Brucella spp. distribution, hosting ruminants from Greece, applying various molecular identification techniques

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Abstract

Background: Brucellosis still remains an endemic disease for both livestock and human in Greece, influencing the primary sector and national economy in general. Although farm animals and particularly ruminants constitute the natural hosts of the disease, transmission to humans is not uncommon, thus representing a serious occupational disease as well. Under this prism, knowledge concerning Brucella species distribution in ruminants is considered a high priority. There are various molecular methodologies for Brucella detection with however differential discriminant capacity. Hence, the aim of this survey was to achieve nationally Brucella epidemiology baseline genotyping data at species and subtype level, as well as to evaluate the pros and cons of different molecular techniques utilized for detection of Brucella species. Thirty-nine tissue samples from 30 domestic ruminants, which were found positive applying a screening PCR, were tested by four different molecular techniques i.e. sequencing of the 16S rRNA, the BP26 and the OMP31 regions, and the MLVA typing panel 1 assay of minisatellite markers.

Results: Only one haplotype was revealed from the 16S rRNA sequencing analysis, indicating that molecular identification of Brucella bacteria based on this marker might be feasible solely up to genus level. BP26 sequencing analysis and MLVA were in complete agreement detecting both B. melitensis and B. abortus. An interesting exception was observed in 11 samples, of lower quality extracted DNA, in which not all expected MLVA amplicons were produced and identification was based on the remaining ones as well as on BP26. On the contrary OMP31 failed to provide a clear band in any of the examined samples.

Conclusions: The present study reveals the constant circulation of Brucella bacteria in ruminants throughout Greece. Further, according to our results, BP26 gene represents a very good alternative to MLVA minisatellite assay, particularly in lower quality DNA samples.

Keywords: Brucellosis, Brucella spp., ruminants, PCR, MLVA, BP26

Background

Brucellosis is globally one of the most severe and debilitating zoonoses affecting livestock and humans [1, 2]. Ruminants are also considered the major natural hosts, maintaining the causative agent of the disease in the environment [3–5]. The disease is transmitted to humans either by direct contact with infected animals or by consumption of unpasteurized dairy products [6]. In this context, high prevalence of the disease is accompanied...
by economic collapse for the stakeholders owing to the produced milk reduction, abortions and forced slaughter. Brucellosis is endemic in countries around the Mediterranean basin, western Asia and parts of Africa as well as Latin America [7] while it constitutes an important occupational disease for breeders, employees in slaughterhouses and veterinarians as well.

The etiological agents of brucellosis are the proteobacteria belonging in the genus Brucella (family: Brucellaceae). Twelve species have been described, with B. abortus mostly infecting cattle while B. melitensis mostly infecting small ruminants. It should be also mentioned that Brucella melitensis is responsible for the vast majority of human cases worldwide [8]. In a relevant study in Greece, more than 90% of Brucella spp. strains from clinical specimens was identified as B. melitensis [9]. It should be noted that in Greece, an ongoing vaccination and monitoring program takes place during the last two decades, in an effort to eradicate the disease by slaughtering infected animals and protecting young ones [10–13].

For the effective monitoring of both brucellosis control programs and human disease, it is important to have reliable tests to differentiate vaccine and field strains. Many molecular approaches have been developed to detect vaccine strains [14–17].

More specifically, many PCR based attempts have been conducted to develop an efficient protocol, able to identify Brucella genus and distinguish species. At genus specific level, Brucella detection molecular tools have been developed targeting various conserved genomic regions, such as the 16S–23S intergenic transcribed [18, 19], the BCSP31 gene [20, 21], the 16S rRNA [22], the perosamine synthetase (per) gene [23], the gene encoding the Omp2a protein antigen [24], the outer membrane proteins (omp2b, omp2a and omp31) [25] and the proteins of the omp25/omp31 family of Brucella spp. [26]. Nevertheless, the above assays vary greatly in sensitivity and specificity [27].

PCR based assays targeting species specific level identification are also numerous [28–33], whereas the multilocus variable-number tandem-repeat analysis (MLVA) assay developed by Le Flèche et al. [30] is considered the gold standard molecular methodology for Brucella typing [34]. An appropriate PCR based detection protocol should be able to discriminate vaccine Brucella strains (RBS1 and Rev1) from pathogenic ones, with many available assays towards this direction [16, 29, 35–37].

Altogether, the main scope of the present study was the investigation of Brucella species in ruminants from Greece, at both species and subtype level. Particularly, our objectives were to identify Brucella species and subtypes of all the strains, which are very important for epidemiologic surveillance and investigation of outbreaks in brucellosis endemic regions such as Greece [38, 39]. Eventually, in an effort to evaluate the advantages and drawbacks of different molecular techniques utilized for detection and discrimination of Brucella species, an additional goal of the study was to evaluate the efficacy and reliability of different Brucella identification methodologies.

