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Detection of *Mycobacterium tuberculosis* Complex Genetic Material in a Free-living Brown Bear (*Ursus arctos*)

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ABSTRACT: *Mycobacterium tuberculosis* complex (MTBC) has rarely been detected in bears (*Ursidae*). We describe detection of MTBC genetic material using a single-tube, high-multiplex PCR and fluorescence-based detection system in a throat swab collected from a free-living, problem individual during immobilization and telemetry collar deployment. Mycobacterial culture was negative in all samples.

Mycobacterium tuberculosis complex (MTBC) infection has been confirmed in many wildlife species, including carnivores (Bruning-Fann et al. 2001; Martín-Atance et al. 2006; Orłowska et al. 2017). Due to their position at the top of the food chain and their scavenging behavior, carnivorous species may act as MTBC bioaccumulators, making them suitable indicators of MTBC in the environment (VerCauteren et al. 2008). Bears (*Ursidae*) are long-living, far-ranging, omnivorous mammals that often feed on ungulate carcasses (e.g., Bojarska and Selva 2012). To date, MTBC infection has only been detected twice in free-living bear species: An American black bear (*Ursus americanus*) and a Marsican brown bear (*Ursus arctos marsicanus*; Bruning-Fann et al. 2001; Fico et al. 2019). Poland is home to a brown bear population of approximately 110 individuals confined to the Carpathians, with most of the population inhabiting its easternmost part, the Bieszczady Mountains (Selva et al. 2011; Śmietana et al. 2014). The species is strictly protected in the European Union under the Habitats Directive (Publications Office of the European Union 1992).

A set of nasal, throat, ear, and rectal dry swabs (three per anatomic site) were collected in 2020 from an immobilized free-living male brown bear, about 5 yr old, in the Bieszczady Mountains. The bear had undertaken increasingly bold behavior involving frequently approaching human settlements. The animal was captured and immobilized by a specialist team qualified to trap problem bears. The swabs were taken by a veterinarian from the Veterinary Clinic in Bukowsko, Poland and were transported refrigerated to the laboratory for mycobacterial culture and molecular testing to screen for MTBC infection. At the time of capture, the animal was in very good physical condition, with no visible signs of disease. During the intervention, the animal was equipped with a telemetry collar and transported to another part of the Bieszczady Mountains, away from human settlements. A few weeks later, the collar was lost and no further observation of the bear was possible. The trapping procedure was performed with the consent of the Regional Director of Environmental Protection in Rzeszów (WPN.6401.105.2019.ŁL.1.).

Mycobacterial culture was performed at the National Tuberculosis and Lung Disease Research Institute, Warsaw, Poland, as described (Klatt et al. 2015). Dry swabs were immersed in saline (0.9% NaCl) and decontaminated with N-acetyl-L-cysteine with 4%

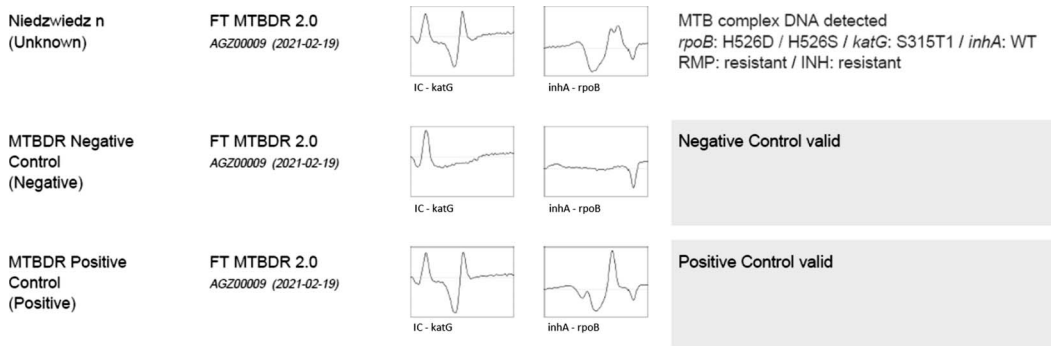

FIGURE 1. The result of a single-tube, high-multiplex PCR and fluorescence-based detection system (FluoroType MTBDR, Hain Lifescience, Nehren, Germany) of a throat swab collected from a male brown bear (*Ursus arctos*, designated here as Niedzwiedz n) from the Bieszczady Mountains, Eastern Carpathians, Poland. The assay detects the characteristic IS6110 MTBC sequence and mutations in the genes that determine resistance to rifampicin (rpoB) and isoniazid (katG, inhA).

