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The potential risk of international spread of *Mycobacterium bovis* associated with movement of alpacas

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Abstract

Introduction: The study highlights the transboundary nature of tuberculosis (TB) in alpacas and the failure of current ante-mortem testing protocols (the tuberculin skin and Enferplex Camelid TB tests) to identify TB-free alpaca herds and individuals for export. Our research and the available literature indicate that the alpaca (*Vicugna pacos*) is extremely susceptible to *Mycobacterium bovis* infection, and that testing periodicity fails to take into account that animals do not manifest disease symptoms for a long time. The skin test failed to identify *Mycobacterium bovis* infection in two alpacas prior to their movement from the UK to Poland. The animals were purchased by a breeding centre in Poland, and were then shown at an international animal exhibition. The last owner of the alpacas before their deaths from TB bought the infected animals unwittingly in order to run rehabilitation activities with disabled children on his farm. **Material and Methods:** Thoracic lymph node, lung and liver tissue samples obtained at necropsy were examined histopathologically after Ziehl–Neelsen staining. Tissue samples were homogenised and mycobacteria present there were cultured on Stonebrink's medium during a 6-week incubation. A commercial test using polymorphism of the chromosomal direct repeat region provided species identification and additional identification was by spacer oligonucleotide typing and mycobacteria interspersed repetitive unit–variable number tandem repeat analysis with a gel electrophoresis protocol. **Results:** The microbiological examination confirmed multiorgan TB caused by the SB0666 spoligotype of *Mycobacterium bovis*. **Conclusion:** Due to the suboptimal performance of current diagnostic tests for TB in alpacas, there is a risk that infected animals may be moved unwittingly. A risk of TB spread associated with the international movement of alpacas is implied by this study.

Keywords: alpaca, tuberculin skin test, Enferplex Camelid TB test, *Mycobacterium bovis*.

Introduction

Tuberculosis (TB) in mammals is caused by mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC) (27). Two of the most common species that infect animals are *Mycobacterium bovis*, found worldwide (36), and *Mycobacterium caprae*, distributed predominantly in Europe (30).

Alpacas (*Vicugna pacos*) are a relatively new species of farm animals in Poland with only little more than a decade of breeding history in the country, but it is estimated that their population already exceeds 5,000 individuals (13). Although they are bred and raised mainly for their soft and luxurious fleece, they are also used in agritourism and recreation, as well as in alpaca therapy (13, 17). These animals are susceptible to TB (16, 17), which is most commonly spread between them by the aerogenic route. Alpacas have the habit of spitting on other alpacas and people in various situations, and this expectoration is also the transmission route of

tubercle bacilli that originate from an alpaca's lungs. The infection is commonly passed between different herds through the movement of asymptomatic mycobacterial carriers. Introducing infected animals into a tuberculosis-free herd usually causes infection in other animals. Infections with *M. bovis* may go unnoticed in camelids until deaths occur in the herd, preceded by upper respiratory tract symptoms. Sudden deaths without prior symptoms of the disease have also been reported (25).

Ante-mortem diagnosis of TB in species other than cattle is difficult to perform for two main reasons, these being the challenge posed by collecting appropriate samples and the lack of reliable tests (1, 28, 33). In many European countries, including Poland, the tuberculin skin test (TST) is still considered the official screening test for bovine tuberculosis (bTB), and a negative result is required to obtain veterinary movement permits.

In Poland, the owners of alpacas infected with TB cull them on their own initiative, and do not typically subject them to thorough laboratory diagnosis. This is a significant limitation on the effective control of TB, since epidemiological information is lacking. The appearance of further tuberculosis outbreaks in alpacas in Poland is a very dangerous phenomenon that may result in the country losing its official status as being free from bTB.

