

Contamination of bovine, sheep and goat meat with *Brucella* spp.

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Abstract

A study was conducted in order to evaluate the contamination by *Brucella* spp. of meat from animals slaughtered because they had resulted positive for brucellosis at some time during their life. After slaughter and before delivery to market outlets, swab samples were taken from 307 carcasses of infected animals: 40 cattle, 60 sheep and 207 goats. The swabs were subsequently analysed by means of polymerase chain reaction (PCR) tests. In addition, bacteriological tests were carried out on the lymph nodes and internal organs of the same animals. *Brucella* spp. was detected by means of PCR in 25/307 carcasses (8%): 1 bovine (2.5%), 9 sheep (15%) and 15 goats (7.2%) and was isolated by means of a cultural method in 136/307 carcasses (44%). Moreover, additional analysis, performed on lymph nodes from the same carcasses that had proved positive by PCR, allowed highlighting type 3 *Brucella abortus* in the bovine carcass and type 3 *Brucella melitensis* in the sheep and goat carcasses. The study shows that cattle, sheep and goats meat of animals slaughtered because they had tested positive for brucellosis may be contaminated by *Brucella* spp. As this could constitute a real risk of transmission to both butchery personnel and consumers, the meat of animals infected by *Brucella* spp. should be analysed before being marketed. In this respect, PCR technique performed on swabs proved to be more useful, practical and faster than the traditional bacteriological method.

Introduction

Brucellosis is a highly contagious zoonosis caused by bacteria of the genus *Brucella* and is listed in Class B animal epidemics by the World Organization for Animal Health (OIE). The genus has six classic species: *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae*. Recently, the marine species *B. ceti*, *B. pinnipedialis*, *B. microti* and *B. inopinata* were included in the genus (Foster *et al.*, 2007; Scholz *et al.*, 2008, 2010). The incidence of human brucellosis is estimated by the OIE at

500,000 new cases per year worldwide. However, official statistics is widely acknowledged to be underestimated and, in a considerable number of cases, the origin of the infection is not identified. The majority of human cases worldwide is attributed to *B. melitensis* (Pappas *et al.*, 2005). In general, *B. melitensis* and *B. suis* are more virulent in humans than *B. abortus* or *B. canis* (WHO, 2006). Other species can cause infection in humans, but only rarely (Diaz Aparicio, 2013). The disease is more prevalent in western parts of Asia, India, Middle Eastern, Southern European and Latin American countries. The transmission of brucella infection and its prevalence in a region depend on several factors, such as dietary habits, methods of processing milk and milk products, social customs, husbandry practices, climatic conditions, socioeconomic status and environmental hygiene (Mantur and Amarnath, 2008). As the infectious dose is very low, infections are an occupational risk for farmers, veterinarians, abattoir workers (Schneider *et al.*, 2013), laboratory personnel and others who work with animals and consume their products (Pepin *et al.*, 1997). Human brucellosis is transmitted by inhalation, animal contact and the consumption of dairy products and undercooked meat products. The consumption of traditional dishes, such as raw liver, can cause human infection (Malik, 1997). Muscle tissue generally contains a small number of organisms. However, it has been estimated that even 10-100 microorganisms are sufficient to cause the disease in humans (Pappas *et al.*, 2006). Thus, contaminated meat and meat products could represent a source of infection, especially if they come from animals slaughtered during the acute phase of the disease (FAO/WHO, 1986) and if they are consumed raw or undercooked. The handling and preparation of infected meat and offal may give rise to the contamination of other foodstuffs and kitchen utensils (WHO/CDS/EPR/2006.7; WHO, 2006). Tests carried out in the US on the carcasses of bovine and swine that were slaughtered because infected revealed that 1.2% of the bovine carcasses and 3.5% of the swine carcasses were contaminated by *Brucella* spp. (Sadler, 1960). In a similar study conducted in India on 100 carcasses of goats, two of the 700 neck muscle samples analysed tested positive for *Brucella melitensis* (Randhawa and Karla, 1970). Other studies have shown that the offal of slaughtered ruminants constitutes a risk for transmission of the infection (Fatma and Mahdey, 2010; Sekulovski, 2008). In order to evaluate the prevalence of contamination of meats and to assess the risk of infection for consumers and professionally exposed workers, an investigation on 307 carcasses of bovines, sheep and goats that were slaughtered because they had resulted positive on serological testing for *Brucella* spp. was performed. The study was carried out within the framework of the national programmes for the

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eradication and monitoring of animal diseases and the prevention of zoonoses in Italy, approved by Decisions 2011/807/UE and 2012/761/UE of the European Commission (2011, 2012).

