Available online at www.sciencedirect.com
SCIENCE DIRECT®
The Veterinary Journal 170 (2005) 249–256
The Veterinary Journal
www.elsevier.com/locate/tvjl

A survey for *Mycobacterium avium* subspecies *paratuberculosis* in the Royal Zoological Society of Antwerp

E. Vansnick [a,c,*], F. Vercammen [b], L. Bauwens [b], E. D'Haese [c], H. Nelis [c], D. Geysen [a]

[a] *Institute of Tropical Medicine, Veterinary Department, Nationalestraat 155, 2000 Antwerp, Belgium*
[b] *Royal Zoological Society of Antwerp, Centre for Research and Conservation, K. Astridplein 26, 2018 Antwerp, Belgium*
[c] *Laboratory of Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, 9000 Gent, Belgium*

Accepted 30 July 2004

Abstract

Paratuberculosis is a chronic intestinal disease of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Very little is known about the status of paratuberculosis in European zoos. In this study, the presence of *Map* in the animal collection of the Royal Zoological Society of Antwerp (RZSA) was investigated. Faecal and post mortem samples from 48 ruminants were used to set up cultures. DNA from faeces, tissue and positive cultures were tested by IS900 polymerase chain reaction (PCR). Additionally, 448 serum samples were tested with an ELISA kit.

All culture samples were negative whereas PCR gave three positives on biopsy samples and one positive on faecal samples. With the ELISA, 21 sera could be classified as positive. There is evidence that *Map* is present in the RZSA but no high level faecal shedders could be detected. Further investigations are required in other European Zoos in order to complete the picture of *Map* infections.

Keywords: Mycobacterium; Paratuberculosis; Zoo animals; PCR; ELISA; Culture

1. Introduction

Johne's disease, caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), is a chronic progressive enteritis affecting domestic and wild animals, mainly ruminants. The disease has a worldwide distribution and results in substantial losses in domestic livestock, wildlife and zoological specimens (Cocito et al., 1994).

Shedding of the organism results in spread of the disease and seeding of the soil with long-persisting organisms (Manning and Collins, 2001). Young animals are at the highest risk of acquiring an infection with *Map* and are typically infected through the faecal-oral route either by ingesting the organism through contaminated milk or by ingestion of the mycobacterium from contaminated surfaces (Harris and Barletta, 2001).

The specific housing in zoo facilities is a potential risk factor for infection. Up to one-third of zoos accredited by the American Zoo and Aquarium Association have reported a minimum of one *Map* infected animal since 1995 (Manning and Collins, 2001). The major risk factor is the introduction of new animals into the herd. Between zoos, animals are exchanged frequently and factors such as concentration of animals and stress during transport enhance the possibilities of *Map* transmission.

In the past, the Royal Zoological Society of Antwerp (RZSA) had some problems with *Map*. In 1979 during a post mortem examination, Johne's disease was diagnosed in a gayal (*Bibos frontalis*). A few years later

* Corresponding author. Present address: Department of Animal Health, Nationalestraat 155, 2000 Antwerp, Belgium. Tel.: +32 3 2476278; fax: +32 3 2476268.
E-mail address: evansnick@itg.be (E. Vansnick).

1090-0233/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2004.07.006

(1982), *Map* was isolated from a pudu herd (*Pudu pudu*) (De Meurichy et al., 1985). In 1999, an okapi (*Okapia johnstoni*) (Spec ID M9326) was exchanged with Blijdorp (Rotterdam Zoo, The Netherlands) as part of a breeding program. After transport, the okapi developed diarrhoea. A faecal sample of this okapi was sent to ID-DLO (Lelystad, the Netherlands) and tested positive on culture for *Map* (Dr. Francis Vercammen, personal communication).

Diagnosis of Johne's disease in non-domestic ruminants is difficult. Culture is considered to be the gold standard but this is slow and labour-intensive. Due to intermittent shedding, faecal cultures can give false-negative results (Barrington et al., 2003). Polymerase chain reaction (PCR) testing could be a rapid and sensitive alternative for culture, but faecal samples remain the most difficult specimens for DNA extraction and amplification (Shames et al., 1995; Widjojoatmodjo et al., 1992). ELISA is a rapid and relatively inexpensive test but has a low sensitivity when tested on sera of subclinical animals and is only validated for cattle and small ruminant samples (Payeur, 1998). Current research focuses on the application of ELISA to detect antibodies in non-domestic species, by using a non-specific binding conjugate (e.g., protein G) or by developing a monoclonal/polyclonal conjugate that can bind antibodies produced by all artiodactyls (Manning and Collins, 2001; Schroen et al., 2002; Kramsky et al., 2003).

