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Short communication

Validation of a Real-time PCR assay for fast and sensitive quantification of *Brucella* spp. in water buffalo milk

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ABSTRACT

Water buffalo milk and derived dairy products, including mozzarella cheese, represent a possible source of *Brucella* contamination for consumers. *Brucella* is a severe pathogen for human health even at low concentrations. It is therefore fundamental to develop an assay that is faster and more sensitive than the traditional bacterial culturing method for the detection of the pathogen in the food matrix. We designed a Real-time PCR assay able to detect as low as 1 CFU/ml of *Brucella* spp. in water (80% probability) and 3 CFU/ml of *Brucella* spp. in buffalo milk (50% probability) in less than 3 h without any enrichment step. The assay was validated by calculating specificity, sensitivity, detection limit, precision, PCR efficiency, DNA extraction efficiency and food matrix inhibition. When this method was employed to detect and quantify *Brucella* spp. in 109 buffalo milk samples, the assay demonstrated a higher sensitivity in comparison to bacteriological analysis (27 positive samples and 2 positive samples, respectively).

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1. Introduction

The European Community has a long tradition of production and consumption of raw milk cheese. Since cheeses made from raw or thermised milk have been responsible for many food poisoning outbreaks in humans by several bacteria, it is fundamental to ensure microbiological safety of these products (Commission Recommendation of 19 December 2003, 2004/24/EC, http://eur-lex.europa.eu). Mozzarella di Bufala Campana (Buffalo Mozzarella Cheese, BMC) is an unripened cheese famous worldwide for its special and unique flavour and taste. It is produced in specific regions of southern Italy from the milk of locally raised water buffaloes. According to its disciplinary of production (Decree of the Ministry of Agriculture and Forestry, Annex 1, 18 September 2003, *G.U. n. 258, 6.11.2003*), this cheese must be exclusively manufactured with raw, thermized or pasteurized whole fresh buffalo milk. The main use of unpasteurized milk, together with a production process that has no step to surely inactivate bacteria, render the product a possible vehicle of pathogens from the raw materials. Of these pathogens, *Brucella* spp. is of great importance because brucellosis is endemic in water buffalo in southern Italy. *Brucella* localizes in the supramammary lymph nodes and mammary glands of infected animals and is consequently spread in milk. Since production of BMC cannot warrant danger (pathogen) elimination, to correctly implement the H.A.C.C.P. system, (mandatory for all the food industries, EC Regulation 852/2004 on the hygiene of foodstuffs) it is essential to pursue the absolute safety of the raw buffalo milk. In this regard, EC Regulation 853/2004 states that raw buffalo milk must come from herds that are officially free of brucellosis. Nevertheless, in order to guarantee food safety it is important to utilize a rapid and sensitive detection method that can lead to the identification of likely-contaminated milk samples coming from previously unrecognized infected animals.

Detection of *Brucella* spp. in milk is commonly carried out by a bacterial culture method (reference standard assay). However, as a class III pathogen, *Brucella* cultures are hazardous, require a 6-week time period for definitive diagnosis, and require a minimal amount of viable *Brucella* in the specimen (Bounaadja et al., 2009). These limitations have led to the development of new PCR-based detection methods that are rapid, less hazardous, and more sensitive. The ability to find pathogenic bacteria in food samples by molecular assays is influenced by PCR-inhibitory substances associated with the food matrix (Rossen, Norskov, Holmstrom, & Rasmussen, 1992) and by the specificity of the genus of the pathogen to be identified. In this study we analysed the ability of different combinations of commercial DNA extraction and Real-time PCR amplification kits to detect *Brucella* spp. in water buffalo milk with absolute specificity and high sensitivity, comparing the

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results with those obtained by traditional culture analysis. With the best combination of kits in terms of efficiency, sensitivity, specificity and precision, we developed a standardized Real-time PCR assay that allowed us to quantify the amount of *Brucella* present in 1 ml of contaminated field milk. This is the first paper, to our knowledge, reporting the validation of an assay for Real-time PCR quantification of *Brucella* in buffalo milk.

2. Materials and methods

2.1. Biological material

The study was carried out on samples of milk and serum collected from 109 buffaloes culled in the district of Caserta, including 89 animals belonging to herds with a known history of brucellosis and 20 animals belonging to herds officially *Brucella*-free. Bacterial strains utilized for cross-reaction assessment are indicated in Table 1. *Brucella abortus* reference strain was kindly provided by the National Reference Laboratory for Brucellosis, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, Teramo, Italy.

2.2. Detection of anti-brucella antibodies (IgGαBr) in buffalo serum and milk

Animal serum-positivity to *Brucella* was established by Rose Bengal test (RBT) and complement fixation test (CFT). The analyses were carried out according to the World Organisation for Animal health (OIE) Terrestrial Manual 2008 (www.oie.int/fr/normes/mmanual/2008/pdf/2.04.03_BOVINE_BRUCELL). Milk samples were tested for the presence of anti-*Brucella abortus* IgG and anti-*Brucella melitensis* IgG by ELISA using a commercial kit (Pourquier ELISA Brucellosis, Institute Pourquier, Montpellier, France).