sodium hydroxide and 2.9% sodium citrate tribasic dihydrate (Chempur, Piekary Śląskie, Poland) for 15 min. The decontaminated material was diluted in phosphate buffer (Chempur) containing 5% Tween 80 reagent solution (Fisher Bioreagents, Fair Lawn, New Jersey, USA) and centrifuged (1,500 × 3G). The resulting supernatant was discarded. The obtained pellet was resuspended in phosphate buffer, inoculated onto solid Stonebrink and Löwenstein-Jensen media (both Becton Dickinson, Franklin Lakes, New Jersey, USA), and incubated at 37 C. Mycobacterial growth (characteristic rough white-yellow colonies) was assessed every 7 d for 10 wk.

The pellet was also inoculated into Middlebrook 7H9 liquid medium in BD BBL mycobacteria growth indicator tubes (MGIT; Becton Dickinson). The inoculated media were placed in a BACTEC™ MGIT™ 960 mycobacterial detection system (Becton Dickinson) and incubated at 37 C. Fluorescence readings were taken continuously using an ultraviolet transilluminator for 8 wk.

The swabs immersed in saline (0.9% NaCl) were also tested directly for MTBC DNA using a single-tube, high-multiplex PCR and fluorescence-based detection system (FluoroType MTBDR, Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. This assay detects the characteristic IS6110 MTBC sequence but does not distinguish between individual species within the complex. The test also detects mutations in the genes that determine resistance to rifampicin (rpoB) and isoniazid (katG, inhA). Briefly, the mycobacterial DNA in the swabs was extracted, amplified using specific primers, and then hybridized with complementary fluorophore and quencher Lights-On and Lights-Off probes (HAIN Lifescience) for MTBC. Melting curves analysis was then performed in the FluoroCycler XT (HAIN Lifescience), with the characteristic fluorescence pattern being detected and interpreted with FluoroSpftware XT software (HAIN Lifescience).

No mycobacterial growth was observed using any of the three different mycobacterial culture methods. However, the FluoroType test confirmed the presence of MTBC genetic material in the throat swab, together with the H526D/H526S mutation in the rpoB gene, which confers the resistance to rifampicin and the S315T1 mutation in the katG gene, which confers the resistance to isoniazid (INH; Fig. 1).

Mycobacterium tuberculosis complex infection has previously been detected in Marsican brown bear (*Ursus arctos marsicanus*), which forms a very small, genetically isolated population and is usually classified as an endemic subspecies (Benazzo et al. 2017). However, this report presents the first detection of MTBC genetic material in the nominal subspecies of the brown bear (*Ursus arcos*). Interestingly, MTBC genetic material was only found in the throat swab. It is possible that the material

originated from an MTBC-infected animal ingested by the bear, because tuberculosis is present in wildlife in the Bieszczady Mountains, particularly in European bison and wild boar (Krajewska et al. 2015; Orłowska et al. 2020), and brown bears prey on and scavenge the carcasses of both these species in this area (Jankowski et al. 2019).

It is also possible that the tested individual was infected with MTBC and was shedding mycobacteria. Unfortunately, the bear lost his collar, therefore follow-up was not possible. It is difficult to assess the susceptibility of bears to MTBC infection because so few cases have been recorded in the literature (Bruning-Fann et al. 2001; Veeraselvam et al. 2013; Marinaik et al. 2022). Nevertheless, our results highlight the value of screening large carnivores for the presence of possible MTBC infections which might result in tuberculosis and the need to follow-up on collared individuals.

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