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Mycobacterium avium subsp. *hominissuis* INMV 51 infection in a Red-crested turaco (*Tauraco erythrolophus*)

Nachweis einer Mycobacterium avium subsp. hominissuis INMV 51-Infektion bei einem Rotschopfturako (Tauraco erythrolophus)

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Summary

A four years old Red-crested turaco (*Tauraco erythrolophus*) was found apathetic sitting on the ground of the aviary and died during the clinical examination. Necropsy of the emaciated bird revealed multiple granulomas within the body cavity and inner organs. Histopathological examinations of these granulomas showed epitheloid macrophages and central necrosis demarcated by giant cells. Using Ziehl-Neelsen staining, massive accumulations of acid-fast bacteria were found within the lesions and the preliminary diagnosis "avian tuberculosis" was made. This infectious disease is mainly caused by *Mycobacterium avium* subsp. *avium (Maa)* or *M. genavense (Mg)*. However, microbiological culture followed by PCR revealed the occurrence of *Mycobacterium avium* subsp. *hominissuis (Mah)*. The isolate was assigned to INMV profile 51, which has been described in Asia and Europe mainly in humans, as well as in pigs and cattle until now. When infected birds have intensive contact with humans, a risk assessment must be carried out based on the identity of the causative pathogen. For this purpose, the determination of the species and if applicable the subspecies is mandatory and an additional molecular typing, e.g. by INMV profiling, helpful.

Keywords: *Mycobacterium avium* subsp. *hominissuis*, Red-crested turaco, *Tauraco erythrolophus*, zoo, avian tuberculosis

Zusammenfassung

Ein vier Jahre alter Rotschopfturako (syn. Rothaubenturako, *Tauraco erythrolophus*) wurde apathisch auf dem Boden der Voliere sitzend aufgefunden und verstarb während der klinischen Untersuchung. Bei der Sektion des abgemagerten Vogels wurden multiple Granulome in der Körperhöhle und den inneren Organen festgestellt. Histopathologische Untersuchungen der Granulome zeigten epitheloide Makrophagen und zentrale Nekrosen, die durch Riesenzellen abgegrenzt waren. Mittels Ziehl-Neelsen-Färbung wurden massive Anhäufungen von säurefesten Bakterien in den Läsionen gefunden und die vorläufige Diagnose „Geflügeltuberkulose" gestellt, eine Infektionskrankheit, die hauptsächlich durch *Mycobacterium avium* subsp. *avium (Maa)* oder *M. genavense (Mg)* verursacht wird. Die mikrobiologische Kultur und PCR ergaben im vorliegenden Fall aber das atypische Vorkommen von *Mycobacterium avium* subsp. *hominissuis (Mah)* bei dem Turako. Das Isolat wurde dem INMV-Profil 51 zugeordnet, das bisher vor allem in Asien und Europa beim Menschen, aber auch bei Schweinen und Rindern beschrieben wurde. Wenn infizierte Vögel intensiven Kontakt zu Menschen haben, muss eine Risikobewertung auf der Grundlage der Identität des ursächlichen Pathogens durchgeführt werden. Hierfür sind die Bestimmung der Spezies und ggf. Subspezies obligatorisch und eine molekulare Feintypisierung, z. B. mittels INMV-Typisierung, hilfreich.

Schlüsselwörter: *Mycobacterium avium* subsp. *hominissuis*, Rotschopfturako, *Tauraco erythrolophus*, Zoo, aviäre Tuberkulose

Introduction

Red-crested turacos are middle-sized birds of the family *Musophagidae*. They are endemic to Angola's forests where these territorial, frugivorous birds live in trees. They occur in smaller groups or pairs and live mostly monogamously. Red-crested turacos can reach an age of five to nine years in the wild and in captivity up to 30 years (Tocidlowski 2012). Because of their colourful plumage, turacos are often kept in zoos and enjoy great popularity (Peat 2017). According to the IUCN Red List of Threatened Species the wildlife population density is decreasing, but the status is still "least concern" (BirdLife International 2016).