Materials and Methods

The study lasted 12 months and was carried out in 10 abattoirs in southern Italy, where brucellosis infection is still widespread on cattle, sheep and goat farms. After slaughter, swab samples were taken from 307 carcasses: 40 cattle, 60 sheep and 207 goats. These animals came from 24 different farms and were slaughtered because they had proved positive on serological tests for *Brucella* spp. In previous studies (Randhawa and Karla, 1970; Fatma and Mahdey, 2010) *Brucella* spp. contamination of the carcasses of slaughtered animals had been evaluated by means of microbiological tests carried out on samples of muscle or organ tissue. In the present study, swab samples were taken in accordance with the procedure indicated by the Decision of the European Commission (2001): duplicate samples were taken by means of swabs – both dry and moistened in a sterile aqueous solution – which were wiped over the external and internal surfaces of the carcasses immediately after slaughter and before storage in refrigerators. A total of 608 swab samples were taken; no transport medium was deemed necessary, as the samples were promptly consigned to the laboratory, where they underwent polymerase chain reaction (PCR) analysis for *Brucella* spp. in accordance with the following procedure.

For DNA extraction, a commercially available kit (QIAamp DNA Mini Kit; Qiagen, Valencia, CA, USA) was used according to the protocol described for the swab matrix. Amplification was carried out by means of a nested PCR technique, based on a published protocol (Romero *et al.*, 1995; Romero and Lopez-Goni, 1999; Tantillo *et al.*, 2001, 2003), involving two successive amplification steps. The second step utilised a pair of oligonucleotides that are internal to those used in the first step and produce a smaller fragment; they prove functional only if the result of the first PCR is specific. The first amplification step used the primer pairs F4 (5'-TCGAGCGCCCGCAAGGGT-GAGCGG-3') and R2 (5'-AACCATAGTGCTC-CACTAACC-3') allowing an amplification product of 905 bp. In the second step of PCR, the primer pairs R0 (5'-TAGCTAGTTG-GTGGGGTAAAGGC-3') and R1 (5'-CAGGCTTGCGCCCATTTGCC-3') were used allowing an amplification product of 144 bp. To prepare the reaction mixture for the first PCR, 12.5 µL of a commercially available PCR master mix (PCR master mix; Promega Corp., Madison, WI, USA) was used at a 1X concentration for each sample. The reaction mixture contains 50 units/mL of Taq polymerase, 400 mM of dATP, dGTP, dCTP, DTTP and 3 mM of MgCl₂. To the reaction mixture, 0.5 µL of each primer – F4 and R2 at a concentration of 0.2 µM – and 6.5 µL of H₂O were added, with a final reaction volume of 25 µL. As a positive control in PCR, we used a type-3 *Brucella melitensis* biovar saved in our laboratory. The negative control was made up only of master mix and water.

To prepare the reaction mixture for the second PCR, the same procedure was carried out, with primers R1 and R0. The thermal profile of the first PCR involved an initial denaturation at 95°C for 4' followed by 35 cycles of denaturation (at 94°C for 1'), annealing (at 54°C for 1') and extension (at 72°C for 1') and a final extension at 72°C for 7'. The thermal profile of the second PCR was the same as the first one except for the cycle number that consisted of 30 cycles in the second PCR. In accordance with Italian law and with the aim of eradicating brucellosis in cattle, sheep and goats, the carcasses of infected animals may be freely marketed if the outcome of post-slaughter examination is favourable, while all the internal organs and the udders must be destroyed. Parts of these organs were therefore removed and subjected to culture tests for the bacteriological identification and typing of *Brucella* spp. by means of the technique described in the Manual of the Office International des Epizooties, ch. 2.4.3 2009 lett. B (OIE, 2015). This involves: streaking on brucella agar medium added with a selective supplement and horse serum; incubation at 37°C in an atmosphere enriched with 5-10% CO₂; and observa-

tion for up to 10 days. Subsequent species typing of the isolated strains was carried out at the National Reference Centre for Brucellosis in Teramo (Italy) by means of the PCR-restriction fragment length polymorphism technique, in accordance with the procedure prescribed in Vol. 1, Part. 2, Sect. 2.4 Ch. 2.4.3. of the OIE Manual (OIE, 2015).

Results

The biomolecular tests revealed the presence of *Brucella* spp. in 25/307 carcasses (8%): 1 bovine carcass (2.5%) of a 9-year-old cow; 9 carcasses of sheep (15%) aged between 18 months and 5 years, all from the same farm; and 15 carcasses of goats (7.2%) aged between 12 months and 6 years and coming from 5 different farms (Figure 1). The 25 carcasses found to be contaminated with *Brucella* spp. belonged to 25 animals slaughtered in 6 different slaughterhouses. The bacteriological tests carried out on the organs of all 307 animals examined allowed isolating *Brucella* spp. in 136/307 cases, *i.e.* 44%. The typing analysis allows detecting type 3 *Brucella abortus* in the cattle organs, and type 3 *Brucella melitensis* in the sheep and goats organs. The organs, specifically the lymph nodes, from all the carcasses that had tested positive on PCR on

swabs, also proved positive on bacteriological testing for type 3 *Brucella abortus* (the bovine carcass) and for type 3 *Brucella melitensis* (the sheep and goat carcasses).

