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Evaluation of a new set of Real-Time PCR for *Brucella* detection within human and animal samples

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ABSTRACT

A quantitative TaqMn Real-Time PCR assay was developed and its diagnostic value on human serum and livestock samples were evaluated. *Brucella* species could be distributed through communities as a biological agent. Rapid detection of biological threat agents is critical for timely therapeutic administration. Quantitative real-time PCR provides a rapid, sensitive and specific tool for molecular identification of this agent. We evaluated a new real-time PCR set by numerous human and livestock samples. For primers and probe designation BCSP31 of B. melitensis 16 M was used. Different sero-positive human samples and tissue samples livestock with suspected brucellosis were then assayed after primary adaptation of Q-PCR in the laboratory. The detection limit of the assay was 10 fg (equivalent to 2 genome). Totally 50 samples (25 sero-positive, 25 sero-negative) of patients and 75 samples from slaughtered livestock (due to brucellosis) were collected and assayed by Q-PCR, along with control samples. Brucella genome were detected by this real-time set from nearly 42% (5 out of 12) of negative samples by conventional PCR. This system also detected brucella contamination in 46 out of 75 animal samples.

Key words: Real-Time PCR, TaqMn, Brucella, BCSP31

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1. Introduction

The genus of *Brucella* are facultative intracellular gram negative bacteria that can cause brucellosis within human communities and animals including livestock (Hosseini Doust *et al*., 2011). Occurrence of relapses and focal complications in brucellosis results from capability of their intracellular survival (Queipo-Ortuno *et al*., 2008). The diagnosis of brucellosis is based mainly on serological responses, which can be unspesific owing to cross-reaction or subclinical prevalence of disease. Early diagnosis of brucellosis plays the most important role in initiating therapy, control and eradication programs, while it requires reliable and sensitive diagnostic tools (Khoramabadi *et al*., 2009). The intracellular location of the bacteria and the disease evolution hamper the usefulness of blood culture (Zerva *et al*., 2001). Although it is gold standard, the isolation of Brucella from blood samples or other sterile body fluids, only has 70% sensitivity (Queipo-Ortuño *et al*., 2005). Serological tests which principally are based on anti-lipopolysacharide antibodies can show false positive owing to cross reaction or sub sensitive or high immunity reactions depending on subclinical or endemic prevalence of the disease (Vrioni *et al*., 2008).

Molecular diagnostic techniques have shown more sensitivity and specificity compared with culture and serological tests (Newby *et al*., 2003). Real-Time PCR systems represent improved sensitivity, specificity and speed due to combine use of fluorogenic dyes and directly detecting their emissions as well as removal of post-amplification detection procedures (Bogdanovich *et al*., 2004).

In addition, quantitative Real-Time PCR is useful tool for discriminate between inactive, serologically positive and active human brucellosis (J D. *et al*., 2005). The goal of our present study was to design quantitative TaqMn Real-Time PCR assay and evaluate it on serum samples from human.

2. Materials and Methods

2.1. Samples

Twenty five samples from sero-positive (brucellosis) patients were collected from health centers of east of Iran. The samples were previously assayed based on clinical, serological and bacteriological findings. Among them, eight samples were obtained from patients who were under therapy, three cases were within relapse phase, and two cases completed their antibiotic therapy four weeks earlier and one patient was under in concluded therapy. Blood cultures were performed for 15 clinical samples from which eight were culture positive. Standard tube agglutination tests were positive (≥1/160) in all samples. Sera from 25 healthy volunteers with no history of brucellosis were also assayed along with other clinical samples.

2.2. DNA Extraction from serum samples

Serum samples were preserved at -20˚C. DNA extraction from serum samples were performed using QIAamp DNA Mini kit (Qiagen), in accordance with the manufacture's instructions. All samples were measured on spectrophotometer (NanoDrop 2000) and stored at -20˚C until tested.