Mycobacteria are aerobic growing rod-shaped bacteria, which are characterized by a waxy mycolic acid lipid layer in the outer cell membrane that mediates the acid-fast phenotype in different staining methods (e.g., Ziehl-Neelsen, Kinyoun, Fite-Faraco). Compared to other bacteria, most *Mycobacterium (M.)* species grow very slowly in vitro, so that cultural detection can take several months. Only very few members of the genus are obligate pathogens for humans, in particular the members of the *M. tuberculosis* complex (MTC) and some non-tuberculous mycobacteria (NTM), e.g. *M. ulcerans* or *M. leprae*, while most NTM can be found ubiquitous in the environment, e.g., in soil and water, and are facultative pathogenic for their respective hosts.

In captive birds and poultry avian tuberculosis (avTB) is a chronic infectious disease that occurs worldwide (Dvorska et al. 2007). The clinical signs remain unspecific in most cases and may include emaciation, apathy, lameness, ruffled feathers and/or diarrhoea. Nevertheless, also cases without any premortal clinical signs are described (Tell et al. 2001). Contrary to what the name "tuberculosis" suggests, the disease is caused by NTM. In poultry avTB is mainly caused by *M. avium* subsp. *avium* (*Maa*), whereas pet birds are mostly infected by *M. genavense (Mg)* (Manarolla et al. 2009). Beside the previous mentioned two major pathogens of avTB, other NTMs (e.g., *M. fortuitum*, *M. intracellulare*, *M. scrofulaceum*, *M. gordonae*, *M. nonchromogenicum*, *M. celatum*, *M. intermedium*) can cause identical pathological lesions in affected birds (Bertelsen et al. 2006, Hoop et al. 1996, Kik et al. 2010, Pfeiffer et al. 2017). Rarely infections with members of the MTC, e.g. *M. tuberculosis* or *M. bovis*, are reported in birds (Hoop et al. 1996, Peters et al. 2007, Sanchez et al. 2016, Steinmetz et al. 2006).

In the early '90s *Mg* was first detected in immunodeficient humans, especially AIDS patients, and in pet birds (Böttger et al. 1993, Hoop et al. 1993). The culture of *Mg* is difficult, as it demands high standards to the media (supplementation of mycobactin J), the incubation atmosphere (5% CO_2, 42°C) and needs long incubation periods (up to several months) (Böttger et al. 1993). Therefore, molecular methods like polymerase chain reaction (PCR) are of high importance in diagnostic workflows. Several protocols are published (Chevrier et al. 1999, Mendenhall et al. 2000, Tell et al. 2003). Mainly pet birds, such as amazon parrots, zebra finches, budgerigars or other psittacids (Ferrer et al. 1997, Gomez et al. 2011, Hoop et al. 1993), but also free-living birds may be infected by *Mg* (Patino et al. 2018). Besides various avian species and humans, *Mg* was found in several other mammalians, e.g. horses, ferrets, squirrels or chinchillas, or even in reptiles, e.g. snakes (Bottcher et al. 2016, De Lorenzi et al. 2018, Huynh et al. 2014, Theuss et al. 2010, Ullmann et al. 2016).

According to genetic examinations, *M. avium* subsp. bacteria belong to a complex comprising 12 species (van Ingen et al. 2018). Nevertheless, in the strict sense the *M. avium* complex (MAC) focussed in the past on the four *M. avium* subspecies *avium*, *silvaticum* (*Mas*), *hominissuis* (*Mah*) and *paratuberculosis* (*Map*), as well as *M. intracellulare* (*Mi*) (Biet et al. 2005, Turenne et al. 2007). Thereof, *Maa* and *Mas* are mainly found in avians, *Mah* and *Mi* in a variety of host species and environmental samples, whereas *Map* causes the so-called Johne's disease in ruminants (Turenne et al. 2007). The MAC members can be differentiated by their dependence of the growth factor mycobactin J, mandatory for in vitro growth of *Map* and *Mas*, and the serological reactivity in serovars 1-3 (*Maa*), serovars 4-6, 8-11 and 21 (*Mah*) and serovars 7, 12-20 and 22-28 (*Mi*) (Wayne et al. 1993). Today, the presence or absence of specific insertion sequences (IS) is tested by different PCRs and allows a much faster assignment to the respective *M. avium* subspecies: IS*1311* (present in all *M. avium* subsp.), IS*1245* (present in *Maa*, *Mas*, *Mah*), IS*901*/IS*902* (present in *Maa*, *Mas*) and IS*900* (present in *Map*) (Turenne et al. 2007).