Discussion

The results obtained show that the carcasses (*i.e.*, the meat) of animals slaughtered due to *Brucella* spp. infection may also be contaminated by the same infective agent. This situation is the same as that seen in the case of other pathogens, like *Salmonella* spp., for which specific laboratory tests are prescribed and measures have to be taken. In the present study, the percentage of meats that proved to be contaminated was far higher than in the studies quoted above (Sandler, 1960; Randhawa and Karla, 1970). Contamination by *Brucella* spp. was detected in a higher percentage of sheep carcasses than cattle or goat carcasses, while the bacteriological tests conducted on the organs detected the highest percentage of contamination among goats. The only *Brucella* serotypes isolated from the organs were type 3 *Brucella abortus* in cattle and type 3 *Brucella melitensis* in sheep and goats. These are the only serotypes that have been isolated in recent years in the geographical area considered (Casalinuovo *et al.*, 2011; De

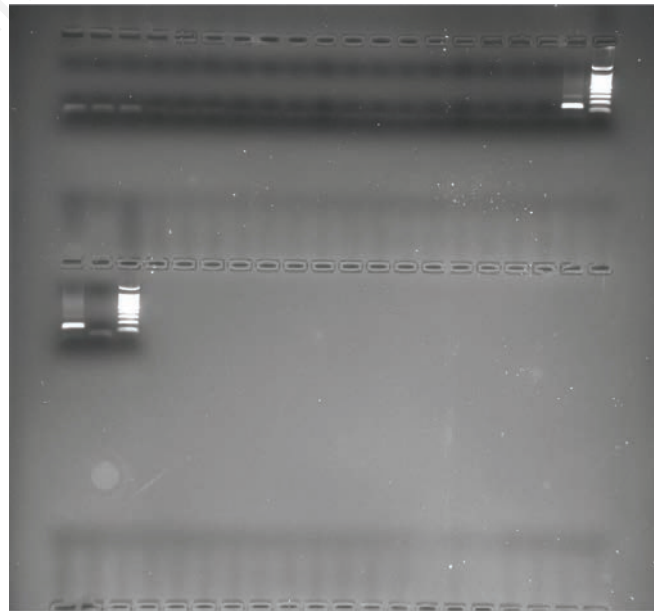


Figure 1. Polymerase chain reaction analysis for *Brucella* spp. Amplification product derived from the analysis of 19 swabs: 10 µL of each reaction mixture was separated by means of 2% agarose gel, observed under ultraviolet light and photographed. A 100 bp marker was used as a reference. Positive and negative controls were loaded in the last two wells of the gel, while the presence of a positive sample (144 bp) can be noted in the 19th well.

Massis *et al.*, 2014). The handling of the carcass entrails in the slaughterhouse has surely caused contamination. Thus, the environments and facilities of slaughterhouses may also be severely contaminated, thereby constituting a risk of contagion for staff involved in butchery and meat handling, and a risk of contamination of the carcasses of other animals that are not infected by brucellosis. Indeed, the positivity of the carcasses involved 6/10 slaughterhouses.

Our samples were taken at the end of the butchery process, when the carcasses were ready for storage in refrigerators or delivery to retail outlets. This means that each contaminated carcass could in turn contaminate equipment and utensils and other meats or food-stuffs. Consequently, even in domestic kitchens, contamination could further involve other equipment, utensils and food products. Indeed, as *Brucella* spp. survives well at the temperatures reached by refrigerators or freezers, other refrigerated or frozen foods may become contaminated. Thus, all meat products must be thoroughly cooked before being eaten.

PCR testing of swab samples taken from the carcasses of animals that had tested positive for *Brucella* proved to be useful. This practice could be applied systematically, since this non-invasive sampling technique does not damage commercial meat cuts, is economically advantageous, and requires far less time (24-48 h) than detection by means of culture media. Carcasses that resulted positive for PCR might be sequestered and potentially submitted to different confirmation tests.

Conclusions

Annex I, section IV, chapter IX, F, of Regulation (CE) N°. 854/2004 (European Commission, 2004) deals with what should be done with meat from infected animals, and lays down preventive measures to be taken with regard to the specific risks of brucellosis. Indeed, the Regulation requires that animals proving positive or uncertain for brucellosis have to be slaughtered separately, and that precautions have to be taken to avoid the risk of contaminating other carcasses, the butchery process and the abattoir staff. The udders, genital organs and blood of such animals are classified as unfit for human consumption, while their meat can be freely marketed. Only if *post-mortem* examination reveals lesions attributable to acute infection by *Brucella* spp. must their meat be declared unfit for consumption and destroyed. Otherwise, the meat of these animals, just like the meat of healthy animals, is allowed to enter the food chain. These measures are justified by the need to avoid the

destruction of large amounts of meat, which is a valuable food source. Nevertheless, the results of the present study show that the carcasses, and consequently the meat, of animals positive for brucellosis may be contaminated by *Brucella* spp. This means that consumers may be exposed to the risk of infection, so they should be sufficiently informed with regard to the origin of the meat at the time of purchase.

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