A rapid minor groove binder PCR method for distinguishing the vaccine strain *Brucella abortus* 104M

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**Abstract**

**Background:** Brucellosis is a widespread zoonotic disease caused by Gram-negative *Brucella* bacteria. Immunisation with attenuated vaccine is an effective method of prevention, but it can interfere with diagnosis. Live, attenuated *Brucella abortus* strain 104M has been used for the prevention of human brucellosis in China since 1965. However, at present, no fast and reliable method exists that can distinguish this strain from field strains. Single nucleotide polymorphism (SNP)-based assays offer a new approach for such discrimination. SNP-based minor groove binder (MGB) and Cycleave assays have been used for rapid identification of four *Brucella* vaccine strains (*B. abortus* strains S19, A19 and RB51, and *B. melitensis* Rev1). The main objective of this study was to develop a PCR assay for rapid and specific detection of strain 104M.

**Results:** We developed a SNP-based MGB PCR assay that could successfully distinguish strain 104M from 18 representative strains of *Brucella* (*B. abortus* biovars 1, 2, 3, 4, 5, 6, 7 and 9, *B. melitensis* biovars 1 and 2, *B. suis* biovars 1, 2, 3 and 4, *B. canis*, *B. neotomae*, and *B. ovis*), four *Brucella* vaccine strains (A19, S19, S2, M5), and 55 *Brucella* clinical field strains. The assay gave a negative reaction with four non-*Brucella* species (*Escherichia coli*, *Pasteurella multocida*, *Streptococcus suis* and *Pseudomonas aeruginosa*). The minimum sensitivity of the assay, evaluated using 10-fold dilutions of chromosomal DNA, was 220 fg for the 104M strain and 76 fg for the single non-104M *Brucella* strain tested (*B. abortus* A19). The assay was also reproducible (intra- and inter-assay coefficients of variation = 0.006–0.022 and 0.012–0.044, respectively).

**Conclusions:** A SNP-based MGB PCR assay was developed that could straightforwardly and unambiguously distinguish *B. abortus* vaccine strain 104M from non-104M *Brucella* strains. Compared to the classical isolation and identification approaches of bacteriology, this real-time PCR assay has substantial advantages in terms of simplicity and speed, and also reduces potential exposure to live *Brucella*. The assay developed is therefore a simple, rapid, sensitive, and specific tool for brucellosis diagnosis and control.

**Keywords:** Brucellosis, *Brucella abortus*, Minor groove binder, SNP-based assay
swine, and *B. melitensis* M5 in sheep and goat. In addition, live, attenuated *B. abortus* 104M has been adopted as a vaccine for use in humans since 1965. This strain, which was first isolated from the foetus of an aborted cow in the former Soviet republic in 1950, exhibits low virulence, high stability and high immunogenicity [7].

However, since it is a live attenuated strain, vaccination with 104M may cause vaccine-related cases of brucellosis, and it may be difficult to differentiate between a vaccine response and a natural infection, which complicates diagnosis. At present, a rapid and reliable method for distinguishing 104M from field strains is not available. The main objective of this study was to develop a PCR assay for rapid and specific detection of 104M.

**Methods**

**Strains and DNA extraction**

*Brucella* strains used in the present study are listed in Table 1. These comprised 18 representative strains of *Brucella* species and biovars, five *Brucella* vaccine strains, and 55 *Brucella* field strains. In addition, four non-target organisms (*Escherichia coli* K99, *Pasteurella multocida* C48–1, *Pseudomonas aeruginosa* DI-1, and *Streptococcus suis* ST171) were included. *Brucella* strains were cultured on tryptose agar at 37 °C with 5–10% CO₂ when required for 48–72 h in a biosafety level 3-equipped laboratory. Bacteria were then washed with normal saline containing 0.5% formaldehyde, and inactivated at 37 °C for 24 h. The four non-*Brucella* species were cultivated as described previously [8], and harvested and inactivated as described above. Unless specified, genomic DNA was extracted with the QIAamp DNA mini kit according to the manufacturer’s instructions (Qiagen GmbH., D40724 Hilden).

