Veterinary Microbiology 163 (2013) 116–123

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Distribution of *Mycobacterium avium* ssp. *paratuberculosis* in a German zoological garden determined by IS900 semi-nested and quantitative real-time PCR

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ARTICLE INFO

Article history:
Received 13 March 2012
Received in revised form 4 December 2012
Accepted 6 December 2012

Keywords:
Germany
Mycobacterium avium ssp. *paratuberculosis*
PCR
Zoological garden

ABSTRACT

Little data concerning the distribution of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in zoological gardens is available. The presence of MAP in captured wildlife might provide further information on non-ruminant hosts and expand the list of animals susceptible to MAP being potential sources of MAP transmission. Therefore, a German zoological garden with recent history of clinical paratuberculosis in Barbary sheep (*Ammotragus lervia*) and an alpaca (*Lama pacos*) was selected to estimate the distribution of MAP infections in 21 mammalian and avian species. Pooled faecal samples from individual animals of each species were tested for the presence of MAP. A previously developed IS900 semi-nested PCR (snPCR) assay, amplifying a 587 bp and a 278 bp fragment, was used for the detection of MAP-DNA. Based on this snPCR, in 14 out of the 21 pooled faecal samples MAP-DNA was detected. MAP positive snPCR results were observed in ruminants and camelids as well as in non-ruminants such as equines, primates, rodents, and birds. Moreover, a quantitative real-time PCR demonstrated that the concentration of MAP-DNA was within the range of 2.2×10^3–9.6×10^6 MAP-DNA equivalents per gram faeces. The highest amount was shed by primates such as Black-and-white ruffed lemurs (*Varecia variegata*) and Cottontop tamarins (*Saguinus oedipus*).

This is the first survey investigating the presence of MAP in a German zoo, which includes non-ruminants. The results of the present study confirm the wide host range of MAP and demonstrate that MAP occurs more frequently in zoo animals than expected. In order to restrict further spread of MAP in European zoos, additional investigations regarding the existing transmission pathways of MAP in zoos are recommended.
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1. Introduction

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the infectious agent of Johne's disease, a degenerative chronic granulomatous inflammation of the intestinal tract causing diarrhoea, weight loss, reduced reproductive performance, and eventually death (Harris and Barletta, 2001). Since paratuberculosis is primarily recognized in domestic ruminants, its greatest economic effect is seen in the dairy and beef industry. Nevertheless, it has also been reported in a wide range of other domesticated, wild, and zoo animals including non-ruminants (Greig et al., 1999; Beard et al., 2001a; Motiwala et al., 2004; Glawischnig et al., 2006; Florou et al., 2008; Stevenson et al., 2009; Fritsch et al., 2012).

The epidemiology of MAP, particularly regarding inter-species transmission and the role of wildlife reservoirs, is

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http://dx.doi.org/10.1016/j.vetmic.2012.12.009

not well understood. There is evidence that free-ranging wildlife including non-ruminants has become increasingly important in the transmission and maintenance of MAP. Known wildlife reservoirs are rabbits in Scotland (Greig et al., 1997; Beard et al., 2001b) and a wide spectrum of free-ranging birds and mammals in the USA (Corn et al., 2005). Previous studies, which employed molecular genetic typing techniques, demonstrated the concept of natural inter-species transmission of MAP between wildlife and domestic ruminants (Greig et al., 1999; Florou et al., 2008; Stevenson et al., 2009; Fritsch et al., 2012).

The presence of MAP in wildlife reservoirs and the possibility of inter-species transmission may have important implications for the control of the disease and may interfere with final stages of bovine eradication programmes. Increased wildlife popularity has also led to a greater translocation of wildlife and the development of captive wildlife industries. Data on MAP occurrence in animals other than ruminants is required prior to the generation of effective and comprehensive paratuberculosis control programmes. Captured wildlife kept in common housing conditions in zoo facilities, including crowding of animals, may provide tentative knowledge in terms of inter-species transmission of MAP. Although studies on the occurrence of MAP in faecal samples of zoo ruminants have already been published (Weber et al., 1992; Erume et al., 2001; Vansnick et al., 2005), little data concerning its distribution in zoological gardens is available. Therefore, the aim of this study was to evaluate the presence of MAP via DNA detection by PCR assays in faeces of captured wildlife and to identify further animal species susceptible to MAP. This is the first survey conducted to investigate the occurrence of MAP in a German zoo including non-ruminants.

