Molecular Detection of *Brucella* spp. in Ruminant Herds in Greece

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Abstract

Brucellosis is a worldwide distributed infectious disease. Ruminants and other animal species (swine, dogs, equids etc), as well as wild mammals can be affected. The disease can be transmitted to humans through the food chain or by direct contact with infected animals. Because of the relatively high economic burden due to abortions within a herd, significant efforts have been employed and hence the disease in most European countries has been eradicated. Accordingly, Greece applies both control and eradication programs concerning small ruminants (sheep and goats) and bovines depending on the geographical area. Current challenges in the standard antibody-based laboratory methods used for *Brucella* detection are the failure to differentiate antibodies against the wild strain from the ones against the vaccine strain Rev1 and antibodies against *B. melitensis* from those against *B. abortus*. The aim of the study was to modify previously published protocols based on PCR analysis and to generate a new diagnostic tool able to confirm the doubtful results delivered from serology and mainly to do easier and more reliable the detection of *Brucella*. For this reason, 264 samples derived from 191 ruminants of the farm and divided in 2 groups (male/female) were examined with a modified DNA extraction and PCR protocol. Molecular examination revealed the presence of *Brucella* spp. in 39 out of 264 samples (derived from 30 animals). In addition, *Brucella* spp. was detected in infected tissues derived from testicles, inguinal lymph nodes, fetal liver and fetal stomach content molecular detection of than other infected tissues.

1. Introduction

Brucellosis, also known as Malta fever, is one of the most common bacterial zoonoses in Mediterranean countries (Wareth, et al., 2019) with intensive farming (Memish, et al., 2004, FAO/Brucellosis in human and animals., 2006). The main causative agent of human Brucellosis is *Brucella melitensis* (Alton, et al., 1996) (Brucellosis in human and animals., 2006). The disease is transmitted mainly from infected ruminants to humans and from the consumption of unpasteurized dairy products (Cooper, 1992, FAO/Brucellosis in human and animals., 2006,Norman, et al., 2016, CDC, 2017) provoking a reported amount of about 500,000 new human cases of brucellosis per year worldwide (Godfroid, et al., 2013, Berger, 2016). It is mostly an occupational zoonosis (International Labour Office, 2010). Even if human mortality rate is low, the burden caused by the disease is substantial because of its chronic nature and the frequent relapses that occur with systemic manifestation (Colmenero, et al., 1996). Therefore, an early and accurate diagnosis is of utmost importance (Pappas, et al., 2005).

The natural reservoirs of the bacterium in the environment are the domestic ruminants of the farm, such as sheep, goats and cattle. Brucellosis in farm animals represents an equally great burden with financial consequences in most parts of the world (Rossetti, et al., 2017). Several countries though in western and northern Europe, Canada, Japan, Australia and New Zealand are believed to be free from the pathogen (OIE, https://www.oie.int/en/disease/brucellosis/ 2021). Given the fact that there is no human vaccine available, the key-player to prevention of human disease is the control of zoonosis in animal populations either by vaccination programs or by eradication strategy.

Since 1977, the basic concept for controlling and eradicating brucellosis in Greece has always been vaccination of herds combined with blood testing and slaughtering of all animals found positive in laboratory tests such as Rose Bengal Test (RBT) and Complement Fixation Test (CFT) (seropositive animals). The State Veterinary Services from the Ministry of Rural Development & Food and from the Ministry of Interior have implemented a control and eradication policy, based on systematic vaccination of female animals over the age of 3 months old and blood-sampling of males over the age of 6 months old on the mainland (vaccination zone-VZ), as well as a test and slaughter policy on most of the islands (eradication zone-EZ). The vaccination of sheep and goats was based on the conjunctival administration of the attenuated live vaccine strain, Rev1 (OCUREV ® CZV), once in their life of female sheep and goats. According to the above program, it is strictly forbidden to move animals from the VZ to the EZ, or to move animals which have not been vaccinated within VZ (Fig. 1). Animals found positive in RBT and/or CFT are slaughtered separately from healthy ones, taking all necessary biosecurity measures to prevent the spreading of infection (Tzani, et al., 2012, Greek Ministerial Decision on sheep & goat brucellosis programme, 3339/117339 2016). Unfortunately, this approach was unsuccessful due to various reasons (Katsiolis, et al., 2018).

