Tuberculosis in wild boar and the risk of human infection by *Mycobacterium bovis* results of a study conducted in Southern Italy.

Francesco Casalinuovo[1*], Lucia Ciambrone[1], Raffaele Grillone[2], Natalino De Gori[2]

[1]Istituto Zooprofilattico Sperimentale del Mezzogiorno, Catanzaro, Italy

[2]Azienda Sanitaria Provinciale di Catanzaro, Italy

Abstract

The presence of tuberculosis infection in wild boar populations in a southern Italian territory was investigated, in order to discern any epidemiological and spatial correlations between the strains of *Mycobacterium tuberculosis* complex isolated in wild boar and humans who share the same territory. We also evaluated the presence and level of contamination by *Mycobacterium tuberculosis* complex in meat from wild boar killed during the hunting season. Although the presence of tuberculosis infection in wild boar has been amply demonstrated, little information is currently available regarding the risk of direct human infection through the handling and consumption of the meat of infected animals. Consequently, the risk of humans being infected through eating raw or insufficiently cooked meats contaminated during butchery, albeit deemed probable, has not yet been sufficiently proved. Culture tests were carried out on organs and lymph-nodes presenting suspected tubercular lesions from 250 slaughtered wild boars; spoligotyping tests were then conducted on the strains of *Mycobacterium bovis* obtained, in order to compare these with the strains of *Mycobacterium bovis* isolated from human patients. The results showed a genetic correlation between the SB1565 genotype isolated from wild boar and the same genotype isolated from a human patient with clinically manifested tuberculosis. Moreover, a genetic correlation was seen between the genotypes SB1565 and SB0120 and the strains of *Mycobacterium bovis* isolated from cattle.

Keywords: Wild boar, Zoonosis, Meat, *Mycobacterium bovis*.

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Introduction

Tuberculosis in wildlife is an increasingly important issue, not only because of the zoonotic character of the disease, but also because of the possible impact it may have on plans for the eradication of bovine tuberculosis (BT) in those areas where the infection is still endemic. Indeed, there is broad consensus that the success of plans for eradicating BT in a given region or state also depends on precise knowledge of the role of the wildlife present. With regard to wild boar *(Sus scrofae),* studies on the presence and diffusion of *Mycobacterium tuberculosis complex* (MtbC), as well as the possible role of wild boar in maintaining tuberculosis infection in cattle herds, in other sensitive species and in the environment, have chiefly been carried out in Europe [1-5] though reports have also come in from other continents [6,7]. Among MtbC species, a high prevalence of *Mycobacterium bovis (M. bovis)* has been observed in wild boar, though *Mycobacterium caprae* has also been reported [8,9]. While it has already been demonstrated that wildlife and cattle occupying the same territory often share the same MtbC strains, this same correlation with humans-especially some particularly exposed categories such as hunters and veterinarians-although firmly hypothesised, has not yet been described [2-11]. Little information is currently available regarding the risk of direct human infection through the handling and consumption of the meat of wild boar infected by MtbC. Consequently, the risk of contracting the infection through eating raw or insufficiently cooked meats contaminated during butchery of the animals, albeit deemed probable has not yet been sufficiently proved [12]. Indeed, the handling procedures and the tools used during evisceration can facilitate the contamination of muscle tissue by pathogenic micro-organisms, including *M. bovis,* already present in the organs of the animal. The aim of this study were to investigate the presence of tuberculosis infection in wild boar populations in a southern Italian territory, to pick out any epidemiological and spatial correlations between the strains of MtbC isolated in wild boar and in humans that share the same territory, and, finally, to evaluate the level of MtbC contamination of meat from animals killed during the hunting season.

