: common vole (*Microtus arvalis*) and lesser white-toothed shrew (*Crocidura suaveolens*). On farms A1 and A2 (animals were shifted between farms A1 and A2), the same four IS*900* RFLP types (A-C10, B-C10, B-C1 and B-C9) of faecal isolates were detected. Three RFLP types (except for RFLP type B-C10) were also detected in the stable environment on both farms. On farms B1 and B2 (animals were shifted between farms B1 and B2), two identical IS*900* RFLP types (B-C1 and E-C1) were detected in faeces and in the stable environment. The risk of *MAP* transmission through water was not confirmed in our study. Animals could be infected with different RFLP types of *MAP* if the environment was highly contaminated with the causative agent. On some cattle farms, *MAP* sources in the environment could help explain the relatively long-term persistence of *MAP* in our animal subjects.

Keywords: *MAP*; cow; cultivation; epidemiology; transmission; persistence; Johne's disease

Paratuberculosis (Johne's disease) is an economically serious, chronic and progressive intestinal disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*; Hasonova and Pavlik, 2006). *MAP* is transmitted spontaneously from adults to juveniles mainly by the faecal-oral route (Whittington et al., 2004). *MAP* infected animals (most often cattle) shed *MAP* not only in faeces (Pavlik et al., 2000b) but also in milk (Ayele et al., 2005) and semen (Ayele et al., 2004). *MAP* does not propagate in the environment (Whittington et al., 2003). This limitation is compensated by its ability to survive outside the host organism for a considerable time period: in river water for up to 270 days, in faeces and black soil for 11 months and in dung-water at 5°C for up to 252 days (Chiodini et al., 1984).

MAP can be spread in the environment by various contaminated materials (e.g. manure, soil etc.) and numerous vectors (Pavlik et al., 2009). In the environment with infected animals (e.g. cattle or wild ruminants), *MAP* has been detected in small terrestrial mammals (Greig et al., 1999; Kopecna et al., 2008a), in dipterous insects at different

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stages of development (Fischer et al., 2001) and in earthworms (Fischer et al., 2003a). Some free living animals can also be considered as potential vectors and probably also sources of *MAP* on pastures and in the wild. These are wild rabbits (*Oryctolagus cuniculus*) in Scotland (Greig et al., 1999) and a variety of free living ruminant species in Continental Europe: red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), mouflons (*Ovis musimon*), wild boar (*Sus scrofa*) and brown hares (*Lepus europaeus*) as published by Pavlik et al. (2000a), Machackova et al. (2003, 2004) and Kopecna et al. (2008b).

Following detection of the specific IS*900* element (Green et al., 1989) present in the *MAP* genome in 15 to 20 copies (Bull et al., 2000) the analysis of strains by the RFLP method was combined with hybridisation to IS*900*. This method was gradually applied to epidemiological studies of paratuberculosis in Europe, USA and New Zealand (Whipple et al., 1989; DeLisle et al., 1992). The method was standardized in 1999 (Pavlik et al., 1999) so that the results obtained from different laboratories could be compared. Subsequently, this method has been used in the studies of the transmission of this pathogen between different infected hosts. It has also been employed for the comparison of *MAP* isolates originating from animal faeces, the environment and earthworms from different localities (Fischer et al., 2003a) or for the investigation of *MAP* passage through the intestinal tract of invertebrates such as nymphs of oriental cockroaches or earthworms (Fischer et al., 2003a,b).

Due to the fact that herds of ruminants (mainly cattle) were infected with one RFLP type of *MAP* in most of these studies, we investigated the frequency of occurrence of different RFLP types in faeces and in the environment on different infected cattle farms. Accordingly, the purpose of the present study was to monitor *MAP* occurrence in both animal faeces and different components of the environment in four herds of infected cattle and to investigate the distribution of different RFLP types with the use of a standardized IS*900* RFLP method.

MATERIAL AND METHODS

Studied cattle farms

The study was performed in two cattle herds on four farms over the course of one year. Herd A was kept on farms A1 and A2 in Eastern Slovakia and herd B on farms B1 and B2 in Western Slovakia. On these four farms, various systems of milking, feeding and calf rearing were applied.

