

Development of a Differential PCR Assay for Detection of *Brucella abortus* and *Brucella melitensis*: an Analytical Approach for Monitoring of *Brucella* spp. in Foods of Animal Origin

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Abstract

Background: Classical bacteriological detection of *Brucella* species from food, and environment is routinely carried out based on morphological and biochemical characteristics. However, for increasing specificity and sensitivity of species identification methods, development of a molecular assay is necessary that was main aim of this study.

Methods: Panel of some reference strains belonging to the phylum *Proteobacteria* were specifically used in this study. Additionally, the panel was enriched with 20 *Brucella* field strains isolated from 13 cattle and 7 sheep (West Kazakhstan region), and six strains from three cattle and three sheep (Almaty region). Bacterial identification before designing was carried out based on *16S rRNA* sequencing with universal primers. Primer design was implemented using the Primer3 program. Finally, specificity and sensitivity of the PCR assay for *Brucella* identification were evaluated.

Results: The sensitivity of the developed conventional PCR assays was assessed with the range of 2×10^5 to 12 genomic copies isolated from *B. abortus* 100 and *B. melitensis* H-12 reference strains. The sensitivity of the developed assays using Ba and Ba-r, Bm and Bm-r primers was determined to be 1.6×10^3 genomic copies.

Conclusion: Quick detection and species identification of *Brucella* strains circulating in Kazakhstan would help local authorities in decision-making and implementation of the most effective strategies for control of these bacteria. Our PCR-based assay was the first step towards developing a novel kit with final aim of standardizing molecular identification of *B. abortus* and *B. melitensis* in foods of animal origin in Kazakhstan and other central Asia countries.

Introduction

Brucellosis is a common food-borne infection posing a significant threat for public health worldwide. Brucellosis is known as a major zoonotic disease that its clinical symptoms in human are often undulant fever, headache, abdominal pain, arthralgia, and myalgia (Falenski et al.,

2011; Gopaul et al., 2014; Winchell et al., 2010). Brucellosis is an endemic disease in Kazakhstan characterized by high infection rates in humans and animals; actually, the current situation of brucellosis in farm animals is an emergency, and the disease is widely spread among cattle

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and sheep in farms of all regions of the country. Infected domestic and wild animals serve as the natural reservoir and source of infection that occurs through direct contact, by inhalation of infected aerosols (Foster et al., 2007; Scholz et al., 2008; Taleski et al., 2002; Whatmore et al., 2014) or consumption of contaminated foods of animal origin (Hoseinpour Ganjaroudi et al., 2015; Ravanel et al., 2009), for example unpasteurized milk or cheese (Al Dahouk et al., 2013). As a consequence, Kazakhstan is one of the ten countries with the highest incidence of human brucellosis in the world.

The causative agents of brucellosis are Gram-negative bacteria belonging to the genus *Brucella*. Among all *Brucella* species, *B. melitensis*, *B. abortus*, and *B. suis* have a particularly critical and extensive impact on public human and animal health (Godfroid et al., 2011). In Kazakhstan, the most common circulating species are *B. melitensis* and *B. abortus*. For detection purposes, serological assays and bacterial isolation are routinely carried out. Commonly employed serological methods are Rose Bengal Test (RBT), Serum Agglutination Test (SAT), Complement Fixation Test (CFT), Enzyme Linked Immunosorbent Assay (ELISA) and Fluorescence Polarisation Assay (FPA). They are sensitive and rapid methods to perform, but sometimes false positive reactions occur with cross-reactive bacteria, such as *Yersinia enterocolitica* O:9, due to the similar structure of the O-chain in the smooth lipopolysaccharide portion (Bounaadja et al., 2009; Hinic et al., 2009). The gold standard for the detection of *Brucella* species is isolation of the bacteria on culture media. However, isolation of *Brucella* bacteria is a time- and resource-intensive task; it requires level-2 bio-containment facilities and highly skilled technical personnel to handle samples and live bacteria for eventual identification and biotyping. Handling live *Brucella* involves risk of laboratory infection and very strict biosafety rules must be observed. To avoid these disadvantages, methods based on Polymerase Chain Reaction (PCR) are very useful and considerable progress has been made recently to improve their sensitivity and specificity, in association to straightforward reaction preparation and affordable costs. *Brucella* detection still is based mainly on classical bacteriological methods in Kazakhstan, *Brucella* identification also relies on phenotyping, i.e. the assessment of morphological and metabolic characteristics. Therefore, the development of a molecular assay for detection and species determination of this pathogen is a compelling issue. In fact, even though several *Brucella* genus- or species-specific PCR assays, using *16S rRNA* or other gene targets (e.g., *bcsp31*, *IS711*, *omp2* genes), have been developed (Bricker et al., 2003; Cloeckert et al., 1995; Ficht et al., 1990; Hinic et al., 2009). Currently, only the kit BRU-COM (AmpliSens, Russia) is officially registered in the

