Original Scientific Article

APPLICATION OF FLUORESCENCE BASED MOLECULAR ASSAYS FOR IMPROVED DETECTION AND TYPING OF *BRUCELLA* STRAINS IN CLINICAL SAMPLES

Kiril Krstevski[1], Ivancho Naletoski[2], Dine Mitrov[1], Slavcho Mrenoshki[1],
Iskra Cvetkovikj[1], Aleksandar Janevski[1], Aleksandar Dodovski[1], Igor Djadjovski[1]

*[1]Veterinary Institute, Faculty of Veterinary Medicine, Ss. Cyril and Methodius
University in Skopje, Republic of Macedonia*
*[2]Animal Production and Health Section, Joint FAO/IAEA Division,
International Atomic Energy Agency, Vienna, Austria*

ABSTRACT

Bacteria from the genus *Brucella* are causative agents of brucellosis – a zoonotic disease which affects many wild and domestic animal species and humans. Taking into account the significant socio-economic and public health impact of brucellosis, its control is of great importance for endemic areas. The chosen control strategy could be successful only if adapted to the current epidemiological situation. This implies that a choice of appropriate diagnostic procedures for detection and typing of *Brucella spp.* strains are of essential importance. Significant advancement of molecular techniques and their advantages compared to classical methods, give strong arguments in promotion of these techniques as a powerful tool for comprehensive diagnostics of brucellosis. Considering this, the major tasks of the study were to select and implement molecular tests for detection and genotyping *Brucella* spp. and evaluate their performances using DNA from cultivated *brucellae* (islolates) and limited number of tissue samples from seropositive animals. The obtained results confirmed that implemented real time PCR for *Brucella* spp. detection, as well as MLVA-16 used for genotyping, have excellent analytical sensitivity (4.2 *fg of Brucella* DNA were successfully detected and genotyped). Furthermore, compared to bacteriological cultivation of *Brucella* spp., real time PCR and MLVA-16 protocols showed superior diagnostic sensitivity and detected *Brucella* DNA in tissues from which *Brucella* could not be cultivated. Based on the summarized study results, we propose a diagnostic algorithm for detection and genotyping of *Brucella* spp. bacteria. Routine use of proposed diagnostic algorithm will improve the effectiveness of infection confirmation and help for accurate evaluation of epidemiological situation.

Key words: brucellosis, clinical samples, DNA, real time PCR, MLVA-16

INTRODUCTION

Bacteria from the genus *Brucella* are causative agents of brucellosis – a zoonotic disease which affects many wild and domestic animal species and humans. Currently, ten *Brucella* species are officially recognized: *B.melitensis, B. abortus, B. suis,* *B. canis, B. ovis, B. neotomae, B. pinnipedialis, B. ceti, B. Microti* and *B. inopinata.* This classification of the genus *Brucella* is based on the differences in host preference, pathogenicity, phenotypic traits and genetic structure. However, it is very likely that novel species will be included in the genus soon, mainly as a result of advancement and increased use of molecular diagnostic tools (1).

Infections in animals result in significant economic losses due to induced abortions, infertility, reduced milk and body weight production. Moreover, the presence of brucellosis in animals represents a constant risk for human infections, which are commonly characterized with fever, malaise, sweating and lymphadenopathy (2). In the absence of vaccine for human use, control of human brucellosis remains largely dependent on the control of the disease in animals (3).

Corresponding author: Assist. Prof. Kiril Krstevski, DVM, MSc, PhD
E-mail address: krstevski@fvm.ukim.edu.mk
Present address: Veterinary Institute, Faculty of Veterinary Medicine,
Ss. Cyril and Methodius University in Skopje,
Republic of Macedonia
Phone: +389 2 3240 754

Taking into account the significant socio-economic and public health impact of brucellosis, its control is of great importance for endemic areas. Different scientifically approved approaches are available for brucellosis control. However, the chosen control strategy could be successful only if it is adapted to the current epidemiological situation, as well as to the economic and political situation in the country or the region (4, 5). Epidemiology of brucellosis is very complex due to the possible involvement of different mammal (animal) and different *Brucella* species. This fact highlights the importance of the diagnostic procedures for detection and typing of *Brucella*, as powerful epidemiological tools which are indispensable for a successful control program.

The only unequivocal confirmation of *Brucella* spp. infections is laboratory detection and identification of the *brucellae* in the affected organism. Until recently, isolation of *Brucella* has been considered as a gold standard assay for brucellosis diagnosis (6, 7). However, significant advancement of molecular techniques and their increased application in the diagnostic laboratories during the past two decades, has led to the formal recognition of these techniques as valid assays for definite diagnosis of brucellosis (8). Unlike isolation, PCR based molecular tests do not require the presence of vital bacteria in the samples, provide quick and objective results and are safe and relatively easy to perform (7, 9). Moreover, due to the possibility for detection of bacterial DNA even in the samples with small number of *Brucellae*, they have better sensitivity compared to isolation (6, 7, 10). Numerous molecular assays using different targets for *Brucella* spp. identification have been published in the last twenty five years (11-16). Different methods are available for determination of species (17-21), as well as for typing at subspecies level (22-25). Molecular typing based on the analysis of variable number of tandem repeats analysis (VNTRs) in multiple loci (MLVA), provides valuable epidemiological results and is of great value for investigating outbreaks. MLVA testing scheme consisting of 16 genetic loci (MLVA-16) has high discriminatory power at subspecies level and enable differentiation of unrelated *Brucella* spp. strains, which could not be differentiated by classical microbiological methods (26-35). The existence of public database with MLVA-16 allelic profiles (http://mlva.u-psud.fr/brucella/) allows comparison of the typed *Brucella* spp. strains at regional and international level (36). In addition, allelic profiles could be used for species identification or confirmation, if necessary.

