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Pulmonary mycobacteriosis of sitatunga antelope caused by *M. avium* ssp. *hominissuis*

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Abstract

Introduction and Objective. Mycobacteriosis are diseases caused by acid-fast mycobacteria other than *M. leprae* and tuberculous mycobacteria. Animal mycobacteriosis is often caused by *M. avium* ssp. *hominissuis*. Many species of animals are susceptible to infection with this bacterium, even those kept in Zoological Gardens. The aim of the study was to determine the species of bacterium responsible for causing the disease in the tested animals.

Materials and method. Tissue samples of two male sitatunga antelopes (Tragelaphus spekii) were analyzed. Lymph node and lung samples were subjected to anatomical examination and Ziehl-Neelsen staining. Real-time PCR was performed to confirm or rule out tuberculosis mycobacteria infection. In order to isolate the bacterial strain, tissue samples were inoculated on both solid and liquid media. HainLifescience CM tests, mass spectrometry and New Generation Sequencing were used to determine the mycobacterial species.

Results. Results showed that atypical mycobacteria are responsible for the antelope disease. The results of the HainLifescience CM test and mass spectrometry indicated that the mycobacterium responsible for causing mycobacteriosis was *M. avium*. New Generation Sequencing helped to identified a subspecies that was *M. avium* ssp. *hominissuis*.

Conclusions. The sitatunga antelope is an animal susceptible to infection by *M. avium* ssp. *hominissuis*. Considering the wide range of hosts and the easiness of interspecies transmission of the pathogen, as well as its zoonotic nature, the mycobacteriosis induced by this microorganism should not be underestimated.

Key words

zoonosis, mycobacteriosis, *M.avium* ssp. *hominissuis*, sitatunga antelope

INTRODUCTION

Mycobacteriosis are diseases caused by acid-fast mycobacteria other than *M. leprae* and mycobacteria from the *Mycobacterium Tuberculosis* Complex group (MTBC). These mycobacteria are defined as non-tuberculous mycobacteria (NTM), mycobacteria other than tuberculosis (MOTT), or atypical. To-date, over 200 species of atypical mycobacteria have been described in the literature, of which about 30 have the ability to infect humans and animals. Among these microorganisms is *M. avium* ssp. *hominissuis* (MAH), belonging to the *Mycobacterium avium* Complex group (MAC) [1, 2].

The MAC members are non-spore-forming, gram-positive, non-chromogenic, acid-fast mycobacteria. MAC presence has been recorded in Asia, North and South America and Europe. According to literature sources, bacteria belonging to the *Mycobacterium avium* group have been isolated from soil, aerosol-forming water, bathrooms, house dust, birds, livestock, hot water systems and cigarette ingredients. The group includes several species of atypical mycobacteria, the differentiation of which is possible only with the use of molecular methods [3]. These bacteria belong to the III group according to Runyon's classification, and their growth optimum temperature range is wide, ranging from 28 °C – 38.5 °C. The optimum temperature for *M. avium* culture is 34.5 °C, but most of these bacteria can survive even at 49 °C [4].

M. avium, commonly known as the avian mycobacterium, consists of four subspecies: *M. avium* ssp. *avium* (MAA), *M. avium* ssp. *silvaticum* (MAS), *M. avium* ssp. *paratuberculosis* (MAP) and *M. avium* ssp. *hominissuis*. It was first isolated in 1933 from chicken tissues with tuberculosis-like lesions, and cases of the disease in humans were identified a decade later [5]. Among the subspecies of *M. avium*, *M. avium* ssp. *hominissuis* is the most widespread. Turenne et al. proved that MAH has the highest level of genomic heterogeneity within MAC; they also concluded that MAP, MAA and MAS evolved independently of MAH [6].

MAH is an environmental bacterium, often found in water, soil, dust or straw, and its main hosts are humans and pigs. This mycobacterium is also an opportunistic pathogen for other mammals, including cattle, from which it is one of the most frequently isolated NTM. This zoonotic pathogen in humans, especially in developed countries, is considered as a common cause of respiratory diseases that may have a severe course [6, 7]. It affects other organs much less frequently, causing inflammation of the lymph nodes, skin and soft tissues, and it may be disseminated in people with impaired immunity [8]. People infected with HIV, who are not properly treated, are particularly at risk of infection. According to studies, the incidence of MAC mycobacteriosis ranges from 20–40% in this group of people [9].