The objective of this study was to investigate the presence of *Map* in the RZSA animal collection. This is the first time in Europe that the results of an extensive paratuberculosis survey in zoo and animal parks have been reported. Different diagnostic methods that are complementary were used. For herd screening, a commercial ELISA that can detect multiple host species antibodies was used.

2. Materials and methods

2.1. Sample collection

2.1.1. Sera

Four hundred and forty-eight sera samples (all from different animals) from 49 different ruminant species were randomly chosen from the sera collection bank of the Royal Zoological Society of Antwerp. The sera were collected between 1976 and 2002 and kept at −20 °C (Table 1).

2.1.2. Faeces and tissue

Faeces, mesenteric lymph node and intestinal tissue (ileum and rectum) were collected from 48 ruminants during routine post-mortem examination (Table 2). Culture of samples ($n = 192$) was done immediately and all PCR samples ($n = 192$) were kept frozen for several weeks until processed. Additionally, 22 faecal samples of an okapi (Spec ID M9326) were taken monthly from November 2000 until August 2002.

2.1.3. Spiked samples

The detection limits of the different methods (culture and PCR) were determined using *Map* spiked samples. *Map* cells were cultured on Löwenstein–Jensen supplemented with mycobactin J (1 mg/L). Culture tubes were harvested in 2 mL of a 10% Tween 20 solution containing glass spheres. The bacteria were subsequently mixed on a vortex mixer to destroy the hydrophobic clumps, followed by the preparation of tenfold dilutions in physiological saline. The serial dilution was confirmed with solid phase cytometry (ChemScan, Chemunex) which allows rapid enumeration of microorganisms at single cell level. *Map* culture negative faeces and tissue were mixed with the defined suspension containing *Map* organisms.

2.2. Decontamination

The double incubation method of Whitlock and Rosenberger (1990) was used as decontamination protocol. Briefly, 2–5 g faeces were mixed with 10 mL of sterile saline and kept for 30 min at room temperature. Five millilitres of the surface fluid was transferred to a fresh tube containing 25 mL of a 0.9% hexadecylpyridinium chloride solution (Sigma) in half-strength brain-heart-infusion broth (LabM). The sample was incubated for 24 h at 37 °C. After centrifugation at 900*g* for 30 min, the pellet was resuspended in 1 mL of an antibiotic mixture (100 μg vancomycin + 100 μg nalidixic acid and 50 μg amphotericin B in 1 mL distilled water, all Sigma reagents). The samples were incubated for 48–72 h at room temperature.

For tissue samples, the decontamination was slightly different. Five grams of tissue were homogenised in a blender for 30 s using sterile physiological water. Twenty-five millilitres of a 0.75% hexadecylpyridinium-chloride solution was added and the sample was left standing at room temperature for 48–72 h, filtered through sterile gauze cloth, centrifuged at 900*g* for 30 min and the pellet suspended in 1 mL antibiotic mixture. The sample was incubated for 48 h at room temperature and the sediment was used for culture.

2.3. Culture

Radiometric culture 0.1 mL of the sediment was inoculated in BACTEC12B-vials (Becton–Dickinson). The BACTEC12B-vials were supplemented with 200 μL of PANTA-PLUS (Becton–Dickinson), 1 mL Egg Yolk (Becton–Dickinson), 5 μg of mycobactin J (Synbiotics) and 0.7 mL of water and incubated at 37 °C. Every week, for one year, the growth index (GI) was

Table 1
Animal sera tested with the Herdchek M.ptb ELISA kit (Idexx)

