Presence of DNA of Mycobacterium bovis in Beef Aimed for Human Consumption

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ABSTRACT

Background: Human tuberculosis due to Mycobacterium bovis is largely caused by the consumption of raw milk or by products based on non-pasteurized milk. However, the consumption of raw or undercooked contaminated meats could be an element of risk. The worrisome recrudescence of cases of human tuberculosis in countries where the disease is not endemic implies the need for thorough and definitive assessment of the risk to human health of eating meat contaminated by M. bovis.

Aim: The aim of the present study was to investigate the possible presence by DNA M. bovis in meat beef at the time when it is released onto the food market.

Method(s): We utilized a polymerase chain reaction (PCR) test to detect the DNA of M. bovis in the meat of 48 cattle which had been slaughtered because they were positive results to intradermal tuberculin test (IDT) and on the carcasses of 12 regularly slaughtered healthy cattle; all these meats were ready to be supplied to the commercial market.

Result: DNA of M. bovis was detected in 27% of the carcasses of the IDT-reactive animals and in 58% of the carcasses of the healthy animals. A significant difference was seen between the results obtained from swab samples and those yielded by sponge bag samples taken at the same time.

Conclusions: The results obtained confirmed the presence of M. bovis on the production line as a result of the presence of meat from IDT-reactive animals. The meat of healthy animals that are butchered in facilities that practise the deferred butchery of cattle infected by tuberculosis is also a risk factor.

INTRODUCTION

Despite the progress made in recent years in terms of prevention and treatment, human tuberculosis (TB) remains one of the main threats to health worldwide. Disease hits 10 million people in media and in 2015, it caused 1.8 million deaths. (1) The Mycobacterium tuberculosis complex (MTBC) includes M. tuberculosis (the cause of most human tuberculosis), M. bovis, bacillus Calmette-Guérin (BCG, the vaccine strain), M. africanum, M. microti, M. caprae, M. canetti, M. pinnipedii, M. orygis, M. suricattae and recently recognized M. mungi. (2) M. bovis is the main cause of tuberculosis (TB) in cattle, deer, and other mammals, it can also cause the disease in humans, of which in 2015 there were an estimated 149,000 cases of zoonotic caused by M. bovis, with an estimated mortality of 13,400 persons. (1) Worldwide, M. bovis causes less than 1.4% of pulmonary tuberculosis cases, with peaks of 2.8% of cases in within Africa, while in the EU are estimated to be 0.4%. (1, 3) This figure is certainly underestimated, as the mycobacterium responsible is not isolated and identified in all cases diagnosed. (4) Cases of human tuberculosis caused by M. bovis are mainly due to the consumption of raw milk. Zoonotic tuberculosis may either be transmitted indirectly through contaminated dairy products or directly through contact with infected animals (farmers, veterinarians, and slaughterhouse personnel are the main professionals at risk of infection). However, the consumption of raw or undercooked contaminated meats also constitutes an element of risk. (5) Likewise, meat from cattle that prove reactive to the intradermal tuberculin test (IDT) cannot be considered free from contamination by M. bovis, even in the absence of visible organ lesion. (6) The lack of reliable data on the true prevalence and incidence of infection by M. bovis in humans, in addition to the persistently high levels of incidence of tubercular infection observed cattle in many developing countries, implies the need for thorough and definitive assessment of the risk to human health of eating meat contaminated by M. bovis. Although it has not yet been documented, M. bovis infection can theoretically be transmitted from meat to humans. (7) This risk is acknowledged by the international scientific community, (8) albeit at a low level on account of the fact that meats are cooked, (8, 9) and because the muscles and blood are not infected by infection. However, the possible cross-contamination of other foodstuffs, utensils, and work surfaces should not be underestimated. Moreover, it must...
be borne in mind that certain food products found in some culinary preparations may contain raw or lightly cooked meats. The present study investigated the risk of direct or indirect human tubercular infection by *M. bovis* through the consumption or handling of infected meat from cattle by detection of *M. bovis* DNA in meat. The investigation was conducted by means of a polymerase chain reaction (PCR) test. The test was carried out on swabs and sponge bags taken from meats that were ready to be supplied to the market for free consumption; these meats were from 60 cattle, 48 of which had proved positive on the IDT and 12 of which were healthy, but were butchered in the same facility, where the deferred butchery of cattle infected by TB is practiced. The 48 IDT-reactive cattle came from infected farms situated in an area of southern Italy where the mean prevalence of TB infection is 0.37%, of the mean incidence 0.35%. (10)

