Original Article

Development of an interferon-γ release assay (IGRA) for detection of *Brucella abortus* and clinical diagnosis of brucellosis

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Abstract
Introduction: Brucellosis, caused by *Brucella abortus (B. abortus)*, is an important zoonosis posing a great risk to both livestock and humans. Currently, most assays for clinical diagnosis of brucellosis have been developed based on serological principles; however, these assays have a number of limitations and disadvantages.
Methodology: To address this concern, the aim of this study was to develop a gamma interferon (IFN-γ) release assay (IGRA) for the diagnosis of brucellosis. Towards this end, the stimulating effect induced by different somatic antigens of *B. abortus* on the secretion of IFN-γ was evaluated.
Results: The best antigen candidate, *B. abortus* strain 2308, able to induce high levels of IFN-γ expression in peripheral blood (PB) cells from cattle, was used for the development of the IGRA. The optimal concentration for stimulation was determined as 1.0×10^7 CFU/mL. This study demonstrated that IFN-γ was detectable on day 5 post infection (p.i.) and peaked on day 14 p.i.. Finally, the IGRA developed was used for detection of *B. abortus* in clinical samples, and a higher level of IFN-γ was detected in *Brucella*-infected samples compared to vaccination samples and negative controls.
Conclusions: The optimal somatic antigen for *B. abortus* was identified and used to establish a robust IGRA. The IGRA developed is suitable for clinical diagnosis of brucellosis, especially in the early stages of infection.

Key words: *Brucella*; IGRA; brucellosis; IFN-γ; diagnostics.

Introduction

Brucellosis is a critical zoonotic disease caused by *B. abortus*, potentially leading to chronic multiple organ dysfunction and posing a high risk to livestock and public health [1]. Brucellosis is widely prevalent around the world, and nearly 170 countries or districts have so far reported an epidemic of this disease [2]. In the past decade, brucellosis re-emerged in China, as evidenced by increasing reports on epidemics of this disease. Although the rate of infection of *B. abortus* in animals and humans has been decreasing in recent years, it is still a great threat to animal husbandry and public health [3]. It is thought that prevention and control of brucellosis relies largely on reliable diagnosis. The existing methods for the diagnosis of brucellosis, however, have been developed based mainly on serology, such as the Rose Bengal test (RBT), standard tube agglutination test (SAT), complement fixation test (CFT), fluorescence polarization test (FPA) and enzyme-linked immunosorbent assay (ELISA) [4]. Non-specific reactions are common in these approaches since specific epitopes of *Brucella* and parasitic bacterium are localized in the cell [5].

Brucella is an intracellular bacterium, and this property elicits the host cellular immune response and accordingly, detection of the cellular immune response could be used to develop methods for the diagnosis of brucellosis. The most common method used in the clinic based on host cellular immune response is the *Brucella* skin delayed-type hypersensitivity (SDTH) test, which was developed to detect the allergic response in the host after subcutaneous injection of brucellins. It was reported that the rate of detection was 88% and 95% for the SAT and CFT assays [6]. It was demonstrated that the specificity of SDTH was higher than RBT and CFT when used in animals without vaccination with brucellosis vaccine [6]. IFN-γ is a significant cytokine that plays a crucial role in the host immune response. Previous studies demonstrated that the number of CD4^{+} cells and the expression of IFN-γ increased in the early stages of *B. abortus* infection [7-8]. Interestingly, the level of CD4, CD8 and CD21 in the blood of cattle increased when animals received the

Brucella S19 vaccine and were stimulated by the *Brucella* 2308 antigen. Meanwhile, the IFN-γ expression mediated by CD4 was dramatically increased [7,8].

Similarly, *Mycobacterium bovis* is an intracellular bacterial pathogen inducing high levels of IFN-γ in PB cells when animals are stimulated by tuberculin [9]. Therefore, the secretion of IFN-γ is an indicator for *Mycobacterium* infection, and was used to establish an IGRA, which was subsequently applied clinically for the detection of bovine tuberculosis [10]. At the present time, a commercial kit based on the detection of IFN-γ expression for diagnosis of *Mycobacterium bovis* is available.