territory of the Republic of Kazakhstan. This kit can identify the causative agent of brucellosis limited to the genus. Additionally, the so-called Bruce-ladder PCR assay have been developed, being able to differentiate *Brucella* at the species level (Kang et al., 2011; Lopez-Goni et al., 2008; Mayer-Scholl et al., 2010), but set up of this assay is very expensive (the commercial cost in Kazakhstan is about 1000 \$ for 24 reactions) and not available to regional laboratories.

In this study, we reported the development of a new conventional PCR assay to identify *B. abortus* and *B. melitensis* from other *Brucella* species. This conventional PCR assay, made available to Kazakh regional laboratories, would implement rapid detection of *B. abortus* and *B. melitensis* infection with high sensitivity and improved accuracy detection of the *Brucella* species in food as well as environment.

Materials and methods

Bacterial strains and DNA samples

A panel of some reference strains belonging to phylum *Proteobacteria* were specifically used in this study including *B. melitensis* 16 M, *B. melitensis* 565, *B. melitensis* H-12, *B. abortus* 544, *B. abortus* S 19, *B. abortus* 100, *B. abortus* 960, *B. ovis* 8, *B. ovis* 63/290, *B. suis* 1330, *B. canis* 1066, *Salmonella abortus equi* E 841, *S. typhimurium* 371, *S. enteritidis*, *Escherichia coli* ATCC 25922, *E. coli* 0-15, and *Pasteurella multocida bovis* 216. Reference strains were provided by the National Veterinary Reference Centre, Astana, Kazakhstan. Additionally, the panel was enriched with 20 samples from 13 cattle and 7 sheep (West Kazakhstan region), and 6 samples from 3 cattle and 3 sheep (Almaty region). These samples were tissues (liver, spleen, lymph nodes) and blood animals reared for food provision that resulted positive to *B. abortus* and *B. melitensis* isolation during routine monitoring. All strains were identified as *Brucella* species on the basis of classical identification procedures including colony morphology, CO₂ requirement, H₂S production, inhibition of growth by basic fuchsin and thionine, oxidase, catalase, and urease activity, lysis by phages, and also agglutination with monospecific sera (anti-A, anti-M, as well as anti-R sera), following the international recommendations (OIE, 2015).

Bacterial identification was carried out based on *16S rRNA* sequencing with the universal primers 8F (5'-AGAGTTTGTATCTGGCTCAG-3') and 806R (5'-GGACTACCAGGTATCTAAT-3') (Edwards et al., 1989). Sequence analysis was carried out also to identify possible bacterial contamination in the samples (De Vegas et al., 2006). Species-level identification was per-

formed with OOM-Screen Brucellosis-RT Kit (Syntol, Russia) by PCR real-time. Total genomic DNA was extracted using a Pure Link Genomic DNA Kit (Invitrogen, USA) according to the manufacturer's instructions and stored at -20°C until required.

