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VALIDATION OF RT-QPCR TECHNIQUE FOR DETECTION OF BRUCELLA GENOME IN MILK SHEEP AND GOAT IN WEST BANK PART OF PALESTINE

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Abstract

Brucella melitensis is a severe pathogen for human and animals, even at low concentrations. The milk of sheep and goat and the fresh dairy products, including white cheese, are the main source of consumers' contamination. Early detection, using reliable validated diagnostic tools, is crucial for the control and eradication of the disease. The aim of this study was to develop fast molecular in-house techniques, such as RT-qPCR, to detect Brucella genome in milk. The validation of the method was carried out according to the specifications of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organization for Animal Health OIE in chapter 1.1.5: Validation and quality control of polymerase chain reaction methods used for diagnostic of infection disease and with the requests of the ISO/IEC 17025:2005: General requirements for the competence of testing and calibration laboratories. Tests for the RT-qPCR validation have proved his ability to detect 3.4 copies of Brucella's genome, into 5µl amplification product. Furthermore, DNA from non-Brucella microorganisms was not detected by developed method. While the identification of Brucella melitensis by traditional methods is time-consuming and may impair the outbreaks control, the RT-qPCR proposed can be used as a complementary, rapid and sensitive diagnostic tool for Brucella spp *in Palestine, contributing to properly implement the control policy of authorities.*

Key words*: Brucella genome, real-time PCR optimization, validation, protocol in-house*

INTRODUCTION

Brucellosis is a zoonotic disease and an important public health problem worldwide, especially in Mediterranean countries [Doganay et al., 2003; Gul et al., 2007].
The brucellosis is produced in different animal species by different *Brucella* species. The most significant and important, as zoonotic pathogen, in this genus is *Brucella melitensis* [Scholz et al., 2013; Mayer-Scholl et al., 2010].
Brucella infections are causing tremendous economic losses due to the decrease of the productivity as a result of abortion weakness of offspring and reduced milk production and may be associated with the loss of trade opportunities [FAO, 2010].
The human infections usually occur due to the ingestion of the contaminated dairy products or following the close contact with infected small ruminants [Saleem et al., 2010; Kaoud et al., 2010; Doganay et al., 2003; Zvizdic et al., 2006].
Milk of animals is the foremost source of humans' infection with *Brucella* and its bacteriological isolation has low sensitivity (Ning et al., 2013), depending on the viability and number of *Brucella* in the sample, as well as the nature of the sample that is usually cross-contaminated with various bacteria species. Thus, culture methods are not always successful, moreover, they are time-consuming and their handling could be hazardous [Hinic, 2009; Refai et al., 2002].

Serologic methods are rapid but inconclusive, because not all infected animals produce detectable levels of antibodies, and the cross-reactivity against other antigens can give false-positive results [Gwida et al., 2011]. Early detection of *Brucella* genome, by using valid diagnostic tools, is crucial for the control and eradication of this disease [Al-Garadi et al., 2011; Bricker et al., 2002] The molecular diagnostic techniques represent an important breakthrough in the diagnostic practice. The most of the authors confirmed that real-time PCR is a highly sensitive method for the *Brucella* detection from various samples [Doosti et al., 2011; Safarpoor Denkordi et al. 2014; Newby et al., 2003; Al-Garadi et al., 2011; Mirnejad et al., 2012; Foster, 2008].

Laboratory tests for any infectious agent, by molecular diagnostic techniques, are requesting standardization, optimization and quality assurance [Sloan, 2007]. This is the request of the international quality standard for veterinary laboratories in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organization for Animal Health (OIE) chapter 1.1.5: Validation and quality control of polymerase chain reaction methods used for diagnostic of infection disease and the guide ISO/IEC 17025:2005: General requirements for the competence of testing and calibration laboratory which demands for verification and validation procedures for each in-house assay (OIE, 2008; ISO/IEC 17025, 2005).

The real time quantification is based on the relationship between initial template amount and Ct value, obtained during amplification, an optimal qPCR assay absolutely essential for accurate and reproducible quantification of samples.