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In animals, the disease is most often manifested by the formation of mineralized granulomas in the lungs or lymph nodes located in the chest, and purulent inflammation of these organs, which is also characteristic of tuberculosis caused by *M. bovis* and *M. caprae* [10]. However, tuberculous-like lesions can also be caused by bacteria outside the genus *Mycobacterium,* such as *Rhodococcus equi*, *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp. or *Trueperella pyogenes*. Therefore, many additional diagnostic tests are often required to establish the cause of infection [11].

OBJECTIVE

The aim of the study was to determine the species of bacterium responsible for causing the disease in the tested animals. Due to the clinical symptoms of the respiratory system, as well as the type of the pulmonary lesions of the first tested antelope, it was decided that the samples should first undergo diagnostic tests for diseases caused by bacteria of the genus *Mycobacterium*. In the case of the second tested antelope, the research direction was determined with reference to the herd history with prior isolation of *M. avium* ssp. *hominissuis*. The obtained results may help to understand that purulent lung inflammation may be the result of infection with bacteria of the genus *Mycobacterium,* and that some atypical mycobacteria are capable of causing a disease resembling tuberculosis.

MATERIAL AND METHODS

Tissue samples of two male sitatunga antelopes (*Tragelaphus spekii*) were analyzed. The animals were kept in the Zoological Garden in Gdańsk, Poland, and belonged to the same herd. Their deaths occurred 1.5 years apart. The first antelope, 1.5 years old (male A), died for unknown reasons, showing clinical signs of respiratory disease the day before his death. The second, a 7-year-old animal (male B) died 3 months after the onset of disease symptoms, which were characterized by neck twisting. An anatomopathological examination was performed to visually assess the tissues. Subsequently, microscopic slides were prepared from the tissues with visible lesions and stained with the Ziehl-Neelsen method.

In the culture study, approximately 3g of lung and mediastinal lymph node tissue samples were cut into small pieces and placed in sterile filter bags (Interscience, Schaffhausen, Switzerland). 15 ml of 5% oxalic acid was added to the sample placed in the bag and homogenized for 3 minutes. The filtrate was then poured into a Falcon tube and incubated for 20 minutes at 37 °C. After incubation, the supernatant was centrifuged for 10 minutes at 3,500 × g, after which the supernatant was removed and the pellet washed twice in saline and centrifuged again for 10 minutes at 3,500 × g. The obtained material was plated on 4 Petragniani and 4 Stonebrink media and incubated for 6 weeks at 37 °C with weekly readings. At the same time, the samples were tested in an automated liquid culture system – Mycobacteria Growth Indicator Tube (MGIT). All samples were prepared using the MycoPrep kit (Becton Dickinson, Frankin Lakes, USA) used to decontaminate samples containing mycobacteria, according to the manufacturer's instructions.

The prepared samples were inoculated into a liquid medium with 7H9 agar and incubated at 37 °C. In order to fast exclude or confirm the infection of the animal with tuberculosis mycobacteria, real-time PCR was performed (Tab. 1; Tab. 2). DNA isolation was performed using the DNeasy blood & tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The species identification of the strain was based on the performance of the Hain Lifescience CM test (Hain Lifescience, Nehren, Germany), in accordance with the manufacturer's instructions and MALDI-TOF mass spectrometry of the Bruker system (Bruker, Billerica, USA). In mass spektrometry, the extraction method with formic acid and acetonitrile for mycobacteria was used in accordance with the manufacturer's instructions. The subspecies were determined using next generation sequencing (NGS) and analysis of IS901, IS1245, IS311 genes sequence.

Table 1. Sequences of primers and probe used in the study

Probe/Primer	Sequence (5′ -> 3′)*
Forward primer	GGT AGC AGA CCT CAC CTA TGT GT
Reverse primer	AGG CGT CGG TGA CAA AGG
Probe	FAM-CAC GTA GGC GAA CCC- MGB NFQ

*Sequences of primers and probe were obtained from the European Union Reference Laboratory for Bovine Tuberculosis in Madrid, Spain.

Table 2. PCR conditions

Temperature	Time	No. of cycles
95 °C	5 min.	1
95 °C	15 s	45
60 °C	1 min.	

