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Presence of *Mycobacterium bovis* in slaughterhouses and risks for workers

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Highlights

- *Mycobacterium bovis* is able to contaminate meats and slaughterhouses.
- Slaughterhouse workers are exposed to the risk of infection by *M. bovis.*
- Measures to prevent infection by *M. bovis* in slaughterhouses are inefficacious.

Abstract

An investigation was carried out to detect the presence of *Mycobacterium bovis* in slaughterhouses where intradermal tuberculin test positive cattle were slaughtered, and to evaluate the risk of contamination by *M. bovis* among exposed slaughterhouse workers. Swabs were taken from the carcasses of slaughtered animals showing autoptic signs of non-generalized forms of tuberculosis, thus authorized for free consumption. Swabs were also taken from the hands and clothes of the staff employed in the butchery production line. Environmental samplings were conducted on the slaughterhouse air using filters and air aspiration devices, and on water used to wash the carcasses after slaughter. Samples from the carcasses of healthy animals were also taken on a following slaughtering session. The swabs were analysed by means of Polymerase Chain Reaction for the detection of mycobacteria. *M. bovis* was detected on meats, on the hands of one worker, and in the washing water. The results obtained from this study confirm that workers are highly exposed to infection by zoonotic tuberculosis, and that cleaning procedures were ineffective in our setting.

Keywords: bacterial zoonosis; biological risk; cattle; *Mycobacterium bovis*; slaughter.

1.Introduction

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Prophylactic measures aimed at eradicating bovine tuberculosis caused by *Mycobacterium bovis* (*M. bovis*) have been compulsory in the European Union for many years (Regulation 429/2016/EU; SANCO/10067/2013; Council Directives 78/52/EEC and 82/400/EEC). Nevertheless, in several member states, not only the disease is still present, but it has actually shown worrying signs of upsurge (Allen et al., 2018; Amato et al., 2018). For some decades, the disease received little attention, especially because the incidence of zoonotic tuberculosis due to *M. bovis* was far lower than that of human tuberculosis, accounting for only a small percentage (0.5-7.2%) of all patients with a bacteriologically confirmed diagnosis of tuberculosis (de la Rua-Domenech, 2006). In Italy, the disease is still present in southern regions with a worrying prevalence. In the Calabria region, for instance, in the 2-year period 2017-2018, 42 outbreaks of bovine tuberculosis in as many herds were officially notified (prevalence 0.35-0.42%; incidence 0.33-0.38%), and 334 animals were slaughtered because they were reactive to the intradermal tuberculin test (IDT) (Italian Ministry of Health, Reports from PNI 2017 and PNI 2018). Of note, IDT is routinely performed on cattle within the framework of the official plan for the eradication of bovine tuberculosis in Italy. Both European and Italian legislation impose that IDT-positive cattle must be regarded as infected and, as such, slaughtered in authorised facilities in accordance with the principle of the so-called deferred slaughter (Italian Ministerial Decree n. 592, 1995; Council Directive 64/432/CEE). In practice, IDT-positive cattle must be slaughtered on different days from healthy cattle, or on the same day after the healthy cattle have been slaughtered. These slaughterhouses are a major source of occupational exposure to zoonotic pathogens and therefore represent a serious biological risk along the entire butchery production line. Accordingly, the Italian legislation classifies slaughtering as an activity at high risk, owing to the continuous contact of slaughterhouse staff with biological material from a large number of animals which may potentially be infected by or carriers of dangerous biological agents (Italian Law Decree n. 81/2008). Moreover, the "animal product manufacture" section of the

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2017 "Roadmap for zoonotic tuberculosis" published by the World Health Organization (WHO), identifies butchers and slaughterhouse workers and the staff of meat-storage facilities as individuals at high risk of zoonotic infection (WHO 2017). A recent systematic review of the literature on this subject (Vayr et al., 2018) has revealed that the data on professional exposure to bovine tuberculosis are somewhat scant. The present study was therefore intended to gain greater knowledge of the occupational risk posed by *M. bovis*. To this end, the presence and dissemination of *M. bovis* in some authorised facilities for the deferred slaughter of IDT-positive cattle to was evaluated in an endemic area of bovine tuberculosis in Southern Italy.