**Minor groove binder (MGB) PCR**

In this real-time PCR assay, a pair of short TaqMan 5’-labelled, 3’- MGB probes defining the single nucleotide polymorphism (SNP) were used to interrogate the 104M strain and non-104 M *Brucella* strains. Use of the MGB protein raises the melting temperature of probes meaning that a single base mismatch causes more destabilisation than would be the case with a longer probe [9]. This facilitates accurate SNP detection. For distinguishing *B. abortus* 104M, the SNP C₂₂₈⁻T₂₂₈ in NL70_10085 was selected. This SNP was identified by comparison of the *B. abortus* 104M draft genomic sequence with the *B. abortus* 9–914, *B. melitensis* M28, *B. suis* S1330, *B. canis* ATCC 23365, and *B. ovis* ATCC 25840, *B. pinnipedialis* B2/94, *B. microti* CCM 4915. One set of primers and probes was designed based on this SNP (Table 2).

The assay was performed using the TransStart Green qPCR SuperMix kit (TransGen Biotech Co., Beijing, China) in a reaction volume of 25 μL containing a reaction mixture volume of 12.5 μL with the working concentrations of primers and probes listed in Table 2, together with the DNA template (2 μL). For detection of the 104M strain, the VAC probe was labelled with 6-carboxyfluorescein (FAM) at the 5’-end and MGB eclipse at the 3’-end. For detection of non-104 M *Brucella* strains, the NON probe was labelled with the fluorophore 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC) at the 5’-end and MGB eclipse at the 3’-end.

PCR cycling parameters were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 56 °C for 10 s, and 72 °C for 10 s. Amplification was performed using the Bio-Rad MiniOpticon system (Bio-Rad Laboratories, Inc., Hercules, CA).

**Sensitivity, specificity, and reproducibility**

For assay sensitivity tests, the minimum detection limit of MGB PCR was evaluated using 10-fold serial dilutions of genomic DNA from *B. abortus* 104M for FAM fluorescence, and *B. abortus* A19 for VIC fluorescence. Each dilution was included in the assay to determine the minimum discriminatory amount of genomic DNA detected in the assay.

For assay specificity tests, we evaluated whether MGB PCR could distinguish the 104M strain from common species and other vaccine strains of *Brucella* using the representative and vaccine *Brucella* strains listed in Table 1. These strains included almost all common species and biovars of *Brucella* and the vaccine strains currently used in China. The four non-*Brucella* spp. (*Escherichia coli* K99, *Pasteurella multocida* C48–1, *Streptococcus suis* ST171, and *Pseudomonas aeruginosa* DI-1) were also tested.

Assay reproducibility was determined by calculating the intra- and inter-assay coefficients of variation (CV), using at least three replicates of each of the 10-fold serial dilutions of genomic DNA to generate a standard curve. The efficiency of the assay was determined using the following calculation: Efficiency = 10 (− 1/slope) – 1.

**Detection of clinical field strains**

A further 55 *Brucella* spp. field isolates (see Table 1) that were isolated from different animal species and areas, identified and provided by China Veterinary Culture Collection Centre were also tested.

**Results**

**Assay sensitivity**

Using 10-fold serial dilutions of *B. abortus* 104M genomic DNA ranging from 1.1 ng/μL to 0.11 fg/μL, the minimum discriminatory sensitivity for detection of
Table 1 *Brucella* spp. strains used in the present study

