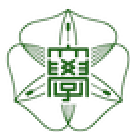
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Ultra-fast detection and differentiation of *Brucella* genus bacteria, *B. abortus*, *B. melitensis*, and *B. suis*, using multiplex convection polymerase chain reaction

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Abstract

Brucellosis is a global zoonotic disease caused by facultative intracellular bacteria from the genus *Brucella*. *Brucella* spp. are gram-negative bacteria that are pathogenic to humans and a variety of animals. Rapid detection and timely treatment of brucellosis is important for increasing the curative rate, to prevent spread among animals, and to reduce the risk of transmission to humans. In this study, we developed a rapid multiplex convection polymerase chain reaction (cPCR) to detect and differentiate *B. abortus*, *B. melitensis*, and *B. suis*. In the ultra-fast cPCR method, a universal primer IS711 and species-specific primer sets specifically detected each target species after a 24 min amplification reaction. Multiplex detection was performed with a mixture of the universal primer and all species-specific primers. Species-specific DNA amplicons were clearly identified by their expected sizes within the same amplification time. When sensitivity of detection was tested, approximately 28 genome equivalents (0.1 pg of genomic DNA) were detected using the cPCR system. The cPCR operation time could be reduced to 20 min, 25 cPCR cycle, without losing the sensitivity of detection. The assay developed in this study worked well in the presence of corresponding animal genomic DNA.

The results revealed that *Brucella* species can be detected and differentiated in singleplex and multiplex regimes of ultra-fast cPCR. The ultra-fast speed and the sensitivity of molecular detection and differentiation of *Brucella* species developed in this study may be useful in laboratory and field applications.

Key Words: *Brucella* species, Molecular diagnostics, Ultra-fast convection PCR

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Introduction

Brucellosis is a global bacterial zoonotic disease. It affects a variety of livestock and wildlife, causing abortion, fetal death, and genital infections of animals, thus resulting in their decreased reproductive efficiency. *Brucella* species are considered to be potentially pathogenic to humans. Human brucellosis is the most common zoonotic infection[23], with diverse symptoms in humans. Most human cases of brucellosis have been attributed to *B. abortus*, *B. melitensis*, and *B. suis*.

Brucella are facultative gram-negative non-spore forming, non-motile coccobacilli. Species from the genus *Brucella* show high degrees of genetic similarity[6,10]. Phenotypical identification and differentiation of *Brucella* is a lengthy process requires level 3 biosafety cabinet and frequently associated with a risk of laboratory-acquired infection[1,22]. To overcome these issues, various approaches, including polymerase chain reaction (PCR)-based assays, to detect and identify *Brucella* species have been attempted[5,9,14]. These include PCR assays, microarray analysis, and sequencing[25]. Microarray analyses and sequencing are highly sensitive, but these are high-cost and/or relatively complicated methods. Among these approaches, the PCR-based method is generally accepted in *Brucella* diagnostics[30].

Rapid molecular detection of infectious diseases, including brucellosis, has recently attracted attention[4,15,17,18,20,29]. However, the undertaken studies involved complicated primer design and/or relatively long reaction times (~1.5–2 h). Furthermore, simultaneous detection of *Brucella* species also became important and several studies reported on the subject[20,26]. Here, we introduce a rapid, simple, and economical method for detection and differentiation of *Brucella* species in singleplex and multiplex modes using convection PCR (cPCR).

Materials and Methods

Bacterial strains and growth conditions: Bacterial strains used in this study were *B. abortus S19*, *B. melitensis M5*, and *B. suis S2* from the Institute of Veterinary Medicine at National Center for Disease Control (Changchun City, Jilin, China). They were grown on tryptic soy agar (TSA; BD, USA) supplemented with 5% fetal bovine serum (FBS; Gibco, USA) for 3 days at 37°C under 5% CO_2[11]. Single colonies were used to inoculate 3% tryptic soy broth and cultivated for 60 h. Bacteria were killed by suspending in methanol:saline (2:1) solution and stored at 4°C.