The hallmarks of an optimized qPCR assay are: linear standard curve (R^2> 0,980 or r > 1-0.9901), high amplification efficiency 90-105% and consistency across replicate reactions [Bio Rad laboratories, 2006; Applied Biosystem, 2003].

The main objective of this study was to develop and validate an in-house Real-Time qPCR protocol, in order to provide a sensitive diagnostic tool for rapid detection of *Brucella* genome in sheep and goat milk. The most important epidemiological target was to implement a rapid preventive tool against brucellosis in Palestine.

MATERIALS AND METHODS

Samples preparation

Control strains

The reference control strains were: different non-*Brucella* bacteria used for optimization and validation RT-qPCR, were retrieved as loops from Oxoid Company; vaccine strain *Brucella melitensis* Rev 1 from Ovejero Company (Spain) and *Brucella melitensis* pure bacteria derived from our laboratory.

In this study were also used wild *Brucella melitensis* strains and positive and negative milk samples (Table 2). All milk samples were obtained from animals during their lactation period: 10 ml of each milk sample was used for detection of *Brucella* genome by Real-Time PCR assay.

Preparation of control reference strains

Each reference strain of bacteria was inoculated onto specific nutrient agar. Plates were then incubated overnight at 37ºC under different specific condition, up to the requirements of each bacterium. After 24 hours each bacterium strain was confirmed by specific biochemical test; then few colonies were harvested from nutrient agar and immersed in 200 µl of phosphate buffer saline. From this, *Brucella melitensis* was inoculated on specific agar and harvested 48 hours later.

DNA extraction

All reference strains of non-*Brucella* and *Brucella* bacteria, *B. melitensis* Rev 1 vaccine strain, *B. mellitensis* wild strain, positive and negative milk samples, were extracted using a commercial kit QIAamp RNA Mini Kit (Qiagen, 52906) according to the manufacturer's instructions. Before extraction milk samples were centrifuged at 8000 rpm for 15 min to settle out the bacteria [Khan et al., 2011; Romero et al., 1999]. The fatty top layer and supernatant were discarded and 200µl pellet were used for the extraction procedure.

Determination of DNA concentration of Brucella melitensis Rev 1 vaccine

In this study, for validation procedure and calculation was used *Brucella melitensis* Rev 1 vaccines strain (Ovejero Company, Spain), as reference material. The DNA concentration was evaluated by using NanoDrop® ND-1000 Spectrophotometer Genomic (Thermo Scientific, USA). The concentration was given in ng/µl and then converted into gene copies per µl by using URI Genomics & Sequencing Center - dedicated software, created by Andrew Staroscik (2004).

Validation of RT-qPCR

Specificityand sensitivityof RT-qPCR

Brucella spp. was identified using the primers and probe targeting the bcsp31 gene (GenBank accession number M20404) [Probert et al., 2004]. The specificity of the primers and of the probes used in this study (Table 1) were analysed by using Standard Nucleotide BLAST (Basic Local Alignment Search Tool) administered by the National Centre for Biotechnology Information (NCBI).The sensitivity and specificity of each qPCR assay were studied using different dilutions of DNA the *Brucella melitensis* Rev 1 and DNA the different non-*Brucella* bacteria, positive and negative *Brucella* milk samples and negative control (Table 2).

Table 1. Specific real-time PCR oligonucleotides primers and probe for *Brucella* group (Probert et al., 2004)

PCR identification	Primer sequence	5'Fluorophore/3' quencher
***Brucella* spp F**	5 –GCTCGGTTGCCAATATCAATGC-3	-
***Brucella* spp R**	5- GGGTAAAGCGTCGCCAGAAG-3	-
***Brucella* spp Probe**	5-AAATCTTCCACCTTGCCCTTGCCATCA-3	6-FAM/BHQ1

Limit of detection (LOD) and standard curve preparation

The evaluation of LOD was performed by standard suspension of genomic DNA of *Brucella melitensis* Rev 1 vaccine strain, as initial stock, and 11 four-fold dilutions with three PCR replicates. Dilutions were chosen within the linear dynamic range of the assay and expected concentrations of DNA within possible specimens. Ct values were determined by running the RT-qPCR using 5µl of each dilution together with known standard PCR in the same run. The log-linear regression analysis, standard deviation and correlation coefficient of the Cq-values of each concentration was performed using Microsoft Excel 2007 software.