Results

16S rRNA sequencing and phylogenetic analysis

Initially all 39 examined samples were subjected to amplification, sequencing and neighbor joining phylogenetic analysis of 16S rRNA. Sequencing results revealed only one identical haplotype in all derived sequences, confirming that all samples belonged to the genus Brucella with 100% similarity with other species of Brucella (Fig. 1). This haplotype was deposited in the GenBank database and given the accession number OM570553. Those inferences demonstrate that 16S rRNA based molecular identification of Brucella bacteria may be feasible solely up to genus level.

MLVA assay

The panel 1 of MLVA minisatellite markers was applied afterwards in the 39 samples with each PCR being performed at least twice. Firstly, three pairs of primers (Bruce06, Bruce11, Bruce42) were used since they provide an easy to observe differentiation method between the species. Bruce06 amplifies an expected amplicon of 408 bp in case of Brucella melitensis and 542 bp in Brucella abortus, Bruce11 a product of 257 bp and 383 bp, and Bruce42 539 bp and 289 bp respectively. Afterwards, the pairs of Bruce08 and Bruce12 were used, followed by the remaining three markers i.e. Bruce42, Bruce 43 and Bruce55, which were applied only for samples in which a positive result was observed with at least one of the first five markers, as Bruce42, Bruce 43 and Bruce55 result in an identical amplicon for Brucella melitensis and Brucella abortus.

The majority of the samples (28 out of the 39) was successfully identified with all the 8 primer pairs of the MLVA assay Panel 1 (Supplementary Material 1). The remaining 11 samples gave the expected amplicon with two or three primer pairs (from the first five markers) and failed to give an amplicon or gave non-specific bands with the rest of the first five primer pairs (Table 1). Interestingly, these samples were of lower DNA quality in terms of purity as measured by the 260/280 absorbance ratio (Table 1).

BP26 and OMP31 analysis

Identification based on the BP26 marker was in complete agreement with MLVA results, as only two haplotypes
were defined, one assigned to *B. melitensis* and one to *B. abortus*, clearly differentiated from each other and identical with conspecific sequences obtained from GenBank (Fig. 1). These two haplotypes were deposited in the GenBank database and given the accession numbers OM628689 and OM628690, for *B. melitensis* and for *B. abortus* respectively. Nevertheless, this was not always the case regarding the identification with the screening PCR, where there were two samples in which the two methods did not agree (Table 1). Finally, OMP31 marker failed to provide a clear band or sequence in any of the examined samples.

**Brucella** infection summarized results

In total, concerning billy goats all of the examined samples were identified as *Brucella melitensis*, whereas in rams and bulls, only *B. melitensis* and *B. abortus* were identified, respectively. Furthermore, in 3 billy goats and in 1 ram which were PCR positive for vaccine strain Rev1, it was confirmed by MLVA. Regarding females, in 2/3 goats *B. melitensis* was detected, with the remaining on being *B. abortus*, whereas 5/6 ewes hosted *B. melitensis* with again the remaining one being *B. abortus*, and all 17 examined cows hosting *B. abortus*.

**Discussion**

In Greece, brucellosis still remains an endemic zoonosis infecting a wide range of animal species with an influence both in public health and in national economy. Although in 1977, a national program against ruminants’ brucellosis was designed and initiated, the disease is not eradicated yet. The generally accepted pathogenic agent of bovine brucellosis is *B. abortus*, whereas *B. melitensis* is only occasionally detected. Respectively, the common pathogenic agent of caprine and ovine brucellosis is *B. melitensis*, whereas *B. abortus* is only rarely found in small ruminants. Thus, small ruminants are considered as the main hosts for *B. melitensis* [5, 40]. Nevertheless, even though Giantzis et al. [41, 42] detected *B. melitensis* in bovine aborted fetuses, they found no *B. abortus* in aborted fetuses from ewes and goats during a five-year period of 1976-1981. The clinical, pathological and epidemiological picture of caprine brucellosis due to *B. melitensis* is similar to *B. abortus* infection in cattle. The dominant strain for human brucellosis is *B. melitensis* [43–46]. On the other hand, Giannakopoulos et al. [47] referred human cases in Western Greece where *B. abortus* was identified as an equally frequent pathogenic agent. On top of that, it is of high importance to investigate whether interspecies transmission of *B. melitensis*, *B. abortus* and the vaccine strain Rev1 may occur naturally and cause clinical disease in domestic ruminants or may perplex the standard laboratory exams (RBT & CFT). It should be emphasized that control and eradication policies may have to be sometimes readapted to the new data.