Single cases of mycobacterial infections are described in turacos from zoological gardens in the U.S.A., with only unspecific clinical signs such as poor body condition or soft subcutaneous swellings in the birds (Brannian 1993, Stamper et al. 1998, Tocidlowski 2012, Wilson and Carpenter 1994). Thereby, the frequency with which mycobacterial infections are claimed to be responsible for the death of individuals in the turaco populations varied between the zoos from 3% to 66%, indicating that turacos are not particularly susceptible to mycobacteria (Stamper et al. 1998, Tocidlowski 2012). The diagnosis avian tuberculosis was mainly based on gross and histopathological findings and only in individual cases confirmed by mycobacterial culture, subsequently resulting in the species diagnosis MAC. In the remaining cases, a mycobacterial species more difficult to cultivate, e.g. *Mg*, may have been the causative agent, likewise it was shown in one Great Go-Away-Bird, also a member of the family *Musophagidae* (Pfeiffer et al. 2017, Stamper et al. 1998, Wilson and Carpenter 1994).

The current case report describes the occurrence of avian tuberculosis in a Red-crested turaco, atypically caused by the mycobacterial subspecies *Mah*.

Case description

Animal

A four years old, female, Red-crested turaco (*Tauraco erythrolophus*) was found apathetic sitting on the ground of its enclosure and died during the clinical examination. The bird hatched in 2016 at a zoological institution in Great Britain, which was approved according to the Council Directive 92/65/EEC for the trade of animals within the EU, and imported at the end of the year 2018 into the Berlin Zoological Garden. There it was housed in a subunit of an aviary called the "Fasanerie" together with its male partner, 30 Java sparrows (*Lonchura oryzivora*), two Harlequin quails (*Coturnix delegorguei*), two Golden-breasted starlings (*Lamprotornis regius*), two Spotted thick-knees (*Burhinus capensis*),

TABLE 1: *Detection of mycobacteria in necropsies of birds deceased from avian tuberculosis. The birds lived in the zoological garden and died in the years between 2014 and 2020.*

Isolation date	Bird species and order			Habitat name and housing conditions	Clinical signs	Detected mycobacteria
Jun 2014	Carmine bee-eater	*Merops nubicus*	Coraciformes	Bird house "Afrikaloop" inside/outside	Emaciation, weakness	*Maa*
Dec 2015	Hoopoe	*Upupa epops*	Bucerotiformes	Bird house "Afrikaloop" inside/outside	Sudden death	*Maa*
Apr 2017	Sarus crane	*Antigone antigone*	Gruiformes	"Fasanerie" outside	Killed by a Red fox	*Maa*
May 2017	African silverbill	*Euodice cantans*	Passeriformes	Bird house "Afrikaloop" inside/outside	Sudden death	*Mg*
May 2017	Hoopoe	*Upupa epops*	Bucerotiformes	Bird house "Afrikaloop" inside/outside	Sudden death	*Mg*
Aug 2017	Spotted thick-knee, spotted dikkop	*Burhinus capensis*	Charadriiformes	Bird house "Afrikaloop" inside/outside	Emaciation, dyspnea	*Mg*
Oct 2017	Inca tern	*Larosterna inca*	Charadriiformes	"Strandvogelvoliere" outside	Sudden death	*Maa*
Nov 2017	Blue-throated piping guan	*Pipile cumanensis*	Galliformes	"Fasanerie" inside/outside	Sudden death	*Maa*
Mar 2018	Greater flamingo	*Phoenicopterus roseus*	Phoenicopteriformes	"Fasanerie" and "Flamingohaus" inside/outside	Emaciation, weakness	*Maa*
Nov 2018	Cinnamon teal	*Spatula cyanoptera*	Anseriformes	"Fasanerie" inside/outside	Sudden death	*Maa*
Oct 2019	Green-backed twinspot	*Mandingoa nitidula*	Passeriformes	Bird house "Afrikaloop" inside/outside	Sudden death	*Mg*
Nov 2019	Green-backed twinspot	*Mandingoa nitidula*	Passeriformes	Bird house "Afrikaloop" inside/outside	Sudden death	*Mg*
Dec 2019	Orange-bellied fruit dove	*Ptilinopus iozonus*	Columbiformes	Bird house "Afrikaloop" inside/outside	Sudden death	*Mg*
Jun 2020	Sharp winged teal	*Anas flavirostris oxyptera*	Anseriformes	"Sumpfvogelvoliere" outside	Sudden death	*Maa*
May 2020	Red-crested turaco	*Tauraco erythrolophus*	Musophagiformes	"Fasanerie" inside/outside	Emaciation, weakness	*Mah*