DNA samples for PCR analysis: Genomic DNA from all strains was extracted using commercial MiniBest genomic DNA isolation kit DNA (TaKaRa, Japan) according to the manufacturer's instructions. Meat samples, beef, lamb, and pork, were purchased from commercial sources in Korean markets. For raw meat samples, 50 mg of each sample was chopped into small pieces and subjected to DNA extraction using a DNeasy Blood and Tissue Kit (Qiagen, Germany), as recommended by the supplier. The concentration of each DNA sample was determined with NanoDrop (Thermo Scientific, USA). Isolated DNA (1 ng) and 10-fold serial dilutions were used as templates in cPCR reactions. Mixed DNA samples were prepared by combining equal amounts of individually prepared genomic DNA samples from each *Brucella* strain. Each meat genomic DNA (1 ng) was mixed with corresponding genomic DNA (100 pg) from *Brucella* species where indicated. Copy numbers of genomic DNA in the samples were calculated from 1 ng of DNA, based on the molecular weight of double stranded DNA and chromosomal DNA size (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr), as 2.8×10^5 copies/ng *Brucella* chromosomal DNA.

Species-specific primer design and cPCR: Primers used in this study are shown Table 1. For

Table 1. Sequences of oligonucleotide primers used in this study

Name	Sequence (5'→3')	Tm (°C)	Amplicon size (bp)
IS711	TGCCGATCACTTAAGGGCCTTCAT	60.5	–
BA	GGACGAACGGAATTTTTCCAATCCCATC	60.0	495
BM	AAATCGCGTCCTTGCTGGTCTGA	61.0	733
BS	GCGCGGTTTTCTGAAGGTTCAGG	60.9	285
CF	GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA	64.2	–
Beef-R	CTAGAAAAGTGTAAGACCCGTAATATAAG	53.2	274
Lamb-R1	AAACATAGCCTATGAATGCTGTGGCTATTGT	60.3	340
Pork-R2	CTGTTCCGATATAAGGGATAGCTGATAGTAGA	58	418

Brucella detection, primers are designed as follows. Insertion sequence IS*711*-specific primer was used as a universal primer designed to anneal to the specific insertion (IS) element IS*711* of *Brucella* species. Other species-specific primers, specific to *B. abortus*, *B. melitensis*, or *B. suis*, were designed to anneal downstream of a specific IS*711* element: for *B. abortus*, the IS element downstream of the *alkB* gene was targeted (GenBank accession number AF148682); for *B. melitensis*, the IS element downstream of BMEI1162 (GenBank accession number AE008917.1); and, for *B. suis*, the IS element downstream of BR1674 (GenBank accession number AE014291.4)[2]. Animal meat species-specific primers for beef, lamb and pork detection were used as reported in previous studies[27,28].

The cPCR mixture (20 μL) contained 1× *PalmTaq* HS buffer (including 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.4 U *PalmTaq* High-speed DNA polymerase (Ahram Biosystems, Inc., Korea), and primers for single or multiple species detection. For single *Brucella* species detection, primer concentrations were as follows: 0.25 μM universal IS711 primer and 0.25 μM species-specific primers for *B. abortus* (BA), *B. melitensis* (BM), or *B. suis* (BS). For multiplex detection, primer concentrations were as follows: 0.25 μM IS711 primer, BA primer, and BM primer, and 0.15 μM BS primer. Genomic DNA (∼3 pg) was used as a template.

cPCR was performed with a convection thermal cycler Palm PCR device (G2-12, Ahram Biosystems, Inc., Korea). The cPCR uses three heating plates for denaturing, annealing and polymerization for generation of convection in PCR tube. Thus this method does not require ramp from on temperature to another temperature like other conventional thermocycles. Hence, it dramatically reduces PCR running time. Further details are described in elsewhere[12,13]. The speed level was set to F3, and the annealing temperature was set to 60°C. cPCR reactions were run for 30 cycles in 24 min, unless stated otherwise. Upon completion, an aliquot of the PCR mixture was analyzed by 1.5% agarose gel electrophoresis for 30 min at 100 V. PCR products were visualized by fluorescence after ethidium bromide staining, with an imaging system (Ultra-Lum Imaging System, USA). All experiments were performed at least in triplicate.