2. Materials and methods

2.1.Sampling

Among slaughterhouses authorised to deferred slaughter in the Calabria region (15,222 km²), four were selected based on: i) their willingness to participate to the study, and ii) the presence of infected animals to be slaughtered. They are indicated with numbers, from 1 to 4, and details about their location and working capacity are presented in Table 1. In each slaughterhouse, one session of deferred slaughter of cattle which had tested positive on IDT was attended. The presence of *M. bovis* was sought in: i) the environmental air in the facility, ii) the wastewater derived from washing the carcasses, iii) the meat products, iv) the hands and, v) the clothing of the staff working along the entire butchery production line. In Slaughterhouse no. 4 also a following session involving healthy animals was attended, in order to verify the effectiveness of procedures intended to disinfect the workplace and equipment to avoid cross-contamination of the carcasses from *M. bovis*. During this second session only meat products were sampled, while air, water and staff were not investigated.

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Environmental air was sampled as described in the National Institute for Occupational Safety and Health (NIOSH) Manual of Analytical Methods 0900 (NMAM, 1998). A total of three or four samplers, depending on the slaughterhouse (Table 2), were positioned along the production line at 1.5 mt from the floor, and operated for the whole deferred slaughter session (about 180 min.). Two of the air-sampling modules were set at a flow rate of 2 L/min, and the other modules were set at a flow rate of 4L/min. The two aspiration systems consisted of Inhalable Sampler Stainless Steel model XR2000 and model XR5000 (SKC Inc.), respectively, both connected to IOM pre-selectors equipped with 25 mm diameter sterile PTFE membrane filters (SKC Inc.). At the end of each session, filters were placed in refrigerated containers for transport to the laboratory. The wastewater from the slaughterhouses was sampled by taking four 3L samples of the water after the final washing of the carcasses. Water samples were filtered through 0.20 μm nitrocellulose filters (Millipore), and the retained material from both air and water samples was eluted and tested for the presence of *M. bovis*.

M. bovis was searched on the inner and outer surfaces of carcasses of a total of 65 IDT-reactive cattle slaughtered at different times in the 4 facilities. Post-slaughter inspection deemed meats from these animals to be fit for human consumption. In all four slaughterhouses, IDT-positive animals were slaughtered after the healthy animals had been slaughtered. After slaughter, and before the carcasses were placed in the refrigeration room, each half-carcass underwent sampling, which consisted of extensive wiping the external and internal surfaces with dry sterile rayon swabs (FL Medical). For each carcass a total of four swabs was collected, two from the external surfaces of thigh and chest regions, respectively, and two from surfaces of thoracic and pelvic cavities, respectively. If sampling areas were very dry, a further sample from the external neck surface was taken. The same swabbing procedure was used to sample the carcasses of 12 healthy cattle which were regularly slaughtered day after the IDT-positive cattle.

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A total of eleven workers deployed on the production line of the slaughterhouses (see Table 2 for details) were sampled by extensive swabbing of their hands and work clothes at the end of the working day. A total of 5 swabs were obtained from each worker: one sample from each of their hands and three samples from their working clothes. The use of individual protection devises (IPD) was also recorded.

Immediately after collection, swabs were placed in refrigerated containers and promptly transferred to the laboratory. A summary of sampling activities is shown in Table 2.

The swabs, air filters and wastewater filters underwent Polymerase Chain Reaction (PCR) and bacteriological testings (BT) for the detection of *M. bovis* according to the methods described below. A worker was considered positive for the presence of *M. bovis* if at least one out of four/five swabs gave a positive result to PCR or BT or both tests.

2.2. Molecular detection of mycobacteria and M. bovis

Briefly, each sample was placed in a tube containing 400µl of phosphate-buffered saline (PBS) and 10 mg of glass microspheres (100-200 µm diameter), and incubated at room temperature for 2 h. Samples were then vortexed and tubes were transferred to a tissue lyser (Qiagen s.r.l.), subjected to mechanical disintegration for 5 min at 30Hz, and centrifuged at 8,000 rpm for 30 s. Total DNA was extracted from the pellet and purified using the commercially available Qiamp DNA Mini Kit (Qiagen s.r.l.), following the manufacturer's indications. For PCR-based identification of *Mycobacterium* at the species level, a multiplex PCR test was performed, targeting specific regions of mycobacterial genes (Kulski et al., 1995): i) the MPB70 gene encoding for a specific protein of the *M. tuberculosis* complex (MTC), including *M. bovis* (372 bp amplicon) and, ii) different species-specific regions of the mycobacterial 16S rRNA gene (*Mycobacterium* spp., 1,030 bp amplicon; *Mycobacterium intracellulare*, 850 bp amplicon; *Mycobacterium avium*, 180 bp amplicon). DNA samples (5.4 ng/ml) were amplified in a C1000 Touch Thermal Cycler (Bio-Rad inc.) set to appropriate

