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Polymerase chain reaction and bacteriological comparative analysis of raw milk samples and buffalo mozzarella produced and marketed in Caserta in the Campania region of Italy

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Summary

To help identify an epidemiological link between the consumption of buffalo mozzarella prepared with raw milk (not heat-treated) and cases of human brucellosis, 80 samples of raw buffalo milk and 315 samples of mozzarella were tested for the presence of *Brucella* spp. Samples originated from Caserta, the province with the highest number of *Brucella*-positive buffalo herds in Campania, the region in which 96.02% of all cases of human brucellosis reported in 2000-2005 were recorded. To take into account possible seasonal variations, between February 2006 and March 2007, samples were purchased directly from 72 dairy outlets that were representative of the province. Samples were tested for *Brucella* spp. using the polymerase chain reaction (PCR) and bacterial isolation. Samples tested negative for *Brucella* using both methods. Spiked samples were then prepared and tested by PCR and bacterial isolation and the sensitivity, specificity, repeatability, reproducibility and limit of detection were determined. The PCR limit of detection was below 1 colony-forming unit (cfu)/g. The repeatability and reproducibility of the method were 100% ($p = 0.95$), the sensitivity was 96.7% ($p = 0.95$) and the specificity was 100% ($p = 0.95$).

Keywords

Brucella, Brucellosis, Buffalo, Campania, Caserta, Italy, Microbiology, Milk, Mozzarella, Polymerase chain reaction.

Introduction

Approximately 500 000 new cases of human brucellosis recorded worldwide each year reveal that this disease is still an important zoonosis, especially in the European countries of the Mediterranean, East Africa and parts of Asia and South America (3). Although European legislation (4) safeguards public health against brucellosis by controlling the movement and use of milk for the production of dairy products, 1 218 cases of human brucellosis were reported in the European Mediterranean Basin in 2005, corresponding to an incidence of 0.2 cases per 100 000 inhabitants (6). Between 2000 and 2005, the highest incidence was reported in Portugal (1.4), Italy (1.1) and Spain (0.5), although these incidence figures were lower than those observed in previous years, due to disease eradication programmes in animal populations in these countries (6). In Italy in 2004, 92.18% of human brucellosis notifications came from the regions of Campania, Puglia, Calabria and Sicily, with a similar figure for 2005 (92.7%) (1). Official

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data published by Italian Ministry of Health, reported total brucellosis human cases but did not delineate the biovars involved. However, all human strains sent to our National Reference Centre for brucellosis over the last 10 years were *Brucella melitensis* biovar 3.

In Europe, human infections are mainly caused by *B. melitensis* (90.1%) (3) that is attributable to the consumption of contaminated products, such as fresh milk, butter, ice cream and fresh cheese made with unpasteurised milk (1, 2, 9). In Italy, cheeses awarded the 'DOP' (*denominazione di origine protetta*: protected designation of origin) play an important role in the dairy economy; the label was created with the intention of protecting products originating from a specific geographic area where production requires the use of local raw materials and traditional techniques. These products include buffalo mozzarella which is prepared with raw milk and the sales of which have boomed in recent years, with a sales increase of 7-10% a year for the period 2005-2006. There is a significant presence of brucellosis in buffalo populations in Campania – and especially the province of Caserta, where there is a high density of infected buffalo farms. This study evaluated the presence of *Brucella* in samples of bulk milk destined for dairy outlets and in buffalo mozzarella samples taken in the province of Caserta. Samples were tested for the presence of *Brucella* spp. by polymerase chain reaction (PCR) and bacterial isolation and a bacterial count was performed to determine the contamination level. The sensitivity, specificity, repeatability, reproducibility and limit of detection of the PCR detection method used were also established using artificially spiked samples.

Materials and methods

Sampling of raw buffalo milk and mozzarella

A total of 315 buffalo mozzarella samples were taken directly from the producers in the province of Caserta. The products were packaged in sterile bags, sent by refrigerated courier and tested within 24 h of delivery to the laboratory. Each sample was accompanied by a sampling sheet that gave the origin of the product and various parameters concerning the principal processing stages (pH, salt concentration, temperature).

An analysis was also made of 80 samples of bulk milk destined for mozzarella production that had originated from dairy outlets in the same province.

Diagnostic methods

Mozzarella

Samples weighing 25 g were homogenised 1:10 w/v in a stomacher in *Brucella* broth and the extracts were cultured for *Brucella* isolation from the mozzarella samples using *Brucella* broth and *Brucella* agar (Oxoid, Basingstoke) that included a mixture of antibiotics (Oxoid, Basingstoke) and equine serum (12). A most probable number (MPN) count using the official United States Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) Microbiology Laboratory Guidebook (MLG) 2.03 method (11) was performed simultaneously on each sample.