Species		No. tested	No. positive	No. suspected
Alces alces alces	Elk/Moose	7	0	0
Ammotragus lervia	Barbary Sheep	3	3	0
Anoa depressicornis	Lowland anoa	1	0	0
Bibos frontalis	Gayal	1	1	0
Bibos javanicus domesticus	Banteng	5	0	0
Bison bison	Bison	43	1	2
Bison bonasus	European bison	19	3	1
Boocercus euryceros	East African Bongo	1	0	0
Bos primigenius taurus linnaeus	Wild Ox	4	0	0
Bos taurus	Domestic ox	16	0	1
Boselaphus tragocamelus	Nilgai	11	1	1
Bubalus depressicornis (H. Smith)	Anoa	5	0	0
Capra hircus Fl	Goat	6	0	0
Capra hircus linnaeus	Goat	5	0	0
Capra ibex ibex Linnaeus	Alpine ibex	4	0	1
Cephalophus monticola	Blue duiker	3	0	0
Cervus elaphus elaphus Linnaeus	Red deer	1	0	0
Cervus elaphus nelsoni Bailey	North American Elk	15	0	0
Connochaetes gnou	White-Tailed Gnu	3	0	0
Connochaetes taurinus	Blue gnu	8	0	0
Dama dama	Fallow deer	1	0	0
Damaliscus dorcas phillipsi Harper	Blesbok	3	3	0
Elapurus davidianus Milne-Edwards	Père David's Deer	29	0	1
Gazella leptoceros	Slender-horned gazelle	6	0	0
Giraffa camelopardalis	Giraffe	2	0	0
Hemitragus jemLahicus jemLahicus	Himalayan Tahr	14	0	0
Hippotragus niger	Sable antilope	4	0	0
Okapia johnstoni	Okapi	25	0	1
Oreamnos americanus	Mountain Goat	3	0	0
Oryx dammah (Cretzschmar)	Scimitar Oryx	2	0	0
Oryx leucoryx	Arabian Oryx	10	0	0
Ovibos moschatus Wardi	Musk Ox	11	0	1
Ovis ammon musimon	European Mouflon Sheep	6	0	0
Ovis aries L.	Domestic sheep	27	2	0
Ovis canadensis canadensis Shaw	Bighorn	7	0	1
Ovis dalli	Dall's sheep	1	0	0
Poëphagus mutus grunniens	Yak	23	3	2
Pudu pudu	Pudu	7	2	0
Rangifer tarandus tarandus	Reindeer	22	0	1
Saiga tatarica tatarica	Saiga	3	0	0
Sika hortulorum	Dybowski's Sika Deer	4	0	0
Syncerus caffer brachyceros	Savannah buffalo	1	0	0
Syncerus caffer caffer	African cape buffalo	20	0	0
Syncerus caffer nanus	Forest buffalo	7	0	0
Tapirus indicus	Tapir	1	1	0
Taurotragus oryx	Eland	33	1	2
Taurotragus oryx Derbianus	Derby eland	2	0	0
Tragelaphus spekei gratus Sclater	Sitatunga	12	0	0
Tragulus javanicus	Lesser Malayan cevrotain	1	0	0
Total		448	21	15

determined with an automatic ion chamber (BACTEC 460). Smears were made from all vials with a GI > 20 and stained using the Ziehl–Neelsen method. If acid-fast bacilli were detected, the samples were processed for PCR-testing.

Culture on solid medium was started with the inoculation of 0.1 mL of the sediment onto four tubes with Löwenstein–Jensen medium supplemented with 1 mg mycobactin J, 40 mL Panta-Plus and 4 g of sodium pyruvate per litre. The tubes were incubated at 37 °C. Growth was determined weekly (during one year) and Ziehl–Neelsen coloration was performed on positive tubes. If acid-fast bacilli were detected, the samples were processed for PCR-analysis.