Herd A – farm A1. On farm A1 (140 cows), animals grazed on adjacent pastures. During milk nutrition (for two to three months), calves were kept in pens with five to ten animals in each pen. After the change in diet – milk for plant nutrition (between two and three months after the birth), the feeding of bulls continued until the age of 18 to 20 months when they were sold to the abattoir. Heifers were moved to a stable on farm A2.

Herd A – farm A2. On farm A2, the animals were kept in three pens with 50 animals in each (150 cows in total). During milk nutrition, young bulls and heifers were kept in pens with five to ten in each pen. Before the change from milk to plant nutrition, all bulls were moved to one stable on farm A1. Young heifers were moved and shared the same stable (situated on A2 farm) with heifers from farm A1. If necessary, the late-pregnant heifers (irrespective of the farm where they were born) were moved at random to the stables on farms A1 and A2.

Herd B – farm B1. On farm B1 (170 cows), the animals were kept in four boxes per 40 to 50 animals. Cows were tied up at troughs with drinkers all the year round. During milk nutrition, each calf was kept in a separate pen (nose contact possible). After the switching from milk to plant nutrition, the bulls and heifers were moved into two separate stables on farm B2.

Herd B – farm B2. On farm B2 (400 cows), the animals were kept in stables with 50 to 60 animals in each all the year. Each calf was kept in a separate pen (nose contact possible) for two to three months during milk nutrition. On this farm, all reared (fattened) bulls receiving plant nutrition and all heifers were kept in two separate stables. Before parturition, cows in advanced stages of pregnancy were randomly (irrespective of the farm where they were born) moved to farms B1 or B2.

Examined samples

Faeces were collected individually from the rectum of animals older than 18 months using disposable gloves. Sampling was performed on all farms twice a year. A total of 1 735 faecal samples were collected into disposable pre-labelled 20-ml

plastic vials. After collection, faecal samples were cooled to 6°C and delivered to the laboratory within 24 hours.

Along with the collection of animal faeces on the farms, a total of 636 samples from the farm environment were taken each time: 346 scrapings from the internal environment of the stables, 260 soil samples from paddocks and 30 samples of drinking water from the drinkers. Thirty-nine small terrestrial mammals, i.e. 35 common voles (*Microtus arvalis*) and four lesser white-toothed shrews (*Crocidura suaveolens*) were trapped around shrubs and straw stacks on pastures and in the farm surroundings.

Culture examination

Faecal and environmental samples were stored in the laboratory at –20°C for up to six weeks before culture examination. Approximately 1 g of sample was transferred to a 50 ml flask containing 30 ml of sterile distilled water and agitated in a horizontal shaker for 30 min. Subsequently, the bottle was left undisturbed for a further 30 min so that the large particles might settle; 5 ml of the supernatant was transferred to a 50 ml bottle containing 25 ml of 0.9% HPC (Hexadecyl Pyridinium Chloride: N-cetylpyridinium chloride monohydrate, Merck, USA) solution (Whipple et al., 1991; Pavlik et al., 2000b). After 72 hours the sediment (200 µl) of each decontaminated sample was cultured on four slopes of different Herrold's egg yolk media (HEYM) with Mycobactin J (2 mg/l of medium) and incubated at 37°C. The cultures were evaluated after a period of 2–4 months.

Soil from the pastures/pens and scrapings from the stables. Approximately 10 g of soil and scrapings from the stables (scrapings from the floor, corridors, wall, doors and barriers) were transferred to a 50 ml flask containing 30 ml of sterile distilled water and agitated in a horizontal shaker for 30 min. Further processing has been described above.

Water samples. Twenty-five ml of each water samples were left to settle for 24 hours at room temperature. After decantation of supernatant, 2 ml of 4% NaOH was added to the pellet. The sample was left undisturbed for 15 min at room temperature and centrifuged for 15 min at 3 000 rpm. After the decantation of the supernatant, 2 ml of 5% oxalic acid was added to the pellet for 15 min at room temperature and centrifuged again for 15 min at 3 000 rpm. The pellet was inoculated on three slopes of HEYM with, and one without, Mycobactin J.