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thermal profile. Amplicons were separated by 2% agarose gel electrophoresis in TBE buffer, stained with ethidium bromide, and visualized by UV trans-illumination in a Chemidoc XRS (Bio-Rad inc.).

2.3 Bacteriological tests

Samples were processed according to OIE standard procedures (OIE manual, 2015), and bacteria were isolated on Stonebrink and Löwenstein-Jensen solid media with pyruvate, after a decontamination step. After 60 days incubation at 37°C, suspected mycobacterial colonies were identified by two PCR reactions specific for the *Mycobacterium* genus and for the MTC group, as described above, and by PCR/RFLP analysis of the *gyrB* gene after digestion with the restriction enzyme RsaI (Niemann et al., 2000).

3. Results and discussion

The results of the PCR tests carried out on swabs, filters and water are shown in Table 3. The DNA of *Mycobacterium* spp. was detected in 32% (21/65) of the carcasses, and in 14 of them (14/21, 66%) it was subsequently identified as *M. bovis*. Careful scrutiny of the results revealed that the frequency of contamination of the carcasses both by *Mycobacterium* spp and *M. bovis* increased with the progression of slaughtered cattle. Indeed, samples taken from the carcasses at the beginning of the slaughtering session tested negative, while in subsequent carcasses mycobacterial DNA was increasingly detected. An example of this trend is shown on Fig. 1, and the analysis of samples from other slaughterhouses followed a similar trend. This was probably due to increasing environmental contamination during slaughter.

Interesting results were obtained from sampling the carcasses of 12 healthy cattle regularly slaughtered the day after the deferred slaughter of IDT-positive animals in slaughterhouse no. 4 (Table 3). Impressively, PCR testing revealed the presence of *M. bovis* DNA in the carcasses

of 58% (7/12) of the healthy animals, suggestive of local persistence of *M. bovis* in the environment, likely due to ineffective slaughterhouse cleaning.

On air testing, 69% of filters (9/13) yielded the DNA of *Mycobacterium* spp. upon PCR screening, but in none of them was *M. bovis* subsequently identified. The tests carried out on water samples from the final washing of the carcasses, taken before the water entered the slaughterhouse drainage system, revealed the presence of *M. bovis* in 50% (2/4) of wastewater samples.

The DNA of *Mycobacterium* spp. was also detected on the hands and clothes of 81.8% (9/11) of workers, though *M. bovis* could be identified only on the hands of a single worker (9.1%). The use of adequate IPD by the sampled personnel employed in the butchery production line at the moment of sampling is presented in Table 4.

All attempts to isolate *M. bovis* by BT failed, probably due to a low contamination level of sampled matrices, or to the presence of either dormant or non-viable mycobacteria.

As shown in Table 5, high positivity rates were observed for slaughterhouses no. 2 and 4. In slaughterhouse no. 2, 10.7% of the carcasses were contaminated with *Mycobacterium* spp. and 7.6% with *M. bovis*, whereas in slaughterhouse no. 4, 12.3% of the carcasses were contaminated with *Mycobacterium* spp. and 9.2% with *M. bovis*. Such high contamination level was consistent with the presence of *M. bovis* in the wastewater of both slaughterhouses, and of *Mycobacterium* spp. in air filters and work clothes. Lower contamination levels were observed in slaughterhouses no. 1 and 3, likely due to their smaller size and lower slaughtering activity. Notably, the detection of *M. bovis* on the hands of a worker from slaughterhouse no. 3 is attributable to the poor compliance with hygiene rules and the lack of protective equipment observed in this plant.