In our study, the presence of *B. abortus* was revealed in two such cases, detected from one ewe and one goat. This could be explained by several reasons such as the existence of mixed livestock pastures, the coexistence of sheep and goats in the same shelter with cattle, or occasionally owing to herd movements with infected but non-detected animals. The results suggest cross-species infection of *B. abortus* from cattle to small ruminants raised in close contact [48].
| N  | Sample ID | Farm animal species | Originating tissue | Collection site | Screening PCR | MLVA Variants | BP26 | OMP31 | DNA quality (26/280 ratio) |
|----|-----------|---------------------|--------------------|-----------------|---------------|---------------|-------|-------|--------------------------|
| 1  | 1751      | Billy goat          | Testicle           | Xanthi          | B. melitensis REV1 | B. melitensis REV1 (2/8) | B. melitensis | –     | < 1.2                     |
| 2  | 1761      | Billy goat          | Testicle           | Xanthi          | B. melitensis REV1 | B. melitensis REV1 (8/8) | B. melitensis | –     | 1.8 – 2.2                  |
| 3  | 1771      | Billy goat          | Testicle           | Xanthi          | B. melitensis | B. melitensis (3/8) | B. melitensis | –     | < 1.2                     |
| 4  | 3111      | Billy goat          | Testicle           | Xanthi          | B. melitensis REV1 | B. melitensis REV1 (8/8) | B. melitensis | –     | 1.8 – 2.2                  |
| 5  | 3131      | Ram                 | Testicle           | Xanthi          | B. melitensis | B. melitensis (8/8) | B. melitensis | –     | 1.8 – 2.2                  |
| 6  | 3141      | Billy goat          | Testicle           | Xanthi          | B. melitensis | B. melitensis (8/8) | B. melitensis | –     | 1.8 – 2.2                  |
| 7  | 3171      | Ram                 | Testicle           | Xanthi          | B. melitensis REV1 | B. melitensis REV1 (8/8) | B. melitensis | –     | 1.8 – 2.2                  |
| 8  | 3142      | Billy goat          | Spleen             | Thessaloniki    | B. melitensis | B. melitensis (2/8) | B. melitensis | –     | < 1.2                     |
| 9  | L81       | Bull                | Lymph nodes        | Thessaloniki    | B. abortus | B. abortus (3/8) | B. abortus | –     | < 1.2                     |
| 10 | L84       | Bull                | Lymph nodes        | Thessaloniki    | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 11 | L85       | Bull                | Lymph nodes        | Thessaloniki    | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 12 | L111      | Bull                | Lymph nodes        | Thessaloniki    | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 13 | L112      | Bull                | Lymph nodes        | Thessaloniki    | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 14 | 31A       | Cow                 | Embryonic rumen    | Thiva           | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 15 | 853       | Cow                 | Embryonic liver    | Kalavryta       | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 16 | 3201      | Cow                 | Embryonic rumen    | Pella           | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 17 | L11       | Cow                 | Embryonic rumen    | Xanthi          | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 18 | L12       | Cow                 | Embryonic liver    | Xanthi          | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 19 | L21       | Goat                | Embryonic rumen    | Thiva           | B. melitensis | B. melitensis (8/8) | B. melitensis | –     | 1.8 – 2.2                  |
| 20 | L22       | Goat                | Embryonic liver    | Thiva           | B. melitensis | B. melitensis (3/8) | B. melitensis | –     | < 1.2                     |
| 21 | L31       | Cow                 | Embryonic liver    | Kozani          | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 22 | L32       | Cow                 | Cotyledonary placenta | Kozani       | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 23 | L61       | Cow                 | Embryonic rumen    | Xanthi          | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 24 | L62       | Cow                 | Embryonic liver    | Xanthi          | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 25 | L72       | Ewe                 | Embryonic liver    | Lesvos island   | B. melitensis | B. melitensis (3/8) | B. melitensis | –     | < 1.2                     |
| 26 | L93       | Ewe                 | Embryonic liver    | Volos           | B. melitensis | B. melitensis (8/8) | B. melitensis | –     | 1.8 – 2.2                  |
| 27 | L121      | Cow                 | Embryonic rumen    | Xanthi          | B. abortus | B. abortus (2/8) | B. abortus | –     | < 1.2                     |
| 28 | L122      | Cow                 | Embryonic liver    | Xanthi          | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 29 | L13       | Ewe                 | Cotyledonary placenta | Larisa      | B. melitensis | B. melitensis (8/8) | B. melitensis | –     | < 1.2                     |
| 30 | L141      | Ewe                 | Embryonic rumen    | Larisa          | B. melitensis | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 31 | L201      | Ewe                 | Embryonic rumen    | Fthiotida       | B. melitensis REV1 | B. melitensis REV1 (8/8) | B. melitensis | –     | 1.8 – 2.2                  |
| 32 | L202      | Ewe                 | Embryonic rumen    | Fthiotida       | B. melitensis | B. melitensis (3/8) | B. melitensis | –     | < 1.2                     |
| 33 | L221      | Cow                 | Embryonic rumen    | Kozani          | B. abortus | B. abortus (3/8) | B. abortus | –     | < 1.2                     |
| 34 | L222      | Cow                 | Embryonic liver    | Kozani          | B. abortus | B. abortus (8/8) | B. abortus | –     | 1.8 – 2.2                  |
| 35 | L223      | Cow                 | Cotyledonary placenta | Kozani      | B. abortus | B. abortus (3/8) | B. abortus | –     | < 1.2                     |
administration in females, errors may have occurred. As a result of the accidental vaccination, the male animals may be characterized as false seropositive during the control program. Finally, due to the aforementioned vaccine administration errors, male animals end up to the slaughterhouse while the farm comes under quarantine for a period of at least 2 to 6 weeks. For these reasons, it is important to improve molecular methods to detect the exact pathogenic agent from samples from alive seropositive animals or in case of slaughter to detect it in a rapid and effective way.