Table 2
Animals tested with PCR and culture

No.	Species (Latin)	Species (English)	Age (years)	Remark	Results
1	*Bison bison*	American bison	1	Culling	–
2	*Bison bison*	American bison	16	Culling	–
3	*Bison bonansus*	European bison	2	Culling	–
4	*Gazella leptoceros*	Slender-horned gazelle	7	Pneumonia	–
5	*Cervus elaphus*	Wapiti/Elk	1	Culling	–
6	*Cervus elaphus*	Wapiti/Elk	2	Culling	Lymph node: culture of Maa
7	*Bison bison*	American bison	1	Culling	–
8	*Synercus caffer*	African buffalo	21	Culling	–
9	*Oryx leucoryx*	Arabian Oryx	1	Emaciated, worms	–
10	*Gazella leptoceros*	Slender-horned gazelle	1	Skinny	–
11	*Tragelaphus spekii*	Sitatunga	11	Skinny	–
12	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	8	Heart-insuffiency	–
13	*Bos mutus*	Yak	1	Culling	–
14	*Oryx leucoryx*	Arabian oryx	2	Culling	–
15	*Cervus nippon dybowskii*	Sika deer	4	Culling	–
16	*Dama dama*	Fallow deer	5	Culling	–
17	*Syncerus caffer nanus*	Dwarf forest buffalo	2	Culling	–
18	*Rangifer tarandus*	Reindeer	12	Died of old age	–
19	*Dama dama*	Fallow deer	4	Culling	Faeces: 4 culture tubes contaminated
20	*Boselaphus tragocamelus*	Nilgai	7	Culling	–
21	*Tragelaphus spekii*	Sitatunga	5	Culling	–
22	*Bos taurus*	Domestic cow	4	Culling	Ileum, faeces and rectum: 4 culture tubes contaminated
23	*Bison bison*	Bison	3	Culling	Ileum and rectum: 4 culture tubes contaminated
24	*Bison bison*	Bison	3	Culling	–
25	*Bison bison*	Bison	3	Culling	–
26	*Bos taurus*	Domestic ox	1	Culling	–
27	*Bison bison*	Bizon	3	Culling	–
28	*Okapia johnstoni*	Okapi	18	Anemia	–
29	*Tragelaphus spekii*	Sitatunga	5	Culling	–
30	*Connochaetes gnou*	White tailed gnu	3	Culling	–
31	*Bison bison*	American bison	3	Culling	–
32	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	2	Culling	Faeces: 4 culture tubes contaminated
33	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	2	Culling	–
34	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	2	Culling	–
35	*Hemitragus jemlahicus jemlahicus*	Himalayan tahr	2	Culling	–
36	*Rangifer tarandus*	Reindeer	12	Trauma	–
37	*Capra ibex*	Rock goat	1	Trauma	–
38	*Tragelphus spekii*	Sitatunga	4	Trauma	–
39	*Oryx leucoryx*	Arabian Oryx	5	Skinny, dehydrated	–
40	*Alces alces alces*	European Elk	16	Enteritis	–
41	*Okapia johnstoni*	Okapi	14	Colitis	Lymph node PCR positive
42	*Oryx dammah (Cretzschmar)*	Scimitar Oryx	18	*Clostridium* enterotoxaemia	–
43	*Oryx dammah (Cretzschmar)*	Scimitar Oryx	14	Kidney insufficiency	–
44	*Rangifer tarandus*	Reindeer	12	Gastro-enteritis	–
45	*Bison bonansus*	European bison	20	Culling	Rectum PCR positive
46	*Bibos javanicus domesticus*	Banteng	19	Skinny	Ileum PCR positive
47	*Ovis canadensis canadensis Shaw*	Bighorn	7	Skinny	–
48	*Taurotragus oryx*	Eland	10	Skinny	–

PCR, polymerase chain reaction; Maa, *Mycobacterium avium* subspecies *avium*.

2.4. Tissue DNA extraction

The tissue samples were cut in 1–3 mm^2 pieces and transferred to a 1.5 mL microcentrifuge tube. Two hundred and fifty microlitres of lysis buffer (60 mM Tris, pH 7.4, 60 mM EDTA, 10% Tween 20, 1% Triton-100, 1.6 M Guanidin–HCl), 50 μL of proteinase K (20 mg/mL, Boehringer–Mannheim) and 500 μL of 0.1-mm-diameter glass microspheres were added. The tubes were incubated for 1 h at 60 °C. The samples were sonicated for 5 min at room temperature and 40 μL of diatomaceous-earth solution (Sigma) (0.2 g/mL) were added. The tubes were incubated at 37 °C for 60 min in a thermomixer (Eppendorf).

Next, each tube was centrifuged for 20 s and the supernatant was discarded. The pellet was washed twice with 900 μL of ethanol (70%) and once with acetone. After drying in a thermoblock at 50 °C for 20 min, 90 μL of TE-buffer (10 mM Tris and 1 mM EDTA, pH 8) were added and the mixture incubated for 20 min at 60 °C under constant shaking at 3.36 g (1000 rpm, Thermomixer, Eppendorf) The tube was centrifuged for 40 s and 60 μL of the supernatant was transferred to a new tube for PCR.

2.5. BACTEC DNA extraction

The ethanol extraction method described by Whittington et al. (1999) was used for BACTEC DNA extraction.

