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Evaluation of a real-time PCR in combination with a cultivation method for the detection of *Brucella abortus* in organs of infected cattle in southern Italy

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Abstract

Introduction: Brucellosis is a widespread zoonosis of great economic importance for livestock farming in many areas of the world. It is a highly infectious disease which is diagnosed using conventional serological and microbiological methods. The aim of this study was to assess the efficiency of a specific real-time PCR in combination with broth cultivation in detecting *Brucella* spp. in organs of infected cattle, in order to compare the sensitivity of the two approaches and the time needed in them until a correct diagnosis is made. **Material and Methods:** We examined 67 organs collected from 10 cattle slaughtered following a brucellosis outbreak which occurred in February 2016 in southern Italy. The research was carried out by enrichment broth cultivations in combination with a real-time PCR every week for six weeks. **Results:** *Brucella* strains were isolated by cultivation from 44 enrichment broths of organs. All isolates were later identified as *Brucella abortus* by real-time PCR. Using this method in combination with cultivation made it possible to identify the same percentage of infected animals faster than by cultivation alone. Moreover, the same diagnostic results were obtained, on average two weeks before they would have been using only cultivation. In almost all cases, *Brucella* was detected by real-time PCR after the first week of cultivation in pre-enrichment *Brucella* broth, while the bacterial growth was evident usually after 2 or 3 weeks. **Conclusion:** Real-time PCR has allowed results to be obtained faster than in the classical microbiological method, reducing the response times to identify positive animals by half.

Keywords: *Brucella abortus*, diagnosis, real-time PCR.

Introduction

Brucellosis is a zoonotic disease caused by Gram-negative coccobacilli of the genus *Brucella*, which affects different mammals, including humans. *Brucella* spp. is a facultative intracellular pathogen the ability of which to replicate and persist in host cells is directly associated with its capacity to cause persistent disease and to circumvent the innate and adaptive immunity of the host (12). The *Brucella* genus is composed of eight terrestrial species and at least two marine species (24). Each may infect different host species but has a preferred host (4). Terrestrial *Brucella* spp. include *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (pigs), *B. ovis* (rams), *B. canis* (dogs), *B. neotomae* (the desert woodrat – *Neotoma lepida*), *B. microti* (the common vole – *Microtus arvalis*) (19) and *B. inopinata* (originally isolated from a human patient, but its preferential host is not known) (20). The *Brucella* species isolated from marine mammals are *B. ceti* (porpoises and dolphins) and *B. pinnipedialis* (seals) (5). Recently, *B. papionis* and *B. vulpis* were isolated from the baboon and red fox, respectively (21, 22).

Bovine brucellosis, caused mainly by *B. abortus*, is a major economic problem due to the extensive damage it inflicts on commercial animal husbandry (15).

Although bovine brucellosis has been eradicated in most of the developed countries, it is still prevalent in southern Italy, particularly on Sicily, Basilicata, Campania, Calabria and Apulia regions. Different genetic lineages spread as a result of frequent introductions of infected livestock in past centuries (6, 7). From a public health point of view, brucellosis is considered an occupational disease that mainly affects slaughterhouse workers, butchers, laboratory personnel and veterinarians, but it is also a travel-associated disease (8, 17).

The gold standard for the diagnosis of brucellosis is microbiological isolation of the pathogen from the host tissue or its secretions and subsequent identification of the species. However, the isolation of *Brucella* spp. is considered a hazardous procedure, requiring high-security laboratory facilities of biological containment level 3 and highly skilled personnel, and is also known to be a technique with an extended turnaround time for results (24). In order to overcome these limitations, molecular detection by real-time PCR can be employed. This technique is becoming a very important method for the routine identification of *Brucella*, because it requires minimum biological containment and can provide results in a very short time (2, 10, 18, 24).

Material and Methods

Sample collection. In February 2016, in a herd from the Apulia region of southern Italy composed of 40 cows, seven animals tested positive for *Brucella* spp. when evaluated by routine serological testing. This followed several sporadic cases of brucellosis, and consequently, the entire herd was slaughtered in compliance with the current regulations in Italy. The serological status of the animals was evaluated by the rose Bengal test (RBT) and complement fixation test (CFT), the official methods used in the European Union countries (2). The carcasses of ten animals from the infected herd were taken and target organs were subjected to microbiological procedures for *Brucella* isolation. Seven of these animals were seropositive (RBT+/CFT+) and three seronegative (one RBT+/CFT− and two RBT−/CFT−). The following organs were collected: the submandibular, supramammary, retropharyngeal and internal iliac lymph nodes, udder, spleen and cotyledons. The foetus was taken from one pregnant cow in the fourth and from another in the seventh month of gestation. Amniotic fluid, the brain pleural fluid, a lung, the liver, spleen, abomasum and cotyledons were collected from each foetus. All samples were stored at 4°C after sampling and quickly transported to the laboratory.