| Species (biovar) | Strain | Type | Host | Region |
|------------------|--------|------|------|--------|
| *B. abortus* (1) | A544 (CVCC790, ATCC23448) | Reference strain | Bovine | United Kingdom |
| *B. abortus* (2) | 86/8/59 (CVCC12, ATCC23449) | Reference strain | Bovine | United Kingdom |
| *B. abortus* (3) | Tula (CVCC13, ATCC23450) | Reference strain | Bovine | United Kingdom |
| *B. abortus* (4) | 292 (CVCC16, ATCC23451) | Reference strain | Bovine | United Kingdom |
| *B. abortus* (5) | B3196 (CVCC14, ATCC23452) | Reference strain | Bovine | United Kingdom |
| *B. abortus* (6) | 870 (CVCC17, ATCC23453) | Reference strain | Bovine | United Kingdom |
| *B. abortus* (7) | 63/75 (CVCC15, ATCC23454) | Reference strain | Bovine | United Kingdom |
| *B. abortus* (8) | C68 (CVCC11, ATCC23455) | Reference strain | Bovine | United Kingdom |
| *B. abortus* (9) | C72–62 (CVCC887) | Field strain | Bovine | Inner Mongolia |
| *B. abortus* (10) | C72–63 (CVCC888) | Field strain | Bovine | Inner Mongolia |
| *B. abortus* (11) | C72–61 (CVCC886) | Field strain | Bovine | Inner Mongolia |
| *B. abortus* (Unknown) | SHDeer-74 (CVCC780) | Field strain | Cervine | Shanghai |
| *B. abortus* (Unknown) | C72–387 (CVCC785) | Field strain | Bovine | Heilongjiang |
| *B. abortus* (Unknown) | C72–10 (CVCC786) | Field strain | Bovine | Heilongjiang |
| *B. abortus* (Unknown) | 2308 (CVCC788) | Field strain | Bovine | Heilongjiang |
| *B. abortus* (Unknown) | HBCow-1 (CVCC2408) | Field strain | Bovine | Hubei |
| *B. abortus* (Unknown) | HBCow-2 (CVCC2409) | Field strain | Bovine | Hubei |
| *B. abortus* (Unknown) | C72–12 (CVCC3621) | Field strain | Bovine | Heilongjiang |
| *B. abortus* (Unknown) | C72–8401 (CVCC3622) | Field strain | Bovine | Inner Mongolia |
| *B. abortus* (Unknown) | C72–8403 (CVCC3623) | Field strain | Bovine | Inner Mongolia |
| *B. abortus* (Unknown) | NMCow-2 (CVCC3635) | Field strain | Bovine | Inner Mongolia |
| *B. melitensis* (1) | 16 M (CVCC7002, ATCC23456) | Reference strain | Caprine | United Kingdom |
| *B. melitensis* (2) | 63/9 (CVCC21, ATCC23457) | Reference strain | Caprine | United Kingdom |
| *B. melitensis* (3) | Ether (CVCC20, ATCC23458) | Reference strain | Caprine | United Kingdom |
| *B. melitensis* (1) | Goat-901 (CVCC3627) | Field strain | Caprine | Inner Mongolia |
| *B. melitensis* (2) | CVCC3620 | Field strain | Caprine | Inner Mongolia |
| *B. melitensis* (Unknown) | C71–1257 (CVCC928) | Field strain | Caprine | Inner Mongolia |
| *B. melitensis* (Unknown) | C71–13 (CVCC929) | Field strain | Caprine | Inner Mongolia |
| *B. melitensis* (Unknown) | C71–35 (CVCC936) | Field strain | Caprine | Qinghai |
| *B. melitensis* (Unknown) | C71–44 (CVCC938) | Field strain | Caprine | Xinjiang |
| *B. melitensis* (Unknown) | Goat-963 (CVCC952) | Field strain | Caprine | Inner Mongolia |
| *B. melitensis* (Unknown) | M54–8 (CVCC3624) | Field strain | Caprine | Inner Mongolia |
| *B. melitensis* (Unknown) | Goat-866 (CVCC3625) | Field strain | Caprine | Inner Mongolia |
| *B. melitensis* (Unknown) | Goat-872 (CVCC3626) | Field strain | Caprine | Inner Mongolia |
| *B. melitensis* (Unknown) | Goat-865 (CVCC3628) | Field strain | Caprine | Inner Mongolia |
| *B. suis* (1) | S1330 (CVCC70524, ATCC23444) | Reference strain | Porcine | United Kingdom |
| *B. suis* (2) | Thomsen (CVCC22, ATCC23445) | Reference strain | Porcine | United Kingdom |
| *B. suis* (3) | 686 (CVCC23, ATCC23446) | Reference strain | Porcine | United Kingdom |
| *B. suis* (4) | 40 (CVCC24, ATCC23447) | Reference strain | Porcine | United Kingdom |
| *B. suis* (5) | KP6 (CVCC3651) | Field strain | Porcine | Guangdong |
| *B. suis* (6) | ZCS (CVCC3653) | Field strain | Porcine | Guangdong |
| *B. suis* (7) | ZC1 (CVCC3655) | Field strain | Porcine | Guangdong |
| *B. suis* (8) | ZC6 (CVCC3649) | Field strain | Porcine | Guangdong |
| *B. suis* (9) | KP1 (CVCC3658) | Field strain | Porcine | Guangdong |
| *B. suis* (10) | KP2 (CVCC3659) | Field strain | Porcine | Guangdong |
| *B. suis* (11) | KP3 (CVCC3660) | Field strain | Porcine | Guangdong |
### Table 1 Brucella spp. strains used in the present study (Continued)