Our results provide evidence that the environment of facilities where cattle are slaughtered are contaminated by mycobacteria. However, the geographical diversity and composition of the reservoirs of environmental mycobacteria have not yet been thoroughly described, despite

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the need to tackle the risk of exposure to bovine tuberculosis. In all four of the slaughterhouses examined, carcasses were found to be contaminated by *M. bovis*; this demonstrates that slaughtering infected animals predictably causes contamination of the environment and, owing to the handling of slaughtered animals, also of workers and meat. Moreover, the carcasses of healthy animals that were slaughtered the day after the deferred slaughter of infected animals were also found to be contaminated.

This is proof of the poor efficacy of the cleansing and disinfection operations which should be carried out after the slaughter of infected animals, as prescribed by Regulation n. 854/2004/CE (Regulation (EC) No 854/2004 of the European Parliament). Contaminated carcasses that are deemed fit for consumption on post-slaughter visual inspection enter the regular chain of distribution and marketing of meats, finally reaching the consumer. Moreover, it must be pointed out that, in the region where the present study was conducted, 90% of outbreaks of bovine tuberculosis are diagnosed in the slaughterhouse by means of post-mortem inspection of cattle from herds that are officially unaffected. In addition, the measures that need to be adopted in order to avoid contamination following the detection of tubercular lesions during normal slaughter are not prescribed by national and European regulations governing the inspection of meats and the prevention of occupational exposure to risk. The detection of *M. bovis* on the hands of one worker, even after washing, denotes actual exposure of workers to contagion through the skin. Professional exposure due to aerosols generated by mechanical handling processes and/or to contact with contaminated water cannot be ruled out, since mycobacteria were detected in 50% of water samples and ca. 70% of air filters.

Human infection by *M. bovis* often occurs through the ingestion of foodstuffs, particularly contaminated milk and cheese. However, with regard to occupational exposure, a major role is played by the inhalation of infected aerosols and direct contact with skin wounds on the hands and arms of workers. In such cases, the minimum infective dose is much lower than in

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the case of contagion through the alimentary route (Humblet et al., 2009). Moreover, according to an Italian occupational health report, most wounds (35% of the total) incurred by slaughterhouse workers are caused by knives (ISPSEL Report 2000). In all the slaughterhouses investigated in the present study, the use of personal protection devices by workers during all the phases of butchery was almost non-existent (Table 4). In all the four sampled slaughterhouse none of the employed personnel wore mandatory individual protection devices, such as protective headgear/helmet, cut-protecting safety gloves, eyeshade/face mask, safety boot, disposable coverall. Disposable latex gloves and waterproof aprons were the only used devices. This indicates that the perception of biological risk during slaughterhouse activities is still low among workers, managers and health authorities responsible for inspections.

The results of our analyses of the water used to wash the carcasses confirmed the presence of contamination in all the phases of butchery. Moreover, it should be stressed that the environmental conditions encountered in slaughterhouses, namely: *i*) humidity due to the abundant use of water for washing the carcasses, entrails, equipment and work areas; *ii*) the presence of residual organic material, and *iii*) the low temperatures, can prolong the survival of *M. bovis*, and therefore the duration of exposure in slaughterhouses (Fine et al., 2011).

4. **Conclusions**

Workers involved in the slaughter of cattle and the handling of meats are at high risk of being into contact with animal-borne pathogens, such as *M. bovis*, and therefore of developing an occupational zoonosis. In industrialised countries, meat from cattle infected by *M. bovis* can be freely marketed, provided that the result of post-slaughter veterinary inspection of the carcasses is favourable. However, this policy, which is dictated by purely economic considerations, is no longer sustainable and should be reconsidered to prevent the risk of

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infection among exposed slaughterhouse workers. Moreover, the risk also extends to consumers, given that *M. bovis* contamination of meats that are ready to be marketed has been ascertained. In this regard, consumers are not provided with any information on the state of health of the cattle from which meats are derived. For years, the question of whether cattle that prove positive on the IDT should be destroyed has been matter of discussion (ACM/981b). In developing countries, by contrast, where the risk of zoonosis due to *M. bovis* is much higher, a very different approach is required, in view of the different cattle rearing and slaughter practices, and the lack of plans for the eradication of the disease.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Ethics

Any procedure involving human subjects has been carried out in accordance with the relevant institutional laws and guidelines, and has been approved by the institutional Ethics Committee