The purpose of this study was to establish and evaluate a diagnostic algorithm (flowchart) for *Brucella* spp. detection and typing, which will exploit the advantages of fluorescence-detection based molecular techniques in terms of sensitivity, safety and testing time and hence provide improved brucellosis diagnostics when clinical samples are tested.

MATERIAL AND METHODS

Samples

One isolate of *B. abortus* (field strain MKD-1027), derived from culture collection of the Faculty of Veterinary Medicine-Skopje, and organs from 11 slaughtered seropositive ruminants (Table 3) were used for extraction of genomic DNA. Organs from one animal (spleen, supramammary, iliac and inguinal lymph nodes) were grinded and macerated together in a form of suspension suitable for cultivation, inoculated on selective (*Farrell's*) media and archived at -80°C as "tissue sample". Eleven tissue samples used in the study were randomly selected from the FVMS's collection of tissue samples kept at -80°C.

DNA extraction

Pure bacterial DNA used for implementation and optimization of the test protocols for detection and molecular typing was obtained from the MKD-1027 isolate using *PureLink Genomic DNA kit* (Life Technologies, USA), according to the manufacturer's instructions for Gram negative bacteria. Concentration and purity of extracted bacterial DNA was determined spectrophotometrically (A_{260} and A_{280}) using NanoDrop 2000c (Thermo Scientific, USA). Total genomic DNA was extracted from tissue samples using the same commercial kit, according to the manufacturer's instructions for tissues. The genomic DNA extracted from tissue samples was used to evaluate the performance of implemented testing scheme.

Real time PCR

Genomic DNA from field strain MKD-1027 was used for optimization and performance evaluation of implemented real time PCR for *Brucella* spp. detection. Primers and probe used in the test are targeting *IS711* and are described elsewhere (11). Eight tenfold dilutions corresponding to a DNA concentration range between 1.7 ng/µL and 0.17 fg/µL were prepared from the purified DNA sample and tested in duplicate. Real time PCR

reaction was prepared in a final volume of 25 µL using 12.5 µL 2x RT PCR reaction mix for probes (Bio-Rad, USA), 8 µL nuclease-free water, 2 µL primer-probe mix and 2.5 µL of DNA sample. Final concentrations of primers and probe were 0.5 µM and 0.2 µM, respectively. Amplification was performed in IQ5 Thermal Cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 95°C for 5 min and 42 cycles of denaturation at 95°C for 15 s, and combined annealing-extension at 60°C for 30 s. Efficacy of the reaction (E), coefficient of determination (R^2) and slope of the standard curve were calculated using IQ5 software. One of the DNA dilutions with high quantification cycle (*Cq*) value has been defined as a future "positive control sample" and was independently tested ten times. Obtained *Cq* values were used for calculation of coefficient of variation (CV) standing for inter-assay reproducibility and upper and lower limits of the positive control (95% and 99% confidence levels).

MLVA-16 genotyping

For achieving the best analytical sensitivity and repeatability of the capillary electrophoresis system used, all loci from the MLVA-16 panel (22, 24) were amplified in singleplex PCR reactions using 5' - fluorescently (6-FAM) labeled forward primers and *Taq PCR Master mix kit* (Qiagen, USA). Depending on the amount of input DNA, three different protocols for PCR reaction set-up and cycling were used (Table 1).

PCR reactions were set in a total volume of 15 µL (PCR-1 and PCR-2) and 25 µL (PCR-3), containing 1 x Qiagen master mix and 0.5 µM of each primer. All PCR amplifications were performed in a TC412 thermal cycler (Techne, UK).

The size of the PCR products was determined using capillary electrophoresis on ABI 310 system (Applied Biosystems, USA). Briefly, undiluted (PCR-3) or diluted (PCR-1, PCR-2) products have been prepared for electro kinetic injection by adding deionized formamide (Applied Biosystems, USA) and *GS LIZ500* or *GS LIZ1200* (Applied Biosystems, USA) size standards. Amplified loci from referent *B. abortus S19* and *B. melitensis Rev-1* strains were used as a control strains. Fragment sizing was performed by *GeneMapper* software ver. 4.0, according to a generated standard curve. Obtained raw data for the size of each locus was recorded in an excel sheet. Measured fragment size was corrected for the previously determined measuring aberration of the instrument. Corrected values were converted in tandem repeat units using *Table for allele assignment* freely available at MLVA Net for *Brucella* website (http://mlva.u-psud.fr/brucella/spip.php?article93). Aberration in size measuring was determined for each locus by sequencing. Complete sequences of all 16 loci for referent *B. abortus S19* and *B. melitensis Rev-1* strains were obtained using *ABI Prism Big Dye Terminator (v3.1) cycle sequencing ready reaction kit* (Applied Biosystems, USA).

RESULTS

Real time PCR – culture dilutions

Bacterial DNA was detected in seven out of eight tenfold DNA dilutions when tested with the real time PCR assay for detection of *Brucella* spp., corresponding to analytical sensitivity of 4.2 fg bacterial DNA (Fig. 1, Table 2). The implemented test protocol amplified broad range of DNA concentrations (4.2 ng to 4.2 fg) with excellent linearity ($R^2= 0.997$) and 85.5% reaction efficacy (Fig. 2).