2.3. Primers and Probe

TaqMan probe and primers were designed using AlleleID, version 7.0 (PREMIER Biosoft). TaqMan Real-Time PCR assay was designed based on genus-specific bcsp31 gene which encodes an immunogenic membrane protein of 31 kDa (Ratushna *et al*., 2006). 166-bp amplicon was amplified by following primers: sense primer, Mbm1, (5'-ATCGTTCTTGAAGCCTAC-3'), antisens primer, Mbm2, (5'-AA ATACCGTTCGAGATGG-3'). A 24-bp TaqMan probe was designed, Mbm, (5'- ATATCAA GGCTGAACACCTGAAGC- 3') and the 3'-end is blocked with a phosphate group to prevent extension of probe in the PCR reaction. Mbm TaqMan probe was fluorescence labeled at the 5'-end with 6-carboxyfluorescein phosphoramidite (FAM) as the reporter dye, and the 3'-end with 5-carboxytetramethylrhodamine (TAMRA) as the quencher. The theoretical specificity of the primers and probe was determined by comparison with the Gene Bank database using the Basic Local Alignment Search Tool (BLAST).

2.4. Assay conditions

Real-Time PCR amplification were carried out in a total reaction volume of 20 μl containing 10 μl Premix Ex Taq™ (Perfect Real Time) reagent (TAKARA BIO INC, Shiga, JAP), a 100 nM concentration of each primer, a 250 nM concentration of the probe and 50 ng of template DNA. The Real-Time PCR was performed on a 7500 Real-Time PCR system (Applied Biosystems; ABI) using the following profile: initial template denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Data analysis was performed using 7500 system SDS software (ABI). The 2nd derivative maximum algorithm was used to calculate the cycle threshold (C_t) value.

All runs included negative and positive control. The positive control contained 5.5×10^4 copies of plasmid per reaction. All samples were tested in duplicate. A negative result was considered when no amplification occurred or C_t value was greater than 38 cycles and in positive result, both replicate had to be positive. The bacterial DNA load per milliliter of serum was calculated from the standard curve.

2.5. Statistical analysis

The student t-test and χ^2 were used to compare continuous variables and categorical variables respectively. A p-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Specificity and sensitivity

The analytical sensitivity of Real-Time PCR assay was performed by amplifying ten-fold serially diluted *B. melitensis* 16M genomic DNA (from 10 ng to 1 fg). The detection limit of the assay was 10 fg (equivalent to 2 genome), with a linear regression equation of C_t = -3.17 log (copy no.) + 37.7, correlation coefficient (R^2) value of 0.99, and PCR efficiency (E) of 2.0.

Amplification of 5-fold serial dilution of a plasmid with the cloned fragment of 166 bp from *B. melitensis* 16M showed a linear detection range of 10^5- 10^1 copy per reaction mixture, with an R^2 value of 0.99 and a PCR efficiency of 2. The linear regression equation was: C_t = -3.16 log (copy no.) + 37.2.

Amplification results for 14 non-*Brucella* strains (data not shown) were negative with *brucella*-specific TaqMan Real-Time PCR assay evaluated in this study.

3.2. Detection and quantification of brucella DNA in different samples

All human clinical samples were positive by standard tube agglutination test (STA) with titer between 1/160 and 1/1280 unit. Brucella was isolated in blood cultures from 8 out of 15 (53%) samples. These samples were positive by both serology test and also real-time PCR. All serum samples obtained from 25 healthy donors (control group) were negative for real-time PCR. Therefore, the specificity of our assay was 100%. Among sera from 25 patients, eighteen (72%) were positive by Real-Time PCR with mean bacterial DNA load 3.7×10^3 copies/ml (range, $6.4 \times 10^1 - 5.8 \times 10^4$ copies/ml). None of 75 livestock samples were positive by standard culturing method. In contrast, all of them (100%) were serologically (SAT) positive. The Q-real time PCR was able to detect brucella DNA within up to 62% of the animal samples (Figure 1).

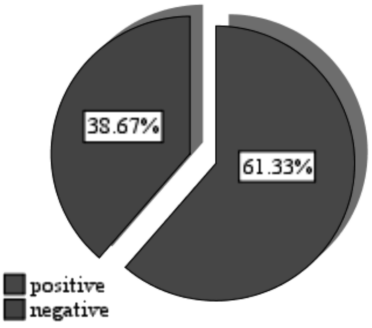

Figure1. Clinical samples that were positive by Q-real-time PCR

4. Discussion

The aim of this study was to develop a quantitative TaqMan Real-Time PCR assay and evaluate its diagnostic use for the detection of *Brucella* DNA in human serum samples. The sensitivity of Q-PCR were 100% based on result of the 8 patients who were positive by both serology and blood culture. Eighteen (72%) of 25 cases were positive only by Real-time PCR and serology. Because none of the 25 control samples was positive, the specificity of the assay was 100%. Similar findings have been reported by Krishna *et al*. (2008).