IFN-γ is an indictor cytokine in the infection process of *Brucella* and *Mycobacterium*. The practical value has been confirmed for detection of *Mycobacterium*. The applicability of the IGRA test for detection of brucellosis will be investigated.

This aim of this study was to screen *B. abortus* antigens capable of stimulating PB cells to secret IFN-γ. The final goal was to establish an IGRA based on alterations in IFN-γ expression for the detection of *B. abortus*. To do this, four somatic antigens, including *B. abortus* 2308, *B. melitensis* 28, *B. suis* 1330 and *B. suis* 2 (vaccine strain), were prepared and used to stimulate PB cells collected from healthy and brucellosis-positive and -negative cattle. The stimulation of IFN-γ by the four antigens was compared and the optimum antigen was selected and used to establish the IGRA. Finally, clinical samples were screened using the IGRA to evaluate the developed method.

Methodology

Preparation and inactivation of somatic antigens

Four *B. abortus* strains, including *B. abortus* 2308, *B. melitensis* M28，*B. suis* S1330 and *B. suis* S2, were purchased from the China Veterinary Culture Collection Center (CVCC) and were grown in Tryptic Soy Broth media (Difco, Sparks, USA) at 37ºC with agitation (150 rpm) for three to four days. The bacteria were harvested and the cell pellet was re-suspended in phenolic-normal saline (0.9% NaCl buffer, 0.5% phenolic), and then inactivated at 80ºC for 30 minutes. Incubation of the inactivated *B. abortus* for three days on agar plates confirmed that the inactivation was successful.

Sample collection

The bovine peripheral bloods were provided by the Dairy Cow Center, Shandong Academy of Agricultural Science, China. The *Brucella*-positive bloods, verified by RBT, were collected from cattle without vaccination. *Brucella*-negative bloods were collected from healthy cattle at the Dairy Cow Center, Shandong Academy of Agricultural Science, China. Cattle were infected with 1×10^8 CFU of *B. abortus*, and bloods were collected on day 1, 3, 7, 10, 14, 21, 35 and 42 p.i.

Determination of the optimal concentration of antigen for IGRA

A serial dilution of the antigen was prepared and the concentration was determined, as previously described in the methodology. Subsequently, the PB cells collected from cattle were added to a 24-well plate with a volume of 1mL blood per well. The blood cells were stimulated by different concentrations of the aforementioned four somatic antigens, ranging from 1×10^5 CFU/100μl to 1×10^8 CFU/100μl of the final concentration. The blood cells were incubated for 18-24 h at 37ºC, and the culture supernatant was collected and tested for the expression of IFN-γ using the bovine IFN-γ indirect ELISA test kit (Bovigam 2G, Prionics, 63330, Schlieren-Zurich, Switzerland), according to the supplier's instructions. Briefly, the culture supernatant was diluted in sample dilution buffer in a ratio of 1:1 (supernatant:buffer), and was added into the antigen-coated plate in a volume of 100 μl per well. The plates were incubated at room temperature (25ºC) for 60 minutes and then washed 4 times, and 100 μl of conjugate reagent was added to each well, and then incubated for 60 minutes at room temperature. Plates were washed 4 times, and 100 μl of substrate solution was added to each well. After incubation for 30 minutes in a dark room at room temperature, 50 μl of stop solution was added. Reaction index (RI) values were measured using an automatic microplate reader. An RI higher than 0.8 was considered positive and an RI less than 0.2 was considered negative.