Repeatability, reproducibility and efficiency of the test

The efficiency of the RT-qPCR assays was evaluated in one run with 11 serial dilutions. The repeatability was evaluated by testing all dilutions in three replicates, in three PCR runs, and the assay was repeated in three different days. Tests of reproducibility were performed through running RT-qPCR protocol by another technician. The efficiency of the PCR was calculated from the slope of the logarithmic regression of Ct values plotted against DNA concentrations by $E = e(-1/slope)-1$. The efficiency of the assay was then given by equation: $E (100\%) = (10\text{-}1/k – 1) \times 100$, where k is the slope of the standard curve obtained by linear regression with calculation y-intercept which corresponds C_t value for a single copy of the target molecule and coeficientul of determination (R^2) such that $0 \leq r^2 \leq 1$, which denotes the strength of the linear association between *x* and *y* and represents the percent of the data that is the closest to the line of best fit and is a measure of how well the regression line represents the data [Eurogentec, 2013, Life technologies, 2012, Bio-Rad laboratories, 2006].

Robustness and gel electrophoresis

Robustness was evaluated by running all dilutions of *Brucella melitensis* Rev 1 strain on three different real time instruments: two instruments Real-Time PCR of SmartCycler® - Cepheid and LightCycler® - Roche. PCR products were analysed by 2% agarose (Promega, UK)gel electrophoresis.

RESULTS AND DISCUSSIONS

Concentration of DNA

The concentration of genome stock *Brucella melitensis* Rev 1 vaccine was 2.5 ng/µl and equal to 3.52×10^6 copies of genomic DNA, considering the approximate size of *Brucella* genome 3290000000 bp [DelVecchio, 2002] and represents only one copy of the bcsp31 gene on the *Brucella* genome.

Evaluation specificity and sensitivity of RT-qPCR

The BLAST search showed that the primers and the probes did not have identity with other organisms. The primers sequences were

identical 100% to their respective target. In order to perform the validation of RT-qPCR detection of the gene bcsp31 of *Brucella*spp. were used: different dilutions of the vaccine strain *Brucella melitensis* Rev 1 as positive control and *Brucella* wild strains, and, as negative controls, negative milk samples and non-*Brucella* bacteria. Real time PCR was performed with a set of primers and probe showed in Table 1. DNA amplification mixture was composed of 7.12µl of nuclease free water, 5 µl Qiagen 1 step RT PCR buffer x5 (Qiagen, Cat. No: 210212), 0.8µl dNTPs [200µM] (Qiagen, Ct. No: 210112), 0.5 µl forward and reverse primer [20µM/µl], 0.5 µl [5µM/µl] probe (Syntheza, Israel), 1µl Taq polymerase enzyme (Sigma, USA, Cat. No D4545) and 5µl of DNA product. Amplification was performed in SmartCycler, Cepheid. Reaction was initiated with denaturation at 95°C for 3 minutes followed by 50 cycles: 95°C for 15 sec, annealing and extension at 60°C for 35 minutes. No amplification products were observed in real-time PCR of negative controls, whatever is the targets the non-*Brucella* micro-organisms tested, *Brucella melitensis* negative milk or water samples [Table 2].

Limit of detection (LOD), preparation of standard curve and calculation of efficiency

The stock suspension and 11 four-fold dilutions of template DNA *Brucella melitensis* Rev 1 vaccine strain, ranging from $3.52x10^6$ to 0.8 gene copies per reaction indicate that 3.4 copies of bacterial genomes in 5µl of DNA the sample detected by developed RT-qPCR assay protocol. At these concentrations all *Brucella* positive bacteria and *Brucella melitensis* positive milk samples were positive. In this assay was calculated standard deviation and coefficient of variation [Table 3].