In 2013, the Bovine Tuberculosis Reference Laboratory in Madrid (Spain) issued the Santé et Consommeurs/Directorate General Health and Consumers (SANCO)/7034/2013 Working Document: Diagnosis of tuberculosis in camelids, describing the ante-mortem diagnosis of tuberculosis in alpacas (7). In line with the SANCO guidelines and EU regulations, and to improve the detection of infected animals, it is recommended that the TST is accompanied by serological tests. However, the TST often has unreliable results in alpacas. Studies in naturally *M. bovis*-infected alpaca herds have shown that the TST results are often false negative (16). In this study, 100% of the tested TB alpacas failed to produce skin test reactions. Similarly, a decade earlier, Lyashchenko *et al.* (21) observed no reaction to the TST in more than 95% of tested alpacas with evidence of TB. Ante-mortem diagnosis of TB in non-bovid species, including alpacas, is challenging due to the serious limitations of the existing diagnostic methods, the lack of species-specific reagents, and the insufficient number of animals available for test development (16). For camelid diagnosis, the future seems to lie in tests based on host immune responses, such as serological and cytokine-based assays, and these seem to have utility for the detection of TB. The available literature indicates that promising results have been obtained by tests such as the immunochromatographic lateral flow rapid test (RT) VetTB Stat-Pak kit, the Dual-path platform (DPP) VetTB assay or the Multi-antigen Print Immunoassay (MAPIA) (all from Chembio Diagnostic Systems, Inc., Hauppauge, NY, USA) (16, 21, 26).

Rhodes *et al.* (26) found interferon gamma (IFN-γ) and RT and DPP antibody tests to have similar sensitivities (Se) (ranging from 57.7% to 66.7%), and that the IFN-γ test demonstrated lower specificity (Sp) (89.1%) than the antibody tests (ranging from 96.4% to 97.4%). Similar results were obtained by Krajewska-Wędzina *et al.* (16): the DPP VetTB assay correctly identified 12/20 (60% Se) alpacas infected with *M. bovis* and 19/20 (95% Sp) uninfected controls. The present study's authors (16) and Rhodes *et al.* (26) agree that greater Se could be obtained by combining two antibody tests. The performance of these tests should be further validated in field trials and the tests considered for incorporation into TB management and control strategies for camelid species (16, 21, 26).

Due to suboptimal performance of current commercial diagnostic tests for TB in alpacas, there is a risk that infected animals are moved unknowingly. This study describes an example of the risk of TB spread associated with the movement of alpacas.

Material and Methods

Animals. Two male alpacas (alpaca no. 1, aged approximately 1 year and alpaca no. 2, aged approximately 2 years) were imported to Poland in March 2018 from the UK. Confirmation of their place of origin was the presence of UK ear tags. A TST had been performed in March 2018 in the UK and had failed to identify *Mycobacterium bovis* infection in the animals prior to their movement to Poland. However, these alpacas were subsequently found to be infected with the bacterium. The alpacas were brought to the purchasing breeding centre and then shown on April 8, 2018 at the largest pet fair in Poland: the Warsaw Animal Days in Nadarzyn. The infected alpacas were unwittingly purchased there by the second owner in order to conduct activities with children with disabilities such as Down's syndrome or childhood cerebral palsy on his farm. When alpaca no. 1 died, the owner did not suspect TB. The owner is a veterinarian and he performed a necropsy himself and sent fragments of the diseased organs (the thoracic lymph node, lung and liver) to a private laboratory for histopathological examination. When he observed that TB was suspected in the comments accompanying the results, alpaca no. 2 was subjected to the Enferplex test and the result was negative. This result later transpired to be false. When alpaca no. 2 died, a necropsy was performed also on this occasion by the owner and the same tissue samples were collected for laboratory diagnosis. The methods described below were performed for alpaca no. 2 at the National Veterinary Research Institute (NVRI) in Puławy, Poland.

Histopathological examination. The histopathological examination of the samples from alpaca no 1 was performed in the ALAB Veterinary Laboratory (Warsaw, Poland). The sections were stained using the

Ziehl–Neelsen method, a typical method for acid-fast bacillus staining. The survey methodology was not available to private clients.

Histopathological examination of the samples from alpaca no. 2 was performed on tissue fixed in 10% neutral-buffered formalin and processed and embedded in paraffin blocks using a TP 1020 tissue processor (Leica Biosystems, Buffalo Grove, IL, USA) according to the manufacturer's protocol. The paraffin-embedded tissues were then cut into 4 µm-thick sections with a microtome (Microm, Walldorf, Germany). The tissue sections were stained using the haematoxylin and eosin method (22) and examined using an Axiolab light microscope (Zeiss, Oberkochen, Germany) for the presence of histopathological lesions. For the detection of acid-fast bacteria, the sections were stained using an acid-fast bacillus (AFB)-Color Staining Kit (Merck, Darmstadt, Germany) based on the Ziehl–Neelsen staining method, following the manufacturer's protocol.

