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Short communication

Suspicion of bovine tuberculosis in sheep in the Małopolskie Voivodeship (southern Poland)

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Abstract

Bovine tuberculosis (BTB) in sheep (*Ovis aries*) is caused by *Mycobacterium bovis* and *Mycobacterium caprae*. Even though sheep have been considered less sensitive to BTB than other ruminants, they have been subject to increasing numbers of tuberculosis cases and it has been suggested that they may act as a disease reservoir in some regions. Aim of the study: Following a report of tuberculous-like gross lesions (repeated cases of purulent or caseous lymphadenitis and a single case of serosal tubercles on the peritoneum) from veterinarians working in a slaughterhouse in the Małopolskie Voivodeship, southern Poland, the aim of this study was to conduct ante-mortem BTB diagnostics in three flocks with suspected BTB. The animals for testing were selected randomly from the flocks; a blood sample for interferon-γ release assay (IGRA) and a single tuberculin skin test (TST) was performed on each sheep. All TST results were negative. The IGRA result was positive in two ewes from the same flock (four and five years of age); these two sheep were tested microbiologically using Stonebrink and Löwenstein-Jensen media. No gross lesions suggesting BTB were observed, and the culture results were negative. Based on the positive IGRA results, together with its high specificity in sheep, and the potential risk to humans posed by consuming local unpasteurized dairy products, we recommend introducing serological BTB screening in sheep from this area, and subjecting the positive results to confirmation by culture.

Key words: bovine tuberculosis, dairy products, IGRA, sheep, tuberculin skin test

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Introduction

Bovine tuberculosis (BTB) is a highly contagious bacterial disease caused by the acid-fast bovine mycobacteria *Mycobacterium bovis* and *Mycobacterium caprae* (Schiller et al. 2011, Rodriguez-Campos et al. 2014, Verdugo Escárcega et al. 2020). BTB has a global spread and affects both livestock and wildlife (Fitzgerald and Kaneene 2013, Sichewo et. al 2020, Verdugo Escárcega et al. 2020). Furthermore, *M. bovis* and *M. caprae* can also cause disease in humans (Prodinger et al. 2014, O'Connor et al. 2019); however, this risk seems to be underestimated due to the common lack of Mycobacterium tuberculosis complex (MTBC) species identification (Olea-Popelka et al. 2017). The problem is particularly acute in regions of the world where unpasteurized milk is consumed on a large scale. In addition, the European Food Safety Authority (EFSA) report did not take into account human infections with *M. caprae*.

Although Poland has been an officially tuberculosis-free (OTF) country since 2009, cases are still noted in both livestock and wildlife (Lipiec et al. 2019, Orłowska et al. 2020). It has been suggested that BTB may be a re-emerging problem in Poland (Didkowska et al. 2021); however, to the best of our knowledge, no cases of BTB have been confirmed in sheep in this country so far. Despite this, BTB has been confirmed in this species in other European countries, and the number of reports has increased over the last 20 years (Muñoz-Mendoza et al. 2012, van der Burgt et al. 2013). Additionally, the choice of animals from this region of Poland was not accidental. Since 2008, cattle tuberculosis has been confirmed in this area every year (Krajewska 2016).

Recently, a report was received from veterinarians working in the field about tuberculosis-like gross lesions, consisting of repeated cases of purulent or caseous lymphadenitis and a single case of serosal tubercles on the peritoneum, in a slaughterhouse in one region of the Małopolskie Voivodeship, southern Poland. It was therefore decided to conduct a thorough microbiological examination of the lymph nodes in sheep in this region (Didkowska et al. 2020).

This study also presented an opportunity to use *ante-mortem* diagnostics to check the epidemiological status of BTB in three sheep flocks from this region. These flocks were selected based on the BTB epidemiological situation of BTB in cattle in this region (in previous years, positive results of the tuberculin skin test were found on neighboring farms) and on the suggestions of veterinarians working in the slaughterhouse as being most likely to demonstrate BTB (a single sheep from those farms was found to have tuberculosis-like lesions in *post-mortem* examination).

Materials and Methods

Animals

The study was conducted in 2017 in the Nowy Targ powiat, i.e. county, in the Małopolskie Voivodeship following a report of suspected BTB in three flocks of meat-dairy type sheep (*Ovis aries*). Ethics Committee approval was not needed in this case as the tests were carried out as part of a veterinary service after consultation with the animal owners, who wanted to determine the BTB status of their animals.

Ante-mortem diagnostics were conducted on 50 ewes from three flocks. The sheep ranged in age from three to eight years (mean: five years). The animals were selected randomly for the study. From each animal, blood was collected and a single tuberculin skin test (TST) was performed.

Blood was collected from the jugular vein (*vena jugularis externa*) in 6-ml heparin tubes and transported at room temperature within 24 hours to the laboratory. All individuals with a positive interferon-γ release assay (IGRA) result were selected for further microbiological examination; in these cases, material in the form of lungs and tracheobronchial and retropharyngeal lymph nodes was collected postmortem. None of the animals were killed for the purpose of the study: the material was collected when they were slaughtered.

Interferon-γ release assay (IGRA)

The IGRA test was performed using a commercial Bovigam® TB Kit (Prionics, Switzerland) according to the manufacturer's instructions. Whole blood was stimulated with bPPD 3000 (Prionics, Switzerland) and aPPD 3000 antigens (Prionics, Switzerland) with PBS used as a control. After incubation (37°C, 24 hours), 500 µl of plasma was collected from each of the three tubes taken from each animal; the INF-γ concentration in each was checked by ELISA. Readings were performed using an EPOCH spectrophotometer (BioTek Instruments Inc., USA). The test result was determined by comparing the INF-γ levels in the three plasma samples, as specified in the instructions.