2.6. Faecal DNA extraction

One gram of faeces was suspended in 10 mL of a 2% Tween-solution and glass beads were added. The samples were mixed vigorously on a vortex mixer for 1 min. The sample was kept at room temperature for 30 min before transferring 1 mL of the supernatant to a new microcentrifuge tube. The tube was centrifuged at 6000*g* for 1 min. Supernatant was transferred to a new microcentrifuge tube and centrifuged at 14,000*g* for 10 min. The pellet was suspended in 500 μL of a 100 mM Tris–HCl solution containing 150 mM NaCl and 50 mM EDTA. Five hundred microlitres of 0.1-mm-diameter glass microspheres and 50 mL of 20 mg proteinase K (Boehringer–Mannheim) per milliliter were added. The samples were agitated for 50 s, allowed to digest overnight and agitated again for 50 s. The supernatant was recovered by centrifugation and transferred to a new tube before being heated at 100 °C for 10 min and then cooled to 0 °C on ice.

Two hundred microlitres of a 3.75 M NaCl solution containing 2.5 pmol of biotinylated capture oligonucleotides IS900SB (5′ biotine-gtt cgg ggc cgt cgc tta ggc t 3′) and IS900RB (5′ biotine-gag gat cga tcg ccc acg tga 3′) were added. Tubes were incubated under agitation at 60 °C for 3 h to allow hybridisation. Ten microlitres of streptavidin Dynabeads (washed according to the manufacturer's instructions; Dynal) were added and the incubation was continued for 2 h at room temperature. The magnetic beads were captured, washed twice with 10 mM Tris–HCl–0.1 mM EDTA (pH 8) and resuspended in 20 μL water. Ten microlitres were used as template for amplification.

2.7. PCR

A nested PCR was performed. Primary PCR after tissue and Bactec extraction was performed in a final volume of 25 μL containing 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.6 mM $MgCl_2$, 200 μM of each dNTP, 20 pM of each primer, 0.5 U Taq polymerase and 5 μL sample. The primary PCR after faecal extraction was performed in 50 μL containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.6 mM $MgCl_2$, 200 μm of each dNTP, 20 pM of each primer, 0.5 U Taq polymerase and 10 μL of sample. The primers used in the primary PCRs were IS900 S1 (5′ ggg ttg atc tgg aca atg acg gtt a 3′) and IS 900 R3(2) (5′ agc gcg gca cgg ctc ttg tt 3′) and the predicted amplicon length was 572 bp.

The tubes were incubated in a thermocycler under the following conditions: one cycle of denaturation at 94 °C for 4 min followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 68 °C for 45 s and extension at 72 °C for 45 s followed by a final extension at 72 °C for 10 min.

The secondary PCR was performed in a final volume of 25 μL containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.6 mM $MgCl_2$, 200 μm of each dNTP, 20 pM of each primer, 0.5 U Taq polymerase and 1 μL of the first PCR solution. The primers used in the secondary PCR were IS900S2 (5′ gga ggt ggt tgt ggc aca acc tgt 3′) and IS900R1 (5′ cga tca gcc acc aga tcg gaa 3′). This nested PCR amplifies a fragment of 452 bp. The tubes were incubated in a thermocycler under the following conditions: one cycle of denaturation at 94 °C for 4 min followed by 25 cycles of denaturation at 94 °C for 45 s, annealing at 68 °C for 45 s and extension at 72 °C for 45 s and a final extension at 72 °C for 10 min. A PCR mixture with water as template was used as negative control. Bacterial DNA of the *Map* reference strain ATCC19698 was used as positive control.

The PCR conditions for IS901 amplification were the same as described for IS900. The primers used in the first PCR were IS901S (5′ gca acg gtt gtt gct tga aag gaa t 3′) and IS901R1 (5′ gcg cac gca tga tga gtg gac tta c 3′). The primers used in the semi-nested PCR were IS901S and IS901R2 (5′ caa ccc cgc caa cag gtc ctt aga g 3′). The predicted amplicon lengths were 215 bp for the first round and 118 bp for the semi-nested PCR. Genomic DNA of *M. avium* subspecies *avium* was used a positive control.

PCR products were analyzed by electrophoresis in 2% (w/v) agarose gels stained with ethidium bromide.