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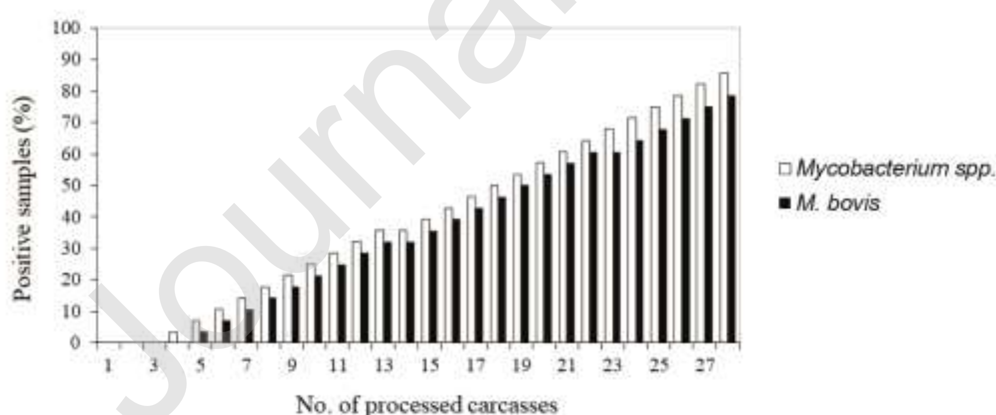

Figure 1. Trend of PCR-positive tests on carcasses sampled in slaughterhouse no. 2 in relation with the progression of butchery activities.

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Table 1. Location and characteristics of selected slaughterhouses.

Slaughterhouse no.	Province	Working capacity*	Slaughtered species**
1	Catanzaro	medium	C, P, S, G
2	Catanzaro	high	C, P, S, G
3	Vibo Valentia	low	C, P, S, G
4	Cosenza	high	C, P, S, G

*low = up to 200 slaughtered animals per week; medium = up to 500 slaughtered animals per week; high = more than 500 slaughtered animals per week

**C= cattle; P= pigs; S= sheeps; G= goats

Table 2. Number of sampled workers and matrices for each facility.

Matrix	Slaughterhouse no.				Total
	1	2	3	4	
Bovine carcasses	18	28	7	24*	77
Swab from carcasses	72	130	35	105	342
Swabs from workers	1	5	2	3	11
Air filters	3	4	3	3	13
Wastewater samples	1	1	1	1	4

*includes 12 carcasses from healthy cattle and related samples.

Table 3. Results of PCR tests for *Mycobacterium* spp. and *M. bovis* in different samples.

Sample(s)	No.	Positive	
		Mycobacterium spp.	*M. bovis*
Carcasses (IDT-positive animals)	65	21	14
Carcasses (healthy animals)	12	9	7
Ambient air	13	9	0
Workers	11	9	1
Wastewater	4	4	2
Total	105	52	24

Table 4. Use of different kinds of individual protection devices (IPD) by sampled personnel during their work in the slaughterhouse.

IPD	Worker no.										
	1	2	3	4	5	6	7	8	9	10	11
Protective headgear	no	no	no	no	no	no	no	no	no	no	no
Protective goggles	no	yes	no	no	no	no	no	no	no	no	no
Face mask (mouth protection)	no	no	no	no	no	no	no	no	no	no	no
Safety gloves (cut-protecting)	no	no	no	no	no	no	no	no	no	no	no
Disposable coverall	no	no	no	no	no	no	no	no	no	no	no
Safety boot	yes	yes	no	no	no	no	no	yes	yes	no	yes
Protective waterproof apron	yes	yes	yes	yes	no	yes	yes	yes	yes	no	yes

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Disposable latex gloves	no	yes	yes	yes	no	yes	yes	yes	yes	no	no

Table 5. Positive PCR results for the detection *Mycobacterium* spp. and *M. bovis* in different matrices and slaughterhouses.

Slaughterhouse	Carcasses		Workers		Ambient air		Wastewater	
	Mycobacterium spp.	*M. bovis*	*Mycobacterium* spp.	*M. bovis*	*Mycobacterium* spp.	*M. bovis*	*Mycobacterium* spp.	*M. bovis*
1	2	1	1	0	1	0	1	0
2	7	5	4	0	3	0	1	1
3	4	2	2	1	3	0	1	0
4	8	6	2	0	2	0	1	1
Total	21	14	9	1	9	0	4	2

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