Materials and Methods

The carcasses and organs of 250 wild boars were examined. These animals had been killed in the regular manner during the hunting season, from October to December 2016, in an area of about 300 km² in southern Italy (Calabria region), where the species is fairly abundant owing to the favourable pedoclimatic conditions. In the laboratory, analytical tests for MtbC were conducted on organs and lymph-nodes which, on examination of the carcasses, had presented lesions attributable to MtbC. The genetic variety of the strains of *M. bovis* isolated from the boar was correlated by means of spoligotyping with the strains isolated from cattle and those isolated between 2013 and 2014 from humans residing in the same territory where the investigation was carried out. Finally, we checked for the

possible contamination by MtbC of 15 carcasses stored in a refrigerator at a temperature of +2°C and ready to be supplied for free consumption. The carcasses were sampled by means of dry swabs wiped over the external and internal surfaces. The organs with suspect lesions and the swab samples taken from the carcasses underwent Polymerase Chain Reaction (PCR) molecular tests and bacteriological examinations (BE) by culturing in selective media, according to the following procedures:

PCR on tissues

DNA was extracted by subjecting the tissue to mechanical lysis; the supernatant was then collected and processed by means of a commercially available extraction kit (QIAamp DNA mini kit Qiagen, Germany). The DNA extracted was subjected to a PCR reaction based on specific amplification of the IS6110 gene insertion region, which is highly repeated in the genome of strains belonging to the genus *Mycobacterium;* for this purpose, we used primers complementary to the target sequence which amplify a fragment of 209 bp: EXT-1 5'-CCCGGACAGGCCGAGTTT-3' INT-1 5'-CCCCATCGACCTACTACG-3'. The reaction mixture was prepared by using a commercially available master mix (Hot Start TAQ Master Mix Qiagen; final concentration 2.5U HotStarTaq.

Master mix, containing 1.5 mM of magnesium chloride and 200 µM of each dNTP). The primers were used at an initial concentration of 10 µM. The thermal profile for amplification involves an initial phase of Taq activation of 15 min at 95°C, followed by 45 cycles of: denaturing for 30 sec at 95°C, annealing for 30 sec at 58°C and an extension for 45 sec at 72°C, with a final extension for 10 min. at 72°C.

PCR on swabs

For the extraction of MtbC DNA, a commercially available kit (QIAamp DNA mini kit, Qiagen, Germany) was used in accordance with the protocol described for swabs. For amplification, the Kulski was applied; this involves carrying out a Mycobacter Genus-specific PCR (Target 16S rRNA): 1030 bp, and a Tuberculosis Complex-specific PCR: TBCX (Target: MP70): 372 bp [13].

A commercial master mix (PCR Master Mix, Promega Corporation, Madison, WI, USA) was prepared at a 1X concentration, containing 50 units/ml of TAQ Polymerase, 400 Mm of dATP, dGTP, dCTP, dTTP and 3Mm of $MgCl_2$, while the primers utilised were at an initial concentration of 20 µM. The thermal profile for MTC involved a cycle at 94°C for 5 min. and 35 cycles set as follows: 94°C for 30 sec., 57°C for 30 sec., 72°C for 70 sec., and 72°C for 7 min.

Bacterial isolation and identification

Samples were processed according to OIE standard procedures, and bacterial isolation was carried out by means of seeding on Stonebrink and Löwenstein-Jensen solid media with pyruvate. The isolates were identified by means of two PCR reactions specific for the *Mycobacterium* genus and for the MTC group, and, following PCR/RFLP of the Gyrb gene, by using the RSaI restriction enzyme for digestion [13].

Results/Observations

On examination, the carcasses displayed a mean prevalence of 11.2% (28/250) of lesions attributable to tuberculosis infection.

From an anatomo-pathological standpoint, the predominant lesions were constituted by caseous and calcified forms in the lymph-nodes, mainly the mandibular lymph-nodes, but also the bronchial, mediastinal, hepatic, pre-scapular, pre-crural and inguinal lymph-nodes. The PCR and BE tests carried out on the organs presenting lesions proved positive in 21/28 animals (75%); the results are shown in Table 1.

Table 1: *Results of laboratory tests conducted on wild boar organs presenting lesions attributable to/suspect for MTC.*

Animal	organs with lesions	PCR for MTC1	CT2	PCR strain3	Strain identification
1	retropharyngeal lymph.	+	+	+	*M. bovis*
2	retropharyngeal/bronchial lymph.	+	+	-	-
3	Lungs	+	+	+	*M. bovis*
4	Lungs	+	-	-	-
5	retropharyngeal lymph.	+	+	+	*M. bovis*
6	retropharyngeal lymph.	+	+	-	-
7	retropharyngeal lymph.	+	+	-	-
8	retropharyngeal lymph.	+	+	+	*M. bovis*
9	retropharyngeal/bronchial lymph.	-	+	-	-
10	Bronchial	+	-	-	-
11	retropharyngeal/mediastinal lymph.	+	+	+	*M. bovis*