2.8. ELISA

The serum samples were tested for the presence of antibodies *Map* using the Herdchek ELISA test kit (IDEXX). This is an indirect ELISA performed in *Map* antigen coated microtitre wells. The detection of bound antibodies is based on a horseradish peroxidase (HRPO) protein-G conjugate, which reacts with antibodies of multiple animal species. The kit has only been validated for small ruminants and bovine serum samples. For these animals, the specificity of the kit is 99%

but the sensitivity is affected by the stage of infection. The sensitivity on low faecal shedders is only 15%, whereas for clinically affected animals it is 87%, with an overall sensitivity of 45% (Collins, 1996).

All samples were tested according to the manufacturer's instructions supplied with the kit. Fifteen microlitres of sample were mixed with 135 μL of adsorbent solution and incubated for 60 min at RT. One hundred microlitres of each pre-incubated sample were transferred into a well of an antigen coated microtitre plate and incubated for 60 min at RT. The wells were then rinsed five times with diluted wash solution. One hundred microlitres of HRPO conjugate were added to each well and incubated for 30 min at RT. The wells were washed five times with diluted wash solution. One hundred microlitres of substrate solution were added and the plates incubated for 10 min at RT in the dark. The reaction was stopped with 100 μL of stop solution. The absorbance was measured at 450 nm.

3. Results

3.1. Culture

A GI of >20 was recorded in 10 of the 192 Bactec 12B vials. After Ziehl–Neelsen staining, only one vial (a mesenteric lymph node from a Wapiti) showed acid-fast bacilli. A DNA extraction was performed on the positive Bactec vials but the PCR with IS900-primers was negative. To identify the mycobacterium, a panel of mycobacterium-specific primers for the detection of IS901, IS902 and IS6110 was used. A positive PCR was shown using primers amplifying the IS901 locus (an insertion sequence of *M. avium* subspecies *avium*).

One hundred and six out of 768 Löwenstein–Jensen solid media became contaminated (fungal and bacterial). Culture results were not available for seven samples due to bacterial or fungal overgrowth of the four solid culture media (Table 2). The four tubes of the mesenteric lymph node of the wapiti were Ziehl–Neelsen positive. The PCR using the IS900 assay was negative but the IS901 assay gave positive results. The acid-fast bacilli that we could culture were *M. avium* subspecies *avium*. No *Map* could be detected in the faeces cultures of the okapi.

As control, the two culture methods were also tested with spiked samples (positive control) and a positive result was obtained. The detection limit was determined at 1000 *Map* organisms per gram tissue or faeces.

3.2. DNA extraction and PCR

DNA extraction and PCR were undertaken three times on each tissue sample. Only three samples were IS900 positive one okapi lymph node (no. 41), one bison rectum (no. 45) and one Banteng ileum (no. 46) (Table 2). A second PCR on the same sample was performed and again gave a positive result. The faecal DNA extracts of the 48 animals were all negative. Only one faecal sample (June 2002) out of a total of 22 samples from the okapi was IS900 positive.

Map spiked samples were used to test and evaluate the faecal and DNA extraction method. The faecal assay could detect 100 bacilli per gram of faeces and the tissue DNA extraction could detect 1000 *Map* organisms per gram of tissue.

3.3. ELISA

An S/P ratio of >0.400 (the cut-off ratio for small ruminants according to the manufacturer) was considered positive. An S/P ratio of between 0.150 and 0.400 was considered as a suspect. The results of the ELISA on the 448 sera gave 21 positives and 15 suspects (Table 1).

4. Discussion

This study was undertaken to investigate the presence of *Map* in the animal collection of the RZSA using different diagnostic tests. No *Map* positive growth was detected on solid medium (Löwenstein–Jensen + mycobactin) or BACTEC12B. The sensitivity of the *Map* culture method is estimated at 50% (Collins, 1996) and is obviously related to the likelihood of sufficient numbers of *Map* being shed from the intestinal lesions. The sensitivity of culturing assays is also strain dependent, as some strains (sheep and bison) are more difficult to isolate than others (Juste et al., 1991; Garrido et al., 2000).

The negative isolation of *Map* from animal samples may have been due to the limited distributions of focal lesions, sporadic excretion of organisms or the too-high detection limit of the faecal culture methods employed (McDonald et al., 1999). Another possibility could have been the cases of paucibacillary paratuberculosis which characteristically show the presence of few organisms in infected tissues or faeces.