In particular, development and improvement of efficient DNA-based methods is a consistent demand, as well their comparative efficiency. In this context, here we utilized and evaluated four different molecular tools towards this scope. Particularly, apart from the “gold standard” MLVA assay, the 16S rRNA, the OMP31 and the BP26 genes were analyzed. Assays targeting Brucella non-coding genomic tandem repeats, such as the MLVA minisatellites [30] or the microsatellite loci proposed earlier [15, 49], have been developed based on the principle of the high genetic homogeneity levels of the different strains, which fail to be distinguished by classic conserved genomic region markers such as the 16S rRNA. Nevertheless, microsatellites, although are excellent markers for genotyping and genetic differentiation studies, on account of hypervariability and high mutation rates may be inadequate to assign the biovar correctly or an isolate at species level. Minisatellites (MLVA) [30] on the contrary, possess a better species identification capability and are moderately variable higher, resulting in higher discriminatory power. On the other hand, gene sequences possess lower mutation rates. In line with Gupta et al. [50] who concluded that for genus-based identification of Brucella species, 16S rRNA and 16S-23 rRNA gene are the best target, in our case examination of 16S rRNA produced one identical haplotype in all samples, thus not capable to discriminate the different Brucella species. Our results are in agreement with the same study [50] concerning identification based on BP26 gene targets.

Interestingly, BP26 worked better in lower quality samples, where not all minisatellite markers gave product (Table 1). In PCR based methods the quality and purity of Brucella spp. DNA is a crucial prerequisite before performing these methods, especially for multiplex PCR methods. Any inhibitor in DNA samples from any source can affect the result of a PCR based method. Sensitivity and specificity of most PCR-based methods are not well established and their real capability for use with clinical samples and hence diagnosis has not been validated [27]. Specifically, for investigation of Brucella presence and species identification, samples may originate from slaughterhouses or dead animals, sometimes travelling for days and eventually received at the labs highly degraded. Sequencing of the BP26 gene represents a very good alternative to MLVA minisatellite assay that according to our results constitutes an effective alternative marker in lower quality DNA samples.

**Conclusions**

The present research provides clear evidence for the continuing circulation of Brucella species in ruminants throughout Greece, a country that still remains therefore endemic in Brucellosis. All but one molecular techniques tested were proved informative and efficient. However our results clearly demonstrate a better performance of BP26 gene marker in lower quality samples, in terms of optical density ratio.

**Methods**

**Sample collections**

During a period of 3 years (2016-2018), 264 samples were collected from a total of 191 farmed ruminants, originating from farming units located in the mainland of Greece throughout the country. Those samples derived either from positive males to Rose Bengal Test (RBT) and/ or Component Fixation Test (CFT) either from aborted

| Sample ID | Farm animal species | Originating tissue | Collection site | Screening PCR | MLVAa | BP26 | OMP31 | DNA quality (26/280 ratio) |
|-----------|---------------------|-------------------|----------------|---------------|-------|------|-------|--------------------------|
| L231      | Goat                | Embryonic rumen   | Magnesia       | B. melitensis | B. abortus (8/8) | B. abortus | – | 1.8 – 2.2 |
| L301      | Cow                 | Embryonic rumen   | Trikala        | B. abortus    | B. abortus (8/8) | B. abortus | – | 1.8 – 2.2 |
| L312      | Cow                 | Cotyledonary placenta | Florina     | B. abortus    | B. abortus (3/8) | B. abortus | – | < 1.2 |
| L317      | Cow                 | Embryonic liver   | Florina        | B. abortus    | B. abortus (8/8) | B. abortus | – | 1.8 – 2.2 |