Mycobacterial culture and strain identification. Lung, liver and lymph node samples were cleared of fat and cut into small pieces. They were then placed in special sterile bags with a filter (Interscience, Schaffhausen, Switzerland) and processed in a laboratory homogeniser (Interscience, Schaffhausen, Switzerland) for 3 min. After homogenisation, the tissues were flooded with a 5% solution of oxalic acid in twice the volume of the sample and the tissue and acid were thoroughly mixed. The supernatant was pipetted into a separate Falcon 50 mL tube (Bionovo, Legnica, Poland) and set aside in the incubator for 20 min at 37°C. The sample was then centrifuged for 10 min at 3500 × g. The supernatant was removed, and the sediment was washed twice with sterile saline. The resulting sediment was inoculated onto Stonebrink's solid medium (own production) and incubated at 37°C for 6 weeks. All mycobacterial media and reagents were prepared by the NVRI in the Culture Media Unit (15). The tissue samples were subjected to these microbiological procedures in accordance with the instructions from the Chief Veterinary Officer of Poland.

The species identification of the isolates was performed with a commercial GenoType MTBC Test (Hain Lifescience, Nehren, Germany) according to the manufacturer's protocol (29).

The spoligotyping test was achieved using spacer oligonucleotide typing with a commercial kit (Isogen Bioscience, Maarssen, the Netherlands) (10). This method detects polymorphism of the chromosomal direct repeat region present only in the genome of the *M. tuberculosis* complex (12).

The mycobacterial interspersed repetitive unit–variable number of tandem repeats (MIRU-VNTR) analysis of DNA from mycobacterial isolates from tissue samples targeted the 15 loci with the highest variability in the MTBC complex. The pattern was created based on loci analysed in the following order: MIRU-4, MIRU-10, MIRU-16, MIRU-26, MIRU-31, MIRU-40, VNTR 424, VNTR 577, VNTR 2165, VNTR 2401, VNTR 3690, VNTR 4165, VNTR 2163B, VNTR 1955, VNTRB 4052. The result was visualised by gel electrophoresis (2.5% agarose gel, large gel, 65V, 5 h) and interpreted as previously described (32).

Serological assays. The Enferplex Camelid TB test was carried out by SureFarm (Evershot, UK). Seven antigens were used to detect antibodies to MTBC antigens, including four antigens commonly used for serological TB tests in animals, *i.e.* ESAT-6, CFP-10, MPB83 and MPB70 and three proprietary antigens (Enfer Scientific, Naas, Ireland) designated as A, B, and C (Enfer ID TB2108180069). The described Enferplex test carried out on alpaca no. 2 was not affected by a booster effect of any prior skin test.

Results

The first male alpaca (alpaca no. 1) purchased at the fair died in August 2018 with emaciation but no other obvious clinical signs. During necropsy, lesions were observed in multiple organs, including the lungs, liver, spleen, and mesenteric lymph nodes. The lung lesions were consistent with granulomatous pneumonia, with individual granulomas measuring 3–10mm in diameter (as described by the owner, a veterinarian).

Histopathological examination by the ALAB Laboratory in Warsaw revealed granulomatous inflammation characterised by variably sized areas of lytic necrosis with peripheral infiltration of activated macrophages in all the examined tissues. The presence of acid-fast mycobacteria was described in sections of lung lesions, which suggested the cause of pneumonia was mycobacterial infection.

Another male alpaca present on the farm (alpaca no. 2), which did not have clinical signs, was tested at the end of August for antibodies to *M. bovis* using the Enferplex serological test (Enfer Scientific). The test results for the two- and four-antigen panels were negative. However, in early November 2018, this second alpaca was observed to have significant emaciation and shortness of breath, and would cough occasionally; auscultation detected rales in the bronchi, and the animal died 10 days later. The necropsy revealed the presence of pale-grey, variably sized (1–10 mm), multifocal to coalescing lesions in the lungs (Fig. 1), liver and lymph nodes.