Table 2. Control and reference strains of bacteria used in validation of RT-qPCR and specificity evaluation result

Strain	Reference and origin	Type of samples	No of samples	Results
Brucella melitensis Rev 1	Elberg strain of vaccine, Ovejero	Vaccine	2	Positive
Brucella melitensis	Field strain, our laboratory	Pure bacteria	2	Positive
Brucella melitensis positive milk samples	Field strain, our laboratory	Milk	10	Positive
Bacillus cereus	ATCC 11778, Oxoid	Pure bacteria	2	Negative
Campylobacter jejuni	ATCC 29428, Oxoid	Pure bacteria	2	Negative
E.coli	ATCC 12229, Oxoid	Pure bacteria	2	Negative
Salmonella enerica subsp *Enteritidis*	ATCC 13076, Oxoid	Pure bacteria	2	Negative
Listeria monocytogenes	ATCC 7644, Oxoid	Pure bacteria	2	Negative
Staphylococcus aureus	ATCC 33862, Oxoid	Pure bacteria	2	Negative
Yersinia enterocolitica	ATCC 23715, Oxoid	Pure bacteria	2	Negative
Brucella melitensis negative milk samples	Our laboratory	Milk	10	Negative
Negative control		Water	2	Negative

Table 3. DNA concentrations and Ct values, standard deviation and coefficient of correlation obtained from experiment

No of dilution	Concentration (ng/µl)	No of copies in 1 µl of RT-qPCR product	No of genes copies in 5µl	Ct Mean ± SD	CV (%)
Stock	2.5	704000	$3.52x10^6$	19.04 ± 0.07	0.003
Dilution 1	0.625	176000	8.8×10^5	22.20 ±0.28	0.013
Dilution 2	0.156	43900	2.2×10^5	24.37 ± 0.46	0.019
Dilution 3	0.039	11000	$5.5x10^4$	26.72 ± 0.23	0.009
Dilution 4	0.00975	2750	13750	29.52 ± 0.41	0.014
Dilution 5	0.00244	687	3940	31.15 ± 0.26	0.008
Dilution 6	0.000609	171	860	32.91 ± 0.23	0.007
Dilution 7	0.000152	42.8	214	34.85 ± 0.68	0.019
Dilution 8	0.0000380	10.7	53.5	36.21 ± 0.75	0.021
Dilution 9	0.00000952	2.68	13.4	38.13 ± 0.09	0.002
Dilution 10	0.00000238	0.67	3.4	39.04 ± 0.07	0.002
Dilution 11	0.000000595	0.17	0.8	Not detect	-

The LOD for each assay was the lowest concentration consistently detected in all three PCR runs. The first ten dilutions were detectable and produced standard curve with correlation coefficient 0.996881. Analysis of the stock suspension and of the different dilutions of standard DNA revealed that the efficiency of the assay is 96.5%, with y-intercept 18.7 and R^2 value 0.09984 (Figure 1).

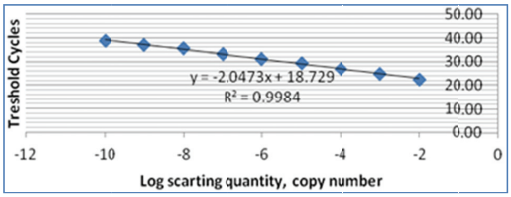

Figure 1.The linearity and linear regression of RT-qPCR standard curve the bscp31 gene of *Brucell*a spp.

Repeatability, Reproducibility, Robustness, and gel electrophoresis

Results were found almost similarly for all three replicates of the dilution and also for the replicates evaluated in three different days and on three different instruments. Given results by following criteria were established in order to validate the analysis: samples were considered positive for *Brucella* spp when their amplification curves were similar to the positive control curve and exceeded the threshold with Ct values lower than 40. The electrophoresis of amplified product revealed a single band, corresponding at the expected size and the decrease of the DNA concentrations of amplicons is showed by the decrease in fluorescence [Figure 2].

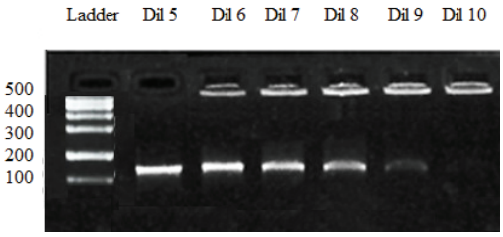

Figure 2. Different concentration of RT-qPCR products are show equal different visibility of bands in gel electrophoresis

DISCUSSION

Despite the intensive ocular vaccination program, brucellosis in Palestine remains a significant problem. Our previously study revealed that during the years 2013 and 2015 several new isolates of *Brucella* spp. were detected in small ruminants from the northern districts of the West Bank [Awwad et al., 2015].