a Parentheses indicate numbers of markers that worked properly in MLVA identification
fetuses of sheep, goats, and cattle. Thirty-nine (39) out of the 264 tissue samples, which derived from 30 animals, were found PCR positive using the screening Multiplex PCR detection molecular methodology of Garcia-Yoldi et al. [29] for the needs of the Greek Ministry of Rural Development [51] and were utilized for the needs of the present study. The 39 samples originated from various organs and tissues from 30 male and female domestic ruminants, i.e. 7 sheep, 7 goats and 16 bovines, including 7 testicles, 1 spleen, 5 lymph nodes, 11 embryonic rumens, 11 embryonic livers, 4 cotyledonary placentas. The above 39 samples were found positive by the Multiplex PCR Assay for Brucella spp. [29] in our laboratory [51]. All samples are displayed in detail in Table 1.

**Extraction of genomic DNA from tissue sample**

All procedures were performed under the Biosafety Level three (BSL3) guidelines [52]. Tissue samples were initially homogenized within 200μl sterile Phosphate Buffered Saline (PBS) Sigma- Aldrich using a tissue grinder. For homogenization, aseptic processing of all samples was performed by removal of extraneous material and further maceration and chopping into small pieces in PBS. DNA isolation was carried out using the High Pure PCR template preparation kit (Roche, Basel, Switzerland) following the manufacturer instruction with a final elution volume of 80 μl in each sample. The concentration and the quality of the extracted DNA were evaluated in a micro-volume Q5000 UV-Vis Spectrophotometer (Quawell, USA).

**Molecular identification of Brucella species**

Since our aim was also to evaluate the applicability of different molecular tools for Brucella detection, four different molecular techniques were applied for identification of bacterial species and assignment of Brucella positive samples to species level.

Initially the 16S rRNA gene was amplified using the universal for bacteria species primer pair 27F-1492R (Table 2) that may identify the greatest majority of bacteria species at least at genus level. This pair of primers amplifies nearly the complete 16S RNA. The 27F and 1492R [53] primers corresponded to positions 8–27 and to positions 1492–1513 of Escherichia coli 16S rRNA, respectively [56].

Positive Brucella samples were then subjected to PCRs targeting the BP26 and the OMP31 genes, using the primer pairs BP26F-BP26R [54] and OMP31F-OMP31R [55] (Table 2). BP26 is a conserved gene capable of distinguishing the different Brucella species [57]. On the other hand,

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| Table 2 | Primers used for Brucella identification |
|---------|----------------------------------------|
| Primer Name | Primer sequence (5'-3') | Size of product (bp)* | Annealing temperature | Reference |
| Bruce06F | ATGGGATGTGGTAGGATATCG | 274, 408, 542* | 51°C | Le Flèche et al. [30] |
| Bruce06R | GCCGTGAATATGCAATTTCGTTTTGTG | 330, 348, 366* | 51°C | Le Flèche et al. [30] |
| Bruce08F | ATATTGCAATTCGAGGCTGATTC | 509, 257, 383* | 51°C | Le Flèche et al. [30] |
| Bruce08R | ACAGAACAGTTTCCAGGTCGTC | 345, 392, 375* | 51°C | Le Flèche et al. [30] |
| Bruce42F | CATGGGCTCACTATACGCGAAT | 170, 182, 182* | 51°C | Le Flèche et al. [30] |
| Bruce42R | GGGCAAATATGCACTACGAGGTT | 538, 539, 289* | 51°C | Le Flèche et al. [30] |
| Bruce45F | ATCTCTGGCCTCTCCTGAC | 187, 151, 151* | 51°C | Le Flèche et al. [30] |
| Bruce45R | TATTTTCCGCTGCCCATAAAC | 234, 273, 273* | 51°C | Le Flèche et al. [30] |
| Bruce55F | TCGAACGATGTTTGGATGATGTT | 1412 | 50°C | Frank et al. [53] |
| Bruce55R | AATCTGGGCTGCTGTTGTTTC | 58°C | Gupta et al. [54] |
| 27F | AGAGGTTATGCCTCAG | 1029 | 55°C | Vizcaino et al. [55] |
| 1492R | TACCGGTTTGTTGATGATGTTT | 670 |
| BP26F | GGGGCTCAGATAAGCCGTCCT | 720 |
| BP26R | GAGCTGCTGCTGCTGCTGCTG |
| OMP31F | TGACAGACTTTTCGGCCGA | |
| OMP31R | TATGGGATGCAATTCGAGG |

* Products are size-specific for B. suis, B. melitensis and B. abortus, respectively
OMP31 gene is only present in *B. melitensis*. Cloning and sequencing of *B. melitensis* 16M OMP31 [55], a gene coding for a major Brucella outer membrane protein, verified that this gene is missing from *B. abortus* strains [58].