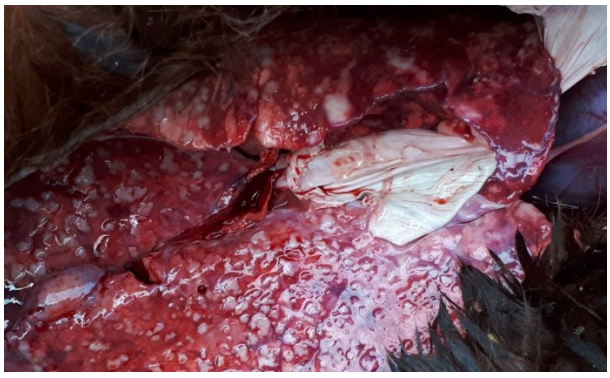

Fig. 1. Lung of alpaca no. 2 showing tubercular lesions

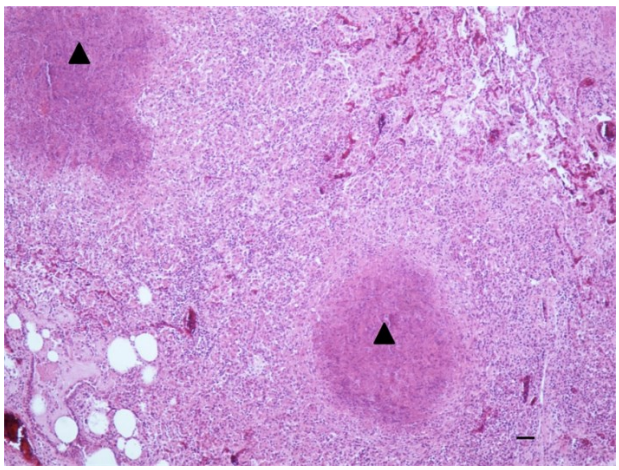

Fig. 2. Lung of alpaca no. 2 showing granulomatous lesions with necrotic centres (triangles) surrounded by macrophages and lymphoid cells infiltrating the lung parenchyma. Haematoxylin and eosin staining. Bar = 50µm

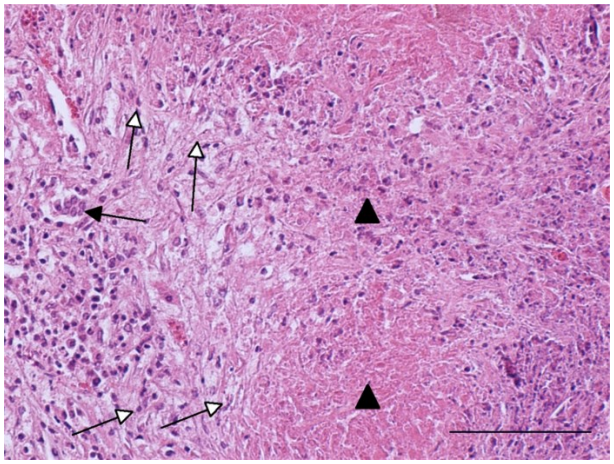

Fig. 3. Lung of alpaca no. 2 showing granulomatous lesions compounded by cellular debris (black triangle) surrounded by palisading spindle-shaped macrophages at the edges of the necrotic areas (white arrows) with rare presence of large multinucleated cells (black arrow). An infiltration of lymphocytes, plasma cells and fewer neutrophils scattered between the macrophages is visible on the left side of the photograph. Haematoxylin and eosin staining. Bar = 50µm