Milk

Samples (50 ml) were transferred to a test tube and centrifuged at 2 000 g for 30 min. Cream and sediment samples were sown directly onto *Brucella* agar and enriched in *Brucella* broth (1 ml of sediment in 9 ml of broth) (12). All samples (enriched and direct) were incubated at 37°C in 10% CO_2. The enriched broths were sown onto *Brucella* agar every week for a total of 42 days. A total of 5 ml of the homogenate was taken for the detection of *Brucella* spp. by PCR. Then, 500 µl of Tween 80 (Becton Dickinson, St Louis, Missouri) was added and the sample centrifuged at 13 400 rpm for 5 min. DNA was extracted using the Ultraclean Microbial DNA Kit (Mobio Laboratories Inc., Carlsbad). DNA concentration and purity were determined by biophotometer (Eppendorf, New York). The primers used (labelled as BB1 and BB2) were designed on the basis of the IS711 structure. Specificity was verified by comparing sequences in the basic local alignment search tool (BLAST) database (8). M13 (MWG BiotecH, Ebersberg) was used as the internal control, as reported by Josefsen *et*

al. (7) to reveal any PCR inhibition. The primer and control sequences are reported in Table I. A GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, California) was used for amplification in the following conditions: 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The amplified product was separated by agarose gel electrophoresis (Eppendorf, Milan) 1% in TBE 1X (BioRad Laboratories, Segrate, Milano Province). The gel was stained with Sybr Safe DNA gel stain (Invitrogen, Foster City, California) and examined by transillumination (Alpha Innotech Corporation, San Leandro, California).

The PCR was run on preparation of the homogenate and after incubation for three and seven days. When negative at 7 days, the test was repeated after incubation lasting six weeks.

Validation of polymerase chain reaction test method

Artificially spiked samples were prepared to determine the limit of detection, sensitivity, specificity, repeatability and reproducibility of the *Brucella* spp. PCR detection method. A suspension of *B. melitensis* biovar 3 (Central Veterinary Laboratory, Weybridge) with an optical density (OD600) of 0.400±0.005 as measured by biophotometer (Eppendorf, Milan), equivalent to approximately 10^8 colony-forming units (cfu)/ml, was prepared in phosphate-buffered saline (PBS) to determine the limit of detection. Ten-fold dilutions down to 10^4 cfu/ml were prepared from the initial inoculation. The 10^4 cfu dilution was used for sample spiking and the bacterial count of the inoculation was measured by titration on brain heart infusion agar (Biolife, Milan).

The sample was prepared by taking 25 g of buffalo mozzarella which had given negative results for *Brucella* spp. on isolation, placing it in a stomacher bag with 225 ml of *Brucella* broth (Oxoid, Basingstoke) and inoculating the prepared suspension to achieve a final count of approximately 10^3 cfu/g. Five ten-fold dilutions in *Brucella* broth were prepared from the homogenate (from 10^3 to 10^{-1} cfu/g) and tested for *Brucella* spp. by PCR.

The sensitivity of the method was determined by examining 30 spiked samples that had been prepared in the same way, so as to obtain a final concentration of 10 cfu/g and specificity by examining 30 mozzarella samples known to be negative for *Brucella*. Negative samples were prepared using the same procedure.

The data was analysed by Beta distribution (s+1, n-s+1), where s is the number of successes and n the number of samples analysed.

Repeatability was calculated by examining a known positive sample, spiked to a final count of 10 cfu/ml, 30 times consecutively.

Reproducibility was determined by analysing 30 artificially spiked mozzarella samples and 30 negative samples. Data were then evaluated using the Cohen k test.

Results

All buffalo mozzarella and bulk milk samples tested by isolation and PCR were found negative for *Brucella* spp. The MPN was found to be <0.3 MPN/g. Tests on spiked samples examined by PCR before and 3 and 7 days after incubation revealed an improvement in the method's limit of detection (Fig. 1). Samples inoculated with a 1:10 dilution of samples proven to have a minimal number of

Table I
Primer and internal control sequences

Target	Sequence
BB1	CATATCTTCCGGGGCGAGTTGGTA
BB2	GGATGACTCATTCTGAGCCGTTGCCTTGAGATTG
M13rev	GAGCGGATAACAATTTCACACAGG
M13uni	AGGGTTTTCCCAGTCACGACGTT