In spite of the IS711-based real-time PCR assay is specific and highly sensitive, its different copy number according to each species can affect to quantification of bacterial load assay (Chen *et al*., 2007). Bounaadja, *et al*. (2009) reported the mean difference of Ct between IS711 and *bcsp*31 (ΔCt IS/bcsp) in all species of *brucella* that was 2.45. Although Most studies of the diagnostic in human brucellosis have been undertaken with whole blood samples, Serum is the preferred clinical specimen due to reduce inhibition by hemoglobin, human DNA and simplicity (Matar *et al*., 1996).

Detection of the wide range of bacterial DNA load (range, $6.4 \times 10^1 - 5.8 \times 10^4$ copies/ml) in different stage of disease (in different patients) showed this system could be applied for diagnosis and differentiation between past and active brucellosis. The largest quantity of *Brucella* DNA load (5.8×10^4 copies/ml) was detected in patient with strong fever. While the low-level brucella DNA load were detected in patients who concluded antibiotic therapy. Interestingly, we observed higher *Brucella* DNA load after the one month treatment in patients who concluded antibiotic therapy. Presence of high titer of antibody and low copy number of organism indicates a robust sustained immune response that results low DNAaemia.

The detection limit of the *bcsp* 31 Real-time PCR in this work was noticeably improved in comparison to Real-time PCR published by Mukherjee *et al*. (2007). Hinic, *et al*. (2009) greatly evaluated previously published real-time PCR assays targeting *bcsp*31, *per*, IS711, he described the limit of detection varied widely among the assays (16–1600 fg) demonstrating that some assays should not be applied to clinical samples. Our study was located on patients with positive serological test with symptoms from mild to strong. It would be useful to fallow up the current patients using Q- PCR techniques in order to evaluate them more acurately.

Real-Time PCR provides an ideal tool for detection and quantification of bacterial DNA load. Use of Real-Time PCR, not only increase. The specificity and sensitivity but also reduce the time required to accomplish the detection (Amalia *et al*., 2008). It does not require

Table 1. Analytical findings of human clinical samples, by Q-PCR compared with conventional PCR and other microbiological methods. * Copy / ml, ND; Not done.

Sample label	Blood culture result	Serological test (SAT)	PCR (Conventional)	Real-Time PCR		
				result	C_T	Count*
HS 1	Negative	Positive	Negative	Negative	37.81	
HS 2	Negative	Positive	Negative	Negative	37.14	64.60
HS 3	ND	Positive	Negative	Negative	37.39	49.30
HS 4	ND	Positive	Negative	Positive	35.72	131.75
HS 5	Negative	Positive	Negative	Negative		0
HS 6	Positive	Positive	Positive	Positive	32.98	760
HS 7	ND	Positive	Negative	Positive	35.90	115.60
HS 8	ND	Positive	Positive	Positive	36.64	85
HS 9	ND	Positive	Positive	Positive	35.10	193
HS 10	Negative	Positive	Negative	Positive	37.00	70
HS 11	Negative	Positive	Positive	Positive	36.10	106.25
HS 12	Negative	Positive	Negative	Positive	36.90	64.60
HS 13	ND	Positive	Negative	Negative		00
HS 14	ND	Positive	Positive	Positive	36.30	89.25
HS 15	ND	Positive	Negative	Negative	37.50	41.65
HS 16	ND	Positive	Negative	Positive	35.79	129.20
HS 17	Positive	Positive	Positive	Positive	32.46	1066
HS 18	ND	Positive	Negative	Negative	37.80	-
HS 19	Positive	Positive	Positive	Positive	35.45	297.5
HS 20	Negative	Positive	Positive	Positive	36.00	203.15
HS 21	Positive	Positive	Positive	Positive	32.40	2040
HS 22	Positive	Positive	Positive	Positive	29.5	57715
HS 23	Positive	Positive	Positive	Positive	31.00	1000
HS 24	Positive	Positive	Positive	Positive	30.70	1500
HS 25	Positive	Positive	Positive	Positive	30.90	1010

post amplification handling of PCR products, therefore reduces the risk of laboratory contamination and false positive results (Hosseini Doust *et al*., 2007). Quantification of microbiological load is valuable for numerous infectious disorders such as hepatitis and HIV infection (Redkar *et al*., 2001). With respect to potentially chronic intracellular infections such as brucellosis, microbiological load could serve as an indirect index of pathogen presence.

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