| Species (biovar) | Strain | Type | Host | Region   |
|-----------------|--------|------|------|----------|
| B. suis (3)     | KP5 (CVCC3661) | Field strain | Porcine | Guangdong |
| B. suis (3)     | HNPig-1 (CVCC3662) | Field strain | Porcine | Hainan   |
| B. suis (3)     | HNPig-2 (CVCC3663) | Field strain | Porcine | Hainan   |
| B. suis (Unknown) | B54 (CVCC1072) | Field strain | Porcine | Russian |
| B. suis (Unknown) | C73–5 (CVCC1080) | Field strain | Porcine | Guangxi |
| B. suis (Unknown) | C73–10 (CVCC1083) | Field strain | Porcine | Guangxi |
| B. suis (Unknown) | C73–11 (CVCC1084) | Field strain | Porcine | Guangxi |
| B. suis (Unknown) | C73–13 (CVCC1085) | Field strain | Porcine | Guangxi |
| B. suis (Unknown) | C73–23 (CVCC1089) | Field strain | Porcine | Guangxi |
| B. suis (Unknown) | C73–25 (CVCC1091) | Field strain | Porcine | Guangxi |
| B. suis (Unknown) | C73–26 (CVCC1092) | Field strain | Porcine | Guangxi |
| B. suis (Unknown) | Br.63/3 (CVCC3639) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.63/142 (CVCC3640) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.86/27 (CVCC3641) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.63/62 (CVCC3642) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.79/224 (CVCC3643) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.Thomsen1720 (CVCC3644) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.Thomsen5 (CVCC3645) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.63/225 (CVCC3646) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.63/32 (CVCC3647) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | Br.64/24 (CVCC3648) | Field strain | Unknown | United Kingdom |
| B. suis (Unknown) | ZC2 (CVCC3656) | Field strain | Porcine | Guangdong |
| B. suis (Unknown) | ZC3 (CVCC3657) | Field strain | Porcine | Guangdong |
| B. suis (Unknown) | DF1 (CVCC3654) | Field strain | Porcine | Guangdong |
| B. suis (Unknown) | SD1 (CVCC3652) | Field strain | Porcine | Guangdong |
| B. suis (Unknown) | ZC4 (CVCC3650) | Field strain | Porcine | Guangdong |
| B. ovis         | 63/290 (CVCC7001S, ATCC25840) | Reference strain | Ovine | United Kingdom |
| B. canis        | RM6/66 (CVCC70701, ATCC23365) | Reference strain | Canine | United Kingdom |
| B. neotomae     | KP4 (CVCC3664) | Field strain | Canine | Guangdong |
| B. abortus (1)  | A19 | Vaccine | – | – |
| B. melitensis (1) | M5 | Vaccine | – | – |
| B. abortus (Unknown) | 104 M | Vaccine | – | – |
| B. abortus (1)  | S19 | Vaccine | – | – |
| B. suis (1)     | S2 | Vaccine | – | – |