Maa = Mycobacterium avium subsp. *avium*; *Mah = Mycobacterium avium* subsp. *hominissuis*; *Mg = Mycobacterium genavense*

and one Malagasy turtle dove (*Nesoenas picturata*). The birds are kept under conditions allowing inside/outside access without contact to visitors. They have free access to food prepared by the zookeepers consisting of apple, pear, orange, grapes, cherries, cooked corn, tomato, cucumber, bell pepper, mealworms and bird minerals (Salvana Tiernahrung, Kl. O.-Sparrieshoop) and water. The aviary has a natural ground allowing exposure to the soil of the enclosure. Direct contact with birds from the other subunits of the "Fasanerie" or other aviaries of the zoo is impossible. However, indirect contact via the zookeepers or equipment used for maintenance of the enclosures can´t be ruled out. In addition, contact with the excretions of wild birds and rodents passing through the mesh fence of the subunits may happen. All living birds are currently without clinical signs. Birds of various other avian species are generally kept in caged aviaries in the zoo (Table 1), except for Sarus cranes (*Antigone antigone)*, which have access to a fenced green land enclosure, allowing contact with wild birds and mammals. At the Berlin Zoological Garden, avTB caused by *Maa* and *Mg* but not by *Mah* has repeatedly been diagnosed in various subunits of the "Fasanerie" and the other aviaries in the past (Table 1). However, the turaco represents the first case of avTB in its subunit and the first case of *Mah* associated avTB in this zoo.

Pathologic-anatomic and histologic examination

Necropsy of the emaciated bird revealed multiple granulomas of up to 2 cm in diameter within the body cavity (Figure 1). In addition, miliary confluating granulomas were present in various inner organs especially spleen, liver and lung with destruction of approximately 80% of the lung parenchyma. Massive amounts of acid-fast bacteria were detected in Ziehl-Neelsen-stained imprint smear preparations of the granulomas. Histopathologically, the lesions were characterized by an infiltration of epitheloid macrophages and the formation of granulomas with central necrosis demarcated by giant cells (Figure 2a). Again, acid-fast bacteria were found in the cytoplasm of the epitheloid macrophages, as well as extracellularly within the necrotic areas by Ziehl-Neelsen staining (Figure 2b). Granulomas were detected neither macroscopically within the whole digestive tract nor in routine histopathological specimens from proventriculus, ventriculus and jejunum.

Microbiological examinations

Concurrent bacterial, mycotic and parasitic infections were ruled out by routine diagnostic workup including bacteriology of heart, lung, liver and intestine using standard culture media, mycology of the lung and coproscopical examination of intestinal contents for the detection of developmental stages of endoparasites. In addition, infections with chlamydiae, Influenza A virus, Avian orthoavulavirus 1, West Nile virus and Usutu virus were ruled out by PCR and virus culture using standard protocols approved by the German veterinarian National Reference Laboratories (Friedrich-Loeffler-Institut 2019).