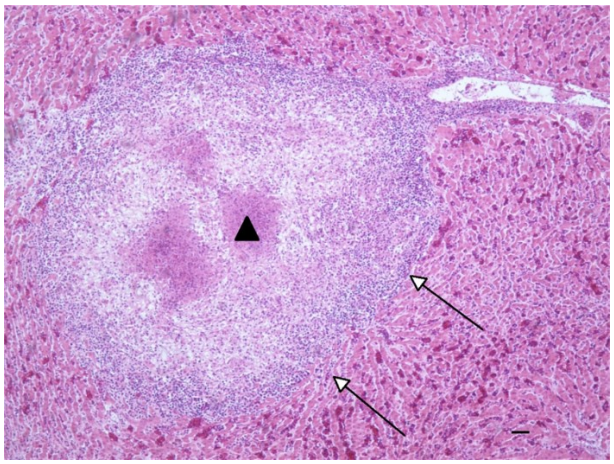

Fig. 4. Liver of alpaca no. 2 showing a granulomatous lesion with a necrotic centre (black triangle) surrounded by macrophages, neutrophils and lymphoid cells infiltrating hepatic parenchyma (white arrows). Haematoxylin and eosin staining. Bar = 50µm

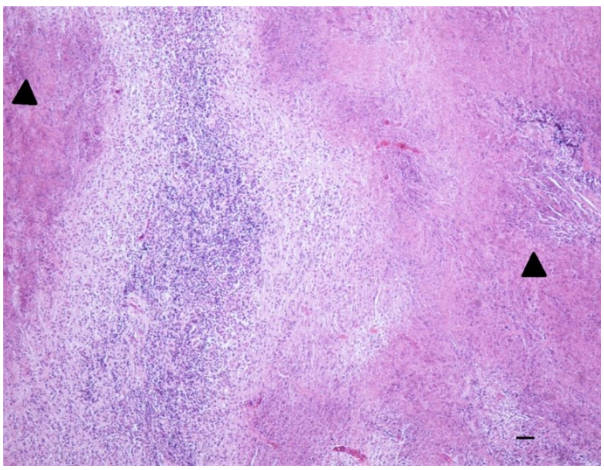

Fig. 5. Lymph node of alpaca no. 2 showing granulomatous lesions with areas of extensive caseous necrosis (black triangles) surrounded by macrophages and lymphoid cells. Haematoxylin and eosin staining. Bar = 50µm

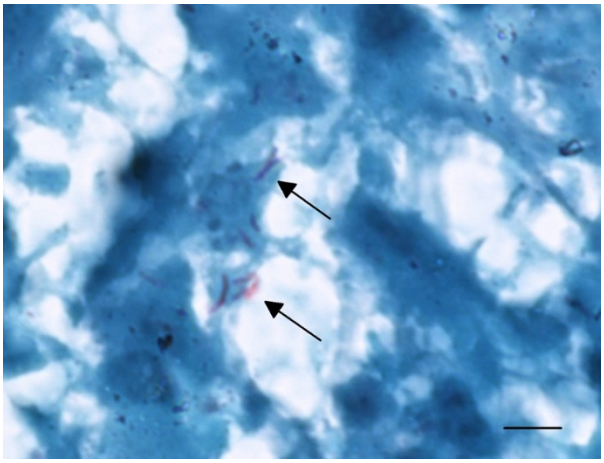

Fig. 6. Lung of alpaca no. 2 showing acid-fast bacilli visible as red-stained rod-shaped microorganisms (arrows). Acid-fast bacillus staining. Bar = 5µm

Histopathological examination revealed granulomatous inflammation in all the examined tissues (lungs, liver and thoracic lymph nodes). The granulomas observed in the lungs and liver consisted of variably sized and shaped eosinophilic, necrotic areas surrounded by neutrophils, epithelioid macrophages and rare multinucleated cells, as well as lymphocytes and plasma cells. The lymphoid cells at the margins of the lesions infiltrated surrounding tissues and there were no fibrous capsules visible around the lesions (Figs 2, 3 and 4). The lymph nodes were characterised by extensive, multifocal to coalescing areas of lytic necrosis surrounded by activated macrophages and lymphoid cells (Fig. 5). Based on the granuloma-formation scoring method by Wangoo *et al.* (37), the developmental stages of the observed granulomas were determined as II (nonencapsulated clusters of inflammatory cells, sporadically showing minimal necrosis) and III (necrotic centres, faint capsule formation). The AFB staining revealed the presence of red stained microorganisms characteristic of acid-fast bacteria, visible both within the cytoplasm of the macrophages and extracellularly,

scattered throughout the sections, in all the examined tissues (Fig. 6).