Detection of *Brucella* spp. by culture method is sometimes unsuccessful. The current golden standard in terms of laboratory techniques for the screening of brucellosis in ruminants are Rose Bengal Test (RBT) and Complement Fixation Test (CFT). Although both methods are officially recommended by the EU, fail to discriminate the produced antibodies between the wild and the vaccinate strains resulting in generation of false positive results (Stournara, et al., 2008) that provoke wrongful slaughtering. Therefore, the generation of a novel, rapid, sensitive and specific molecular diagnostic tool such as PCR directly on tissues of suspected animals (mainly aborted fetuses and associated maternal tissues, spleen of seropositive animals) (Bricker, 2002) should be developed in order to accurately detect the pathogen. The most common difficulties comprise the preparation of samples and purification of PCR inhibitors (Amin, et al., 2001).

For all the above reasons, a reliable PCR test able to detect the bacterium in infected tissues of seropositive animals would minimize the false positive results and would boost the laboratory involvement.

2. Materials And Methods

2.1 Reference strains of *B. melitensis* and *B. abortus* as positive controls

The following *Brucella* reference strains were used as positive controls in this study. Reference strain 16M (*B. melitensis*, ATCC[a] 23456) and Reference strain 544 (*B. abortus*, ATCC 23448) were purchased from Culture Public Health England (Salisbury, UK). The vaccine strains Rev1 and RB51 [produced by CZ Veterinaria (Pontevedra – Spain, the European distributor of the product, under license from the Colorado Serum Company, Denver, Colo, USA)]. Once reconstituted one intraocular dose (1drop ≈ 35μl) of the vaccine Rev1, contains 1-2x10^9 cfu of live attenuated *B. melitensis* strain each dose of the

vaccine Rev1 (smooth phase). Accordingly, each dose (2ml, sc) of the vaccine RB51, contained 10-34x10^9 cfu/ml of live attenuated *B. abortus* strain RB51 (Tittarelli, et al., 2008) (rough phase). These two types of vaccines are widely used in national brucellosis eradication programs in Greece. *B. melitensis* strains (16M, Rev1) were cultured aerobically while the *B. abortus* strains (544, RB51) were cultured under microaerophilic conditions, respectively. For cultivation *Brucella* agar (Oxoid, Hampshire, UK) supplemented with *Brucella* Selective Supplement SR0083A (Oxoid, Hampshire, UK) and Columbia sheep blood agar plates (Oxoid, Hampshire, UK) were used.

2.2 Samples collection

In this study, 264 samples were collected from 191 domestic ruminants from farms/herds located in different regions of Greek mainland, during the period 2016-2018. All samples were immediately forwarded to the Department of Microbiology and Infectious Diseases (Aristotle University of Thessaloniki, Greece) for DNA extraction. The extract was then stored at -20^{o}C for further analysis.

The samples were divided into two different groups. The first group included 121 samples from tissues (49 testicles, 56 spleens and 16 inguinal lymph nodes) that were collected from 83 non-vaccinated, slaughtered male animals which were found positive in RBT and/or CFT (47 rams, 29 billy goats and 7 bulls).

The second group included tissues collected from aborted fetuses of sheep, goats and cattle. In total, 143 samples (5 fetal membranes, 47 fetal livers, 9 fetal spleens, 68 fetal stomach contents, 11 placenta and 3 posterior mammary lymph nodes) derived from 108 cases of abortion (57 sheep, 5 goats, 46 cows) were vaccinated with Rev-1 vaccine.

2.3 DNA extraction

All tissue samples were processed under Biosafety Level three (BSL3) with high personal protections (WHO, 2004). Tissue samples were processed aseptically by removing extraneous material and chopped into small pieces and macerated using a tissue grinder with 200μl of sterile Phosphate Buffered Saline (PBS-Sigma Aldrich, Germany).