Finally, the MLVA typing panel 1 assay of minisatellite markers was applied [30, 59]. For MLVA analysis we worked with the set of primers (Bruce06, 08, 11, 12, 42, 43, 45, 55, Table 2), which amplifies 8 minisatellite markers with a good species identification capability [30]. All PCRs were performed in 20 μl final volumes, containing 0.6 pmol of each forward or reverse primer, 10 μl KAPA 2G Fast Hot Start readymix (Merck, Germany), approximately 50 ng extracted DNA and nuclease free water up to the final volume. Reactions were performed in a FastGene ULTRA Cycler (Nippon Genetics, Japan) and amplification program was as follows: after an initial denaturation at 95°C for 3 min, 35 cycles were performed, of denaturation at 95°C for 30 sec, annealing at 48–60°C (Table 2) for 40 sec, and extension at 72°C for 40-60 sec, depending on the product length (40 sec for products smaller than 1000 bp; 60 sec for products larger than 1000 bp), and final extension at 72°C for 10 min. The amplified products were examined by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (0.5 mg/ml) and photographed by photo documentation system, or in gel stained with silver nitrate in cases of very small size products differences.

Clean-up kit (Macherey-Nagel, Germany) following the manufacturer's recommended protocol and sequenced bidirectionally in an ABI 3730xl automatic sequencer. Sequences produced, were aligned using the software MEGA 7 [60] and compared with conspecific and congeneric ones obtained from the GenBank database in Neighbor Joining phylogenetic trees that were created in the same software applying a bootstrap value of 1000 iterations.

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**Authors’ contributions**

A.K. collected the samples, performed the molecular analyses and wrote the first draft of the manuscript. D.K.P participated in the PCR analyses and wrote the first draft of the manuscript. I.G. corrected and wrote the final draft on the manuscript. K.P. participated in sample collections and in molecular analyses. A.Z. conceived and corrected the paper. N.D.G. coordinated the study and helped to draft the manuscript. E.P. conceived, coordinated the study and supervised the whole experiments. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets generated and analysed during the current study are available in the NCBI GenBank repository, accession numbers OM628689, OM628690 and OM570553 (https://www.ncbi.nlm.nih.gov/nuccore/OM628689, https://www.ncbi.nlm.nih.gov/nuccore/OM628690, https://www.ncbi.nlm.nih.gov/nuccore/OM570553).

**Declarations**

**Ethics approval and consent to participate**

All animal manipulations were carried out according to the EU Directive on the protection of animals’ usage for scientific purposes (2010/63/EU). The research protocol was approved by the General Assembly of the Veterinary Faculty of Aristotle University of Thessaloniki decision 55/27-05-2015. Also, permissions were obtained from all participating farm owners for collecting the samples.

**Consent for publication**

Not applicable

**Competing interests**

The authors declare no competing interests

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**Supplementary Information**

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**Additional file 1.**

**References**

1. Gwida M, Al Dahouk S, Melzer F, Rösler U, Neubauer H, Tomaso H. Brucellosis–regionally emerging zoonotic disease? Croat Med J. 2010;51(4):289–95.
2. Dean AS, Clump L, Greter H, Schelling E, Zinsstag J. Global burden of human brucellosis: a systematic review of disease frequency. PLoS Negl Trop Dis. 2012;6(10):e1865.
3. Blasco JM, Molina-Flores B. Control and eradication of Brucella melitensis infection in sheep and goats. Vet Clin Food Anim Pract. 2011;27(1):95–104.
4. Elzer PH, Hagius SD, Davis DS, DelVecchio VG, Enright FM. Characterization of the canine model for ruminant brucellosis. Vet Microbiol. 2002;90(1-4):425–31.
5. Díaz A. Epidemiology of brucellosis in domestic animals caused by Brucella melitensis, Brucella suis and Brucella abortus. Revue scientifique et technique-Office international des epizooties. 2013;32(1).

6. Atturi VL, Xavier MN, De Jong MF, Den Hartogh AB, Tsolis RM. Interactions of the human pathogenic Brucella species with their hosts. Annu Rev Microbiol. 2011;13(65):523–41.

7. Moreno E. Retrospective and prospective perspectives on zoonotic brucellosis. Front Microbiol. 2014;13(5):213.

8. Corbel MJ. Food and Agriculture Organization of the United Nations, World Health Organization & World Organisation for Animal Health. Brucellosis in humans and animals. World Health Organization; 2006. https://apps.who.int/iris/handle/10665/43597.