Primer design and PCR protocols

Brucella spp. reference sequences were retrieved from the GenBank using the RefSeq database and aligned by BioEdit software. Primer design was implemented using the Primer3 program (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>). Two genomic regions were selected for primer design after an alignment including all *Brucella* species and taking advantage of inter-species nucleotide differences. Specifically, primers were designed for *B. abortus* and for *B. melitensis* targeting the bacterial ABC transporter ATP-binding protein and the sulfate ABC transporter, permease protein CysW genes, respectively. The primer set for *B. abortus* spanned a fragment of 102 bp in size, while the primer set for *B. melitensis* amplified a region of 65 bp in size (Table 1). The newly designed forward and reverse primers were further optimized (Tm selection, lack of stem loop formation, and lack of self-annealing) using the Oligonucleotide Properties Calculator (www.basic.northwestern.edu/biotools/oligocalc.html). The annealing temperature was experimentally optimized for each primer set by performing PCR through an annealing temperature gradient ranging from 53 to 63 $^{\circ}\text{C}$. PCR was performed using 10 μl of 2.5x master mix (Syntol, Russia), 0.5 μl each primer (5 pmol) and 1 μl DNA in a total volume of 25 μl . Amplification was carried out using a Mastercycler PCR machine (Eppendorf, Germany). Initially, PCR amplification was performed with an initial denaturation step at 95 $^{\circ}\text{C}$ for 3 min., followed by 40 cycles at 63 $^{\circ}\text{C}$ for 40 s, and 95 $^{\circ}\text{C}$ for 15 s. The number of cycles was then reduced to 30, while the other parameters were kept unchanged. The amplified products were resolved on 3% agarose gels, the bands stained by ethidium bromide solution, and then visualized in a UV transilluminator. After that, all the banding patterns were carefully compared by visual inspection.

Specificity and sensitivity of the PCR assay

The specificity of the PCR assay was assessed using *Brucella* reference strains, *Brucella* field isolates and non-*Brucella* bacteria included in the panel. The assay sensitivity was determined by a serial 5-fold dilution ranging from 2×10^5 to 12.8 genomic copies of the *B. abortus* 100, *B. melitensis* H-12, and *B. suis* 1330 reference strains. DNA concentration was measured using a Dynamica Halo DNA master spectrophotometer (Switzerland) and used to calculate the number of *Brucella* genomic copies. The results obtained with the novel assay were compared with those of *16S rRNA* (Edwards et al., 1989) and PCR real-time OOM-Screen Brucellosis-RT Kit, which were used to identify *Brucella* species.

Results

PCR amplification performed following a protocol employing 40 cycles of amplification, showed some cross-reactivity with the selected primer sets were submitted to a large number of PCR cycles (data not shown). However, species-specific amplification was obtained by reducing the number of cycles to 30. By employing optimized PCR conditions, the developed conventional PCR assay showed specific positive reactions only to *B. abortus* and *B. melitensis* samples included in the panel (reference strains and tissues/blood), whereas they yielded negative reactions to other *Brucella* species and non-*Brucella* strains (Fig. 1 and Fig. 2).

As shown in Table 2, the results were compared with those obtained on the same samples using the PCR real time OOM-Screen Brucellosis-RT Kit. The complete concordance between two methods confirmed accuracy of the developed conventional PCR assay with newly designed species-specific primer sets. The sensitivity of the developed conventional PCR assays was assessed by means of a 5-fold serial dilution of genomic DNA ranging from 2×10^5 to 12 genomic copies isolated from *B. abortus* 100 and *B. melitensis* H-12 reference strains (Fig. 3). The sensitivity of the developed species-specific PCR assays using Ba and Ba-r, Bm and Bm-r primers was determined to be 1.6×10^3 genomic copies.

Table 1: Species-specific primers for the detection of *B. abortus* and *B. melitensis*

Bacteria	Primer name	Primer sequence (5'-3')	Amplicon size
<i>B. abortus</i>	Forward Ba	5'-TCCAATAATGGCGCTGTGCAAGA-3'	102 bp
	Reverse Ba-r	5'-TCGAGCCAGGCTGTGGTTTCC-3'	
<i>B. melitensis</i>	Forward Bm	5'-TCCAAACGCTTTCCCGGACGA-3'	65 bp
	Reverse Bm-r	5'-GGCGAAACGGAAAAAGGTATCTCCAC-3'	