Evaluation of IFN-γ expression and Brucella antibodies

The level of IFN-γ expression and antibodies specific for *Brucella* were measured. The detection of IFN-γ is described above. The antibodies were detected by indirect ELISA based on *B. abortus* lipopolysaccharides (LPS). Briefly, a flat-bottomed 96-well polystyrene micro-titer plate (Nunc, DK4000, Roskilde, Denmark) was coated with 100 μl of LPS (10 μg/mL), from *B. suis* biotype 2, suspended in 0.05 mM sodium bicarbonate buffer (pH 9.6), and each plate was incubated at 4°C for 18 h. Plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST [pH 7.4]) and then blocked

with 5% gelatin from cold water fish skin (Sigma, St.Louis, MO 63103, USA) at 4°C for 16-18 h. After washing three times with PBST, serum samples were diluted in PBST in a ratio of 1:50 and added to the LPS-coated wells. The plates were incubated for 30 minutes at 37⁰C. For detection of IgG, 100 µl of a 1:10,000 dilution of rabbit anti-bovine IgG peroxidase-conjugated antibodies (A5295; Sigma, St. Louis, USA) was added to each well. Each plate was incubated for 30 minutes at 37°C and then washed twice with PBST. Substrate solution (100µl) containing 3, 3', 5, 5'-tetramethylbenzidine was added to each well and incubated for 15 minutes in a dark room at room temperature. The reaction was stopped by adding of 100 µl 1M HCl to each well. The optical density $(OD)_{450}$ values were measured using an automatic microplate reader (SPECTRA MAX190, Molecular Devices LLC., Sunnyvale, USA). The percentage of inhibition values were calculated using the following formula: $P\% = OD_{450nm}$ sample value$/OD_{450nm}$ positive control$\times 100\%$. The recommended cutoff value was

20% for IgG. Samples with a P% equivalent to or higher than 20% were considered to have specific antibodies against *Brucella* LPS.

Detection of IFN-γ in clinical samples

The clinical bovine PB from *Brucella*-infected, vaccinated or negative control cattle were stimulated respectively with the optimal antigen concentration determined above; a total of 24 samples were collected from three groups and used subsequently to verify the reliability of the IGRA method. The IGRA approach was assessed by comparison of the level of IFN-γ expression. Samples were incubated at 37°C for 16-24 hours, the supernatant collected and the level of IFN-γ release was detected as described above.

Statistical analysis

The level of IFN-γ expression in cattle was analyzed for statistical significance using a repeated measures *t* test (GraphPad 5.0). A p-value of < 0.05 was considered significant.