2. Materials and methods

2.1. Animals and sampling

Faecal samples were taken from mammalian and avian species kept in a zoological garden in North Rhine Westphalia, Western Germany (52°17′46.43″N, 7°25′25.50″E). The 13 ha (32.5 acre) zoo specializes in primate and avian husbandry. The animal park, with 1065 animals representing 102 species, is famous for its natural-looking exhibits. Zoo staff members are grouped into three different working teams responsible for feeding, cleaning, and maintenance in a distinctive district.

The zoo was chosen for this study due to its known clinical paratuberculosis history. In winter 2008/2009, zoo residents such as Barbary sheep (*Ammotragus lervia*) and an alpaca (*Lama pacos*) exhibited clinical signs of paratuberculosis. The alpaca was further examined and tested positive for MAP in faeces and lymphatic tissue by IS900 PCR and rtPCR assays. PCR results were confirmed by culture and Ziehl-Neelsen staining (Münster et al., 2013). For a better risk assessment of MAP presence within the animal collections of the current survey, pooled faecal samples of approximately 10–100 g from each species were collected during a single routine inspection tour guided by professional veterinarians and zookeepers. Sampled species included ruminants (No. 2, 16, 20), camelids (No. 23, 25), equines (No. 17, 19), primates (No. 2, 11, 12, 26), rodents (No. 11, 23), macropods (No. 22), and birds (No. 13, 21, 22, 27). Certain facilities of the chosen zoo harbour diverse species (Fig. 1). To prevent or minimize contaminations by MAP distributed in the environment, fresh faecal samples, clearly related to the respective species, were carefully picked up randomly from the compounds using disposable gloves and transferred immediately to plastic jars kept in a clean separate box. Specimens were stored at −20 °C and kept frozen until tested by PCRs.

2.2. DNA extraction

DNA was extracted for PCR analysis from faecal samples using a modified protocol of the QIAamp Blood Kit (Qiagen, Hilden, Germany). Depending on the species, faecal samples of approximately 10–100 g were mixed 1:1 with PBS, remixed and ultra-sonicated thrice for 10 s at 20 kHz (Bendelin Sonoplus HD 2200, Berlin, Germany). Since a low MAP content was expected, 10 aliquots of 1 g of the 1:1 mixture with PBS were taken from each pooled faecal sample and subsequently transferred to a tube containing 540 μl lysis buffer. Following mixing, the samples were incubated on a shaker at 37 °C and 900 rpm for 30 min, after which 60 μl ready-to-use proteinase K solution (20 mg/ml) and 600 μl lysis buffer were added. The tubes were incubated immediately at 56 °C and 900 rpm for 30 min, and for a further 15 min at 95 °C. The supernatants were transferred into new tubes, before 400 μl ethanol (≥99.8%) were added and the mixtures were centrifuged through a QIAamp Spin Column. Centrifugation and washing procedures were performed according to the corresponding kit manual. In a final step, the extracted total DNA was eluted and re-suspended in 200 μl elution buffer at a temperature of 85 °C and stored at −20 °C prior to use.

2.3. Qualitative IS900 semi-nested polymerase chain reaction

A qualitative snPCR, based on the insertion sequence IS900, was performed as described elsewhere (Schneider, 2003; Münster et al., 2011, 2012). PCR reactions were carried out in 25 μl volumes containing a 2.5 μl DNA template in a reaction mixture with one Ready-To-Go™ PCR bead (Amersham Pharmacia Biotech, Freiburg, Germany), 0.5 μl dimethyl sulphoxide (DMSO), and 0.5 μl upstream and downstream primers (10 pmol/μl), respectively. PCR cycling began with an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 64 °C for 1 min, primer elongation at 72 °C for 1 min, and finished after a final extension at 72 °C for 8 min. The second snPCR step was performed under the same conditions using 2.5 μl of the first PCR mixture as template. The primers MAPfor1 (5′-GTC GGC GTG GTC GTC TGC TGG GTT GAT-3′) and MAPrev (5′-GCG CGG CAC GGC TCT TGT TGT AGT C-3′) amplified a 587 bp fragment in the first run. The primer MAPrev also served as a reverse primer in the second step. Together with the forward primer MAPfor2 (5′-CGG GCG CAC GGT

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