Identification and characterization of the mycobacterial isolate

Ziehl-Neelsen-staining of putative mycobacterial bacterial culture grown on Stonebrink agar slants showed the presence of acid-fast rods. From the colony material DNA was extracted using a heat and ultra-sonic lysis of

FIGURE 1: *Red-crested turaco (Tauraco erythrolophus): tuberculous granuloma within the body cavity (Photo: Christoph Schulze).*

the bacterial cells. For species and subspecies identification the DNA was submitted to PCR analysis targeting *Mycobacterium* sp.-specific 16S rRNA, *M. avium* subsp.-specific insertion sequences (*IS*), which included IS*1245*, IS*900*, and IS*901* (Friedrich-Loeffler-Institut 2019), as well as a *Mg*-specific DNA fragment (Chevrier et al. 1999). Thereby, the isolate was identified to be *Mah*, as it was positive for mycobacterial 16S rRNA and *IS*1245, but negative for *IS*901, *IS*900 and the *Mg*-specific DNA fragment.

The *Mah* isolate was further characterized by genotyping using the Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeat (MIRU-VNTR) analysis, encompassing the loci VNTR 292, X3, 25, 47, 3, 7, 10, and 32, following the protocol of Thibault et al. (2007) and the MAC-INMV-SSR database (http://mac-inmv.tours.inra.fr) (Cochard et al. 2020). The identified pattern of repetitive counts (2-4-2-2-1-1-2-8) matched the profile INMV 51.

Discussion

In captive birds and poultry avTB is a chronic infectious disease that occurs worldwide (Dvorska et al. 2007). In commercial holdings the disease is of decreasing importance, but in the last years poultry in smaller backyard farms and pet birds in private households seem to be affected more often (Tell et al. 2001). In addition, avTB is a long known problem in various avian species from zoological holdings (Keymer et al. 1982, Krause et al. 2015, Montali et al. 1976, Portaels et al. 1996, Witte et al. 2008). Ante mortem diagnosis in birds, based on tuberculin tests, serology, haematology or radiography, is often not conclusive to identify individual animals and, especially the immunological assays, more suitable at herd level (Montali et al. 1976, O.I.E. 2018). The tuberculin test, performed by application of purified protein derivate (PPD) of *Maa* in the skin of the wattle, comb or the propatagium, and assessed 48 h later for inflammation, is unreliable in several avian species and mainly used for pre-movement testing of domestic fowl (O.I.E. 2018, Tell et al. 2001). Consequently, diagnosis of avTB in individuals is often based on the pathological findings during necropsies and detection of the pathogen by culture and/or molecular diagnostics, e.g., polymerase chain reaction (PCR). Even though the pathological findings can be very diverse, the presence of granulomas or accumulations of epitheloid macrophages in single or multiple organs in combination with acid-fast rods within the lesions are pathognomonic. Most often multiple organs, such as liver, lung, spleen and/or intestine are affected in birds suffering from avTB, as in the case described here (Hoop et al. 1993, Tell et al. 2001, Witte et al. 2008). However, disseminated forms without visible granulomas at necropsy or cases with affection of only single organs (e.g., eye-lids, skin or bones) are known (Montali et al. 1976, Portaels et al. 1996).

Data sets from zoos, where dead birds are often routinely necropsied, show that repeated cases of avTB can occur in a certain flock, but often only single individuals are affected (Portaels et al. 1996, Witte et al. 2008). At the Berlin Zoo, 15 animals with avTB, in