Sample analysis. Briefly, tissue samples were prepared by removal of extraneous material (*e.g.* fat), cut into small pieces, macerated using a stomacher (Mayo International, Milan, Italy) with 10 mL of sterile phosphate-buffered saline (pH 7.2) and homogenised for 10–15 min (20). One millilitre of each homogenate was immediately inoculated in duplicate onto blood agar plates containing 5% (v/v) of defibrinated sheep blood and onto *Brucella* agar culture media plates (23). The tissue cultures were incubated at 37°C, both aerobically and microaerobically (5–10% (v/v) CO_2) for at least 7 days. In addition, a 1 mL aliquot of the homogenate was added to 9 mL of enrichment *Brucella* broth and incubated at 37°C in air supplemented with 5–10% (v/v) CO_2 for up to 6 weeks, with weekly subcultures into solid selective medium.

The suspected colonies were subcloned to confirm the presence of *Brucella* spp. and the organisms were identified by conventional procedures such as colonial morphology, Gram staining, catalase, oxidase and urease activities, and by agglutination test with polyclonal antisera.

The real-time PCR was carried out immediately from the homogenates and then every week for six weeks from the enrichment broths. Total DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for Gram-negative bacteria. For real-time PCR analysis, 2 µL of DNA was amplified using the SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, CA, USA), with a 300 nM concentration of each forward and reverse primer and a 200 nM concentration of the probe (all products of Eurofins Genomics, Ebersberg, Germany), in a CFX Connect Real-Time PCR Detection System (Bio-Rad). The primers and probes were designed to target the genus-specific IS711 insertion sequences and the *B. abortus*-specific BruAb2_0168 target, as previously described (9). The reaction conditions were the following: 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, primers annealing at 60°C for 30 s, and elongation at 72°C for 20 s. The cycling was followed by a final extension step at 72°C for 10 min. A negative control (PCR mixture without DNA template) and a positive control consisting of DNA were included in each amplification run.

Results

The results of the real-time PCR and of other diagnostic investigations are summarised in Table 1. *Brucella* spp. were detected in 44 out of 67 broth culture media bearing organ tissue inoculate with both cultivation and molecular methods, while only 4 out of 44 samples were positive by real-time PCR performed directly on the homogenates of the organs. All the positive isolates were subsequently identified as *B. abortus* by real-time PCR. All samples collected from foetuses were negative for *Brucella* spp. by both methods, presumably because the bacteria had not yet crossed the placental barrier.

Table 1. Results of serological, culture and real-time PCR analyses in organs of slaughtered cattle following a brucellosis outbreak

Animal	Serological status	Lymph node								Udder		Spleen		Cotyledon	
		Submandibular		Supramammary		Retropharyngeal		Internal iliac							
		PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture
A	RBT+ / CFT+	+ (•)	+ (•••)	−	−	+ (•)	+ (••)	+ (••)	+ (•••)	−	−	+ (••)	+ (•••)	Not sampled	
B	RBT+ / CFT+	+ (•)	+ (•••)	−	−	−	−	+ (hom)	+ (••)	−	−	+ (•)	+ (•••)	Not sampled	
C	RBT+ / CFT+	+ (•)	+ (••)	−	−	+ (•)	+ (••)	+ (•)	+ (••)	+ (•)	+ (•••)	+ (•)	+ (••)	+ (•)	+ (••)
D	RBT+ / CFT+	+ (•)	+ (•••)	−	−	+ (•)	+ (••)	+ (•)	+ (••)	−	−	−	−	+ (•)	+ (••)
E	RBT+ / CFT+	+ (•)	+ (••)	+ (•)	+ (••)	−	−	+ (•)	+ (••)	−	−	−	−	+ (•)	+ (••)
F	RBT+ / CFT+	+ (•)	+ (•)	+ (•)	+ (••)	+ (•)	+ (••)	+ (•)	+ (•)	+ (•)	+ (••)	+ (•)	+ (••)	+ (hom)	+ (•)
G	RBT+ / CFT+	+ (•)	+ (••)	−	−	+ (•)	+ (••)	+ (•)	+ (••)	−	−	+ (•)	+ (••)	Not sampled	
H	RBT+ / CFT−	+ (•)	+ (••)	+ (•)	+ (••)	+ (•)	+ (••)	+ (•)	+ (••)	−	−	−	−	−	−
I	RBT− / CFT−	+ (••)	+ (•••)	−	−	+ (•)	+ (••)	+ (•)	+ (••)	−	−	+ (•)	+ (••)	+ (hom)	+ (•)
L	RBT− / CFT−	+ (•)	+ (••)	−	−	+ (hom)	+ (••)	−	−	−	−	−	−	+ (•)	+ (••)

Hom – homogenate; RBT – rose Bengal test; CFT – complement fixation test; • – from the study start to identification of *Brucella abortus* was 7 days; •• – was 14 days; ••• – was 21 days