Results

Specificity determination for ultra-fast cPCR

As this was the first time that cPCR was used for *Brucella* species detection, we evaluated the performance of cPCR using the universal IS*711* primer and species-specific primer sets BA, BM, and BS. We used a fragment complementary to the multiple insertion sequence element IS*711* as a universal primer, as this IS is stable with respect to both number and insertion site in *Brucella* chromosomes and is frequently used as a *Brucella* detection

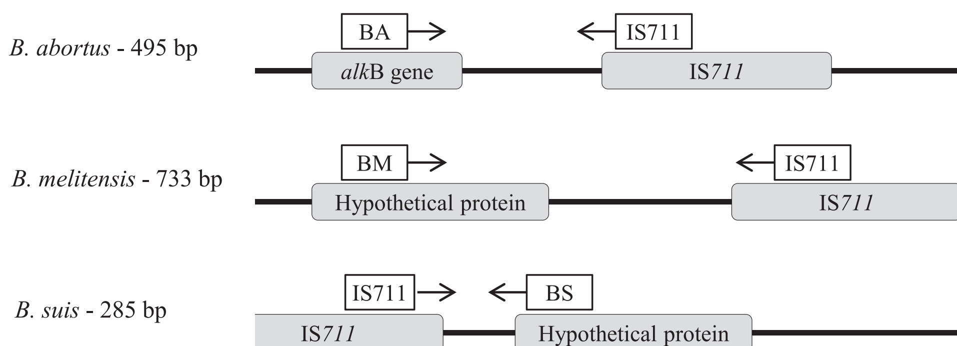

Fig. 1. Location of primers used for detection of *Brucella* genus with cPCR. The universal primer IS711 anneals to IS*711*, and species-specific primers (BA, BM and BS) are derived from species-specific chromosomal locations of *B. abortus*, *B. melitensis*, and *B. suis*. The expected amplicon sizes are given.

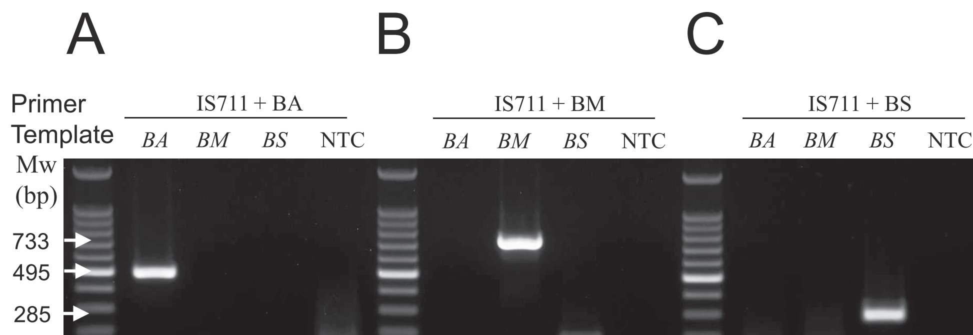

Fig. 2. Detection of *Brucella* species with species-specific primer sets. Genomic DNAs isolated from *B. abortus* (*BA*), *B. melitensis* (*BM*), and *B. suis* (*BS*) were used as templates, with the universal primer IS711 and each species-specific primer set *BA* (**A**), *BM* (**B**) or *BS* (**C**), for ultra-fast cPCR. DNA amplicons of expected sizes were detected. cPCR operation time was 24 min (equivalent to 30 cycles). NTC, no template control; Mw, molecular weight marker.

target[2,3,7,14,19]. Primer sequences and their chromosomal locations are shown in Table 1 and Fig. 1, respectively.

The cPCR reactions were performed for 30 cycles (24 min), with genomic DNA isolated from each *Brucella* species. As shown in Fig. 2, strong DNA amplification was detected with species-specific primers and genomic DNA purified from each *Brucella* species tested (495 bp band in lane BA in Fig. 2A; 733 bp band in lane BM in Fig. 2B; and 285 bp band in lane BS in Fig. 2C). However, since these species-specific primers did not amplify DNA from other related species, no unspecific DNA bands were detected. When BA specific primers were used, only *B. abortus* was detected, not *B. melitensis*, or *B. suis*. Similar results were obtained with *B. melitensis*-specific and *B. suis*-specific primers. No DNA amplification was evident in no template control (NTC) samples.

Multiplex identification and differentiation of Brucella species

Next, multiplex detection of *Brucella* species by cPCR was tested. The universal primer and species-specific primers for *B. abortus*, *B. melitensis*, and *B. suis* were mixed together and used for rapid-PCR identification of *Brucella* species. In this experiment, mixtures of the same amounts of different genomic DNAs were prepared. Each individual genomic DNA sample (Fig. 3, lanes 1–3) and a combination of genomic DNA from two different species (Fig. 3, lanes 4–6), or all three species (Fig. 3, lane 7), were used as templates in cPCR amplification. Approximately 3 pg of each type of genomic DNA was used, and 30 cycles of cPCR were performed

in 24 min. As anticipated, amplified DNA bands of 495 bp, 733 bp, and 285 bp were detected when individual *B. abortus*, *B. melitensis*, or *B. suis* genomic DNA was used, respectively (Fig. 3, lanes 1–3). Similarly, two specific DNA bands were observed when a combination of genomic DNA from two different species (Fig. 4, lanes 4–6) was used as a template. Finally, three DNA bands of expected sizes were detected when combinations of genomic DNA from three species (Fig. 3, lane 7) were used.