Table 2: Results of applying the novel conventional PCR assay (+/-) and the OOM-Screen-Brucellosis-RT Kit (Ct value) on the panel of bacterial strains included in the study (reference and field isolates)

Sample	<i>B. abortus</i>	<i>B. melitensis</i>
<i>B. abortus</i> 544	+(16,2)	-
<i>B. abortus</i> 100	+(16,4)	-
<i>B. abortus</i> 960	+(16,9)	-
<i>B. abortus</i> 19	+(16,2)	-
<i>B. melitensis</i> 16M	+(16,4)	+(19,2)
<i>B. melitensis</i> 565	-	+(17,1)
<i>B. melitensis</i> H-12	-	+(16,7)
<i>B. canis</i> 1066	-	-
<i>B. suis</i> 1330	-	-
<i>B. ovis</i> 8	-	+(16,8)
<i>B. ovis</i> 63/290	+(16,4)	-
<i>S. abortus-egui</i> E841	-	-
<i>E. coli</i> ATCC 25922	-	-
<i>E. coli</i> 0-15	-	-
<i>P. multocida bovis</i> 216	-	-
0011	+(16,9)	-
0044	+(16,4)	-
0013	+(17,1)	-
0041	-	+(16,2)
00192	+(16,2)	-
00194	+(16,7)	-
0037	+(19,2)	-
00055	-	+(16,4)
00134	+(16,8)	-
<i>S. typhimurium</i> 371	-	-
<i>S. enteritidis</i>	-	-
Water	-	-

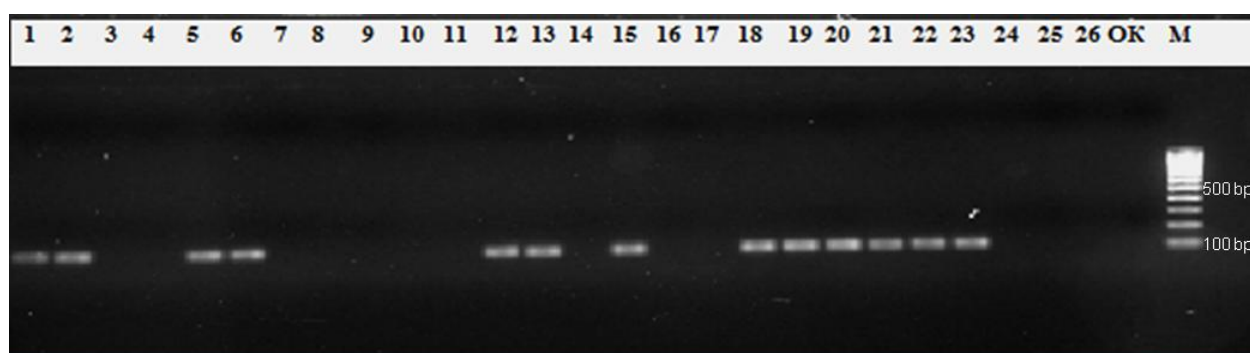


Fig. 1: PCR amplification of *Brucella* spp. isolates with Ba and Ba-r primers. Positive results including lane 1: *B. melitensis* 16 M; lane 2: *B. abortus* 544; lane 5: *B. abortus* 100; lane 6: *B. abortus* 960; lane 12: *B. abortus* 19; lane 13: *B. ovis* 63/280; lane 15: 00134; lane 18: 00192; lane 19: 0044; lane 20: 0013; lane 21: 0011; lane 22: 00194; lane 23: 0037. Negative results including lane 3: *B. melitensis* 565; lane 4: *B. melitensis* H-12; lane 7: *B. ovis* 8; lane 8: *B. canis* 1066; lane 9: *B. suis* 1330; lane 10: *S. abortus-egui* E841; lane 11: *E. coli* ATCC 25922; lane 14: 0041; lane 16: *E. coli* 0-15; lane 17: *P. multocida bovis* 216; lane 24: 00055; lane 25: *S. typhimurium* 371; lane 26: *S. enteritidis*. Lane OK: negative control; lane M: 100 bp GeneRuler DNA ladder