Tuberculin Skin Test (TST)

The single TST was performed in accordance with the Instructions given by the Chief Veterinary Officer (instrukcja_gruzlica.pdf (piwet.net)). Briefly, the TST was performed with Bovitubal 28000 (Bioveta, Czech Republic) in a volume of 0.1 ml (2800 international units of tuberculin) by intradermal application to the hairless site on the medial side of the thigh, about 5 cm above the knee joint and about 5 cm from the edge

of the hairy skin. The TST reading was performed 72 hours after tuberculin injection. The criterion for a positive result was the presence of redness or swelling 10 mm in diameter or greater.

Culture

MTBC diagnostic culture was carried out as described previously (Didkowska et al. 2020). Briefly, the clinical material was decontaminated with a 5% solution/.n of oxalic acid (Sigma-Aldrich, St. Louis, MO, USA) and then homogenized in a stomacher (MiniMix, Interscience, France). After centrifugation (1500 g, 10 minutes), the supernatant was removed and the pellet washed twice with sterile physiological sodium chloride solution (Polfa Lublin, Poland). The obtained pellet was placed on two solid media: Stonebrink (Becton Dickinson, Holdrege, NE, USA) and Löwenstein-Jensen (Becton Dickinson, Holdrege, NE, USA). Both media were incubated at 37°C for 12 weeks and assessed every seven days.

Results

The results of all samples of TST were negative. The IGRA result was positive in two ewes. The animals were four and five years of age and were from the same herd.

Neither of the sheep with the positive IGRA result demonstrated any gross lesions suggesting BTB in *post-mortem* examination, and the culture results were negative.

Discussion

Even though it cannot be ruled out that IGRA results in two sheep were false-positive, based on the reports of veterinarians from this region and the zoonotic nature of BTB, there is a strong justification for continuing this line of research. This is particularly important as some locally-obtained dairy products, such as *bundz* (cheese) and *żętyca* (whey), are produced from unpasteurized sheep milk, and can present a risk of human infection by mycobacteria (Migdał et al. 2020).

While the role of small ruminants in the epidemiology of animal tuberculosis has gained greater attention, the scientific literature lacks standardization of procedures and solid quantitative estimates of the accuracy of BTB diagnostic tests. A recent review found that the sensitivity of tuberculin testing ranges from 0.51 to 0.59 for the single intradermal test and from 0.30 to 0.50 for the comparative intradermal tuberculin test; although IGRA offers higher sensitivity, ranging from 0.66 to 0.72, these values are still lower than in cattle. All tests demonstrate satisfactory specificity, ranging from 0.95 to 0.99 (Roy et al. 2020).

Hence, the *ante-mortem* diagnostics conducted in our study might have insufficient overall sensitivity. Fortunately, *ante-mortem* BTB diagnostics in sheep continue to be improved, and serological tests with 100% sensitivity and 98% specificity have recently been added to the testing armory (Infantes-Lorenzo 2020); as such, this test should be considered for future screening in this area. Also, it may be worth considering the use of comparative TST in the future; despite having lower sensitivity, it may be suitable for evaluating the widespread occurrence of paratuberculosis in sheep (Whittington et al. 2019).Without any data to the contrary, it is reasonable to assume that the paratuberculosis situation in sheep in Poland is similar to the other parts of the world.

The number of reported cases of BTB in sheep has increased recently in Europe, and it has even been suggested that sheep may be a reservoir of BTB in Spain (Muñoz-Mendoza et al. 2016). In addition, molecular studies have raised the possibility of human-sheep transmission (de Val et al. 2021). BTB has been described widely in Africa (e.g. Saad et al. 2020); however, some recent reports also have occurred in Europe (Muñoz-Mendoza et al. 2012, Reis et al. 2020). The latter reports recommend that special attention should be paid to sheep which share grazing fields with cattle and goats which are species considered more susceptible to *M. bovis* and *M. caprae* infection (Vallejo et al. 2018).

It should be noted that the suspicions of field veterinarians did not necessarily have to be correct. While the presence of serosal tubercles on the peritoneum is a common pathognomy for severe BTB, that of purulent caseous lesions in lymph nodes may be caused by a range of other pathogens, such as *Corynebacterium pseudotuberculosis* (Baird and Fontaine 2007), *Staphylococcus aureus* (Macori et al. 2017) and *Truepella pygoenes* (Rzewuska et al. 2021). This has been confirmed in previous studies carried out in this area; in these cases, the most commonly-isolated pathogens were *C. pseudotuberculosis* and *S. dysgalactiae* subsp. *equisimilis*, and no mycobacteria were isolated from the mediastinal or tracheobronchial lymph nodes (Didkowska et al. 2020). Although selecting animals at random, rather than restricting the study to those in poor condition, may be regarded as a limitation, it should be remembered that tuberculosis may develop long before the onset of clinical symptoms and our methodology takes this into account.

The present study is justified for at least two reasons. Firstly, BTB is still present in Poland, and

cases have been observed in both livestock and wildlife (Krajewska 2016, Lipiec et al. 2019, Orłowska et al. 2020), and sheep, grazing in pastures in mountains for most of the year, are particularly vulnerable to contact with possible infected wildlife (Molik et al. 2018). Secondly, due to the specificity of regional dairy production, it is possible that consumers can eat dairy products containing live mycobacteria (Molik et al. 2018).

Conclusion

It was not possible to confirm the presence of MTBC mycobacteria in the sheep from the studied region; however, taking into account the positive results in the IFN-γ release assay, in combination with its high specificity in sheep, and the potential risk to humans by consuming unpasteurized products, we recommend the introduction of serological BTB monitoring in sheep from this area and the potential confirmation of positive results through culture.

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