Strains were identified and provided by the China Veterinary Culture Collection Centre (CVCC)

Unknown = unknown biovar or host

### Table 2 Targets, primers and probes used for the MGB PCR assay with the associated working concentrations

| Target (position) | Gene description | Working concentration (nM) |
|------------------|------------------|----------------------------|
|                  | Probe            | Primer                     |
| NL70_10085 (228) | molecular chaperone DnaK | VAC: CCGTCGTTATGACGTAT (160) |
|                  |                  | NON: CCGTCGTTATGACGAA (160)|

The position of the SNP within each target is shown in parentheses. The target SNP is shown in bold underlined font in both vaccine (VAC) and nonspecific (NON) probes.
104M–specific strains was ~ 220 fg per reaction for MGB PCR (Table 3). Similarly, using 10-fold serial dilutions of *B. abortus* A19 genomic DNA ranging from 3.8 ng/μL to 0.38 fg/μL, the minimum discriminatory sensitivity for detection of non-104 M *Brucella* strains was ~ 76 fg per reaction for MGB PCR (Table 3). These results indicated that the assays were highly sensitive for the detection of 104M and non-104 M *Brucella* genomic DNA in a single reaction.

**Assay specificity**

For evaluating specificity, the representative and vaccine *Brucella* strains listed in Table 1 were tested using the MGB PCR method described. The results showed that 18 representative strains of *Brucella* (*B. abortus* biovars 1, 2, 3, 4, 5, 6, 7 and 9, *B. melitensis* biovars 1 and 2, 3, *B. suis* biovars 1, 2, 3 and 4, *B. canis*, *B. neotomae* and *B. ovis*), and four *Brucella* vaccine strains (A19, S19, S2, M5) gave strong VIC fluorescence and weak FAM fluorescence below the threshold detection level (Fig. 1a and b).

Only the *B. abortus* 104M vaccine strain gave strong FAM fluorescence and weak VIC fluorescence below the threshold detection level (Fig. 1a and b), indicating that the assay was 104M–specific. All four non-*Brucella* species (*Escherichia coli* K99, *Pasteurella multocida* C48–1, *Streptococcus suis* ST171 and *Pseudomonas aeruginosa* DI–1) were negative for both FAM and VIC fluorescence. These results suggest the MGB PCR assay was highly capable of differentiating 104M from non-104 M *Brucella* isolates and non-*Brucella* strains.

**Assay reproducibility**

The standard curve generated using genomic DNA was linear over a wide range of dilutions (R² = 0.997 and slope = −3.645 for FAM fluorescence; R² = 0.982 and slope = −4.342 for VIC fluorescence). The assay was reproducible, with intra-assay CVs ranging from 0.006 to 0.022, and inter-assay CVs of 0.012 to 0.044. The efficiency of the assay was 88.1% for FAM fluorescence and 69.9% for VIC fluorescence. These figures were used to determine the threshold for detection.

**Detection of clinical field strains**

The results demonstrated strong VIC fluorescence and weak FAM fluorescence for all 55 *Brucella* spp. field isolates tested (Table 1), indicating that none were the 104M strain.

**Discussion**

The *B. abortus* vaccine strain 104M is a stable antigenic structure with low virulence and high immunogenicity, hence it has been used in China since 1965 to vaccinate cattle and humans against brucellosis. Because using a live vaccine may lead to severe pathogenic injury associated with allergy, the 104M strain was only recommended for high-risk populations in China [7], such as those at high risk due to their occupation [10]. The scratch vaccination method was used to introduce five billion bacteria, which achieved 90% protection for a 12 month duration [7]. In some areas in China, vaccination intervention in humans had an obvious effect; the reported cases of brucellosis in the Arong Banner declined sharply by 84.17% from 2005 to 2006 following vaccination, and the morbidity rate of brucellosis declined from 34,732 per 100,000 to 5454 per 100,000 [11].