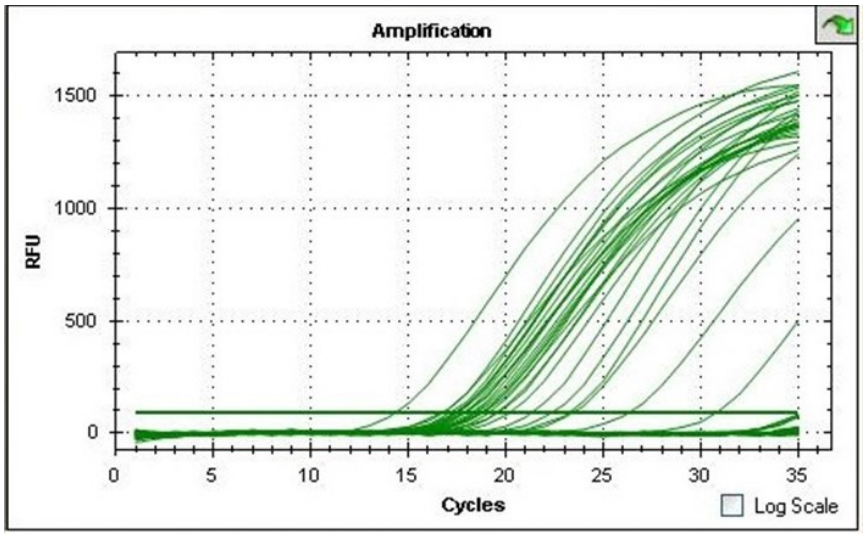

Fig. 1. Real-time PCR analysis performed on DNA extracted from organs cultivated in the *Brucella* enrichment broth after 1 week of incubation

The lymph nodes of the head (submandibular and retropharyngeal lymph nodes) and of the sublumbar region (internal iliac lymph node) were the sites where *Brucella* was most frequently detected. It is worth highlighting that positive cultures and PCR results were also obtained when testing samples from the three seronegative animals (Table 1).

The detection of *Brucella* using PCR in combination with cultivation was possible, on average, two weeks sooner than when cultivation alone was used. In almost all cases *Brucella* was detected by real-time PCR after the first week of cultivation in *Brucella* enrichment broth (Fig. 1), while bacterial growth was usually visually evident on average after 2–3 weeks. In only four cases was bacterial growth from direct seeding of the homogenate possible to discern in one week.

Discussion

Currently, the gold standard diagnostic techniques continue to be based on bacterial isolation from aborted material (foetal tissues, cotyledons and vaginal exudates), lymph nodes, and the udder and spleen followed by microbiological characterisation (16). In recent years, different PCR protocols have been developed and used for the identification of *Brucella* spp. including *B. abortus* (9). Such assays, alone or in combination with microbiology, have proved to be an important alternative fast method that overcomes some problems and disadvantages of traditional microbiological methods.

Even though microbiological isolation is considered the gold standard test for brucellosis diagnosis, and is prescribed by the Terrestrial Manual

of the World Organisation for Animal Health (23), in this study we showed that a real-time PCR in combination with enrichment broth cultivation was able to identify the same percentage of infected animals faster. Moreover, diagnostic results using PCR were obtained, on average, two weeks before they could have been with cultivation alone. In almost all cases, *Brucella* was detected by real-time PCR after the first week of cultivation in *Brucella* enrichment broth, while bacterial growth was visually evident usually after 2–3 weeks. To our knowledge, no comparative studies have been conducted until now on the detection of *Brucella abortus* in cattle samples *via* broth culture using a real-time PCR in combination with it. Most of the studies reported in the literature compared classical microbiological methods and PCR directly from organ homogenates (1, 3, 11, 13, 16) and did not analyse broth cultures of the organs at different times of incubation. We have demonstrated that PCR can have some limitations on its capacity for detection if performed directly on organ homogenates because of low concentrations of *Brucella* in some organs or inaccurate DNA extraction, and moreover, the presence of large amounts of bovine genomic DNA may have inhibitory effects on PCR assays (18). In this study, we showed that PCR is very useful if carried out in combination with cultivation, in order to shorten response times. The molecular approach adopted in this study presents some advantages compared to classical bacteriological techniques with respect to the sensitivity and rapidity of the analyses. In fact, the classical cultivation method shows disadvantages, *i.e*. the long incubation time (six weeks, due to the slow growth of this bacterium), the possible overgrowth of contaminating bacteria and the longer exposure of laboratory personnel to infection risk. In contrast, real-time PCR is a fast and simple method, less hazardous, highly sensitive in this application, extremely specific and relatively inexpensive. Furthermore, the results of this molecular method are not affected by the presence of contaminants.

In conclusion, rapid, definitive and accurate diagnosis of brucellosis is very important for the positive outcome of eradication programmes (14), and the methodology presented here seems to hold promise as such a diagnostic technique. The presented data are preliminary and need to be confirmed by studying a larger number of animals over a longer observation period. However, on the basis of these preliminary results, real-time PCR could be a reliable method for routine identification and differentiation of *Brucella* isolates in addition to classical microbiological methods, providing more rapid and sensitive results.

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