Table 1. Cycling conditions for amplification of VNTR loci for *Brucella* genotyping

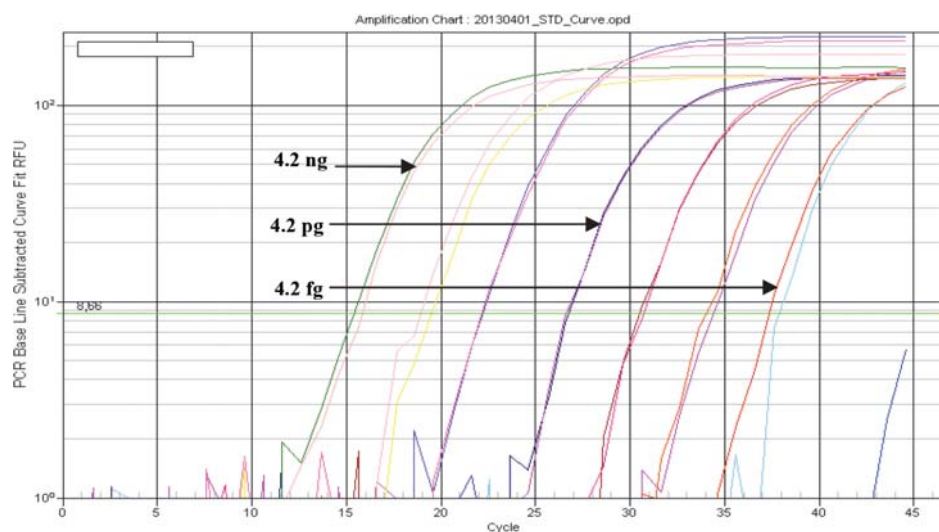| STEP	PCR-1	Cycles	PCR-2	Cycles	PCR-3	Cycles
Initial denaturation	94°C, 5 min	**1**	94°C, 5 min	**1**	94°C, 5 min	**1**
Denaturation	94°C,30 s		94°C,30 s		94°C,30 s	
Primer annealing	60°C,30 s	**30**	60°C, 50 s	**35**	60°C, 50 s	**40**
Extension	72°C, 60 s		72°C, 60 s		72°C, 60 s	
Final extension	72°C,5 min	**1**	72°C, 7min	**1**	72°C, 10min	**1**

Figure 1. Amplification curves of *B. abortus* (MKD-1027) DNA (log view). Tenfold dilutions (from 4.2 ng to 0.42 fg) were tested with the implemented IS711 real time PCR. Fluorescence data is baseline subtracted
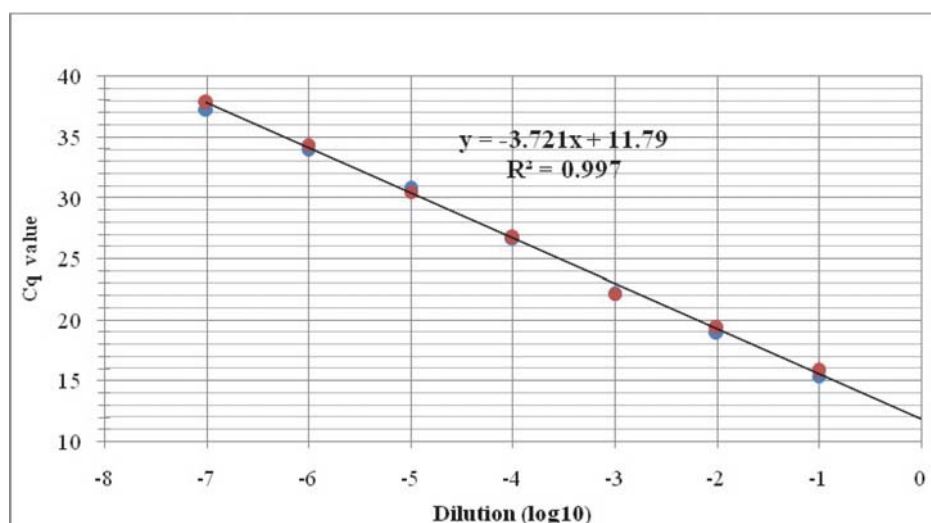

Figure 2. Standard curve obtained after testing tenfold dilutions of *B. abortus* (MKD-1027) DNA with the implemented IS711 real time PCR

Table 2. Tenfold dilutions of *B. abortus* (MKD-1027) DNA with appropriate amounts of DNA in reaction and obtained Cq values (tested in duplicate)

DNA dilution (MKD-1027)	Total amount of DNA in reaction (ng)	*Cq*-1	*Cq*-2	*Cq*-mean
10^{-1}	4.2	15.35	15.78	15.56
10^{-2}	0.42	18.87	19.47	19.17
10^{-3}	0.042	22.07	22.14	22.10
10^{-4}	0.0042	26.63	26.71	26.67
10^{-5}	0.00042	30.71	30.46	30.59
10^{-6}	0.000042	33.97	34.37	34.17
10^{-7}	0.0000042	37.25	37.81	37.53
10^{-8}	0.00000042	/	/	/

Analysis of Cq values obtained in ten independent tests of 10^{-6} *B. abortus* (MKD-1027) DNA dilution, indicated very good reproducibility and inter-assay CV of 1.8%. This DNA dilution has been defined as a positive control for all future runs, with upper and lower Cq limits in the following range: 33 – 34.2 (95% confidence level) and 32.5 – 34.7 (99% confidence level) (data not shown).