Tissue samples from small terrestrial mammals. After gross examination, removed organs were stored at –20°C for up to two months before culture examination. Tissue samples were examined microscopically after staining the smears according to Ziehl-Neelsen (Z-N) for acid-fast rods (AFR) detection. Two samples from each animal were examined by culture: parenchymatous organs (liver, spleen and kidneys together) and the intestinal tract (large and small intestines together). Each tissue sample (about 1 g) was homogenised in a stomacher (Kleinfeld Labortechnik GmbH, Germany) and decontaminated in 0.75% HPC for 72 hours (Pavlik et al., 2000b); 200 µl of the sediment was inoculated as described above.

Isolate examination

Species identification. All 138 mycobacterial isolates were identified by the Mycobactin J dependence test: subculture isolates were isolated on three HEYM with Mycobactin J and one HEYM without Mycobactin J according to Pavlik et al. (2000b) and subsequently identified by the IS*900* polymerase chain reaction (PCR; Bartos et al., 2006).

IS*900* RFLP analysis. A total of 127 randomly selected *MAP* isolates (104 faecal and 23 environmental isolates) were typed by the standardised IS*900* RFLP method (Pavlik et al., 1999). Digestion of DNA was performed by the restriction endonucleases *Pst*I and *Bst*EII.

Statistical evaluation

Two-sided Fisher's test (InStat 3.06, GraphPad software, Inc., USA) was applied to the statistical evaluation of results.

RESULTS

MAP was isolated from 138 (5.7%) of a total of 2 410 investigated samples of biological material (Table 1). The differences between farms in *MAP* detection in both herds were significant ($P < 0.01$): In herd A, *MAP* detection on farm A1 was higher than on farm A2; in herd B, *MAP* detection on farm B1 was higher than on farm B2.

Table 1. *Mycobacterium avium* subsp. *paratuberculosis* isolation from biological material from two herds on four cattle farms

Biological material examined				Herd A						Herd B					
Sample origin	total			farm A1			farm A2			farm B1			farm B2		
	No.	pos.	%	No.	pos.	%	No.	pos.	%	No.	pos.	%	No.	pos.	%
Environment															
Stable scrapings[1]	346	24	6.9	156	15	9.6	50	0	0	50	5	10.0	90	4	4.4
Pasture/pen soil[2]	260	1	0.4	154	0	0	56	0	0	30	0	0	20	1	5.0
Drinking water[3]	30	0	0	5	0	0	5	0	0	10	0	0	10	0	0
Subtotal	636	25	3.9	315	15	4.8	111	0	0	90	5	5.6	120	5	4.2
Individual faecal samples	1 735	111	6.4	279	45	16.1	306	17	5.6	340	23	6.8	810	26	3.2
Small terrestrial mammals[4]	39	2	5.1	13	2	15.4	4	0	0	14	0	0	8	0	0
Total	2 410	138	5.7	607	62	10.2	421	17	4.0	444	28	6.3	938	31	3.3

[1]scrapings from the floors, corridors, walls, doors and barriers were examined
[2]samples from the soil of the pastures/pens were examined
[3]sediment from drinking water from a special reservoir in the pasture/pen was examined
[4]a total of 35 common voles (*Microtus arvalis*) and four lesser white-toothed shrews (*Crocidura suaveolens*) were examined; one common vole and one lesser white-toothed shrew tested positive

By culture, *MAP* was detected in 6.9% of scrapings from stables, 0.4% pasture/pen soil samples and in the organs of 5.1% small terrestrial mammals: one (2.9%) common vole (*Microtus arvalis*) and one (25.0%) lesser white-toothed shrew (*Crocidura suaveolens*). *MAP* was not detected by culture in any of the drinking water samples taken from the farms (Table 1).

On farm A1, significantly more frequent ($P < 0.01$) shedding of *MAP* in faecal samples was observed (16.1% positive samples) than on the remaining farms, i.e. A2 (5.6% positive samples), B1 (6.8% positive samples) and B2 (3.2% positive samples). On this farm, a high detection rate of *MAP* in scrapings (9.6% positive samples) was also observed (Table 1).