RESULTS

In the anatomopathological examination of the first antelope, pathological lesions, characteristic for bovine tuberculosis were observed, including numerous nodules located within the lungs (Fig. 1). However, in the second animal, purulent pneumonia was found without the presence of nodules (Fig. 2). In the Ziehl-Neelsen stained specimens, numerous mycobacteria were observed in each field of view for male A samples, while only single ones for male B. All results

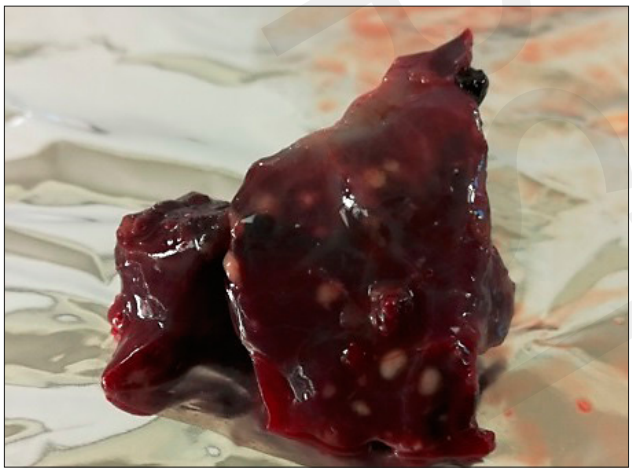

Figure 1. Tuberculosis-like lesions in the lungs of male A

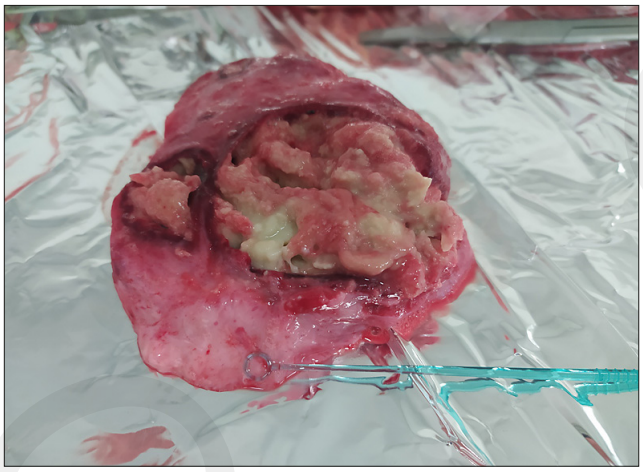

Figure 2. Purulent pneumonia of male B

obtained by performing real-time PCR were negative and clearly indicated that MTBC members were not responsible for the animals disease. The interpretation of real-time PCR results is presented in Table 3.

Table 3. Interpretation of the real- time PCR results

Result	Signal on FAM channel (pathogen)	Signal on HEX channel (internal control)
Positive	X	X
	X	-
Negative	-	X
Unsettled	-	-

In the culture study, visible bacterial growth was obtained on Stonebrink and Petragniani medium in the second week of incubation. In the case of male A, growth was abundant, while in the case of male B, only single colonies grew on the media. The results obtained by using the MGIT system confirmed the presence of *Mycobacterium* in all cases. The Hain Lifescience CM test and MALDI-TOF mass spectrometry showed that the species of isolated bacteria was *M. avium*. Detailed studies of the nucleotide DNA sequence of both strains, performed in order to identify subspecies of *M. avium,* proved that it is *M. avium* ssp. *hominissuis.*

DISCUSSION

The *Mycobacterium tuberculosis* complex (MTBC) is a group of 11 mycobacteria species. MTBC members are obligate human and animal pathogens causing tuberculosis. The exception is *Mycobacterium bovis* BCG (vaccine strains), which is attenuated in the form of *Mycobacterium bovis* [12], which very rarely causes clinical symptoms, and mainly in people with a suppressed immune system. The suppurative lymphadenitis has been noted in 0.1–10% of immunized children under 2 years of age. This disease in cattle is eliminated *ex officio,* and since 2009, Poland has the status of a country officially free from bovine tuberculosis. However, the group of non–tuberculous bacilli is represented by over 200 species. Many of these bacteria, the same as MTBC, have the ability to infect both humans and numerous animal species, which can result in serious economic losses for farmers and breeders [13]. However, the diagnostics of these diseases is much less developed and the diagnosis of mycobacteriosis is usually accidental, during routine tests for tuberculosis. Krajewska et al. described mycobacterial skin infection in domestic cat that were correctly diagnosed four months after the first clinical symptoms appeared. The cat had extensive purulent lesions and fistulas that could potentially be a source of infection for the owners and the veterinarian caring for the animal [14].