**MATERIALS AND METHOD**

We examined the carcasses of 60 cattle. The cattle were slaughtered over 10 months at 6 different authorized slaughterhouses of southern Italy in accordance with European Union regulations (Regulation (CE) n.853/2004) (11) and from 14 different farms of the same territory. Twelve carcasses were regularly slaughtered cattle coming from a herd officially free, while 48 were carcasses of cattle coming from 13 different farms proved positive on IDT. In both cases the meat at the time of collection was ready to be placed on the market prior favourable outcome of the post slaughter inspection. All involved slaughterhouses were authorized to perform deferred slaughter of reactive animals to IDT test in addition to the ordinary ones (Regulation (CE) n.854/2004). (12) Before the carcasses were loaded into refrigerators, samples were taken from each individual half carcass by wiping the outer of shoulder, chest, abdomen, and thigh surfaces with four dry sterile swabs; in some cases, sponge bags were also used. A total of 384 swabs and 72 sponge bags were collected and analyzed. Immediately after sampling, each individual swab was placed back in its own container, while the sponge bags were placed in a sterile sachet containing 25 ml of buffered peptone water. The container, while the sponge bags were placed in a sterile plastic bag, each individual swab was placed back in its own container, while the sponge bags were placed in a sterile sachet containing 25 ml of buffered peptone water. In the samples were promptly refrigerated and delivered to the laboratory, where they underwent PCR testing for *M. bovis* in accordance with the method described below. In the manner described above, we also checked the carcasses of 12 healthy cattle, all regularly slaughtered on the same day in a single plant a distance of 24 hours of slaughter of cattle infected with TB. The culture method for isolating *M. bovis* was also implemented on four sponge bags that had proved positive on PCR testing.

Polymerease Chain Reaction: a commercially available kit (QIaamp DNA mini kit, Qiagen, Germany) was used to extract the DNA, in accordance with the protocol described for the swab matrix. Amplification was carried out by applying the protocol of Kulski et al., (13) which utilizes two PCR: one Mycobacter Genus-specific (target 16S rRNA) 1030 Bp: MYCGEN-F 5’-AGGTTTGATCCTGCGTCAC-3’, MYCGEN-R 5’-TGGACACAGGCCCAAGGGA-3’, and one specific for the group of *Mycobacterium tuberculosis* complex (Mtbc): TBX2 (TARGET: MP70): 372 Bps: TBX-F 5’-GAACATTCGGCGTTGACA-3’, TBX-R 5’-AG CACGCTGCAATCATGTA-3’. To prepare the reaction mixture, 25 µl of a commercially available master mix (PCR Master Mix, Promega Corporation, Madison, WI, USA) a was used for each sample; the master mix contained: 50 units/ml of TAQ Polymerase, 400 Mm of dATP,dGTP,dCTP,dTTP and 3 Mm of MgCl$_2$. To prepare the reaction mixture, we used 0.5 µl of each primer at starting concentration of 100 µM and 19 µl of water up to a final volume of 50 µl. An inactivated strain of *M. bovis* was used as a positive control, while the negative control consisted simply of water and reaction mixture. For *Mycobacterium* spp, the thermal profile entails: a denaturing phase at 94°C for 7 min, followed by 35 cycles that include a denaturing cycle at 94°C for 30 sec, an annealing cycle at 57°C for 30 sec and an extension at 72°C for 70 sec, terminating with a cycle at 72°C for 7 min and then 4°C ad infinitum. The thermal profile for Mtbc entails: a denaturing cycle at 94°C for 5 min, followed by 35 cycles arranged as follows: 94°C for 30 sec, 57°C for 30 sec, 72°C for 70 sec, terminating with a cycle at 72°C for 7 min and then 4°C ad infinitum. In order to identify *M. bovis*, a 242 bp DNA fragment belonging to the IS6110 insertion sequence was amplified (Vitale et al. 1998). Nested PCR was carried out: primers utilised for the first PCR: 295 5’-GGACAACCGCCAATTGCAAAGGC-3’, INS2 5’-CTAGGCGTGCGTGACAAA-3’. For the second PCR, the primers utilised were: IN2 5’-CGTGGGCCGTCGGTGACAAA-3’. To prepare the reaction mixture, we used 12.5 µl of a commercially available master mix (PCR Master Mix, Promega Corporation, Madison, WI, USA). The master mix contained: 50 units/ml of TAQ Polymerase, 400 Mm of dATP,dGTP,dCTP,dTTP and 3 Mm of MgCl$_2$. To prepare the reaction mixture, we used 0.5 µl of each primer at starting concentration of 200 µM and 6.5 µl of water up to a final volume of 25 µl. An inactivated strain of *M. bovis* was used as a positive control, while the negative control consisted of water and reaction mixture. The thermal profile of the first PCR entails an initial denaturing phase of 3 min at 94°C, followed by 25 cycles consisting of denaturing at 95°C for 1 min, annealing at 60°C for 1 min, and polymerization at 72°C for 1 min. This is followed by a final polymerization phase of 7 min at 72°C and maintenance at 4°C ad infinitum. The thermal profile of the second PCR involves initial denaturing for 3 min at 95°C, 30 cycles consisting of denaturing at 95°C for 1 min, annealing at 65°C for 1 min, and polymerization at 72°C for ad infinitum.
Contamination of *M. bovis* in cattle meat