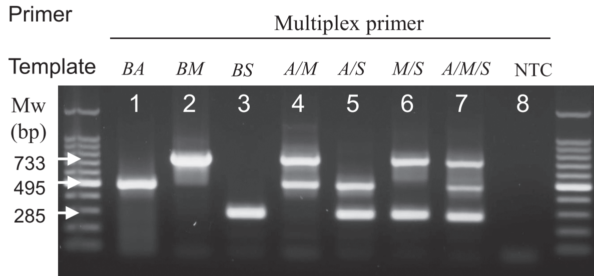

Fig. 3. Multiplex identification and differentiation of *Brucella* species. cPCR was performed with genomic DNAs from *B. abortus*, *B. melitensis*, and *B. suis* mixed together, and mixture of the universal primer and all three species-specific primers. One (lanes 1–3), two (lanes 4–6), and three (lane 7) DNA amplicons of expected sizes were detected, as appropriate. *A/M*, *B. abortus* and *B. melitensis*; *A/S*, *B. abortus* and *B. suis*; *M/S*, *B. melitensis* and *B. suis*; *A/S/M*, *B. abortus*, *B. melitensis*, and *B. suis.* NTC, no template control; Mw, molecular weight marker.

Determination of genomic DNA detection limit of cPCR

To determine the *Brucella* genomic DNA detection limit of the developed cPCR method, genomic DNA from every *Brucella* species tested was diluted from 2.8×10^4 copies (1×10^{-1} ng) to 2.8×10^0 (1×10^{-5} ng), and singleplex (i.e., with one pair of species-specific primers) and multiplex (with all primer sets) cPCR was performed as described in the Methods section (Fig. 4). For *B. abortus*, an amplification signal from 2.8×10^2 genomic DNA copies (1×10^{-3} ng) was clearly visible and a signal from 2.8×10^1 copies (1×10^{-4} ng) was faintly visible, both in singleplex and multiplex cPCR reactions.

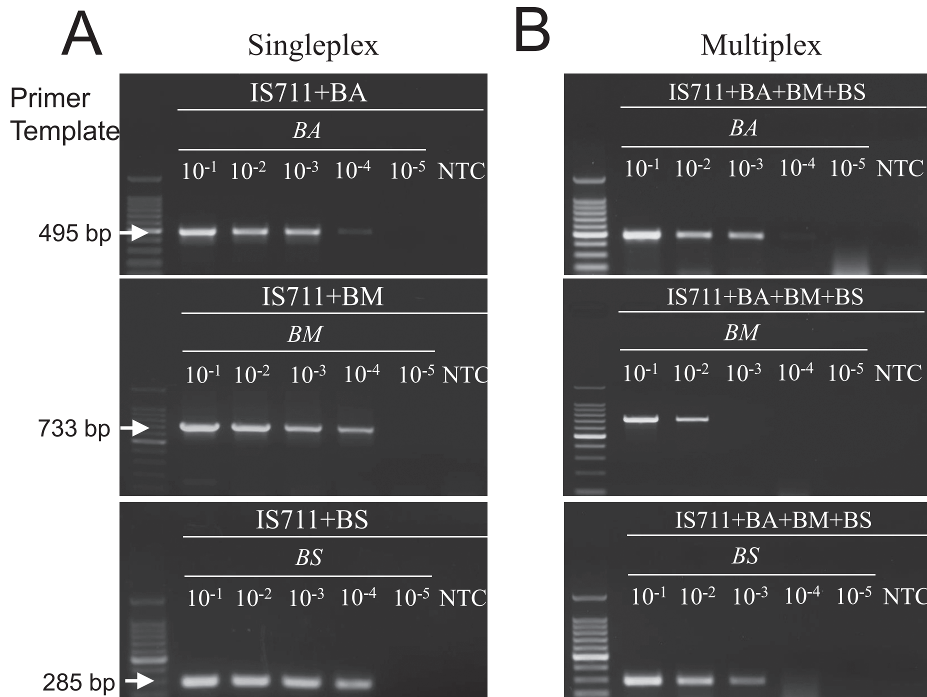

Fig. 4. Determination of the genomic DNA detection limit of singleplex and multiplex mode cPCR. Genomic DNA was serially diluted and used as template. Species-specific primer sets (IS711 and BA, BM or BS) were used in singleplex cPCR (**A**), and a mixture of all primers (IS711, BA, BM and BS) was used for multiplex cPCR (**B**). *BA*, *B. abortus*; *BM*, *B. melitensis*; *BS*, *B. suis*. NTC, no template control; Mw, molecular weight marker.