Fig. 2: PCR amplifications of *Brucella* spp. isolates with Bm and Bm-r primers. Positive results including lane 1: *B. melitensis* 16 M; lane 3: *B. melitensis* 565; lane 4: *B. melitensis* H-12; lane 7: *B. ovis* 8; lane 14: 0041; lane 24: 0055. Negative results including lane 2: *B. abortus* 544; lane 5: *B. abortus* 100; lane 6: *B. abortus* 960; lane 10: *S. abortus-egui* E841; lane 11: *E. coli* ATCC 25922; lane 12: *B. abortus* 19; lane 13: *B. ovis* 63/280; lane 14: 0041; lane 15: 00134; lane 16: *E. coli* 0-15; lane 17: *P. multocida bovis* 216; lane 18: 00192; lane 19: 0044; lane 20: 0013; lane 21: 0011; lane 22: *B. suis* 1330; lane 23: 0037; lane 25: *S. typhimurium* 371; lane 26: *S. enteritidis*. Lane OK: negative control; lane M: 100 bp GeneRuler DNA ladder

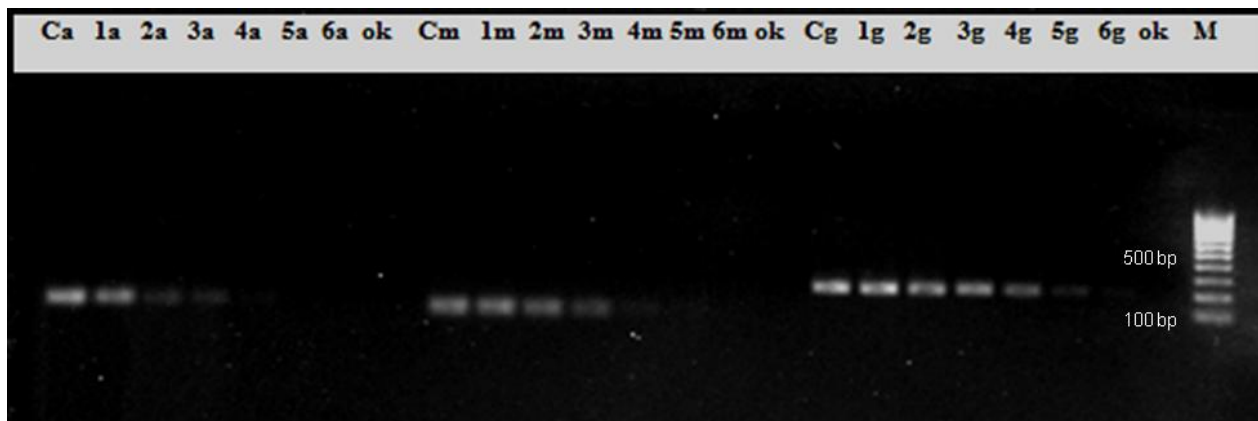


Fig. 3: Sensitivity of the novel PCR assays. PCR with Ba and Ba-r, Bm and Bm-r primers using a 5-fold dilution series of *Brucella* genomic DNA. Lane Ca: concentrated *B. abortus* 2×10^5 copies; lane 1a: *B. abortus* 4×10^4 copies; lane 2a: *B. abortus* 8×10^3 copies; lane 3a: *B. abortus* 1,600 copies; lane 4a: *B. abortus* 320 copies; lane 5a: *B. abortus* 64 copies; lane 6a: *B. abortus* 12 copies. Lane Cm: concentrated *B. melitensis* 2×10^5 copies; lane 1m: *B. melitensis* 4×10^4 copies; lane 2m: *B. melitensis* 8×10^3 copies; lane 3m: *B. melitensis* 1,600 copies; lane 4m: *B. melitensis* 320 copies; lane 5m: *B. melitensis* 64 copies; lane 6m: *B. melitensis* 12 copies. The third panel at the right of the gel shows the same dilution series tested by an in-house PCR targeting *Brucella* genus and usually employed as screening method in our lab