MLVA-16 genotyping

The modified PCR protocol (PCR-3) used for amplification, combined with fluorescence based capillary electrophoresis system for detection, enabled detection of all loci from the MLVA-16 scheme, even in the highest real time PCR positive dilution (10^{-7}) of *B. abortus* DNA (Fig. 3). Next dilution (10^{-8}) was negative, meaning that the lower limit of detection of the implemented MLVA-16 typing system was equal to the lower limit of detection observed in the implemented real time PCR for detection of *Brucella* spp. However, PCR-3 protocol should be used only for samples with minor DNA amounts (close to the lower limit of detection), which could not be genotyped using agar-gel electrophoresis. Otherwise, unspecific fragments are likely to appear (Fig. 4), especially if DNA is extracted from clinical samples. The appearance of unspecific fragments could compromise the interpretation of results in situations when their size is very close to the size of existing alleles.
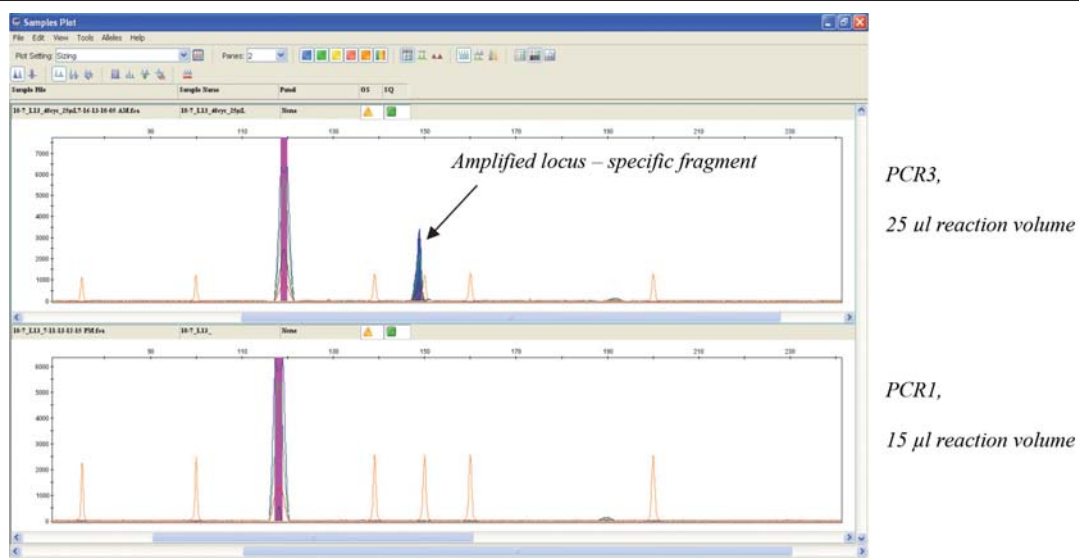

Figure 3. Electropherograms of the products obtained after two different PCR amplifications of bruce30 locus in highest detectable dilution (10^{-7}) of *B. abortus* DNA. Amplified locus was detected only with the modified (PCR-3) protocol (upper electropherogram)
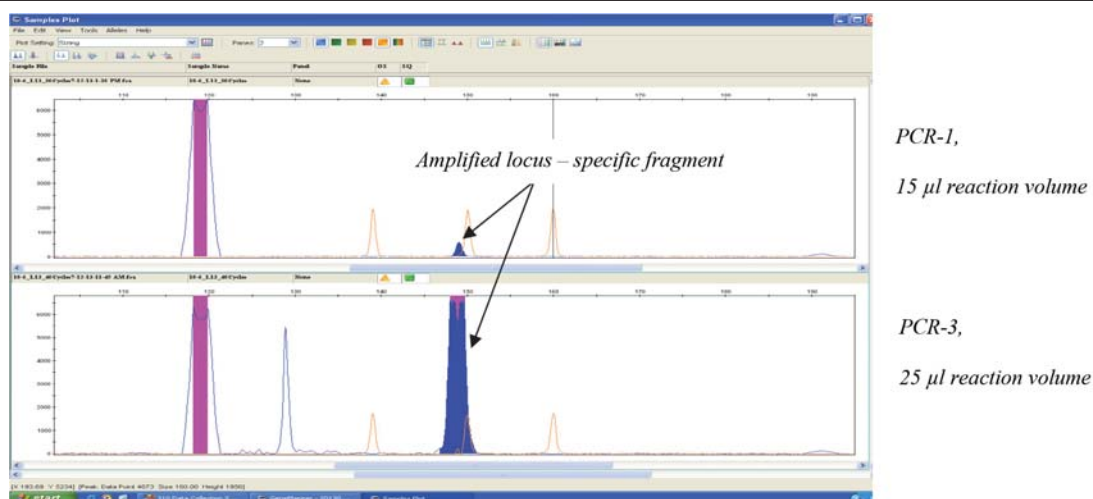

Figure 4. Electropherograms of the products obtained after two different PCR amplifications of bruce30 locus in 10^{-6} dilution of *B. abortus* DNA. Amplified locus was detected with both PCR protocols, but unspecific fragments were present when modified (PCR-3) protocol was used (lower electropherogram)

Real time PCR and genotyping - clinical samples

Brucella spp. DNA was detected in the eleven samples of genomic DNA extracted from the tissues. Obtained *Cq* values varied, indicating different *Brucella* concentrations in the tested samples (Table 3). *Brucella* DNA was detected in seven tissues from which *Brucella* could not be cultivated, although (as expected) with higher *Cq* values. Finally, MLVA-16 profiles were successfully obtained for all samples. There were some reproducibility problems when sample with highest *Cq* value (1-AL) was tested – some of the loci required more than one attempt for obtaining visible signal. This could be explained with low amount of DNA in the 1-AL sample, which was very close to the lower limit of detection of the system.

highly-discriminatory methods for its typing, play important role in the system of eradication.

As previously reported (11), real time PCR targeting *IS711* segment of *Brucella* spp. is a highly sensitive assay and a good candidate for direct testing of clinical samples. In our study, analytical sensitivity of the *IS711* real time PCR assay was 4.2 ng, which is comparable with findings of other authors (11, 37, 38) and proves the robustness of the implemented assay. Theoretically, if we take into account average genome sizes of *Brucella* members, *IS711* real time PCR assay could detect only few bacterial cells. The excellent lower limit of detection is a result of application of real time PCR technology, as well as of the presence of more *IS711* copies throughout the *Brucella* spp. genome.