Genomic DNA from reference bacterial strains was extracted using the High Pure PCR template preparation kit (Roche, Basel, Switzerland) according to the instructions of manufacturer but also with some adaptions as previously described (Karponi, et al., 2019).

2.4 PCR protocol

2.4.1 Reaction mixture and cycling parameters

A conventional PCR was performed to all samples (Garcia-Yoldi, et al., 2006). In every thin walled PCR tube of 50 μl there were 38,25 μl water (nuclease free), 5 μl Dream Taq buffer 10x (Thermo Scientific, California, USA), 1 μl dNTPs (c=10 mM, Invitrogen, Carlbad, CA, USA), 0.25 μl primer f (c=100 μM), 0.25 μl primer r (c=100 μM), 0.25 μl Dream Taq DNA polymerase (1,25 units, Thermo Scientific, California, USA) and 5μl DNA. Pipette tips with aerosol filters were used in order to prevent contaminations. The mix was vortexed briefly to ensure homogeneity of reagents and to avoid bubbles.

For the PCR, a thermal cycler with heated lid was used (Techne, Touchgene, Gradient). After initial denaturation at 95^{o}C for 7 min, the PCR profile was as follows: 1 min of template denaturation at 95^{o}C, 1 min of primer annealing at 59^{o}C and 1 min of primer extension at 72^{o}C, for a total of 40 cycles, with a final extension at 72^{o}C for 10 min. After PCR amplification, 12 μl of PCR product and 4 μl of bromophenol blue (loading buffer) were loaded into wells in 1.5% regular agarose gel (Ultra Pure Agarose, Invitrogen) in Tris base, Acetic acid and Ethylene Diethyl Tetracetic Acid (EDTA) (TAE) buffer in a cuvette flooded with TAE 1x slightly covering the gel. 1 kb DNA ladder (ready to use, GeneRuler, ThermoScientific) was used as molecular marker. Sterile ultrapure water was used as negative control and reference strain 16M, Rev1, reference strain 544 or RB51 were used as positive controls. Bands were visualized by staining with GelRed, Biotium. The electrophoresis equipment was set to run at 110 V for 40 min. Finally, the gel was visualized under UV light and the bands were observed and recorded.

2.4.2 PCR primers

Four pairs of primers were involved in the essay gradually. 1) the pair BMEII0428f/ BMEII0428r (5'-GCC GCT ATT ATG TGG ACT GG-3'/ 5'-AAT GAC TTC ACG GTC GTT CG-3', amplicon size: 587 bp) which detects *Brucella* spp. 2) the pair BMEI0752f/ BMEI0752r (5'-CAG GCA AAC CCT CAG AAG C-3'/ 5'-GAT GTG GTA ACG CAC ACC AA-3', amplicon size: 218 bp) which detects Rev1 strain. 3) the pair BMEII0843f/ BMEII0844r (5'-TTT ACA CAG GCA ATC CAG CA-3'/ 5'-GCG TCC AGT TGT TGT TGA TG-3', amplicon size: 1,071 bp) which detects *B. melitensis* and Rev1. 4) the pair BMEI0998f/ BMEI0998r (5'-ATC CTA TTG CCC CGA TAA GG-3'/ 5'-GCT TCG CAT TTT CAC TGT AGC-3', amplicon size: 1,682 bp) which detects *B. melitensis*, Rev1, *B. abortus* but no RB51. PCR protocol was an adaptation from the previously published paper (Garcia-Yoldi, et al., 2006).

Initially, all samples were tested with primers 428f/428r in order to distinguish those which were positive in *Brucella* spp. as screening method. Then, the positive samples of sheep and goats were tested with the pair 752f/752r in order to discriminate Rev1 strain. The positive samples of cattle were tested with 843f/844r in order to distinguish *B. melitensis* from *B. abortus*, and once negative they were further tested with 998f/997r in order to distinguish *B. abortus* from RB51 (positive in case of *B.abortus*) (Table 1).