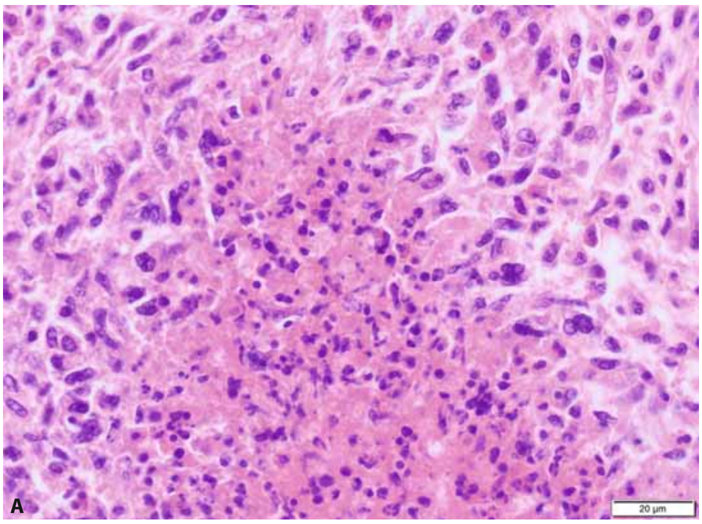

FIGURE 2A: *Red-crested turaco (Tauraco erythrolophus): tuberculous granuloma with central necrosis surrounded by giant cells and epitheloid macrophages; Hematoxylin and Eosin stain (Photo: Christoph Schulze).*

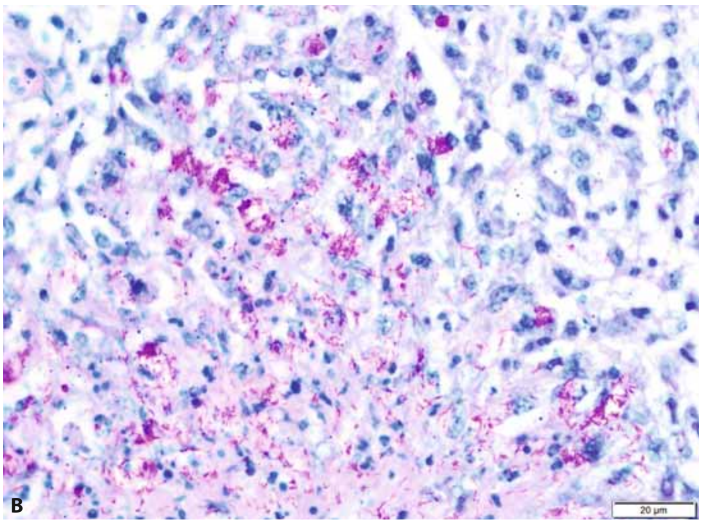

FIGURE 2B: *Red-crested turaco (Tauraco erythrolophus): numerous acid-fast bacilli within the tuberculous granuloma; consecutive histological section to Figure 2a; Ziehl-Neelsen stain (Photo: Christoph Schulze).*

which the mycobacterial species was identified, were diagnosed from 2014 onwards, affecting five different aviaries. Overall, in six birds *Mg* was the causative agent, in eight birds *Maa*, and in the case described here *Mah*. Besides a study of Pfeiffer et al. (2017) at the San Diego Zoo that identified *Mah* in 11.4 % of 105 birds with avTB by whole genome sequencing, only single descriptions of *Mah* infections in birds exist, e.g. in a blue-fronted Amazon parrot (Shitaye et al. 2009) or in a painted quail (Morita et al. 1999). The *Mah* isolates from the quail were subsequently used to infect chickens experimentally with 3-5 x 10^7 cfu/dose (Morita et al. 1999). The infection resulted in dramatic weight-loss and lethargy in all immunocompetent chicken within 44 days, proving the pathogenicity of *Mah* for birds.

The MIRU-VNTR analysis of the isolate from the turaco assigned it to profile INMV 51. This profile was until now only described in isolates from humans, cattle and pigs in Europe and Asia (Iwamoto et al. 2012, Radomski et al. 2010, Scherrer et al. 2018, Starkova et al. 2013). In fact, INMV 51 was the predominant profile within the human isolates tested in a French study (Thibault et al. 2007), demonstrating the zoonotic potential of the *Mah* type detected in the turaco representing the first report of INMV 51 in an avian host. The presence of INMV 51 in several studies on different continents might be a hint for a broad distribution of this *Mah* type in the environment or perhaps in wild birds, which may act as transport vehicles over long distances.