Table 3. Clinical samples in which *Brucella* DNA was detected by IS711 rt-PCR and MLVA-16 profiles were determined using the described protocols

Sample ID	*Cq* value using *IS711* rt-PCR	ISOLATION (Farrell's medium)	Animal Host	Year
70	18.6	POSITIVE	sheep	2010
4	23.8	POSITIVE	sheep	2010
5	26.0	POSITIVE	sheep	2010
69	27.6	POSITIVE	sheep	2010
689	31.0	negative	sheep	2010
388	34.0	negative	goat	2010
652	35.2	negative	sheep	2010
10_ab	35.6	negative	sheep	2013
11/1	31.8	negative	sheep	2013
2-AL	34.8	negative	cattle	2014
1-AL	36.1	negative	cattle	2014

DISCUSSION

Confirmation of *Brucella* spp. infections is very important for successful control of brucellosis in animals. This is especially evident when the eradication programme is in its late stage and the prevalence of disease significantly decreased (4). In this case, rapid confirmation of suspected infections and successful trace-back of its origin and routes, provide significant support to progress towards complete disease eradication. Hence, the selection of proper laboratory methods for rapid, sensitive and specific detection of *Brucella* spp., as well as

Although the study was not designed to compare the performances of molecular detection and isolation, it confirmed the superior *Brucella* spp. detection capability of the *IS711* real time PCR, compared to the isolation when different clinical samples were tested (Table 3). Our results support the conclusion of other authors (39, 40), that isolation itself is not sufficient for confirmation of infected animals during epidemiological investigations. Therefore, we have established a diagnostic flowchart for *Brucella* spp. detection/ identification based on real time PCR, in addition to isolation on selective media (Fig. 5).
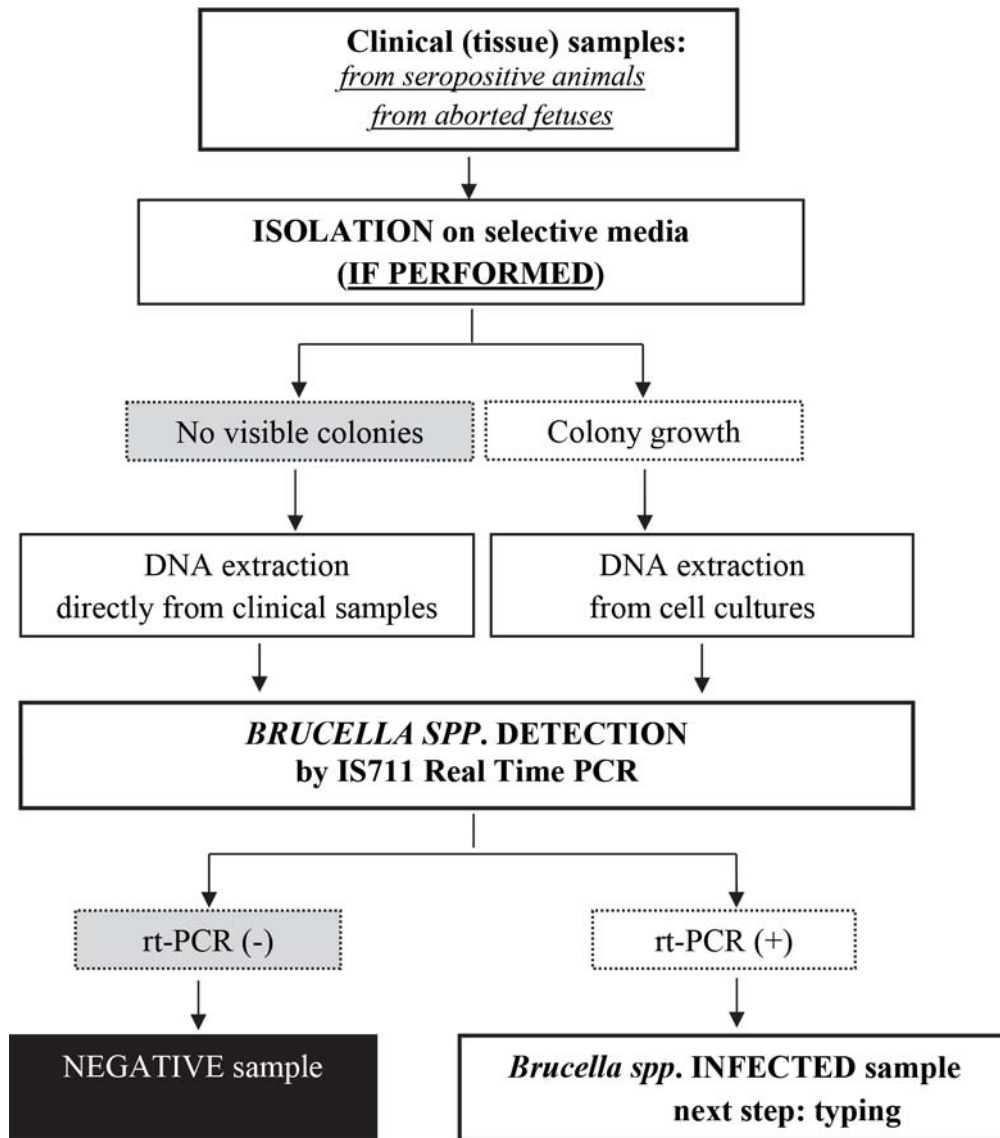
Figure 5. Diagnostic flowchart for *Brucella* spp. detection

Whenever *Brucella* spp. is isolated, all molecular tests for species determination and subspecies typing usually perform well, because a high concentration of bacterial DNA is extracted from colonies. In other words, the analytical sensitivity of molecular methods is not an issue when *Brucella* spp. has been isolated from clinical samples. However, if *Brucella* spp. is not isolated, but is present in the clinical samples in small numbers which are detectable by the *IS711* real time PCR, species and genotype of detected strain could be determined only if highly sensitive technologies are implemented.