Footnote:

[a] ATCC: American Type Culture Collection

3. Results

In total 264 samples were examined derived from 191 animals. Thirty-nine (39) out of 264 derived from 30 animals were found PCR positive (Tables 2, 3, 4 & 5).

In the first group of the 83 seropositive male animals, the results were as follows: 13 out of 121 samples derived from 12 seropositive animals (2 rams, 5 billy goats and 5 bulls) were found positive. In detail, in 1 ram *B. melitensis* and in another ram Rev1 strain were detected respectively, out of the total of 47 seropositive rams (4,26%). In 2 billy goats was found *B. melitensis* and in other 3 ones both *B. melitensis* and Rev1 strain were detected respectively, out of the 29 seropositive billy goats (17,24%) examined, Finally, 5 bulls were found positive to *B. abortus* out of the 7 seropositive ones (71,43%) (Tables 2 and 3).

Regarding the infected tissues of small ruminants, 7 out of 49 testicle samples (14,28%) were found positive by PCR, while only 1 spleen was found positive out of 56 spleen samples (1,78%). On the other hand, regarding the bulls, 5 out of 7 samples from inguinal lymph nodes were found positive (71,43%).

In the second group which included 108 aborted fetuses, the results were the following: 26 samples taken from 18 animals (5 sheep, 2 goats and 11 cows) were found positive on a total of 143 samples. In detail, 4 sheep were found positive for *B. melitensis* and 1 sheep positive for Rev1 strain out of 57 sheep in total (8,77%). Two goats out of 5 were found positive for *B. melitensis* and 11 cows out of 46 were found positive in *B. abortus* (23,91%) (Tables 4 and 5).

Regarding the small ruminants aborted fetal tissues, 4 fetal livers were found positive out of 26 total liver samples (15,38%) and 4 aborted fetal stomach contents were found positive out of 43 (9,30%). On the other hand, regarding the cows, 6 fetal livers out of 21 in total were found positive (28,57%) while 7 out of 25 stomach contents originated from aborted fetuses were found positive (28%). In 10 cases where fetal liver and stomach content were originated from the same animal, the 5 out of them were found PCR positive in both samples. In the 3 of them only fetal livers were positive and in 2 of them only stomach contents were positive.

4. Discussion

Brucellosis is a severe zoonosis, that affects many animal species, with most important the domestic ruminants, such as sheep, goats and cattle. Both breeders and the National Economy deal with grade economic losses such as the reduction of milk production, the trade restrictions, the culling of seropositive animals (and in some cases seronegative, as well), the abortions and stillbirths, the cost of veterinary services, and miscellaneous factors arising from brucellosis on farms (Rossetti, et al., 2017).

Rose Bengal test (RBT) and Complement Fixation Test (CFT) have been used for many years, in brucellosis eradication programs (Garin-Bastuji, et al., 1998, Nagati, et al., 2016). The diagnosis of brucellosis in small ruminants and cattle requires the use of more than one serological test and RBT and CFT are among the most useful tests for routine diagnosis (Baum, et al., 1995). Even if serological cross-reactions have been demonstrated between smooth *Brucella* species and *Escherichia coli* serotypes 0:116 and 0:157, *Salmonella* serotypes of Kaufmann-White group N (0:30 antigen, *Pseudomonas maltophilia* and *Yersinia enterocolitica* serotype 0:9, tend to be of a little significance (FAO/Brucellosis in human and animals., 2006).

The results of our study using the above PCR protocol indicates that detection of *Brucella* spp. DNA directly from testicles and spleen of seropositive male animals was not a convenient procedure confronting with the results derived from serology tests (RBT/CFT) in the first group of 83 seropositive animals. In fact, only 14,45% of the seropositive animals were found positive in PCR analysis. Testes and spleen as biological samples-target for the detection of brucellosis via PCR is mostly rare. According to the literature, the most frequently tested tissues from male animals in order to detect *Brucella* spp. are serum, semen, nasal secretions, feces, lymph nodes and whole blood (Bricker, 2002, Ilhan, et al., 2008, Yu, et al., 2010).