Figure 1. Comparison of IFN-γ expression between brucellosis-negative and -positive peripheral blood cells of cattle stimulated by different antigens. The somatic antigens included *B. abortus* 2308 (A), *B. suis* S2 (B), *B. suis* 1330 (C) and *B. meiltensis* 28 (D).
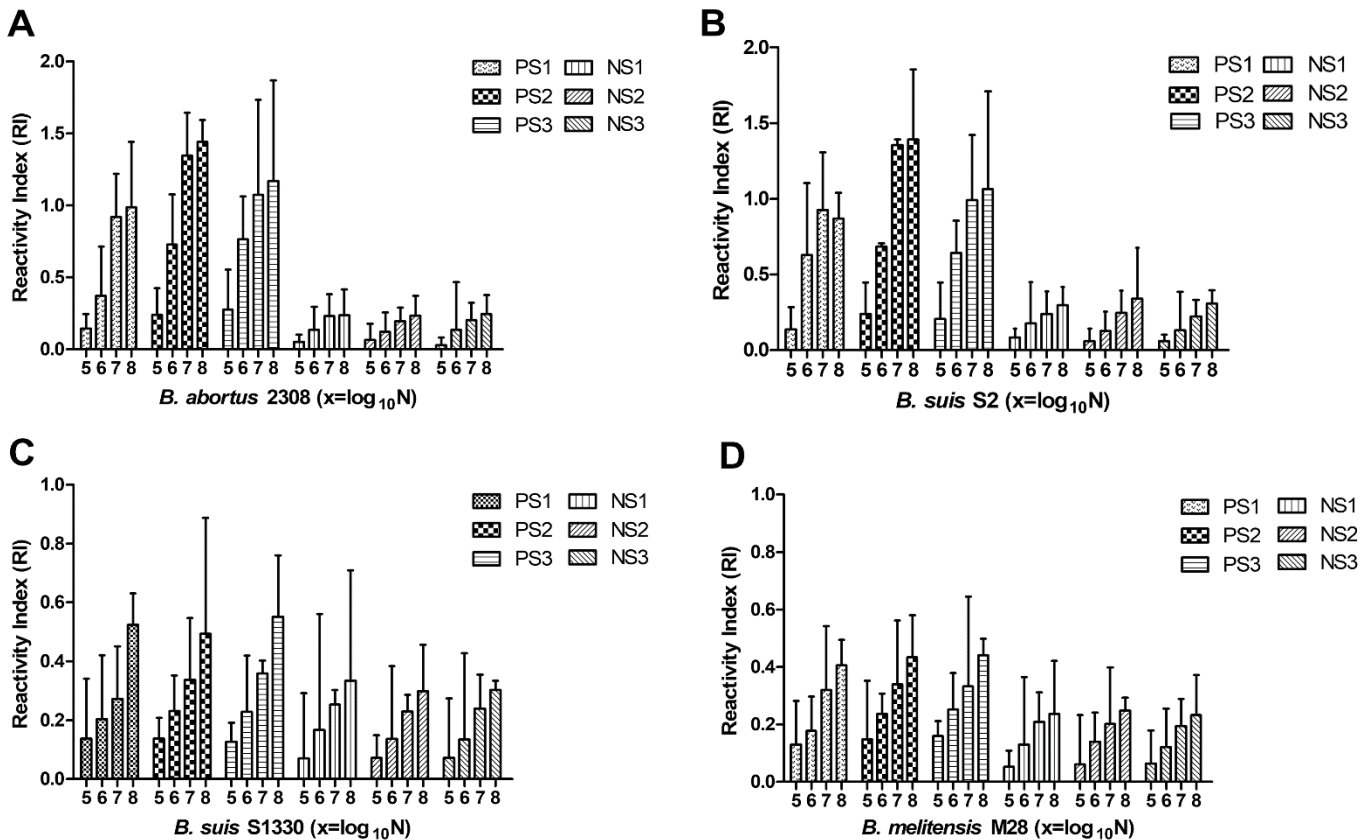
PS represents positive samples, NS represents negative samples. The vertical bars represent 95% confidence interval.

Ethics statement

All experiments were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals published by the Ministry of Science and Technology, China in 2004 and approved by the Animal Care and Use Committee from the China Institute of Veterinary Drug Control, Beijing, China.

Results

Comparison of IFN-γ secretion induced by different B. abortus antigens

In an effort to establish an IGRA for the diagnosis of brucellosis, four *Brucella* strains, including *B. abortus* 2308, *B. melitensis* M28, *B. suis* S1330 and *B. suis* S2 were selected. These strains were derived from *B. abortus*, *B. melitensis* and *B. suis* which are common across the world. The *B. suis* S2 is widely used to control and prevent brucellosis in China. The optimal concentration of these four somatic antigens was first determined. Our results demonstrate that the concentration of *Brucella* antigens was 2.5×10^9 CFU/mL when the optical density value of OD_{600} was 1.0. The somatic antigens were diluted to a concentration of 1×10^9 CFU/mL before use. Subsequently, the antigens were used to stimulate PB cells collected from brucellosis-negative and -positive cattle for secretion of IFN-γ. The stimulatory ability of antigens was represented by their RI, which was indicative of IFN-γ production. As shown in Figure 1, the four somatic antigens were able to induce production of IFN-γ, albeit the level of IFN-γ induced varied across the four antigen groups. As expected, the results indicated that, overall, the level of IFN-γ secretion was dependent on the concentration of antigen, as higher RI values were obtained when higher concentrations of antigens were used (Figure 1). For blood cells collected from *Brucella*-positive samples (PS), the RIs in A2308- or S2-stimulation groups were approximately 2-fold higher than that of S1330- or M28-stimulation groups when the same concentration of antigen was used (Figure 1, compare 1A and1B to 2A and 2B), indicating that *Brucella* strains A2308 and S2 were able to induce higher level of IFN-γ, compared to S1330 and M28. Significantly, when blood cells from *Brucella*-negative samples (NS) were stimulated by the four antigens, all RIs detected in the four groups were significantly lower than those detected in the *Brucella*-positive samples (PS) (Figure 1, compare PS and NS in each panel). Furthermore, for NS samples, the RIs detected in S1330 and M28 groups were slightly higher