The result of the microbiological examination confirmed multiorgan animal tuberculosis caused by *Mycobacterium bovis*, SB0666 spoligotype, 222632237401435 MIRU-VNTR pattern.

Discussion

This article describes two cases of fatal *M. bovis* infection in alpacas imported from the UK. Unfortunately, due to the General Data Protection Regulation (GDPR), from which UK alpaca breeding centre the *M. bovis* individuals came is unclear. According to the Mbovis.org database, the SB0666 spoligotype was first recorded in cattle in the UK in 2003. The spoligotyping results and the mere few months separating the alpacas' deaths from TB, with both having been purchased at the same international exhibition, support the conclusion that the imported alpacas had already been infected at the time of import. The spoligotype pattern concerned, SB0666, is very rare in the UK according to the country's Animal and Plant Health Agency annual bTB surveillance and epidemiology reports. It was identified in seven *M. bovis* UK outbreaks between 2003 and 2010 (34) and was recently isolated from alpacas involved in a TB outbreak during 2017–2018 (unpublished data). The identified spoligotype has never been described in Poland before 2018; however, in that year, several TB outbreaks were identified in alpacas imported from the UK (17). The largest outbreak in Poland was reported in 2020 (16). The alpacas described in this manuscript also come from this alpaca breeding region; in addition, the MIRU-VNTR result obtained in the present study indicates a common source of infection for these animals and the alpacas described by Krajewska-Wędzina *et al*. (16).

That the test results were negative and no clinical signs were noted prior to transport highlights the need for more accurate methods to identify *M. bovis* infected animals prior to import. All imported alpacas have to pass the TST before being imported into Poland. However, TB in alpacas is often asymptomatic until the disease is advanced, and the available tests for ante-mortem diagnosis have suboptimal sensitivity (11). The negative TST and Enferplex results in the described alpaca TB cases suggest the need for more effective serological diagnostic tests in camelids. The best sensitivity of the Enferplex test is 67%. This sensitivity was determined in testing camelids that had received a prior injection of tuberculin 10–30 days earlier to generate an anamnestic boost to enhance test sensitivity (26). Alpaca no. 2 was not administered any such antigen preparation to effect a transient increase in serum antibody level. Unfortunately, the sera examined in this study were not archived for further analyses.

The alpacas described in the study were purchased at a large zoological exhibition in Poland after import from the UK. Such imported alpacas are potential sources of *M. bovis*, which can be a transmission risk for pets, livestock and wildlife (20, 24, 31); in addition, TB being a zoonotic disease, the visitors and owners at the exhibition were subject to potential infection. Transmission of *M. bovis* from an alpaca to a veterinarian has been reported, resulting in cutaneous TB (35). As previously reported, infections in farmers and other animal handlers may be associated with a lack of awareness about zoonoses (2, 14).

The presence of the infected alpacas at the international exhibition was a threat to public health. Compounding the risk of transmission, these alpacas went to a zootherapy centre on an agritourism farm, where the therapist, patients, and alpaca handler interacted directly with the infected animals. The farm also conducted classes for disabled children. However, in this case, the persons in contact with the infected alpacas were tested (TST) and had negative results.

Poland has maintained official TB-free status for over a decade although TB has been sporadically found in cattle and other species (6, 19, 23). The UK is one of the few countries in Europe that does not have TB-free status (5) and alpacas imported from that country jeopardise Poland's TB status. Tuberculosis is present in cattle, as well as in a wildlife reservoir – the European badger (*Meles meles*) (9). In addition, an increase in the number of alpacas diagnosed with TB in the UK was reported in 2018 (18). According to data published by the UK Department for Environment, Food and Rural Affairs (DEFRA), TB is still noted in South American camelids in the UK, in Wales the least frequently (3). Another pointer to the country's problem with this zoonosis is that DEFRA is working to improve TB control in animals other than cattle, particularly South American camelids such as alpacas (4).

There is a global need for stricter methods of TB prevention. The two cases of TB described in this article highlight the risk of *M. bovis* infection associated with the import of alpacas, and the requirement for improved screening tests for this species.

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