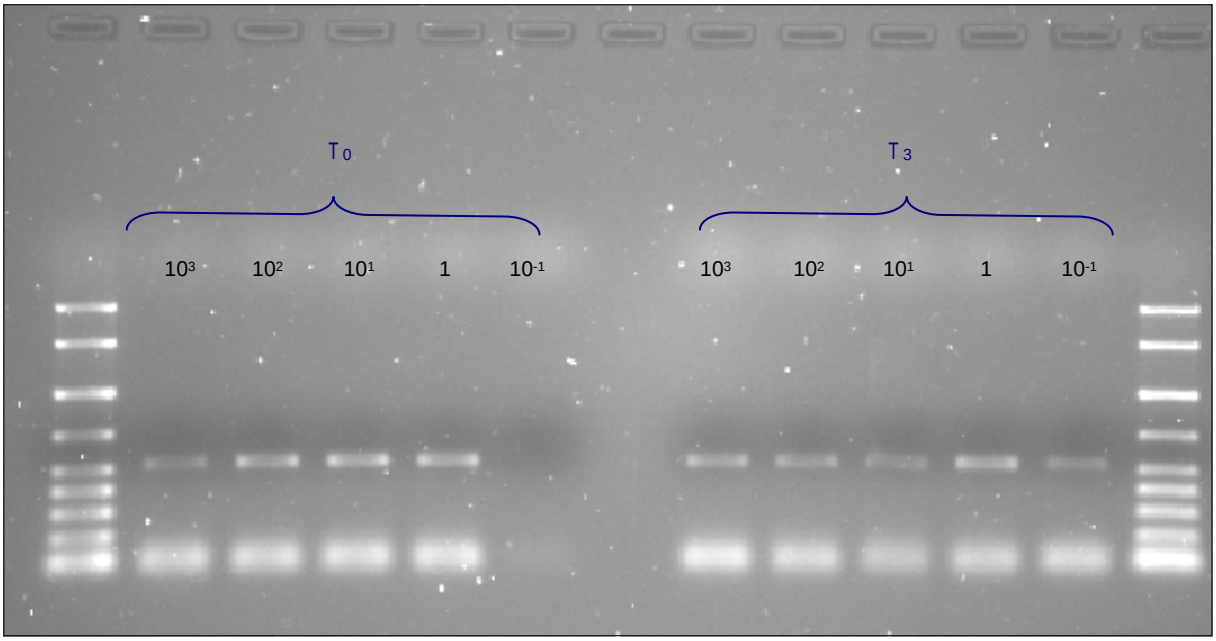

Figure 1
Results of *Brucella* spp. screening by polymerase chain reaction in samples spiked with *Brucella melitensis* biovar 3 at five different inoculation levels, before (T_0) and after incubation for three days (T_3)

organisms tested as a single colony-forming unit per gram and initially found negative tested positive after incubation for 3 days.

Repeatability data were in agreement (100%) with a confidence interval of 90.8%-100% (p = 0.95). With respect to reproducibility, no disagreement was found between the results of two different operators (Cohen k test = 1).

With respect to sensitivity, 29 positive results were obtained, equivalent to 96.7% (lower confidence limit: 88.3%; upper confidence limit: 99.2%; p = 0.95), while specificity was 100% (lower confidence limit: 90.8%; upper confidence limit: (100%; p = 0.95).

Discussion

Despite the adoption of various national regulations and specific regional plans (1, 2), brucellosis in cattle, sheep and goats remains a serious problem, especially in some regions of southern Italy. Buffalo farms are similarly affected: in Campania, with 1 478 farms – of which 994 (65.58%) are located in the province of Caserta alone – tests in 2006 revealed that 28.8% of herds contained serologically positive animals, increasing to 37% in 2007 (1, 2). For this reason, the tests conducted in this study focused on this area. The use of raw milk for the production of buffalo mozzarella, as required by the traditional manufacturing technique, suggests a possible role for this food in human infection. The risk mainly arises from the use of contaminated raw milk destined for dairy production and, less frequently, from secondary contamination that occurs during production and sale (9, 10). Our analyses did not reveal any bulk buffalo milk or mozzarella samples positive for *Brucella* spp., confirming the data reported in other studies (9, 10). However, this result does require further investigation that also includes, on the one hand, the study of the manufacturing processes and, on the other, the practice of heat treatment of milk from negative animals from infected farms.