However, due to lack of serological differentiation, it is difficult to distinguish 104M by serological assay alone. The recent development of SNP-based real-time assays offers a new approach for overcoming this hurdle. SNP-based MGB and Cycleave assays have been used for rapid identification of four *Brucella* vaccine strains (*B. abortus* strains S19, A19 and RB51, and *B. melitensis* Rev1) [12, 13].

In the present study, we developed a new MBG PCR assay that can successfully distinguish 104M strains from other bacterial strains, with a sensitivity of 220 fg, equating to around 60 cells. Furthermore, our MGB PCR assay can detect non-104 M *Brucella* strains in a single reaction.

| Table 3 | Mean quantification cycle (Cq) values resulting from MGB PCR. |
|---------|---------------------------------------------------------------|
| Detection of 104M genomic DNA | Detection of A19 genomic DNA |
| Concentration | Cq of FAM | Cq of VIC | Concentration | Cq of FAM | Cq of VIC |
| 1.1 ng/μL | 24.06 | NA | 3.8 ng/μL | 17.49 |
| 110 pg/μL | 27.20 | NA | 380 pg/μL | 20.89 |
| 11 pg/μL | 30.92 | NA | 38 pg/μL | 24.39 |
| 1.1 pg/μL | 35.27 | NA | 3.8 pg/μL | 31.90 |
| 110 fg/μL | 38.25 | NA | 380 fg/μL | 34.69 |
| 11 fg/μL | NA | NA | 38 fg/μL | 38.11 |
| 1.1 fg/μL | NA | NA | 3.8 fg/μL | NA |
| 0.11 fg/μL | NA | NA | 0.38 fg/μL | NA |

*FAM = 6-carboxyfluorescein; VIC = 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein; NA = not applicable*
reaction with a sensitivity of 76 fg, equating to around 30 cells. This assay allows accurate and reliable discrimination of 104M and non-104 M Brucella strains from common species and biovars of Brucella, Brucella vaccines, and other bacterial strains. Our assay therefore provides a simple, rapid, sensitive, and specific tool for use in the control of brucellosis.

**Conclusions**

A SNP-based MGB PCR assay was developed that could straightforwardly and unambiguously distinguish B. abortus vaccine strain 104M. Results of our study indicate the assay allows accurate and reliable discrimination of 104M and non-104 M Brucella strains from common species and biovars of Brucella, Brucella vaccines, and other bacterial strains. The minimum detection limit of the assay was 220 fg for strain 104M and 76 fg for the single non-104 M Brucella strain tested. Compared to the classical isolation and identification approaches of bacteriology, this real-time PCR assay has substantial advantages in terms of simplicity and speed, and also reduces the potential for exposure to live Brucella. As real-time PCR instruments become more widely used in China, the approach will become widely applicable in routine diagnostics. The assay developed is therefore a simple, rapid, sensitive, and specific tool for brucellosis diagnosis and control.
Abbreviations
B. abortus: Brucella abortus; B. canis: Brucella canis; B. melitensis: Brucella melitensis; B. neotomae: Brucella neotomae; B. ovis: Brucella ovis; B. suis: Brucella suis; FAM: 6-carboxyfluorescein; MGB: Minor groove binder; SNP: Single nucleotide polymorphism-based; VIC: 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
YPC and WN conceived and designed the experiment. WN and YW designed the set of primers and probes. PT cultured all the Brucella and non-Brucella species. WN, LQ, YW and YZ carried out the experiment, including preparation of the bacterial genomic samples, sensitivity assay, specificity assay, reproducibility assay and detection of clinical field strains. WN and YQC analyzed the data and wrote the manuscript. KM and YPC verified the validity and checked the results. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate
Not Applicable

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Not Applicable

Competing interests
The authors declare that they have no competing interests.

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