Discussion

The present study demonstrated that the selected primers are able to provide preferential amplification of perfectly-matched targets when proper PCR conditions are applied. Sensitive and rapid DNA-based techniques for *Brucella* species identification are challenging due to the high genetic interspecies homology, which exceeds 90% (Verger et al., 1985). Several efforts have been carried out to develop primers for *Brucella* DNA detection and the primers were designed targeting different genic and intergenic regions (Bogdanovich et al., 2004; Leal-Klevezas et al., 1995; Mukherjee et al., 2007). Moreover, PCR assays have been described for identification of *Brucella* at the species level and species-specific primers were designed for simplex PCR protocols on *bcs31* and outer membrane proteins (*omp2b*, *omp2a*, *omp31*) (Imoka et al., 2007) and *Brucella*-specific insertion se-

quence IS711 (Hinic et al., 2008). Multiplex PCRs have been also implemented for identification of *Brucella* at the species level. The first multiplex PCR, called AMOS PCR used combined primer pairs; the assay exploits the polymorphism arising from species-specific localization of the genetic element IS711 in the *Brucella* chromosome (Bricker and Halling, 1994; Ewalt and Bricker, 2000; Ocampo-Sosa et al., 2005). An improved multiplex PCR was developed that differentiated all nine currently recognized *Brucella* species, including the recently described species *B. microti*, *B. inopinata*, *B. ceti*, and *B. pinnipedialis* based on multiple targets: a 25-kb DNA deletion leading to the loss of *omp31* gene; a 15-kb deletion comprising *omp25b* and *wboA-wboB* genes; a *wboA* gene disruption by an IS711 element; a 702-bp deletion in the *ery* operon; a specific mutation in the *rpsL* gene; a

976-bp deletion in the chromosome I; a 2.2-kb deletion in chromosome II; a 2.6-kb fragment in *B. suis*, but not in *B. abortus* or *B. melitensis*; as well as an IS711 element downstream of the *bp26* gene in *Brucella* spp. isolated from marine mammals (Mayer-Scholl et al., 2010). Leal-Klevezas et al. (2000) designed novel primers and TaqMan probes for six single real-time and conventional PCRs to identify and differentiate *Brucella* spp. at the species level. These assays exploit the outer membrane protein *omp-2* gene polymorphism arising from species-specific localization of the genetic element IS711 in the *Brucella* chromosome.

Most of these methods have been tested on human samples and differences between primer performances were demonstrated to be significant by statistical analysis for human, animal, and culture samples (Baddour and Alkhalifa, 2008). Moreover, molecular typing techniques (e.g., MLVA) have highlighted that a genetic diversity of *Brucella* isolates exist in relation to their geographic origin (Scholz and Vergnaud, 2013). On these bases, we developed species-specific primers suitable to detect with high sensitivity and specificity the *Brucella* isolates circulating in the Kazakhstan focusing on samples from animal origin. The genomic regions selected for primer design represent another element of novelty in our study. Actually, to the best of our knowledge, no PCR assays based on *Brucella ABC transporter ATP-binding protein* and the *sulfate ABC transporter, permease protein CysW* genes have been published, so far. Therefore, this study enlarges the spectrum of genes suitable for the *Brucella* detection and species differentiation.

The developed conventional *Brucella*-species assays showed a limit of detection equal to 1.6×10^3 genomic copies, comparable to other studies that were able to identify from 9.0×10^2 to 9.0×10^7 bacteria (Baddour and Alkhalifa, 2008; Zamanian et al., 2015). Both PCR assays demonstrated species-specificity and no cross-reaction was observed with different *Brucella* species or other bacterial genus. Therefore, this new PCR method could be a valuable tool for rapid detection and species identification of *B. abortus* and *B. melitensis* in foods of animal origin (especially raw milk) ensuring accuracy, specificity, and sensitivity in detection.

Conclusion

Quick detection and species identification of *Brucella* strains in foodstuff using developed molecular assay would help local authorities of Kazakhstan in decision-making and implementation of the most effective strategies for control of this pathogen. Our PCR-based assay was the first step towards developing a novel kit with the

final aim of standardizing molecular identification of *Brucella* in Kazakhstan and other central Asia countries.

Conflicts of interest

Authors declare that they have no conflict of interest.

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