The first point of particular interest that is especially likely to occur in traditional or non-industrialised dairies and in which the limits established by the hazard analysis critical control point (HACCP) plan for critical control points are not respected may enable *Brucella* to survive processing and still be present in the finished products. The second aspect concerns the lack of strict compliance of the parameters for heat treatment of milk from negative

animals from infected farms, as required by current legislation (5). This is particularly important for fresh dairy products and cheeses (less than 60 days maturation), given that this processing phase is considered as 'sanitising'. Both official and in-house control measures involve the microbiological testing of raw milk for soft cheese production and also of the finished product. A critical point of traditional microbiological testing (10) is the lengthy incubation period and the low percentages of isolation which are difficult to reconcile with official controls, which require products to be blocked while awaiting the result. For this reason, rapid, highly specific and highly sensitive diagnostic tests that can identify infected animals and contaminated foods are increasingly necessary. This study evaluated the performance of a PCR protocol for the detection of *Brucella* spp. in complex matrices, such as cheese, and found it to be easy to perform, rapid, economical and it offered high sensitivity and specificity, both of which are ideal for a rapid screening test.

Conclusion

The problem of brucellosis in buffalo in certain regions of Italy has had a massive impact on social and economic development, so much so that years from now, specific national legislature and regional efforts will still be seeking to reduce infection in livestock to guarantee greater safety for the consumer. While there is evidence that some dairy products, particularly fresh cheese and ricotta cheese that are produced with sheep's milk may represent a source of food infection for humans, buffalo mozzarella would not represent the same risk. The short commercial life of these products is not compatible with the application of traditional methods of finding *Brucella* which are known be time-consuming but which require the application of rapid screening tests, such as PCR. The introduction of such routine methods enables the application of hygienic controls of food of animal origin using rapid, sensitive and specific methods, leading to the the implementation of specific plans to monitor brucellosis, especially in areas with a high incidence of the disease, based on sampling and control of dairy products in time for the the length of the shelf-life of these products to be respected.

References

1. Anon. 2006. Ordinanza ministeriale 14/16/2006: Misure straordinarie di polizia veterinaria in materia di tubercolosi, brucellosi bovina, bufalina e ovicaprina, leucosi in Calabria, Campania, Puglia e Sicilia. *G Uff*, **285**, 7 December.
2. Anon. 2007. Piano triennale per il controllo della brucellosi in provincia di Caserta 2007-2010. Regione Campania, Assessorato alla Sanità, Settore Veterinario, Naples, 17 pp (ec.europa.eu/food/committees/regulatory/scfcah/animal_health/bb_05062007.pdf accessed on 13 April 2009).
3. Boschiroli M.L., Foulongue V. & O'Callaghan D. 2001. Brucellosis: a worldwide zoonosis. *Curr Opin Microbiol*, **4**, 58-64.
4. European Commission (EC) 1990. Council Decision of 21 May 1990 introducing a Community financial measure for the eradication of brucellosis in sheep and goats (90/242/EEC). *Off J*, **L 140**, 01/06/1990, 123-127 (eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=celex:31990d0242:en:html accessed on 13 April 2009).
5. European Commission (EC) 2004. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for the hygiene of foodstuffs. *Off J*, **L 139**, 30.04.2004, 55 (eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:139:0055:0205:en:pdf accessed on 13 April 2009).
6. European Food Safety Authority (EFSA) 2006. *Brucella*. Information on specific zoonoses. *EFSA J*, **94**, 145-288.
7. Josefsen M.H., Cook N., D'Agostino M., Hansen F., Wagner M., Demnerova K., Heuvelink A.E., Tassios P.T., Lindmark H., Kmet V., Barbanera M., Fach P., Loncarevic S. & Hoorfar J. 2004. Validation of PCR-based method for detection of food-borne thermotolerant campylobacters in a multicenter collaborative trial. *Appl Environ Microbiol*, **70** (7), 4379-4383.

8. National Library of Medicine (NLM) 2009. Basic local alignment search tool (BLAST). Blast data base. NLM, Washington, DC (blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 13 April 2009).
9. Serpe L., Battisti A., Alfano F., Scaramuzza A. & Gallo P. 2000. PCR determination of *Brucella* spp. in milk products made and commercialized in the Campania Region. *Industr Aliment*, **39** (388), 5-7.
10. Tantillo G.M., Di Pinto A. & Buonavoglia C. 2003. Detection of *Brucella* spp. in soft cheese by semi-nested polymerase chain reaction. *J Dairy Res*, **70**, 245-247.
11. United States Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) 2008. Most probable number procedure and tables, Appendix 2.03. *In* Microbiology laboratory guidebook 01/28/08USDA/FISIS, Washington, DC, 9 pp (www.fsis.usda.gov/PDF/MLG_Appendix_2_03.pdf accessed on 13 April 2009).
12. World Organisation for Animal Health (Office International des Épizooties: OIE) 2008. Bovine brucellosis, Chapter 2.4.3. *In* Manual of diagnostic tests and vaccines for terrestrial animals OIE, Paris, 624-659 (www.oie.int/eng/normes/mmanual/2008/pdf/2.04.03_bovine_brucell.pdf accessed on 15 March 2009).