Figure 2. Evaluation of IFN-γ secretion in peripheral blood cells infected with *B. abortus* 2308 in IGRA.
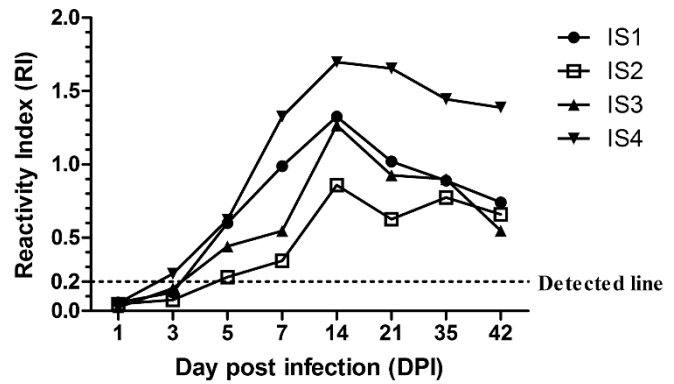
IS represents infected sample. The horizontal dashed line represents the cut-off value (RI > 0.2), above the threshold, IFN-γ was considered present in samples.

than those detected in A2308 and S2 groups (Figure 1, compare C and D panels with A and B panels). This result is contrasts with the results obtained in the PS groups where RI values in A2308 and S2 groups were higher than those of S1330 and M28 groups. As shown in Figure 1, when 1×10^8 CFU/mL of antigen was used, no significant difference in IFN-γ expression was detected among the four groups (Figure 1). In order to avoid false positives and reduce antigen loading, a concentration of 1×10^7 CFU/mL antigen was selected for stimulation.

Detection of IFN-γ expression induced by B. abortus 2308

Currently, the majority of methods for brucellosis detection are based on serological reactions. In order to evaluate the level of IFN-γ expression and verify the effect induced by stimulation, four cattle were infected with *B. abortus* 2308. The results revealed that IFN-γ was detectable on day 5 p.i. (RI>0.2, Figure 2). The level of IFN-γ expression increased rapidly over time, and peaked on day 14 p.i (up to 1.7) before decreasing slowly over time. However, IFN-γ was still detectable on day 42 p.i. (Figure 2). This result suggested that IFN-γ was secreted at an early time from PB cells of cattle after *B. abortus* infection, and the secretion of IFN-γ lasted as long as 42 days.

Detection of antibody produced after B. abortus 2308 infection

Contrary to IFN-γ, the antibody against *Brucella* was detectable (value higher than the cutoff) at a later time after infection with the same *B. abortus* strain 2308

(Figure 3). The results showed that the earliest time the antibody could be detected was 21 days p.i.. However, it increased over time and peaked on day 42 p.i. (Figure 3).

*Evaluation and application of IGRA for clinical samples*

The effect of IGRA for clinical samples, which were infected, vaccinated and non-infected with *B. abortus* was evaluated. A large amount of IFN-γ was produced in the PB cells from infected samples, as illustrated by the high RI value (averaged to 1.0), compared to that of the vaccinated group (with averaged RI value 0.8) ($p < 0.01$, as shown in Figure 4). As expected, IFN-γ was minimal in the negative control group, where PB cells were collected from healthy and non-vaccinated cattle (Figure 4).

Discussion

The role of IFN-γ in the host immune response is diverse, and the importance of this cytokine in the innate immune defense has been well documented [11]. Suboptimal concentration of IFN-γ does not trigger immune cells, instead, it primes for subsequent response upon stimulation, but, in excess, can eventually cause deleterious consequences. This IFN-γ priming effect has been increasingly implicated in the immune response to several infectious diseases, including viral, bacterial, and parasitic diseases [12-14]. For brucellosis, the crucial role of IFN-γ was recognized, as it was shown that IFN-γ knockout mice died due to *B. abortus* infection and IFN-γ producing CD4+ T-cells from infected donors were able to protect the recipient mice against challenge with *B. abortus* [15]. IFN-γ was induced by reactive nitrogen intermediates and reactive oxygen intermediates after mouse macrophages were activated [16]. In addition, IFN-γ persists throughout the whole infection process and is the mechanism by which hosts resisted *B. abortus* infection.