The decontamination and the culture protocols used in this study are well-established routine methods (Cousins et al., 1995; Whittington et al., 1999; Pavlik et al., 2000) and appropriate controls were incorporated using spiked samples. A higher percentage of contamination was found for solid-medium cultures (17.4%) than for BACTEC12B (5.2%) as also has been reported in other studies (McDonald et al., 1999). On the other hand, the use of a stronger decontamination method could affect the growth of *Map* bacterium isolated from some host species (Reddacliff et al., 2003).

PCR provides a rapid, specific test for detecting *Map* in clinical samples. Organisms can be detected

without the need for culture and therefore this method speeds up the process of diagnosis and overcomes problems with fungal or bacterial contamination. Faeces, however, contain lot of PCR-inhibitors, such as bilirubin, bile salts, chelating agents and humic materials, and difficulties are experienced in recovering DNA from small numbers of target organisms in such a complex matrix (Shames et al., 1995; Widjojoatmodjo et al., 1992). Generally, PCR-based tests on faeces have been found to be less sensitive than faecal culture (Harris and Barletta, 2001) but when applied to tissue samples, PCR is more sensitive and reliable. Although the use of biopsy samples is not practical in field studies, the technique is useful in confirming a diagnosis at necropsy. Three autopsy samples were positive on PCR for IS900. Only one faecal sample from the Okapi (Spec ID M9326) was positive on PCR. Thus, there is evidence that *Map* infections are present in the RZSA, but in the form of low level faecal shedders.

Samples from the sera bank were screened by ELISA in order to obtain data on the occurrence of *Map* in the past in the zoo. ELISA was also useful at times when PCR samples could not be obtained. The HerdChek ELISA is an indirect ELISA based on a protein G conjugate. Protein G is a non-species-specific protein binding the Fc region of multi-species IgG in a non-immunological reaction without interfering with the antigen-binding sites (Taatjes et al., 1987). The ELISA has been validated only for small ruminants and bovine serum samples. With this test we observed 21 animals with an S/P ratio >0.400. We assume that these animals were positive when considering the results reported by Stöbel (2002) who described a non-species dependent ELISA for the detection of antibodies to *Borrelia burgdorferi* in zoo animals and clearly demonstrated that protein G was a useful alternative to species-specific secondary antibodies. Kramsky et al. (2003) concluded in their study that protein G conjugates might be useful reagents for serodiagnosis although those authors observed a variation in protein binding of protein G.

A cut-off value has to be established for each species in order to interpret ELISA results correctly but it is nearly impossible to do that for all zoo ruminants. The test kit manual provides S/P ratio cut-off values for two groups of mammals. For bovine samples, an S/P ratio <0.15 is classified as negative, 0.15–0.30 as doubtful and >0.30 as positive. For small ruminants, an S/P ratio between 0.30–0.40 is doubtful and >0.40 is positive. We have used the most conservative cut-off value (small ruminants >0.40) and have classified all samples with a cut-off 0.15–0.4 as suspect. Serum from the gayal (clinical Johne's disease in 1979) and sera from the pudu herd (*Map* positive on culture in 1984) were positive using ELISA.

5. Conclusion

We report here evidence of the presence of *Map* infection in the animal collection of the RZSA. However, no high-level faecal shedders could be detected. A follow-up of the animals using PCR and culture will be necessary, especially for the animal herds with ELISA positive results. Further investigations are also required in other European zoos and animal parks in order to complete the picture of *Map* infections in European collections of non-domestic hoof stock and to initiate control measures if necessary.

Acknowledgements

The authors thank the Flemish Government for financial and structural support of this research at the RZSA. We also thank Idexx (The Netherlands) for providing the HerdChek test kit.

References

Barrington, G.M., Gay, J.M., Eriks, I.S., Davis, W.C., Evermann, J.F., Emerson, C., O'Rourke, J.L., Hamilton, M.J., Bradway, D.S., 2003. Temporal patterns of diagnostic results in serial samples from cattle with advanced paratuberculosis infections. Journal of Veterinary Diagnostic Investigation 15, 195–200.

Cocito, C., Gilot, P., Coene, M., de Kesel, M., Poupart, P., Vannuffel, P., 1994. Paratuberculosis. Clinical Microbiology Reviews 7, 328–345.

Collins, M.T., 1996. Diagnosis of paratuberculosis. Veterinary Clinics of North America: Food Animal Practice 12, 357–371.