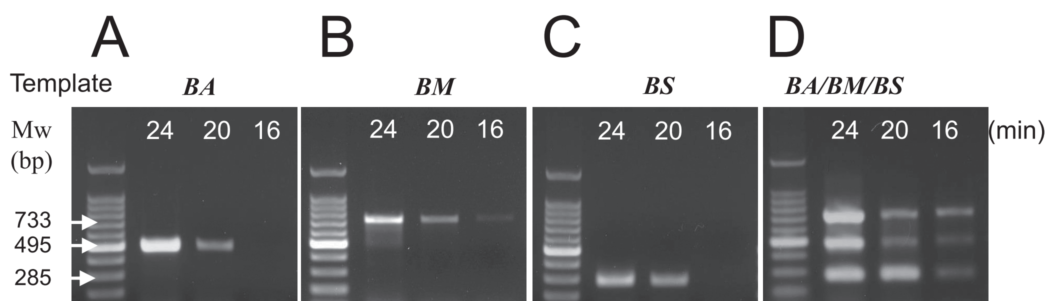

Fig. 5. Rapid detection of *Brucella* species with cPCR. The operation time of cPCR was gradually reduced, and the generated DNA amplicons from template *BA* (**A**), *BM* (**B**), *BS* (**C**) or mixture of *BA, BM* and *BS* (**D**) were analyzed by agarose gel electrophoresis. The concentration of genomic DNA was 10 pg, and a mixture of all four primer sets was used. *BA*, *B. abortus*; *BM*, *B. melitensis*; *BS*, *B. suis*. NTC, no template control; Mw, molecular weight marker.

However, detection sensitivity was different in singleplex and multiplex modes for *B. melitensis*. In singleplex cPCR, an amplified band was clearly visible with a low amount of genomic DNA, 2.8×10^1 copies (1×10^{-4} ng). However, in multiplex mode, an amplified DNA band was detected only with 2.8×10^3 copies of genomic DNA (1×10^{-2} ng). For *B. suis*, at least 2.8×10^1 copies of genomic DNA (1×10^{-4} ng) were clearly detected in singleplex mode and at least 2.8×10^2 copies (1×10^{-3} ng) were clearly detected in multiplex mode. We would like to emphasize that approximately 28 genome equivalents, sub-pg quantities, were detected in singleplex mode by the cPCR method only in 24 min operation time (30 cycles). We anticipate that detection limit would be increased as the number of PCR cycles or operation time are(is) increased.

Rapid detection of Brucella species with cPCR

To test the minimal time required for the cPCR to proceed without losing detection sensitivity, PCR operation time (or the number of PCR cycles) was gradually reduced and the amplicons were analyzed by agarose gel electrophoresis. Experiments were performed in multiplex mode, as described previously. As shown in Fig. 5, each individual target in the singleplex approach, *B. abortus*, *B. melitensis*, and *B. suis*, was clearly detected after 20 min cPCR operation time, equivalent to 25 cPCR cycles. The same results were obtained with multiplex cPCR, as defined amplicons were generated in 20 min. Three distinct DNA bands were detected in the multiplex cPCR experiment even with 16 min of cPCR. These data evidenced that *Brucella* species detection can be achieved with cPCR operation time as short as 20 min.

We tested the assay with the corresponding animal meat genomic DNA background to confirm that the developed assay could be used with real samples. Each *Brucella* genomic DNA was mixed with the genomic DNA prepared from corresponding animal meat and the cPCR was performed. As shown in Fig. 6, *B. abortus*, *B. melitensis*, or *B. suis*, was clearly detected in the presence of 10 times more of beef, lamb or pork genomic DNAs, respectively (Fig. 6A). Genomic DNA prepared from beef, lamb and pork were confirmed to generate meat species-specific amplicons with meat species-specific primers (Fig. 6B).