9. Dougas G, Katsiosl A, Linou M, Kostoulas P, Billinis C. Modelling Human Brucellosis Based on Infection Rate and Vaccination Coverage of Sheep and Goats. Pathogens. 2022;11(2):167.

10. Tzani M, Katsiolis A, Program for the control and eradication of brucellosis in sheep and goats in Greece: HCDCP e-bulletin. 2012;2(4):19-20 ISSN 1792-0016. https://issuu.com/keelpono-hcdd/docs/hcdd_newsletter_april_2012 accessed on 13 March 2021.

11. European Commission 2017. SANTE/10769/2017 Report of the brucellosis Task Force sub-group. Meeting held in Athens Greece 29-31 March 2017. https://ec.europa.eu/food/system/files/2017-06/diseases_erad_bb_summary_greece-athens_20170329.pdf accessed online on 10 Jan 2022.

12. Katsiosl A, Tzani M, Dile C, Korou M, Stournara A, et al. Investigation of the human resources needs for the effective and efficient implementation of the sheep and goat brucellosis program in Greece. Vet J Repub Srp ska (Banja Luka). 2018;18(2):270–96.

13. Straubausch LJ, Berkelman RL. Human illness associated with use of veterinary vaccines. Clin Infect Dis. 2003;37(3):407–14.

14. Clocqueaet A, Grayon M, Grépinet O. Identification of Brucella melitensis vaccine strain Rev. 1 by PCR-RFLP based on a mutation in the rplE gene. Vaccine. 2002;20(19-20):2546–50.

15. Bricker BJ, Ewalt DR, Halling SM. Brucella HOOF-Prints: strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). BMC Microbiol. 2003;3(1):1–3.

16. Lopez-Gori J, Garcia-Yoldi D, Marin CM, De Miguel MJ, Munoz PM, Blasco JM, et al. Evaluation of a multiplex PCR assay (Brucella-ladder) for molecular typing of all Brucella species, including the vaccine strains. J Clin Microbiol. 2008;46(10):3484–7.

17. Gopaul KK, Sells J, Bricker BJ, Crasta OR, Whatmore AM. Rapid and reliable single nucleotide polymorphism-based differentiation of Brucella live vaccine strains from field strains. J Clin Microbiol. 2010;48(4):1461–4.

18. Rijpens NP, Jannes G, Van Asbroeck MA, Rossau R, Herman LJM. Direct detection of Brucella spp. in raw milk by PCR and reverse hybridization with 165–235 RNA spacer probes. Appl Environ Microbiol. 1996;62(5):1683–8.

19. Bricker BJ, Ewalt DR, MacMillan AP, Foster G, Brew S. Molecular characterization of Brucella strains isolated from marine mammals. J Clin Microbiol. 2000;38(3):1258–62.

20. Baily GG, Krahn JB, Drasar BS, Stoker NG. Detection of Brucella melitensis and Brucella abortus in infected animals. J Clin Microbiol. 1995;33(12):3087–90.

21. Bricker BJ, Ewalt DR, Olsen SC, Jensen AE. Evaluation of the Brucella abortus species-specific polymerase chain reaction assay, an improved version of the Brucella AMOS polymerase chain reaction assay for cattle. J Vet Diagn Invest. 2003;15(4):374–8.

22. Peletino A, Nunes A, Grillo T, Isido J, Silva C, Ferreira AC, et al. Genetic characterization of Brucella spp. Front Microbiol. 2021 Nov;12:12.

23. Sangar FJ, Aguero J. Identification of Brucella abortus 819 vaccine strain by the detection of DNA polymorphism at the ery locus. Vaccine. 1994;12(5):435–8.

24. Vemulapalli R, McQuiston JR, Schuring GG, Sirangathan N, Halling SM, Boyle SM. Identification of an IS 711 element interrupting the wboA gene of Brucella abortus vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other Brucella species and strains. Clin Diagn Lab Immunol. 1999;6(5):760–4.

25. Ewalt DR, Bricker BJ. Validation of the abbreviated Brucella AMOS PCR as a rapid screening method for differentiation of Brucella abortus field strain isolates and the vaccine strains, 19 and RB51. J Clin Microbiol. 2000;38(8):3085–6.

26. Al Dahouk S, Le Flèche P, Nöckler K, Amato R, et al. Evaluation of Brucella MLVA typing for human brucellosis. J Microbiol Methods. 2007;69(1):137–45.

27. Marianelli C, Graziani C, Santangelo C, Xibilia MT, Imbriani A, Amato R, et al. Molecular epidemiological and antibiotic susceptibility characterization of Brucella isolates from humans in Sicily, Italy. J Clin Microbiol. 2007;45(9):2923–8.