Respectively, in the second group of 108 suspected animals, 16.67% of the female animals and fetuses were found positive in PCR analysis. The relatively few *Brucella* PCR positive samples from female ruminants with clinical symptoms from the reproductive system in the present study might also suggest the existence of other causes of abortion than brucellosis and it warrants further studies (Petridou, et al., 2018).

PCR-based tests are proving to be faster and more sensitive than conventional methods. Several PCR assays have been described for the detection of *Brucella* DNA using pure cultures or animal or human clinical samples. However, the sensitivity and specificity of the PCR for *Brucella* spp. varies between laboratories, standardization of sample preparation, PCR inhibitors on tissues, target genes, selected primers and clinical status of the animal (acute/chronic) (Amin, et al., 2001, Tekle, et al., 2019). The challenge lies in simplifying the procedure for detecting *Brucella* spp. at the species level in specialized laboratories (Navarro, et al., 2004, Ghorbani, et al., 2012,).

The modified PCR protocol used in our study was time consuming in terms of samples preparation resulting to a minimum of three days for the results to be delivered while the cost was much higher than the one required for serology.

An ideal diagnostic method should be specific, sensitive, rapid, not expensive and easy to perform (Geresu, et al., 2016). According to the presented study, it seems that for the time being this PCR protocol described does not fulfil all these requirements. Finally, the purchase of reagents is fairly expensive

compared with RBT/CFT, but cost may decrease as more PCR diagnostic assays are developed.

In both groups, the prevalence of the *Brucella* strains detected in samples derived from goats (12,5% and 50%) and bovines (71,43% and 25,56%) was much higher than the prevalence of the same detected bacterium in sheep (3,03% and 8,22%). Actually, goats are approximately four times more likely to be infected than sheep (Sintayehu, et al., 2015). Generally, sheep are less susceptible to *Brucella* infection than goats, and this could be partly explained because sheep excrete the bacteria for shorter periods. This may reduce the potential for spread of the disease within and between sheep flocks (Radostits, et al., 2007).

In the first group, preferred tissue for detecting the bacterium in rams and billy goats is testicle (8,7% and 19,23%) instead of spleen (0% and 4,76%), even if it is commonly known that spleen is the most heavily colonized organ, during brucellosis (Silva, et al., 2011). The stage of infection may very well influence the number and location of *Brucella* organisms in white blood cells and lymphoid tissue glands (Gupta, et al., 2006). Concerning bulls, it seems that inguinal lymph nodes give encouraging results (71%), while no other kind of tissues were provided in this study.

The fact that vaccine strain Rev1 was detected in non-vaccinated rams and billy goats, indicates that possibly errors during the vaccine administration in females may occurred resulting in accidental vaccination of the male animals which were found seropositive during the surveillance program. Finally, due to the above vaccine administration errors male animals were slaughtered while the farm was quarantined for a period of at least 2 to 6 weeks. The development of a rapid and cost-effective laboratory method that will discriminate the vaccine antibodies from those caused by wild-type strains is of paramount importance. Alternatively, the development of a new vaccine, like the RB51 strain (Dorneles, et al., 2015), which is implemented in cattle and the produced antibodies are not detectable by RBT/CFT may solve this inconvenience equally.

Among female small ruminants and cattle from the second group which aborted, we suggest as preferred tissues to examine and reach to a positive PCR result tissues from fetal liver or/and stomach content from fetuses than other fetal tissues (fetal membranes, fetal spleens, placenta and posterior mammary lymph nodes).

5. Conclusions

Therefore, the use of the suggested PCR protocol in our study can play an important role in terms of confirmation before the suspected female animals are slaughtered.

B. melitensis and *B. abortus* are common pathogens in Greek small ruminant and dairy cow farms in Greece, while, the Rev-1 vaccine strain can sometimes be implicated in false brucellosis positive diagnosis of small ruminants.

Declarations

Authors' Contributions: A.K. conceived, performed the PCR analyses and wrote the manuscript. E.P. (Eleni Papanikolaou) conceived, designed the methodology, corrected the paper. A.S. corrected the paper.A.Z. conceived and corrected the paper. P.G performed the PCR analyses. E.P (Emmanouil Papadogiannakis) corrected the paper. N.G coordinated the study and helped to draft the manuscript. E.P. (Evanthia Petridou), conceived, coordinated the study and corrected the paper. All authors read and approved the final manuscript.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval: 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 55/27-5-2015.