Farm A1 and A2. In A1 farm *MAP* was not detected in any sample of soil from pastures, which was significantly ($P < 0.01$) lower result comparing to *MAP* detection in faecal samples (16.1% positive

Table 2. IS*900* RFLP types of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolates from four cattle farms

IS*900* RFLP analysis			Herd A						Herd B							
RFLP types found	total number of isolates		farm A1				farm A2		farm B1				farm B2			
			faeces[a]		environment[b]		faeces[a]		faeces[a]		environment[b]		faeces[a]		environment[b]	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
A-C10	38	29.9	23	56.1	9	69.2	6	42.9	0	0	0	0	0	0	0	0
B-C1	42	33.1	9	21.9	1	7.7	2	14.3	4	17.4	0	0	21	80.8	5	100
E-C1	29	22.8	0	0	0	0	0	0	19	82.6	5	100	5	19.2	0	0
B-C9	10	7.9	5	12.2	3	23.1	2	14.3	0	0	0	0	0	0	0	0
B-C10	7	5.5	4	9.8	0	0	3	21.4	0	0	0	0	0	0	0	0
B-C14	1	0.8	0	0	0	0	1	7.1	0	0	0	0	0	0	0	0
Total	127	100	41	100	13	100	14	100	23	100	5	100	26	100	5	100

[a]faecal isolates of *MAP*
[b]environmental isolates of *MAP*

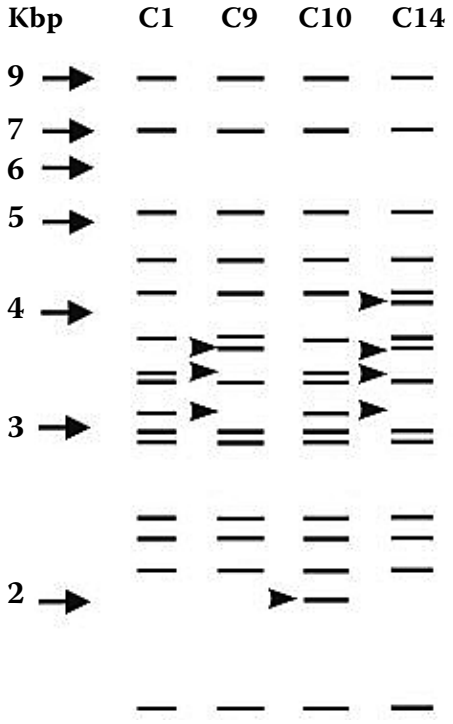

Figure 1. *Bst*EII – IS*900* RFLP profiles C1, C9, C10 and C14 (arrows show the differences between the most common RFLP type C1 and another RFLP types)

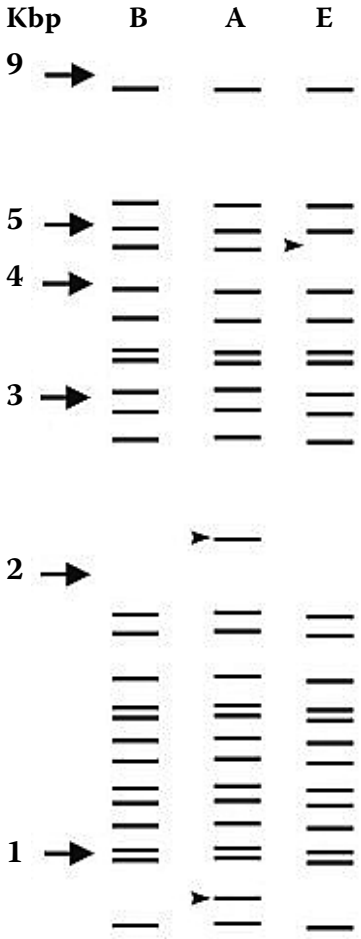

Figure 2. *Pst*I – IS*900* RFLP profiles B, A, E (arrows show the differences between the most common RFLP type B and another RFLP types)

samples), scrapings from stables (9.6%) and organs from small terrestrial mammals (15.4%). Also *MAP* detection in faecal samples was significantly ($P < 0.01$) more frequent (16.1% positive samples) than in the case of environmental (4.8%) samples. *MAP* was detected by culture in 5.6% of cattle faecal samples from farm A2. This finding was significantly ($P < 0.01$) higher than the 0% detection in all 111 samples from the environment (Table 1).