The MLVA-16 typing system that we implemented had equal analytical sensitivity with the above mentioned real time PCR for *Brucella* spp. detection (Fig. 3), meaning that MVLA-16 allelic profiles could be obtained for all detected *Brucella* spp. strains. This theoretical conclusion was practically evaluated with the successful genotyping of all clinical samples from Table 3. Moreover, quantitative capabilities of the real time PCR technology provide precise quantization of *Brucella* DNA loads in the clinical samples, which is very important for selection of the most appropriate MLVA PCR protocol and successful genotyping. Taking into account that DNA sample obtained from the infected tissue contains huge amounts of host DNA, concentration of *Brucella* DNA could not be measured using the common spectrometric method.

As a summary of all results from the study, we established a diagnostic flowchart for molecular typing of *Brucella* spp. strains (Figure 6) detected using the laboratory approach given in Figure 5.
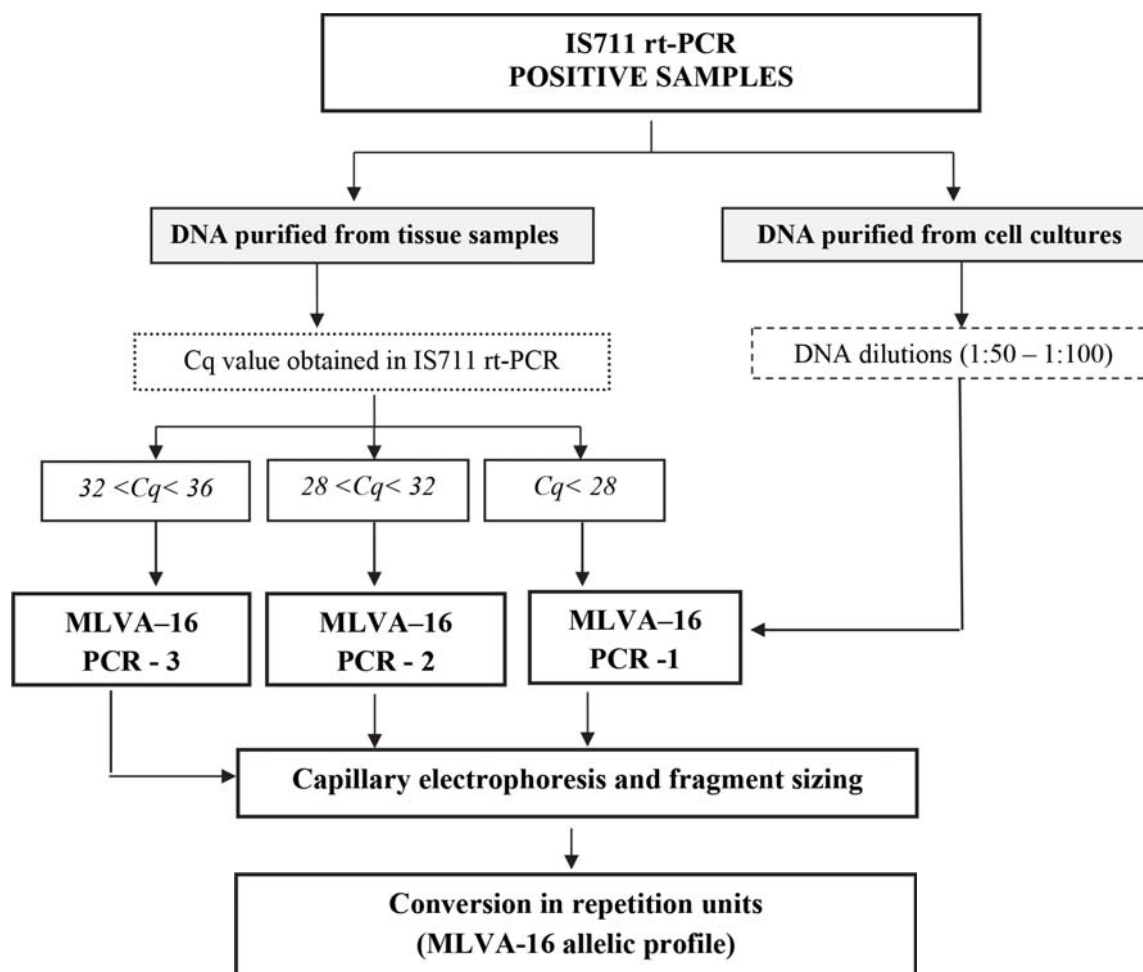
Figure 6. Diagnostic flowchart for genotyping detected *Brucella* spp. strains using MLVA-16

The described testing strategy enables highly sensitive *Brucella* spp. detection, and successful molecular characterization of detected strains. Its application could improve the effectiveness in infection confirmation and provide significant contribution in the accurate evaluation of epidemiological situations.

REFERENCES

1. Scholz, H.C., Vergnaud, G. (2013). Molecular characterisation of Brucella species. Rev. Sci. Tech Off. int. Epiz., 32 (1): 149-162.

2. Whatmore, A. (2011). Current understanding of the genetic diversity of Brucella, an expanding genus of zoonotic pathogens. Infec. Genet. Evol., 9(6): 1168–1184
http://dx.doi.org/10.1016/j.meegid.2009.07.001
PMid:19628055

3. World Health Organization (2005). The control of neglected zoonotic diseases; a route to poverty alleviation. Zoonoses and Veterinary Public Health, WHO, Geneva, Switzerland.

4. European Commission, Directorate General for Health and Consumers (2009). Working Document on Eradication of Bovine, Sheep and Goats Brucellosis in the EU.SANCO/6095/2009. Available at: *http://ec.europa.eu/food/animal/diseases/eradication/eradication_bovine_sheep_goats_brucellosis_en.pdf. last access on 14/7/2015*

5. Scientific Committee on Animal Health and Animal Welfare (2001). Brucellosis in sheep and goat. SANCO.C.2/AH/R23/2001. Available at:
http://ec.europa.eu/food/fs/sc/scah/out59_en.pdf. last access on 14/7/2015

6. Garin-Bastuji, B., Blasco, J.M., Martın, C., Albert, D. (2006). The diagnosis of brucellosis in sheep and goats, old and new tools. Small Ruminant Research 62, 63–70.
http://dx.doi.org/10.1016/j.smallrumres.2005.08.004

7. Poester, F.P, Nielsen, K., Samartino, L.E, Yu, W. L. (2010). Diagnosis of brucellosis. Open Vet. Sci. J., 4, 46-60.
http://dx.doi.org/10.2174/1874318801004010046

8. World Organization for Animal Health – OIE (2014). Chapter 8.4. Infection with Brucellaabortus, B. melitensis and B. suis. In Terrestrial Animal Health Code. OIE, Paris, 2012.