The chosen specific target of *Brucella* - bcsp31 is a conserved, single-copy gene, coding a 31-kDa outer membrane protein and is found in all strains of *Brucella*. This gene is commonly used as target for detection of *Brucella* by PCR, due to the small size of the amplicon (151 bp) that facilitates its use in real-time PCR. [Bounaadja, 2009; Da Costa, 1996; Debeaumont, 2005].

A genus-specific target was chosen to establish in-house RT-qPCR protocols that detect all species of *Brucella* in the milk of the infected animals, in order to increase the possibility of the detection and to improve the control of brucellosis in Palestine. The developed assay performed well in the analytical sense and was able to detect very low concentrations (3.4 copies) of *Brucella* genomic DNA. The test was performed in accordance with requirements OIE and to the requirements of ISO 17025 for optimization laboratory methods. The efficiency of this assay was 96.5%, with y-intercepts 18.7 and R^2 value 0.09984. Although standard curve parameters of genomic DNA *Brucella melitensis* Rev 1 vaccine strain was subtly altered, there was no reduction in percentage amplification at very low bacterial DNA concentrations. A few possible reasons have been taken into consideration for the relatively inconsistent performance of *Brucella* detection by different laboratories. The stage of infection may influence the number and location of bacteria [Alton et al., 1988]; the sample type used for diagnostic purposes may affect the results [O'Leary et al., 2006]; the presence of large amounts of host genomic DNA may inhibit the PCR reaction [Navarro et al., 2002]; the DNA extraction method used may be crucial in determining the ability of the PCR assay to detect the bacterium [Romero et al., 1999and Lopez-Goni et al.,1999]. Factors that may

compromise DNA recovery from milk include difficulties in disrupting bacterial cell walls, loss of DNA template through extraction procedures, or the presence of potential polymerase inhibitors. In addition, the amount of milk used for PCR is much smaller than that required for bacteriological methods, and the number of organisms contained in a sample may thus not reach PCR detection limits [Yousef-Beingi, 2005]. For this purpose the milk samples were centrifuged and only concentrated bacteria (pellet) was used for extraction.

Above of all these reasons, the quality of a laboratorial result is linked to the use of procedures such as validated methods, quality internal controls, participation in inter-laboratorial comparison programs, the proper use of certified reference materials, and the compliance with requirements of standards. Some of the parameters used in validation such as the specificity and sensitivity of the method, the detection limit, linearity, the repeatability, the reproducibility, and the robustness, are crucial to produce reliable in-house method.

In summary, we evaluated the feasibility of molecular assays as improved and very sensitive diagnostic tools for detection of *Brucella* spp. in fresh milk, especially during outbreaks. The advantages of this technique are that it can be performed very quickly, it allows the direct identification of the organism and it decrease the number of false-positive result [Soherbi et al., 2011; Redkar et al., 2001; Yousef-Beingi et al., 2009]. In addition, along with molecular assay, serology must always be performed and, in accord with the goal of the investigation, will be confirmed by bacterial isolation.

CONCLUSIONS

The RT-qPCR for the detection of *Brucella* spp. in fresh milk, above described, proved to be a sensitive and specific tool for the detection of *Brucella* genome.

The efficiency of this assay was 96.5%, with y-intercepts 18.7 and R^2 value 0.09984.

The proposed protocol is fast performed, it allows the direct identification of the organism and it increases the specificity of diagnostic.

The critical step for our RT-qPCR is the quantity of the *Brucella* DNA in the milk samples: to reduce its impact, the milk samples have been centrifuged and the DNA extraction has been carried out only on the sediment bacteria (pellet).

The novel molecular technique such as RT-qPCR in-house is cost-efficiency affordable and is useful as a reliable screening method for the rapid detection of the infectious agent: the use of this technique could be a huge step in order to rapidly implement the measures for the outbreak control, to prevent spread of the disease and to avoid the human infections. This method is designed to be performed using raw milk.

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