28. Karvounarins PA. Aspects cliniques et épidémiologiques de la brucellose bovine, caprine et ovine, en Grèce [Clinical and epidemiological aspects of bovine, caprine, and ovine brucellosis in Greece]. Dev Biol Stand. 1976;31:254–64 French. PMID: 1261740.

29. Gianitsis DG. Brucellosis eradication programs. Course and considerations serological and microbiological tests results. J Hellenic Vet Med Soc. 1984;35(1):19–25.

30. Gianitsis DG, Xenos G, Pashaleri I. Investigation of abortion caused by infective agents in sheep and goats (in Greece). Ellenike Kteniatrike Hel lenic Vet Med. 1984;27:132–41 (in Greek).

31. Galanakis E, Kefallinou A, Kostoulas-Tsiara A, Lapatsanis PD. Childhood brucellosis in Epirus (NW Greece) during the decade 1980-89. Paediatriki. 1993;56:162–8.

32. Galanakis E, Bourantas KL, Levideitouos S, Lapatsanis PD. Childhood brucellosis in north-western Greece: a retrospective analysis. Eur J Pediatr. 1996;155(1):1–6.

33. Tsolia M, Drakonaki S, Messaritaki A, Farmakakis T, Kostaki M, Tsapra H, et al. Clinical features, complications and treatment outcome of childhood brucellosis in central Greece. J Infect. 2002;44(4):257–62.

34. Foukis J, Sandakalis V, Choudrou A, Tsatsaras A, Tsatsaras N, Tselentis Y, et al. The epidemiology of Brucellosis in Greece, 2007–2012: a ‘One Health’ approach. Trans R Soc Trop Med Hyg. 2018;112(3):124–35.

35. Giannakopoulos I, Nikolakopoulou NM, Elloupolou M, Ellina A, Kolonitsiou F, Papanastasiou D. Presentation of childhood brucellosis in Western Greece. Jap J Infect Dis. 2006;59(3):160.

36. Warth G, Meiler F, Tomasi M, Roessler I, Neubauer H. Detection of Brucella abortus DNA in aborted goats and sheep in Egypt by real-time PCR. BMC Res Notes. 2015;8(1):1–5.
49. Bricker BJ, Ewalt DR. Evaluation of the HOOF-Print assay for typing Brucella abortus strains isolated from cattle in the United States: results with four performance criteria. BMC Microbiol. 2005;5(1):1–0.

50. Gupta VK, Shivasharanappa N, Kumar V, Kumar A. Diagnostic evaluation of serological assays and different gene based PCR for detection of Brucella melitensis in goat. Small Ruminant Res. 2014;117(1):94–102.

51. Katsiolis A, Papanikolaou E, Stournara A, Giakkoupi P, Papadogiannakis E, Zdrgas A, Giadinis ND, Petridou E. Molecular detection of Brucella spp. in ruminant herds in Greece. Trop Anim Health Prod. 2022;54:173. https://doi.org/10.1007/s11250-022-03175-x.

52. Zaki AN. Biosafety and biosecurity measures: management of biosafety level 3 facilitates. Int J Antimicrob Agents. 2010;36:S70–4.

53. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. App Environment Microbiol. 2008;74(8):2461–70.

54. Gupta VK, Kumari R, Vohra J, Singh SV, Vihan VS. Comparative evaluation of recombinant BP26 protein for serological diagnosis of Brucella melitensis infection in goats. Small Ruminant Res. 2010;92(2–3):119–25.

55. Vizcaino N, Cloeckaert A, Zygmunt MS, Dubray G. Cloning, nucleotide sequence, and expression of the Brucella melitensis omp31 gene coding for an immunogenic major outer membrane protein. Infect Immun. 1996;64(9):3744–51.

56. Brosius J, Palmer ML, Kennedy PJ, Noller HF. Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc Natl Acad Sci. 1978;75(10):4801–5.

57. Seco-Mediavilla P, Verger JM, Grayon M, Cloeckaert A, Marin CM, Zygmunt MS, et al. Epitope mapping of the Brucella melitensis BP26 immunogenic protein: usefulness for diagnosis of sheep brucellosis. Clin Vaccine Immunol. 2003;10(4):647–51.

58. Vizcaino N, Verger JM, Grayon M, Zygmunt MS, Cloeckaert A, DNA polymorphism at the omp-31 locus of Brucella spp.: evidence for a large deletion in Brucella abortus, and other species-specific markers. Microbiology. 1997;143(9):2913–21.

59. Jiang H, Wang H, Xu L, Hu G, Ma J, Xiao P, et al. MLVA genotyping of Brucella melitensis and Brucella abortus isolates from different animal species and humans and identification of Brucella suis vaccine strain S2 from cattle in China. PLoS One. 2013;8(10):e76332.

60. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–4.

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