Farm B1 and B2. *MAP* was detected by culture in 6.3% samples of biological material in farm B1 and 3.3% in farm B2 (Table 1). The difference between the 6.8% detection of *MAP* in faecal samples and 10.0% detection in scrapings from stables in farm B1 was not significant. Similarly the differences in *MAP* detection in the cattle faeces (3.2% positive samples), scrapings from stables (4.4% positive samples) and samples of the pasture soil (5.0% positive samples) were not significant in farm B2. *MAP* was not detected by culture either in samples of drinking water or in the organs of trapped small terrestrial mammals in both farms. *MAP* contaminated the environment through faeces significantly more frequently ($P < 0.01$) on farm B1 (6.8% positive samples) compared to farm B2 (3.2% positive samples).

Results of RFLP analysis. Six different *Bst*EII-*Pst*I IS*900*-RFLP types were identified after the analysis of 127 *MAP* isolates (Table 2; Figures 1 and 2). RFLP types B-C1 (33.1% isolates), A-C10 (29.9% isolates) and E-C1 (22.8% isolates) represented the most frequent RFLP types. All six RFLP types were identified in *MAP* isolates from faeces in contrast to *MAP* isolates from the environment, in which only four RFLP types were found (Table 2). *MAP* isolates from the environments showed the same RFLP types as *MAP* isolates from faeces. Due to the fact that *MAP* isolates from small rodents repeatedly did not grow in subculture, the obtained amounts of DNA were insufficient for subsequent RFLP analysis.

Herd A. On both farms (A1 and A2), four RFLP types A-C10, B-C1, B-C9 and B-C10 were simultaneously detected in faeces. The first three mentioned RFLP types were also detected in the

environment on farm A1. The RFLP type A-C10 occurred most often in both feacal (56.1% isolates) and environmental samples (69.2%) on farm A1. The RFLP type B-C10 that was least frequently found in animal faeces (9.8%) was not detected in the environment (Table 2). On farm A2 cooperating with farm A1 (all heifers were reared together), *MAP* was detected by culture in animal faeces only. The most frequently identified RFLP type was also A-C10 (42.9%). RFLP type B-C14 (7.1%), which was not found in any of the other *MAP* isolates examined in this study, was observed only in one faecal sample from farm A2 (Table 2).

Herd B. On both farms B1 and B2, only two RFLP types (B-C1 and E-C1) were simultaneously detected in faeces from animals and in the environment. In faecal samples from farm B1, RFLP type E-C1 was the most observed, however on farm B2 B-C1 was most frequently found RFLP type. On both farms the most frequently RFLP type observed in faeces was also found in all environmental samples (Table 2).

DISCUSSION

In contrast to the previously published data (Whittington et al., 2004, 2005), only one *MAP* isolate was detected (on farm B2), out of a total of 260 samples of soil and surface water from pastures and paddocks collected on four farms. No *MAP* isolates were detected in 30 samples of sediment from the reservoirs of drinking water placed on pastures and in paddocks. With respect to the published data, there are four suggested explanations:

1. When *MAP* in faeces becomes mixed with soil, there is a reduction of 90–99% in the apparent viable count of the organism. This is probably caused by the binding of bacteria to soil particles, which are excluded from culture by sedimentation during sample preparation (Whittington et al., 2003). Attachment to soil also occurs with other non-tuberculous mycobacteria (Brooks et al., 1984).

2. The culture method used, in particular the use of antibiotics and disinfectants during sample preparation, further reduces the analytical sensitivity of *in vitro* culture by killing more than 2.7 $\log_{10}$ *MAP* cells (Reddacliff et al., 2003). Sweeney et al. (2006) developed an immunomagnetic capture technique for the recovery of *M. bovis* from environmental samples including soil, faeces and urine and observed higher culture positivity.

3. The third explanation could be the dormancy of *MAP* cells in the environment. This means that *MAP* cells may not be cultured until the environment again becomes favourable and cells regain the ability to divide and thus become detectable (Whittington et al., 2004). The data of Whittington et al. (2004) are consistent with *MAP* being able to enter a dormant or viable, non-culturable state and later reverting to a vegetative form. Dormancy is defined as the state permitting survival of a non-spore-forming bacterial cell without the requirement for replication. It is genetically programmed, reversible, and induced by an unfavourable environment, typically when an essential nutrient required for growth becomes limiting.

4. Inability to detect any *MAP* in water samples could be connected with the methodology used. Due to high content of detritus and organic remains in water, the water samples were left to settle at room temperature firstly. Then the supernatant was discarded and the pellet was used for further experiments. Thus some *MAP* cells in the suspension could be discarded.