Brucella is an intracellular bacterium, and the cellular immune response is the major mechanism for the host to counteract *B. abortus* [17]. However, most of the diagnostic methods available were developed based on the humoral immune responses [18]. However, in the later stage of vaccination or the early period of *B. abortus* infection, antibodies induced by *B. abortus* infection were undetectable. Therefore, it is unlikely that brucellosis antibodies can be detected using serological methods in the early stages of infection. IFN-γ is a significant cytokine that plays a critical role in the host immune response after infection

Figure 3. Measurement of *Brucella* specific antibodies after infection of *B. abortus* 2308 in an indirect ELISA.
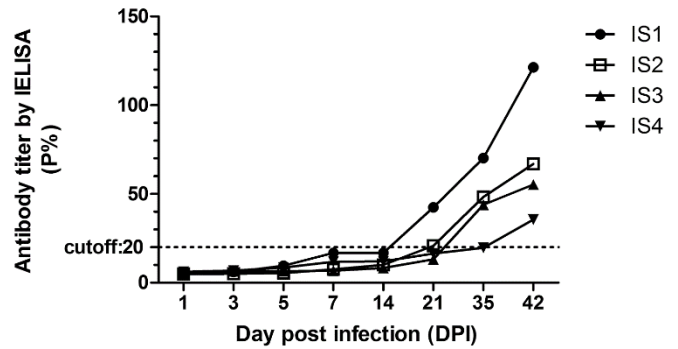

IS represents infected samples collected from different individuals. The horizontal dashed line represents the cutoff value (RI>0.2), above the cutoff, samples were considered brucellosis-positive.

with *B. abortus*, hence a diagnostic method for *B. abortus* detection based on IFN-γ expression has potential.

To develop an IGRA for detecting *B. abortus*, in this study four somatic *B. abortus* antigens were evaluated and the antigen 2308 was optimal, and was subsequently used as stimulator for inducing IFN-γ. An IGRA has been established based on detecting IFN-γ expression. It has remained unclear why *B. abortus* was capable of stimulating host cells to induce a higher level of IFN-γ. This might be related to LPS or some non-structural proteins. In addition, the concentration of somatic antigens was closely related to IFN-γ expression, nevertheless, we demonstrated that the level

Figure 4. The IFN-γ expression in the *Brucella*-infected, -vaccinated and -negative groups.
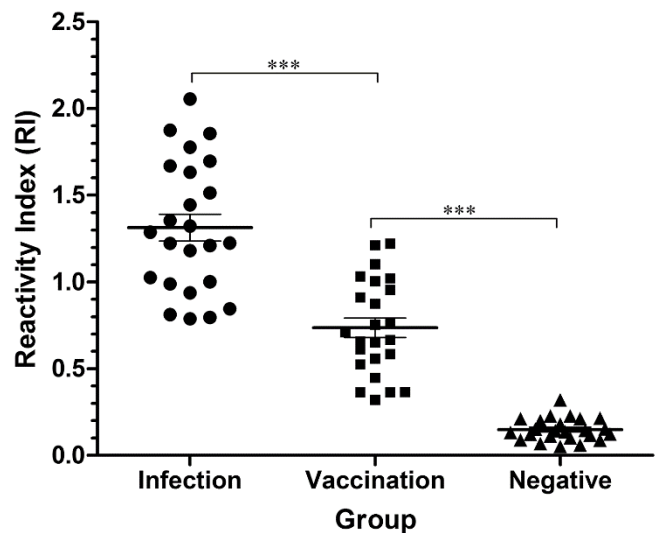
Significant differences between groups are indicated by *p < 0.05, **p < 0.01, ***p < 0.0001 (*t* test). The vertical bars represent a 95% confidence interval.

of IFN-γ expression remained unaltered when the concentration of *B. abortus* was higher than 1×10^7 CFU/mL. Thus, we chose 1×10^7 CFU/mL as the optimal concentration of *B. abortus* 2308 for stimulation and the development of an IGRA.