9. Hornitzky, M., Searson, J. (1986). The relationship between the isolation of *Brucella abortus* and serological status of infected, nonvaccinated cattle. Aust. Vet. J., 63, 172–174.
http://dx.doi.org/10.1111/j.1751-0813.1986.tb02966.x
PMid:3094489

10. Fekete, A., Bantlem, J.A., Hallingm, S.M., Sanborn, M.R. (1990). Preliminary development of a diagnostic test for Brucella using polymerase chain reaction. J. Appl. Bacteriol., 69, 216-227.
http://dx.doi.org/10.1111/j.1365-2672.1990.tb01512.x

11. Bounaadja, L., Albert, D., Chenais, B., Henault, S., Zygmunt, M.S., Poliak, S., Garin-Bastuji, B. (2009). Real-time PCR for identfication of Brucella spp.: A comparative study of IS711, bcsp31 and per target genes. Vet. Microbiol., 137, 156–164.
http://dx.doi.org/10.1016/j.vetmic.2008.12.023
PMid:19200666

12. Leal-Klevezas, D.S. et al. (1995). Single-step PCR for detection of Brucella spp. from blood and milk of infected animals, J. Clin. Microbiol., 12, 3087.

13. Ouahrani-Bettache, S., Soubrier, M.P., Liautard, J.P. (1996). IS6501-anchored PCR for the detection and identification of Brucella species and strains. J. Appl. Bacteriol., 81, 154–160.
http://dx.doi.org/10.1111/j.1365-2672.1996.tb04493.x
PMid:8760325

14. Rijpens, N.P.,Jannes, G., Van Asbroeck, M., Rossau, R., Herman, L.M. (1996). Direct detection of Brucella spp. in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. Appl. Environ. Microbiol., 62(5): 1683-1688.
PMid:8633866   PMCid:PMC167942

15. Romero, C.,Gamazo, C., Pardo, M., López-Goñi, I. (1995). Specific detection of Brucella DNA by PCR. J. Clin. Microbiol., 33(3): 615-617.
PMid:7538508   PMCid:PMC227999

16. Yu, W.L., Nielsen, K. (2010). Review of detection of Brucella spp. by polymerase chain reaction. Croat. med. J., 51(4): 306–313.
http://dx.doi.org/10.3325/cmj.2010.51.306
PMid:20718083   PMCid:PMC2931435

17. Bricker, B.J., Halling, S.M. (1994). Differentiation of Brucella abortus bv.1, 2, and 4, Brucella melitensis, Brucella ovis, and Brucella suis bv.1 by PCR. J. Clin. Microbiol., 32, 2660 –2666.
PMid:7852552   PMCid:PMC264138

18. Bricker, B.J., Halling, S.M. (1995). Enhancement of the Brucella AMOS-PCR assay for differentiation of Brucella abortus vaccine strains S19 and RB51. J. Clin. Microbiol., 33, 1640-1642.
PMid:7650203 PMCid:PMC228233

19. Lopez-Goni, I., Garcia-Yoldi, D., Marin, C.M., de Miguel, M.J., Muñoz, P.M., Blasco, J.M., Jacques, I., Grayon, M., Cloeckaert, A., Ferreira, A.C., Cardoso, R., Corrêa de Sá, M.I., Walravens, K., Albert, D., Garin-Bastuji, B. (2008). Evaluation of a multiplex PCR assay (bruce-ladder) for molecular typing of all Brucella species, including the vaccine strains. J. Clin. Microbiol.,46, 3484-3487.
http://dx.doi.org/10.1128/JCM.00837-08
PMid:18716225   PMCid:PMC2566117

20. García-Yoldi, D., Marín, C.M., de Miguel, M.J., Muñoz, P.M., Vizmanos, J.L., López-Goñi, I. (2006). Multiplex PCR assay for the identification and differentiation of all Brucella species and the vaccine strains Brucella abortus S19 and RB51 and Brucella melitensis. Rev1. Clin Chem., 52(4): 779-781.
http://dx.doi.org/10.1373/clinchem.2005.062596
PMid:16595839

21. Probert, W.S., Schrader, K.N., Khuong, N.Y., Bystrom, S.L., Graves, M.H. (2004). Real-time multiplex PCR assay for detection of Brucella spp., B.abortus, and B. melitensis. J. Clin. Microbiol., 42, 1290–1293.
http://dx.doi.org/10.1128/JCM.42.3.1290-1293.2004
PMid:15004098 PMCid:PMC356861

22. Al Dahouk, S., Le Flèche, P., Nöckler, K. et al. (2007). Evaluation of Brucella MLVA typing for human brucellosis. J. Microbiol. Meth., 69, 137–145.
http://dx.doi.org/10.1016/j.mimet.2006.12.015
PMid:17261338

23. Bricker, B.J., Ewalt, D.R., Halling, S.M. (2003). Brucella "HOOF Prints": strain typing by multilocus analysis of variable number tandem repeats (VNTRs). BMC Microbiol., 3, 15.
http://dx.doi.org/10.1186/1471-2180-3-15
PMid:12857351 PMCid:PMC183870