Culture test: Culture media available in the market were used to perform culture test. The sponge bags underwent a cycle of washing with sterile distilled water; the liquid collected was decontaminated with 2% NaOH and then seeded directly (0.2 ml) in a series of three test tubes containing Stonebrink medium with pyruvate (SM) (BBL™ Stonebrink TB medium + PACT, Becton, Dickinson & C, USA) and three test tubes containing Löwenstein–Jensen medium with glycerol (LJM) (BBL™ Löwenstein–Jensen medium slants, Becton, Dickinson & C, USA), incubated at 37°C and controlled weekly for 60–70 days. In addition, some of the liquid was filtered through a 0.45-µm membrane, transferred onto SM and LJM and incubated by means of the same procedure. With regard to the organs and lymph nodes, 3 g of parenchyma from the sites of lesions was minced and homogenized in physiological solution; then we picked up 2 ml of extract, to which we added 2 ml of 4% NaOH and incubated at 37°C for 30’. After neutralization with H₂SO₄ and centrifugation at 3000 RPM for 15’ at 20°C, the supernatant was eliminated. The sediment was reuspended in a solution of phosphate buffer at pH 6.8 and seeded (0.2 ml) on SM and LJM.

**RESULTS AND DISCUSSION**

The post-mortem inspection has revealed the presence of tuberculosis lesions on the bodies of 45 of the 48 cattle slaughtered that had reacted to the IDT test. However, culture testing and PCR carried out on the organs and lymph nodes of the 48 cattle proved positive for *M. bovis* in all the samples analyzed. The culture tests carried out on the sponge bags did not detect *M. bovis*. As regards the PCR tests carried out on swabs and sponge bags detected the DNA of *M. bovis* in 27% (13/48) of the carcasses examined; Table 1 reports the results of culture tests and PCR tests carried out on organs, swabs and sponge bag samples taken from the carcasses of 9 animals belonging to the group of 48 IDT-positive cattle. All these nine animals came from the same herd and underwent deferred butchery.

From the table, it can be seen that contamination of the carcasses by *M. bovis* increases as the butchery process progresses. Indeed, the carcasses of the first two animals proved negative on PCR testing carried out on swabs and sponge bags. In the animals N°5, N°6, and N°7, the DNA of *M. bovis* was detected on swabs, while sponge bag samples remained negative. Towards the end of the butchery process, both swab and sponge bag samples taken from the carcasses of N°8 and N°9 tested positive. The group of 12 healthy slaughtered cattle in one plant a distance of 24 hours of deferred slaughter of a group of reactive cattle to the IDT test have reacted positively to the PCR test performed on swabs taken. It was found the presence of *M. bovis* DNA on 7 of the 12 carcasses analyzed (58%) (Table 2).

The results of the tests carried out confirm that meat from IDT-reactive cattle that has been authorized for free consumption has probability of being contaminated by *M. bovis*. Both national and European legislation, particularly Regulation N° 854/2004/CE, (12) contain provisions regarding the handling and utilization of the carcasses of cattle slaughtered after proving positive on *intra-vitam* diagnostic testing for infection by *M. bovis*. Specifically, animals displaying a positive or inconclusive reaction to IDT are to be butchered 1 min. This is followed by a final polymerization phase of 5 min at 72°C and maintenance at 4°C *ad infinitum*.

Culture test: Culture media available in the market were used to perform culture test. The sponge bags underwent a cycle of washing with sterile distilled water; the liquid collected was decontaminated with 2% NaOH and then seeded directly (0.2 ml) in a series of three test tubes containing Stonebrink medium with pyruvate (SM) (BBL™ Stonebrink TB medium + PACT, Becton, Dickinson & C, USA) and three test tubes containing Löwenstein–Jensen medium with glycerol (LJM) (BBL™ Löwenstein–Jensen medium slants, Becton, Dickinson & C, USA), incubated at 37°C and controlled weekly for 60–70 days. In addition, some of the liquid was filtered through a 0.45-µm membrane, transferred onto SM and LJM and incubated by means of the same procedure. With regard to the organs and lymph nodes, 3 g of parenchyma from the sites of lesions was minced and homogenized in physiological solution; then we picked up 2 ml of extract, to which we added 2 ml of 4% NaOH and incubated at 37°C for 30’. After neutralization with H₂SO₄ and centrifugation at 3000 RPM for 15’ at 20°C, the supernatant was eliminated. The sediment was reuspended in a solution of phosphate buffer at pH 6.8 and seeded (0.2 ml) on SM and LJM.