In contrast to the low numbers of *MAP* isolates detected in pasture soil, the numbers of *MAP* isolates obtained from the scrapings collected in stables highly exposed to faeces from infected cattle were significantly higher ($P < 0.05$). According to Whittington et al. (2003) it is easier to isolate the *MAP* from such sites than from soils with a high content of organic matter. The effect of UV sunlight is an important factor for long-term survival of *MAP* in the environment. The highest survival rate of the organism in faecal and soil materials was detected (55 weeks) in a fully shaded environment and was the lowest when the examined material was fully exposed to the weather and where vegetation was also removed.

MAP was isolated from two of 39 small terrestrial mammals trapped on the four investigated farms. The positive animals were detected within a two kilometre radius of farm A1, which had significantly more cattle shedding *MAP* in their faeces. The potential for exposure of these mammals to *MAP* would therefore have been greater on farm A1 than the other farms in the study. A previous study by Fischer et al. (2000) suggested that small insectivores and rodents might be involved in the spread of causative agents of mycobacterial diseases between wild and domestic animals. However, exposure of susceptible cattle to *MAP* contaminated cattle faeces poses a much greater risk of infection than *MAP*-infected small terrestrial mammals.

Six different RFLP types were detected in 127 isolates examined. More than one RFLP type was detected on a single farm; five were detected on farm A2, four on farm A1, two on each of farms B1 and B2. Molecular analyses of field mycobacterial isolates may demonstrate variations in the ability of the organism to adapt to specific environmental niches (Kamerbeek et al., 1997; Sweeney et al., 2006). Using a standardized RFLP method, we revealed the presence of the same RFLP types of *MAP* in faecal samples and scrapings from stables on three farms (A1, B1 and B2.) This provided evidence of the shedding of *MAP* by infected cattle into the environment. Fewer RFLP types were detected in samples from the environment of all three farms in comparison with faecal samples (Table 2). This discrepancy was probably due to two factors:

1. The numbers of analysed samples from the environment were lower in comparison with the numbers of faecal samples. Thus the probability of the detection of the remaining *MAP* RFLP types was decreased.

2. Generally, mycobacteria are known to be able to survive for long periods in the environment. However, the rate of survival may vary between *MAP* isolates and some isolates may not survive in the environment for as long as others. The concentration of viable *MAP* in the environment can also be reduced by the effect of different physical factors (desiccation, dilution, UV radiation etc).

RFLP type B-C14 was only detected in one faecal sample. According to epidemiological data, cow spreading this *MAP* isolate was imported to farm A2 from another farm (not A1, B1 or B2) six months before our testing. The time period between the natural infection and the *MAP* shedding by faeces was determined more than 20 months as reviewed by Whittington and Sergeant (2001). This may be reason why we were not able to detect B-C14 RFLP type in faecal samples of other animals and why contamination of environment by this RFLP type was too low to be detected.

MAP of different RFLP types is introduced into a herd by infected cattle and it is spread by the faecal-oral route. Generally, animals are infected by the consumption of faecal material from pasture, by drinking water contaminated with faeces or from teats in the case of calves. Transmammary and intrauterine transmission was also described (Sweeney et al., 1992; Lambeth et al., 2004). Identification of the six RFLP types of *MAP* confirmed the anamnestic data and gave evidence of the fact that A1 and B1 farms were linked with A2 and B2 farms by rearing their heifers together, respectively.

Pathogenic bacteria, including *MAP*, are known to survive in the soil and other environmental substrates (Whittington et al., 2004). *MAP* might employ interaction with protozoa (Mura et al., 2006) and insects, dormancy, biofilm formation and aeroionisation to survive and disseminate in the environment (Rowe and Grant, 2006). *MAP* – with respect to long-term persistence – can survive on a farm for a considerable time when the infected cattle are no longer present (Whittington et al., 2005). The presence of an environmental source of *MAP* may help explain *MAP* persistence in cattle.

In summary, we confirmed the presence of identical RFLP types in faecal and in the environmental samples except for two RFLP types that were isolated only from faecal samples. In contrast to other studies (Whittington et al., 2004, 2005) only one *MAP* isolate was detected from soil samples and no *MAP* isolate was detected from the reservoirs of drinking water placed on pastures and in paddocks.

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