24. Le Flèche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F., Nockler, K., Neubauer, H., Guilloteau, L.A., Vergnaud G. (2006). Evaluation and selection of tandem repeat loci for a Brucella MLVA typing assay. BMC Microbiol., 6, 9.
http://dx.doi.org/10.1186/1471-2180-6-9
PMid:16469109 PMCid:PMC1513380

25. Whatmore, A.M., Perrett, L.L., Macmillan, A.P. (2007). Characterisation of the genetic diversity of Brucella by multilocus sequencing. BMC Microbiol., 7, 34.
http://dx.doi.org/10.1186/1471-2180-7-34
PMid:17448232 PMCid:PMC1877810

26. Ferreira, A.C., Chambel, L., Tenreiro, T., Cardoso, R. et al. (2012). MLVA16 typing of Portuguese human and animal Brucella melitensis and Brucella abortus isolates. PLoS One 7, e42514.
http://dx.doi.org/10.1371/journal.pone.0042514
PMid:22905141 PMCid:PMC3419166

27. Garofolo, G., Di Giannatale, E., De Massis, F., Zilli, K., Ancora, M., et al. (2013): Investigating genetic diversity of Brucella abortus and Brucella melitensis in Italy with MLVA-16. Infect. Genet. Evol.19, 59–70.
http://dx.doi.org/10.1016/j.meegid.2013.06.021
PMid:23831636

28. Her, M., Kang, S.I., Cho, D.H., Cho, Y.S., Hwang, I.Y., et al. (2009). Application and evaluation of the MLVA typing assay for the Brucella abortus strains isolated in Korea. BMC Microbiol., 9, 230.
http://dx.doi.org/10.1186/1471-2180-9-230
PMid:19863821 PMCid:PMC2774859

29. Jiang, H., Fan, M., Chen, J., Mi, J., Yu, R., et al. (2011). MLVA genotyping of Chinese human Brucella melitensis biovar 1, 2 and 3 isolates. BMC Microbiol., 11, 256.
http://dx.doi.org/10.1186/1471-2180-11-256
PMid:22108057 PMCid:PMC3233519

30. Jiang, H., Wang, H., Xu, L., Hu, G., Ma, J., et al. (2013). 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, 8, e76332
http://dx.doi.org/10.1371/journal.pone.0076332

31. Kang, S.I., Heo, E.J., Cho, D., Kim, J.W., Kim, J.Y., et al. (2011). Genetic comparison of Brucella canis isolates by the MLVA assay in South Korea. J. Vet. Med. Sci.73, 779–786.
http://dx.doi.org/10.1292/jvms.10-0334

32. Kiliç, S., Ivanov, I.N., Durmaz, R., Bayraktar, M.R., et al. (2011). Multiple-locus variable-number tandem-repeat analysis genotyping of human Brucella isolates from Turkey. J. Clin. Microbiol. 49, 3276–3283.
http://dx.doi.org/10.1128/JCM.02538-10
PMid:21795514 PMCid:PMC3165627

33. Marianelli, C., Petrucca, A., Pasquali, P., Ciuchini, F., Papadopoulou, S., Cipriani, P. (2008). Use of MLVA-16 typing to trace the source of a laboratory-acquired Brucella infection. J. Hosp. Infect. 68, 274–276.
http://dx.doi.org/10.1016/j.jhin.2008.01.003
PMid:18289724

34. Menshawy, A.M., Perez-Sancho, M., Garcia-Seco, T., Hosein, H.I., García, N., Martinez, I., Sayour, A.E., Goyache, J., Azzam, R.A., Dominguez, L. (2014). Assessment of genetic diversity of zoonotic Brucella spp. recovered from livestock in Egypt using multiple locus VNTR analysis. Biomed. Res. Int., 2014:353876.

35. Mick, V., Le Carrou, G., Corde, Y., Game, Y., Jay, M. et al. (2014). Brucella melitensis in France: Persistence in wildlife and probable spillover from alpine ibex to domestic animals. PLoS ONE, 9(4): e94168.
http://dx.doi.org/10.1371/journal.pone.0094168
PMid:24732322 PMCid:PMC3986073

36. Bosilkovski, M. (2015). Brucellosis: It is not only Malta! In A. Sing (Ed.), Zoonozes – Infections Affecting Humans and Animals (pp. 287-316). Springer Science+Business Media Dordrecht.
ISBN 978-94-017-9456-5.
http://dx.doi.org/10.1007/978-94-017-9457-2_11

37. Newby, D.T., Hadfield, T.L., Roberto, F.F. (2003). Real-time PCR detection of Brucella abortus: a comparative study of SYBR green I, 59-exonuclease, and hybridization probe assays. Appl. Environ. Microbiol., 69, 4753–4759.
http://dx.doi.org/10.1128/AEM.69.8.4753-4759.2003
PMid:12902268 PMCid:PMC169142

38. Redkar, R., Rose, S., Bricker, B., Del Vecchio, V. (2001). Real-time detection of Brucella abortus, Brucella melitensis and Brucella suis. Mol. Cell. Probes, 15, 43–52.
http://dx.doi.org/10.1006/mcpr.2000.0338
PMid:11162079

39. Hinić, V., Brodard, I., Thomann, A., Holub, M., Miserez, R., Abril, C. (2009). IS711-based real-time PCR assay as a tool for detection of Brucella spp. in wild boars and comparison with bacterial isolation and serology. BMC Vet. Res., 5, 22.
http://dx.doi.org/10.1186/1746-6148-5-22
PMid:19602266 PMCid:PMC2719624

40. Ilhan, Z., Aksakal, A., Ekin, I.H., Gulhan, T., Solmaz, H., Erdenlig. S. (2008). Comparison of culture and PCR for the detection of Brucella melitensis in blood and lymphoid tissues of serologically positive and negative slaughtered sheep. Lett. Appl. Microbiol., 46(3): 301–306.
http://dx.doi.org/10.1111/j.1472-765X.2007.02309.x
PMid:18179446