### Table 1

<table>
<thead>
<tr>
<th>Animal N°</th>
<th>Culture test on organs</th>
<th>PCR on organs</th>
<th>PCR on swabs</th>
<th>PCR on sponge bags</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>9</td>
<td>+</td>
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Note: the numerical order indicates the order in which the animals were butchered.
separately from their healthy counterparts. Moreover, every precaution is to be taken in order to avoid the risk of contaminating other carcasses, the production line and the staff working in the facility. All carcasses in which post-mortem inspection reveals diffuse tubercular lesions in various organs or zones must be declared unsuitable for consumption and therefore destroyed. If, however, the tubercular lesion involves only a single organ or the lymph nodes of a single organ or a part of the carcass, only these portions are to be declared unsuitable for consumption and destroyed; the rest of the organs and the carcass may be authorised for free consumption. In the case of the 48 reactive cattle studied, post-mortem inspection revealed tubercular lesions of the complete and incomplete primary complex of the IDT-reactive animals and the contaminated carcasses of the group of 12 healthy cattle butchered. In this case, too, more than half of the carcasses (57%) proved to be contaminated. This means that meat of the carcasses of IDT-reactive cattle before they are deemed suitable for marketing, every time that a portion of the carcass is declared fit for human consume. The PCR testing of samples taken from muscle surfaces by means of dry swabs is suited to this purpose, in that it is rapid and economical and enables the carcasses to be kept refrigerated until the results become available, without causing any significant commercial damage.

CONCLUSIONS

In the EU, there is no system for registering IDT-reactive animals slaughtered and subsequently destroyed because they are deemed unfit for human consumption. In Britain, however, where the incidence of TB is 10%, (14) about 30,000 cattle were slaughtered in the last year, including test reactors, direct contacts and inconclusive reactors, within the framework of a programme for the control of tuberculosis; about half of these animals were declared unfit for human consumption. This means that meat from about 15,000 animals with ascertained or suspected TB infection is placed on food market each year. (15) In the territory where the present investigation was carried out, about 400 IDT-reactive cattle were slaughtered in the three-year period 2013–2015 (mean prevalence of the disease 0.37%, mean incidence 0.35%); from a survey conducted in the establishments of the territory, no more than 5% of the carcasses were destroyed after being deemed unfit for human consumption. In the EU setting, while the supply of milk from TB-infected dairies is regulated by specific legislation, no particular provisions are made with regard to meat; this is probably because the risk of human contamination by M. bovis through

<table>
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<th>Carcasses</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<td>PCR SWAB</td>
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<td>+</td>
<td>+</td>
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<td>–</td>
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Note: the numerical order indicates the order in which the animals were butchered.
meat consumption is considered negligible. (8, 9) With regard to contamination of the muscle portions of carcasses during butchery, a particularly important role is played by the instrumental and manual operations carried out, including the incision of organs and lymph nodes during post-mortem inspection. Within the current framework of the process of meat inspection, it has been suggested that the routine incision of lymph nodes during post-mortem inspection after slaughter should be omitted, in order to avoid contaminating the meat with other pathogens, such as Salmonella spp. and pathogenic VTEC. Escherichia coli. (7, 9) In the case of M. bovis, however, this option does not seem feasible, in that traditional post-mortem inspection is fundamental to the diagnosis of bovine disease. Indeed, in the territory where the investigation was carried out, about 80% of cases of bovine tuberculosis are diagnosed after slaughter. By contrast, it may be reasonable to consider banning the marketing of fresh beef from animals in which post-mortem inspection detects any form and number of visible tubercular lesions. In the absence of macroscopically evident lesions, the meat of IDT-reactive animals should undergo adequate heat treatment before being released onto the market. There remains, however, the need for more effective enforcement of the obligation to implement the hygiene and sanitary measures prescribed for facilities that practise deferred butchery. Finally, we must acknowledge the consumer’s right to be informed of the provenance of meats and of the risk, albeit minimal, connected with M. bovis.

REFERENCES


Core tip: We utilised a polymerase chain reaction test to detect the DNA of *Mycobacterium bovis* in the meat of 48 cattle which had been slaughtered because they were intradermal tuberculin test (IDT)-positive and on the carcasses of 12 regularly slaughtered healthy cattle. The DNA of *M. bovis* was detected in 27% of the carcasses of the IDT-reactive animals and in 58% of the carcasses of the healthy animals. The results obtained confirmed the presence of *M. bovis* on the production line as a result of the presence of meat from IDT-reactive animals which was authorized for free consumption after passing post-mortem inspection. The meat of healthy animals that are butchered in facilities that practise the deferred butchery of cattle infected by tuberculosis is also a risk factor.

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