Consent to participate: All authors have given their consent for participation

Consent for publication: All authors have seen and approved the final version of the submitted manuscript. The article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

Conflicts of Interest: The authors declare no conflict of interest.

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Tables

Table 1
Primers, amplicon size and specificity.

Primers	bp	*B. melitensis*	Rev-1	*B. abortus*	RB-51
1) 428f/428r	587	+	+	+	+
2) 752f/752r	218	-	+	-	-
3) 843f/844r	1.071	+	+	-	-
4) 998f/997r	1.682	+	+	+	+* 2,524

Table 2
First group (seropositive in brucellosis male animals).

Animal	Number of seropositive, male animals	Number of positive animals in PCR	Number of samples	Number of positive samples in PCR	Testicles	Positive testicles in PCR	Spleen	Positive spleens in PCR	Inguinal lymph nodes	Positive inguinal lymph nodes in PCR
Rams	47	2 (4,26%)	66	2 (3,03%)	23	2 (8,7%)	35	0	8	0
Billy goats	29	5 (17,24%)	48	6 (12,5%)	26	5 (19,23%)	21	1 (4,76%)	1	0
Bulls	7	5 (71,43%)	7	5 (71,43%)	0	0	0	0	7	5 (71,43%)
	83	**12 (14,46%)**	**121**	**13 (10,74%)**	**49**	**7**	**56**	**1**	**16**	**5**

Table 3
First group. Detected *Brucella* strains on infected tissues.

Animal	Number of samples (from seropositive animals)	Number of positive samples in PCR	Brucella species found in samples	Infected tissues
Rams	66	2 (3,03%)	• 1 *B. melitensis*, • 1 Rev1	• 2 testicles
Billy goats	48	6 (12,5%)	• 3 *B. melitensis* • 3 Rev1	• 5 testicles, • 1 spleen
Bulls	7	5 (71,43%)	• 5 *B. abortus*	• 5 inguinal lymph nodes
	121	**13 (10,74%)**	**13**	**13**

Table 4
Second group (female animals that aborted and aborted fetuses).

Animal	Number of animals which aborted	Number of positive animals in PCR	Number of samples	Number of positive samples in PCR	Fetal membranes	Positive fetal membranes in PCR	Fetal liver	Positive fetal liver in PCR	Fetal spleen	Positive fetal spleen in PCR	Stomach contents of the fetus	Positive stomach contents of the fetus in PCR	Placenta	Positive placenta in PCR	Posterior mammary lymph nodes	Positive posterior mammary lymph nodes in PCR
Sheep	57	5 (8,77%)	73	6 (8,22%)	2	0	24	3 (12,5%)	2	0	40	2 (5%)	5	1 (20%)	0	0
Goats	5	2 (40%)	6	3 (50%)	0	0	2	1 (50%)	0	0	3	2 (66,67%)	1	0	0	0
Cows	46	11 (23,91%)	64	17 (25,56%)	3	0	21	6 (28,57%)	7	1 (14,29%)	25	7 (28%)	5	3 (60%)	3	0
	108	**18 (16,67%)**	**143**	**26**	**5**	**0**	**47**	**10**	**9**	**1**	**68**	**11**	**11**	**4**	**3**	**0**

Table 5
Second group. Detected *Brucella* strains on infected tissues.