In the analyzed cases, *M. avium* ssp. *hominissuis* was responsible for the disease of sitatunga antelopes. The extent of the lung lesions of the tested animals may indicate the high sensitivity of this species to MAH infection and the transmission of the pathogen between antelopes by aerosol. The sitatunga antelope is an animal that often wades in shallow areas of water reservoirs in which the zoo was also equipped. Wild birds present in the same pond could be a vector for transmission of the pathogen from the outside environment to the zoo, and the animals were probably infected by contact with contaminated water. This may also be confirmed by the fact that the infected animals had been in the zoo for a long time, with the simultaneous lack of introducing new animals into the herd, which excludes the transmission of the germ by the introduction a sick animal into the enclosure.

Antelope mycobacteriosis caused by MAH are very rare. To-date, only one such case has been reported in the Bongo antelope (*Tragelaphus eurycerus*). Moravkova et al. (2013) [15] found MAH infection in five individuals in the zoo. Researchers have described tuberculosis-like lesions located in the lungs of each tested individual. Tissue lesions were observed in the form of nodules, similar to the case of a 1.5-year-old sitatunga – male A described in the current study. The older male B also had lung lesions, but they were manifested by purulent inflammation of this organ. The puzzle is the atypical development of the disease with clinical symptoms in the form of a twist of the neck. Perhaps this was due to a mechanical injury which weakened the animal and facilitated the infection with MAH.

Mycobacteriosis targeted at lung infection may promote disease spreading and transmission of the pathogen to other animals or humans. This is dangerous because infection of an immunocompromised person can be severe and may even lead to death [16]. In extreme cases, amfiksenosis may also occur, i.e. infection of a human being in contact with a sick animal, and then transmission of the pathogen from human to the next lower vertebrate [17]. This is of great importance for cattle, which, like the antelope, belong to the *Bovidae* family and may also be susceptible to infection with this species of *Mycobacterium*. Additionally, the infection of cattle with atypical *Mycobacterium* may make the diagnosis of tuberculosis difficult. It has been proven that mycobacteria belonging to the MAC are the cause of false-positive results of the tuberculin skin tests, resulting in the wrong decision to slaughter positively reacting individuals [18, 19].

Considering the wide range of MAH hosts and the easiness of interspecies transmission of the pathogen, as well as its zoonotic nature, the mycobacteriosis induced by this microorganism should not be underestimated. However, it should be taken into consideration that the occurrence of tuberculosis-like lesions in animal tissues will not always be associated with a disease caused by bacteria of the genus *Mycobacterium*, and an appropriate differential diagnosis

should be performed. Didkowska et. al. [20] found 49 cases of caseous limfadentitis in a *post-mortem* examination of 284 sheeps. Subsequent microbiological studies indicated infection with *Corynebacterium pseudotuberculosis* (34.7%), *Streptococcus dysgalactiae* ssp. *equisimilis* (34.7%), *Staphylococcus aureus* (8.2%), *Enterococcus* spp. (2.0%), *Trueperella pyogenes* (2.0%), and β-haemolytic strains of *Escherichia coli* (2.0%). The authors also provide examples of creating similar lesions by gram negative bacteria, such as *Pseudomonas aeruginosa* and *Moraxella* spp. *Rhodococcus equi*, which is responsible for the infection of many species of farm and wild animals, can also be the bacteria causing purulent pneumonia and granulomatous lesions. Moreover, *R. equi* is a bacterium often isolated together with *M. avium* ssp. *avium*, which may indicate that *Mycobacterium* ssp. infection could predispose farm and wild animals to *R. equi* infection [21, 22, 23].

Animals kept in Zoological Gardens or on private farms should be observed for clinical signs of respiratory disease. If a disease is suspected, the animal should be isolated from the rest of the herd and subjected to intravitally diagnostic tests. Animals in contact with individuals with mycobacterial lesions should be examined to exclude the possibility of transmission of mycobacteriosis. In the case described in the current study, the remaining sitatunga antelopes in contact with males A and B were subjected to a comparative skin tuberculin test. No animal showed a positive reaction.

CONCLUSIONS

1. The sitatunga antelope is an animal susceptible to infection by MAH.
2. Lung lesions of the antelope in the case of MAH infection may take the form of nodules indicating tuberculosis as well as purulent pneumonia.
3. NTM infections in both humans and animals are difficult to diagnose and treat.
4. The occurrence of MAH in an antelope in a zoo is a serious problem in terms of public health protection.

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