The origins of infection in most cases of avTB are usually not known, but the environment and/or other animals are the most likely sources. Members of the MAC are known to survive for long time periods in the environment, especially in soil and water (Lahiri et al. 2014). Furthermore, the environment and tap water are suspected reservoirs of *Mg* (Portaels et al. 1996). The aviaries in the Berlin Zoo have soil floors to promote the natural behaviour of the birds, and all affected holdings contain inside and outside areas, where contact with droppings of wild birds or rodents is possible. This means that by keeping the birds under near natural conditions, which is urged by reasons of animal welfare, they have a risk to come in contact with environmental pathogens. *Maa*, *Mah* and *Mg* have been detected in faecal samples or gastrointestinal tract of several bird species (Bercovier and Vincent 2001, Morita et al. 1999, Patino et al. 2018, Shitaye et al. 2009, 2010, Smit et al. 1987) and rodents (*Maa*) (Fischer et al. 2000). Therefore, the faecal excretion of NTM is an important source of infection for other birds, animals or humans directly or indirectly through contamination of the environment, food or water. Already Witte et al. (2008) showed that birds living together with individuals suffering from intestinal mycobacteriosis have a higher infection risk. More specifically, *Mah* was shown to be present in the intestine of a painted quail and a blue-fronted Amazon parrot as well as in the feces of experimentally infected chickens (Morita et al. 1999, Shitaye et al. 2009) and the introduction of new birds into an existing flock was a risk factor to develop avTB in zoo birds (Witte et al. 2008). In this context, comparable to Trojan horses, seemingly healthy birds can be mycobacterial carriers and may develop clinical disease long time after their import or infect other birds in the flock. In addition, stress that occurs when new animals are introduced into an existing flock can trigger an immunosuppression and an already present pathogen can cause clinical disease. Since *Mah* is a nearly ubiquitous pathogen that potential hosts have constantly to deal with, a lowered immune response is often a prerequisite for a clinically apparent infection. Summarizing, the exacerbation of a subclinical infection with *Mah* was considered to be the most likely explanation for the case of *Mah* related avTB presented here, although, the definitive source of the infection cannot be determined anymore.

An interesting observation in the zoo is that *Mg* infections were only detected in birds of the "Afrika-loop" of the bird house "World of Birds", while *Maa* infections were found in all locations with cases of avTB. The concentration of *Mg* infections in only one aviary can be interpreted as an indication of an animal-to-animal transmission. This hypothesis is supported by data from whole genome analyses of *Mg* and *Maa* isolates from birds within San Diego Zoo (Pfeiffer et al. 2017), where 48 *Mg* isolates showed a significant lower genome variability among each other than the parallel tested *Mah* (n=12) and *Maa* (n=37) isolates possessed even on subspecies level. Whereas, the genetic heterogeneity of the MAC isolates suggested a repeated entry of the pathogens into the population and, accordingly, less frequent animal-to-animal transmission (Pfeiffer et al. 2017, Schrenzel et al. 2008).

Finally, avTB can be caused by different mycobacterial species and subspecies. If infected birds have intense contact to their owner or in zoological gardens with animal care takers or even visitors, a hazard assessment should be carried out based on the identification and typing of the causing pathogen. While *Maa* infections in humans are rare, *Mah* and *Mg* can be isolated from immunosuppressed and -competent humans (Cornelis et al. 2018, Martin and Schimmel 2000, Möbius et al. 2006, Tran and Han 2014). Accordingly, the zoonotic risk may be considered higher for *Mah* or *Mg* than for *Maa*.

Ethical approval

The authors assure that they have followed the general rules of good scientific practice.

Conflict of interest

The authors assure that there are no proprietary, professional or other personal interests in any product or company that could influence the content or opinions expressed in this publication.

Authors contribution

Concept: SAB, CS.
Data collection: AO, CS, NW, SB, SAB, PM.
Analysis and interpretation: AO, CS, SB, NW, SAB, PM.
Draft manuscript: SAB.
Critical revision: CS, PM, AO.
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