12	retropharyngeal lymph.	+	-	-	-
13	retropharyngeal lymph.	+	+	+	*M. bovis*
14	lung/mediastinal lymph.	+	-	-	-
15	Lung	+	-	-	-
16	retropharyngeal lymph.	+	-	-	-
17	mediastinal lymph.	+	-	-	-
18	retrophar./mediastinal/hepatic lymph	+	-	-	-
19	retrophar./prescap./inguinal lymph	+	-	-	-
20	retrophar. lymph/lung	+	-	-	-
21	lung/pleura	+	-	-	-

Notes: [1]PCR performed on organ tissues; [2]culture test performed on organ tissues; [3]PCR performed on the MTC strains isolated

As can be seen, 95% of the samples proved positive on PCR testing for MtbC; only one sample proved negative. By contrast, bacterial isolation was obtained in only six samples (28.5%), probably because of the marked prevalence of chronic calcified lesions. In all, bacteriological examination led to the isolation of 6 strains of MtbC from the organs of 6 wild boar (21.4%); subsequently, these were all identified as *M. bovis:* 3 belonging to the genotype SB1565/VNTR 5,5,5,3,3,10,4,4,4,3,11,5, and 3 belonging to the genotype SB0120 VNTR 4,5,5,3,3,10,4,4,4,3,6,5. Spoligotyping tests conducted on the strains of *M. bovis* isolated from the boar and on those isolated from human patients revealed a genetic correlation between the genotype SB1565 of the animals and the same genotype isolated from a human patient with a clinically manifested tuberculosis infection.

Table 2: *Epidemiological correlation of SB1565 strains of M. bovis isolated in the territory considered.*

Species	A	B	C	D	E	11a	11b	26	1895	15	3232	Miru26	year
bovine	5	5	5	3	3	10	4	4	4	3	11	5	2011
man	5	5	5	3	3	10	4	4	4	3	11	5	2014
bovine	5	5	5	3	3	10	4	4	4	3	11	5	2015
bovine	5	5	5	3	3	10	4	4	4	3	11	5	2016
bovine	5	5	5	3	3	10	4	4	4	3	11	5	2016
wild boar	5	5	5	3	3	10	4	4	4	3	11	5	2017
wild boar	5	5	5	3	3	10	4	4	4	3	11	5	2017
wild boar	5	5	5	3	3	10	4	4	4	3	11	5	2017

This same genotype also proved to be correlated with some strains of *M. bovis* previously isolated from cattle present in the area, which had reacted positively to the intradermal tuberculosis test (IDT) carried out *in vivo.* Table 2 reports the results, which indicate a close epidemiological correlation among wild boar, cattle and humans with regard to the presence of the same SB1565 strain of *M. bovis.* This genotype accounts for 2.5% of all the genotypes of *M. bovis* isolated so far in the territory considered. Table 3 reports the epidemiological correlation between cattle and wild boar with regard to the SB0120 strain of *M. bovis.* The PCR tests conducted on swabs taken from the surfaces of 15 wild boar carcasses that had not presented lesions attributable to, or suspect for, MTC on visual inspection revealed contamination by MtbC DNA in two cases, subsequently identified by means of PCR as *M. bovis.*

Table 3: *Epidemiological correlation of SB0120 strains of M. bovis isolated in the territory considered.*

Species	A	B	C	D	E	11a	11b	26	1895	15	3232	miru26	year
bovine	4	5	5	3	3	10	4	4	4	3	11	5	2007
bovine	4	5	5	3	3	10	4	4	4	3	11	5	2008
bovine	4	5	5	3	3	10	4	4	4	3	11	5	2010
bovine	4	5	5	3	3	10	4	4	4	3	11	5	2011
bovine	4	5	5	3	3	10	4	4	4	3	11	5	2012
bovine	4	5	5	3	3	10	4	4	4	3	6	5	2013
bovine	4	5	5	3	3	10	4	4	4	3	6	5	2013
wild boar	4	5	5	3	3	10	4	4	4	3	11	5	2017
wild boar	4	5	5	3	3	10	4	4	4	3	11	5	2017
wild boar	4	5	5	3	3	10	4	4	4	3	11	5	2017