Table 1
List of non-*Brucella* strains analysed in this study.

Species	Strain
Bacillus cereus	Field strain
Campylobacter jejuni	Field strain
Campylobacter coli	Field strain
Listeria innocua	ATCC 33090
Listeria ivanovii	ATCC 19119
Listeria monocytogenes	ATCC 7644
Ochrobactrum anthropi	ATCC 49687
Ochrobactrum anthropi	ATCC BAA749
Rodococcus equi	ATCC 6939
Salmonella enterica ser. Typhimurium	ATCC 14028
Salmonella Poona	NCTC 4840
Streptococcus bovis	Field strain
Vibrio parahemoliticus	Field strain
Yersinia enterocolitica	ATCC 9610
Bacteria isolated from water buffalo milk	
Aerococcus viridans	Field strain
Aeromonas hydrophila	Field strain
E. coli O157	Field strain
Pseudomonas aeruginosa	Field strain
Serratia marcescens	Field strain
Staphylococcus aureus	Field strain
Staphylococcus cromogenes	Field strain
Staphylococcus epidermidis	Field strain
Streptococcus agalactie	Field strain
Streptococcus disgalactie	Field strain
Streptococcus parasanguinis	Field strain
Streptococcus pyogenes	Field strain
Streptococcus uberis	Field strain

2.3. Detection of Brucella spp. in milk by culturing

Isolation and identification of *Brucella* spp. in milk was carried out by examination of cultures according to the OIE Terrestrial Manual 2008 (http://www.oie.int/fr/normes/mmanual/2008/pdf/2.04.03_BOVINE_BRUCELL). Positive colonies were sent to Brucellosis National Reference Laboratory for typing.

2.4. Extraction of DNA

DNA from bacteria was extracted using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Warrington, UK). Bacterial DNA was extracted from milk samples with two commercial kits: (N) NucleoSpin® Food (Macherey–Nagel GmbH & Co., Düren, Germany) and (Q) QIAamp® DNA Mini Kit (Qiagen GmbH, Hiden, Germany) according to the manufacturer's instructions.

2.5. Real-time PCR analysis

DNA was amplified by Real-time PCR using two commercial kits: (AB) TaqMan® *Brucella* species detection kit (Applied Biosystems, Warrington, UK) and (BIO) BioDect *Brucella* spp. detection kit (Biodiversity, Brescia, Italy). Kit AB, specific for *Brucella melitensis, abortus, canis, suis, neotomae and ovis* (Applied Biosystems, technical support), amplifies the gene *rnpB*, which codes for an RNA component of Ribonuclease P, an essential endonuclease that acts early in the tRNA biogenesis pathway (Xiao, Scott, Fierke, & Engelke, 2002). Kit BIO identifies *Brucella* spp. by amplification of a sequence of the gene *bcsp31*, which codes for a 3l1 kDa cell surface protein found in all *Brucella* species and biovars (Baily, Krahn, Drasar, & Stoker, 1992). Amplifications were performed on a StepOnePlus™ Real-time PCR system (Applied Biosystems, Foster City, CA, USA) following kit specific protocols. All the samples were analysed in triplicate. Positive and negative test controls included in this study are indicated in Table 2.

2.6. DNA extraction and Real-time PCR performance evaluation

Extraction efficiency of the two DNA extraction kits under analysis was determined by performing extraction and Real-time PCR on samples of both DNAse/RNAse-free water (control) and certified *Brucella*-free water buffalo milk (sample), each contaminated with a known amount of *Brucella*. Efficiency was then calculated according to the equation $R = 2^{ct_{sample} - ct_{control}}$ (Fleige et al., 2006). Food matrix inhibition was measured by performing the assay on two controls (DNAse/RNAse-free water and *Brucella*-free

Table 2
Amplification controls.

Internal positive control (IPC)	Chimeric non-relevant DNA already contained in the reaction mix together with its primers and probe. Its amplification indicates the absence of PCR inhibitors in the sample.
Process positive control (PPC)	*Brucella*-free buffalo milk sample spiked with *Brucella* and processed throughout the protocol
Process negative control (PNC)	*Brucella*-free buffalo milk sample processed throughout the protocol
Positive PCR control (PC)	Reaction containing a known concentration of extracted *Brucella*
Negative PCR control (NC)	Reaction performed with DNA/RNA-free water instead of DNA extracted
Premises control	Tube containing all reagents and left open in the PCR set up room. Its amplification indicates presence of contaminating DNA in the environment (quality control carried out at regular intervals).

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