Cousins, D.V., Evans, R.J., Francis, B.R., 1995. Use of BACTEC radiometric culture method and polymerase chain reaction for the rapid screening of faeces and tissues for *Mycobacterium paratuberculosis*. Australian Veterinary Journal 37, 1077–1083.

De Meurichy,W., Portaels, F., Hoorens, J., Bauwens, L., 1985. Outbreak of a paratuberculosis-like disease in a captive pudu herd (*Pudu pudu*) Verhandlungsbericht des 27 Internationalen Symposiumüber die Erkrankungen der Zootiere, St. Vincent/Torino, Italy, 9 June–3 July 1985, pp. 469–480.

Garrido, J.M., Cortabarria, N., Oguiza, J.A., Aduriz, G., Juste, R.A., 2000. Use of a PCR method on fecal samples for diagnosis of sheep paratuberculosis. Veterinary Microbiology 77, 379–386.

Harris, N.B., Barletta, R.G., 2001. *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. Clinical Microbiology Reviews 14, 489–512.

Juste, R.A., Marco, J.C., Saez de Ocariz, C., Aduriz, J.J., 1991. Comparison of different media for the isolation of small ruminant strains of *Mycobacterium paratuberculosis*. Veterinary Microbiology 28, 385–390.

Kramsky, J.A., Manning, E.J., Collins, M.T., 2003. Protein G binding to enriched serum immunoglobulin from nondomestic hoofstock species. Journal of Veterinary Diagnostics Investigation 15, 253–261.

Manning, E., Collins, M., 2001. *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. Revue Scientifique et Technique de l'Office International des Epizooties 20, 133–150.

McDonald, W.L., Ridge, S.E., Hope, A.F., Condron, R.J., 1999. Evaluation of diagnostic tests for Johne's disease in young cattle. Australian Veterinary Journal 77, 113–119.

Pavlik, I., Matlova, L., Bartl, J., Svastova, P., Dvorka, L., Whitlock, R., 2000. Parallel faecal and organ *Mycobacterium avium* subsp. *paratuberculosis* culture of different productivity types of cattle. Veterinary Microbiology 77, 309–324.

Payeur, J., 1998. Overview of Johne's disease diagnostics. In: Proceedings of the Workshop on Diagnosis, Prevention and Control of Johne's Disease in Non-domestic Hoofstock, White Oak conservation Center, Yulee, Florida, June 26–28,1998, pp. 27–30.

Reddacliff, L.A., Vadali, A., Whittington, R.J., 2003. The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subspecies *paratuberculosis* isolated from tissues and faeces. Veterinary Microbiology 95, 271–282.

Schroen, C., Gwozdz, J., Roche, M., Bradley, T., McDonald, W., Condron, R., 2002. Evaluation of diagnostic tests for the diagnosis of Johne's disease in deer. In: Proceedings of the 7th International Colloquium of Paratuberculosis, 11–14 June, Bilbao, Spain, pp. 154–161.

Shames, B., Fox, J.G., Dewhirst, F., Yan, L., Shen, Z., Taylor, N.S., 1995. Identification of widespread *Helicobacter hepaticus* infection in feces in commercial mouse colonies by culture and PCR assay. Journal of Clinical Microbiology 33, 2968–2972.

Stöbel, K., Schönberg, A., Staal, C., 2002. A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi s.l. in zoo animals. International Journal of Medical Microbiology 291 (suppl. 33), 88–99.

Taatjes, D.J., Chen, T.H., Ackerstrom, B., Bjorck, L., Carlemalm, E., Roth, J., 1987. Streptococcal protein G–gold complex: comparison with staphylococcal protein A–gold complex for spot blotting and immunolabeling. European Journal of Cell Biology 45, 151–159.

Whitlock, R., Rosenberger, A., 1990. Fecal culture protocol for *Mycobacterium paratuberculosis*. A recommended protocol. In: Proceedings of the 94th Annual Meeting of the US Animal Health Association 1990, pp. 280–285.

Whittington, R.J., Marsh, I., McAllister, S., Turner, M.J., Marshall, D.J., Fraser, C.A., 1999. Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. Journal of Clinical Microbiology 37, 1077–1083.

Widjojoatmodjo, M.N., Fluit, A.C., Torensma, R., Verdonk, G.P., Verhoef, J., 1992. The magnetic immuno polymerase chain reaction assay for direct detection of *salmonellae* in fecal samples. Journal of Clinical Microbiology. 30, 3195–3199.