Discussion

In this study, we demonstrated that the specific detection and differentiation of strains from *Brucella* genus, *B. abortus*, *B. melitensis*, and *B. suis*, can be achieved with cPCR with ultra-fast speed and high sensitivity. The detection can be achieved using singleplex and multiplex regimes. *Brucella* species can be detected and differentiated in 24 min (30 cycle)

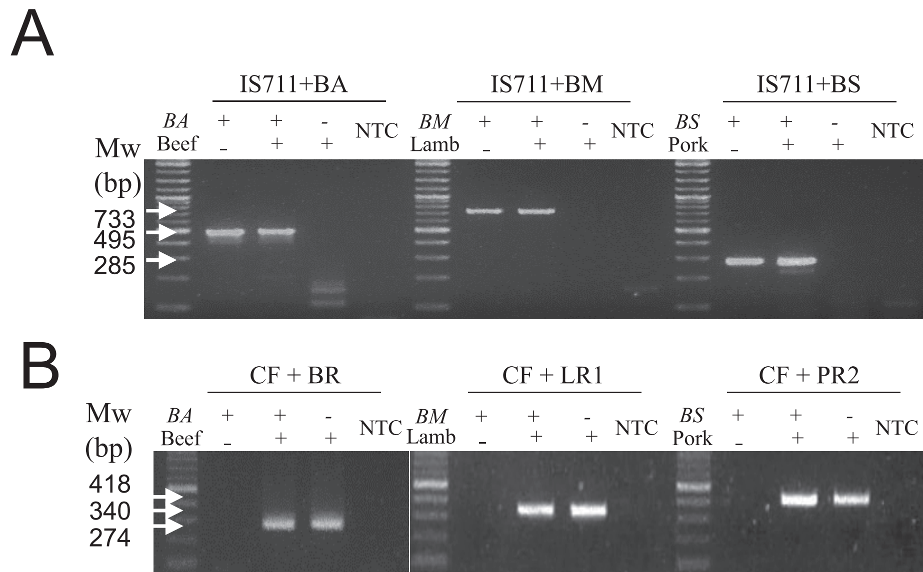

Fig. 6. Detection of *Brucella* species in the presence of genomic DNA from corresponding animal meat. cPCR was performed with 100 pg of each genomic DNAs from *B. abortus*, *B. melitensis*, and *B. suis* in the presence of 1 ng of each genomic DNA from beef, lamb or pork, respectively (**A**). Each genomic DNA prepared from corresponding animal meat, beef, lamb or pork were confirmed with beef, lamb or pork-specific primers (**B**). *BA*, *B. abortus*; *BM*, *B. melitensis*; *BS*, *B. suis*. CF, common forward primer; BR, beef reverse primer; LR1, lamb reverse primer; PR2, pork reverse primer; NTC, no template control; Mw, molecular weight marker.

cPCR. The detection sensitivity was as low as 28 genome equivalents (1×10^{-4} ng genomic DNA). The sensitivity is comparable with and/or higher than other reported methods[7,8,14,19,21,24,26]. Detection limits of previously reported end-point PCR methods are approximately 2–0.2 ng of DNA[21] or 1–10 pg of DNA[16,19]. It is generally accepted that real-time PCR is more sensitive than end-point PCR. However, our data revealed method sensitivity comparable with real-time PCR analyses, where the sensitivity was reported to be ~200–300 genome equivalents[26] or 16–25 genome copies[24].

It was shown that the operation time could be reduced to 20 min (25 cycles). The operation time could be reduced to 20 min (25 cycles). These comprise a significant advance in terms of PCR time reduction compared with previously published studies, where 1.5–2 h were required for unambiguous bacterial detection[14,16,19]. Generally, a single strong band is generated when ultra-fast speed cPCR is performed because, once a primer (or a set of primers) has annealed, amplification begins very rapidly, with lesser chance of other primers annealing to the template. Therefore, it tends to generate unbalanced multiplex cPCR results where the intensity of each DNA band is not similar or equal. However, our data showed that reproducible, similar levels of DNA amplification were obtained under the experimental conditions used. Our assay could detect each *Brucella* species in the presence of the genomic DNA of corresponding animal meat. This results suggested that the assay developed in this study can be applied to real samples in the field.

The ultra-fast multiplex introduced in this study is again cost-saving method compared to other real-time multiplex PCR methods which require more expensive machine and reagents. We believe that the ultra-fast speed and sensitivity of the molecular detection and differentiation method developed in this study for *Brucella* species may be useful in the rapid detection of these bacteria.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflicts of interest.

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