Animal	Number of samples	Number of positive samples in PCR	Brucella species found in samples	Infected tissues
Sheep	73	6 (8,22%)	• 5 *B. melitensis*, • 1 Rev1	• 3 fetal livers, • 2 stomach contents of the fetus, • 1 placenta
Goats	6	3 (50%)	• 3 *B. melitensis*	• 2 stomach contents of the fetus, • 1 fetal liver
Cows	64	17 (25,56%)	• 17 *B. abortus*	• 7 stomach contents of the fetus, • 6 fetal livers, • 3 placentas, • 1 fetal spleens
	143	**26**	**26**	**26**

Figures

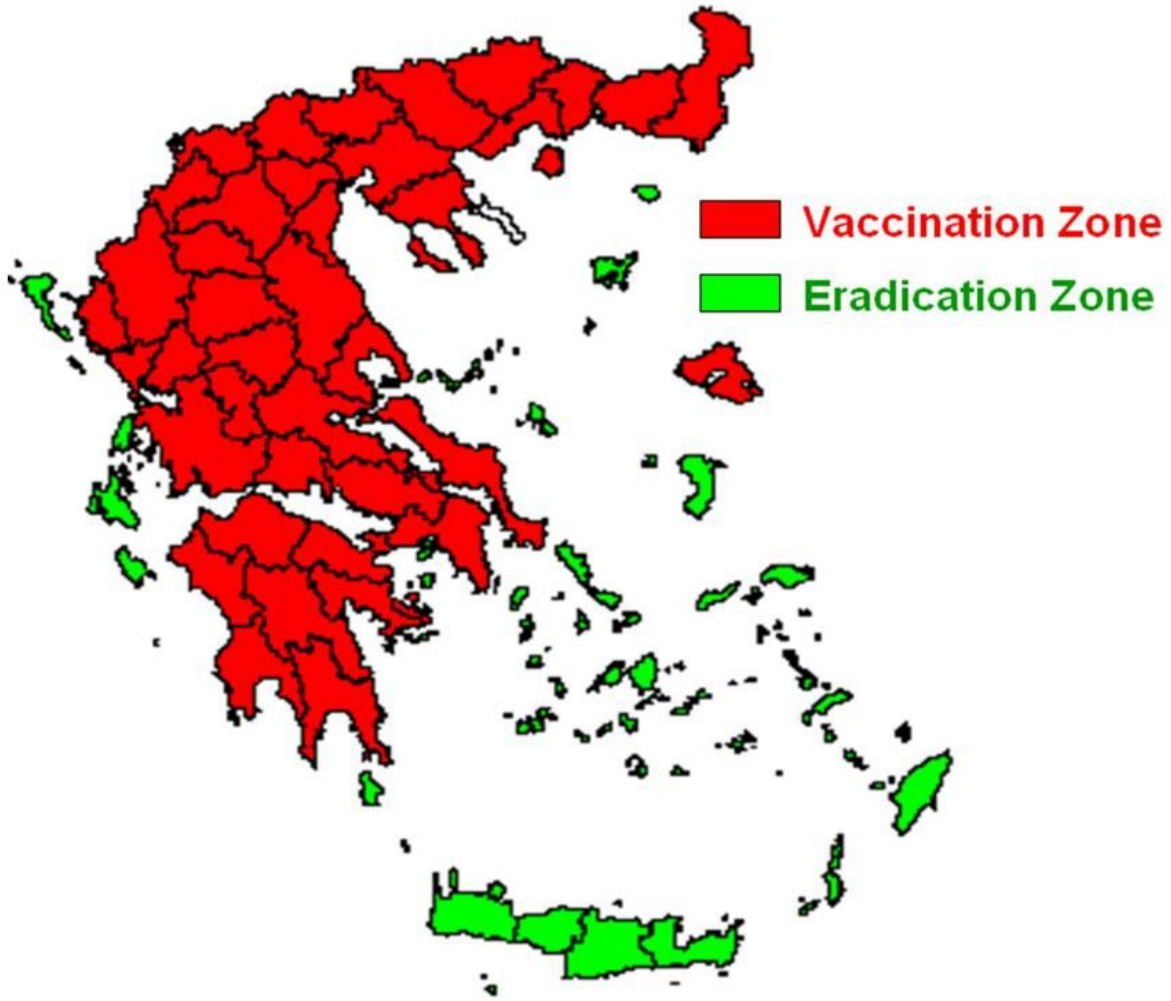

Figure 1

Map of Greece with the 2 different zones for the implementation of sheep & goat brucellosis programme.