Discussion

The results obtained indicate that tuberculosis infection due to *M. bovis* is endemic in the wild boar present in an area of southern Italy, even though no cases of the disease in cattle have officially been recorded for about two years. This contrasts with the claims made by other authors to the effect that the incidence of tuberculosis infection in wild animals diminishes when the infection is controlled or eradicated in livestock [12]. In the present case, wild boars constitute a reservoir from which the infection may spread to bovine herds from which it has previously been eradicated. This is the first

time that epidemiological correlations carried out on the genotypes of *M. bovis* isolated in a given territory have revealed the contemporary presence of the same spoligotype (SB1565) in wild boar, cattle and humans (Table 2). In the human case, the SB1565 genotype of *M. bovis* was detected in a sample from a patient with clinically manifested tuberculosis who resided in the same area where the investigation was conducted. This patient's history recorded the absence of any contact with any type of bovine, ovine, caprine or other sensitive species of ruminant. However, it emerged that the patient had habitually engaged in the poaching of wild boar for his own consumption and for sale, thereby eluding hunting regulations and any form of health control of the meat procured. The ascertained endemic presence of *M. bovis* infection in wild boar populations in the same territory may have caused this human infection through the consumption of raw or undercooked meats that had been contaminated during the processes of evisceration and butchery, which had very probably been carried out without observance of the most elementary norms of hygiene. To date, infection by *M. bovis* has had a scant incidence in cases of human tuberculosis [14]. Nevertheless, the spread of the infection in wildlife could lead to the greater exposure of certain categories, such as hunters and veterinarians. In agreement with other authors, we found that the forms of BT detected in wild boar often involved the lymph-nodes of several regions: maxillary, mediastinal, hepatic, pre-scapular, inguinal and pre-crural [15]. This implies that, in order to exclude tuberculosis infection in wild boar, post-butchery visual inspection should not be limited to the classic locations of infection, such as the mandibular and retropharyngeal lymph nodes, but should be carried out on the greatest possible number of explorable lymph nodes. Microbiological contamination of the meat of wild boar almost always originates from the micro-organisms present on the skin, in the gastrointestinal tract, in other organs or in the muscle tissue itself at the time of butchery [16].

It may also arise as a result of firearms wounds inflicted during the hunt, or occur during skinning and evisceration; these latter operations are generally carried out some time after the kill, and in the absence of the means of rapid cooling and of a subsequent cold chain, which is essential in order to inhibit microbial multiplication [16]. In our study, *M. bovis* contamination of the muscle masses of wild boar carcasses allocated for free consumption was due both to the fact that some of the animals killed were carriers of tubercular lesions and to the lack of the necessary hygiene conditions during butchery and evisceration. The marked growth of wild boar populations has greatly increased the availability of boar meat, which is destined not only for private consumption but also for sale. Consequently, the risk of tuberculosis infection in humans as a result of the handling and consumption of these meats has likewise risen. This outlook is of particular relevance in those regions where BT is endemic in wild boar and where, on account of the enormous increase in populations of this species, there is considerable availability of boar meat for human consumption. Nor is this consumption restricted to the domestic setting, since current legislation permits the public marketing of game meats (CE Regulation n. 852 of 29 April, 2004; CE Regulation n. 853 of 29 April, 2004). Wildlife management must respond to the requirements of ethics, the environment, health and food supply. With regard to health, the results of our investigation indicate that the wild boar is not merely a dead-end host for *M. bovis,* but a veritable reservoir for the maintenance of tuberculosis infection and its possible spread to bovine herds. It is well known that wild boar and cattle can share the same spoligotypes of *M. bovis*. Now, however, it must be acknowledged that humans can contract the infection not only from cattle but, as this investigation shows, also from wild boar through the handling and consumption of meat from infected animals. This implies the need to consider, in the case of large-sized game animals, mandating examination by the official veterinarian and laboratory testing in the case of suspected cross-contamination of meats destined for free consumption.

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***Correspondence to:**

Francesco Casalinuovo

Istituto Zooprofilattico Sperimentale del mezzogiorno

viale Crotone snc

Catanzaro, 88100

Italy .

Tel: 39+ 0961-737763

E-mail: francesco.casalinuovo@cert.izsmportici.it