Intriguingly, there was a significant difference in the initiation of the host cellular immune response and humoral response. This study showed that the host cellular immune response initiated earlier than the humoral response (compare Figure 2 and Figure 3). Some researchers have reported that the level of antibodies, IgG1 subtype in particular, peaked 28-42 days after vaccination with *B. abortus* S19 [19]. However, in this study, for infected individuals, the level of induced antibodies continued to increase, but in the acute infection period, the antibodies were not detected. This indicated that detection methods based on the humoral response have some limitations. Comparatively, IFN-γ secreted in the period of acute *B. abortus* infection peaked as early as 14 days p.i. Therefore, IGRA can be used to detect *B. abortus* at an early stage, including acute infection.

In addition, an interesting finding in this study was that the level of IFN-γ expression in the infected group was significantly higher than the vaccinated group (Figure 4). The explanation for this could be that: 1) the virulent *B. abortus* wild type strain replicated more efficiently than that of the vaccine strain; 2) The wild type strain persisted for a longer time in the host cell, resulting in a stronger immune response, compared to the vaccine strain. This biological property of *B. abortus* facilitates the detection of IFN-γ induced in the host cell after infection, leading to the establishment of IGRA, as described in the present study. It should be noted that the host immune response induced by *B. abortus* is weaker than that induced by *Mycobacterium bovis*. Therefore, the bovine IFN-γ indirect ELISA test kit, which was developed for *Mycobacterium bovis* detection, might not be sensitive enough for detection of *B. abortus* detection. As there was no commercially available kit, we used it as an alternative. There is an urgent need to develop a reliable approach for clinical detection of brucellosis. The IGRA established in this study provides a robust method for diagnosis of brucellosis. Additionally, our IGRA could serve as a potential method to differentiate *B. abortus* from natural infection and vaccination.

Conclusion

B. abortus 2308 somatic antigen is an ideal stimulator for induction of IFN-γ, and the optimal concentration of 2308 for stimulation is 1.0×10^7 CFU/mL. IFN-γ was secreted on day 5 p.i. and peaked on day 14 p.i.. The level of IFN-γ expression in the *Brucella*-infected group was highest, followed by the vaccinated group. IFN-γ expression was not detected in the *Brucella*-negative group.

Authors contributions
YF performed the experiments, analyzed the data (including statistical analysis), and drafted the manuscript. LQZ analyzed the data, drafted the manuscript, HJ and XWP treated the samples and performed the experiments. GZ prepared and provided the reagents exclusively for this study. YXD and JBD performed the experiments, analyzed the data, and edited and finalized the manuscript. All authors read and approved the final manuscript.

References
1. Corbell MJ (1989) Brucellosis: Epidemiology and prevalence worldwide. In Young EJ, Corbel MJ, editors. Brucellosis: Clinical and Laboratory Aspects, Boca Raton: CRC Press. 25-40.
2. Avila-Calderón ED, Lopez-Merino A, Sriranganathan N, Boyle SM, Contreras-Rodríguez A (2013) A history of the development of Brucella vaccines. Biomed Res Int 2: 743509.
3. Zhang X, Wang Z, Mu G, Wang T (2015) Brucellosis control in northeast China: a long way to go. Public Health 129: 1132-1134.
4. Corrente M, Desario C, Parisi A, Grandolfo E, Scaltrito D, Vesco G, Colao V, Buonavoglia D (2015) Serological diagnosis of bovine brucellosis using B. melitensis strain B115. J Microbiol Methods 119: 106-109.
5. Wareth G, Eravci M, Weise C, Roesler U, Melzer F, Sprague LD, Neubauer H, Murugaiyan J (2016) Comprehensive identification of immunodominant proteins of *Brucella abortus* and *Brucella melitensis* using antibodies in the sera from naturally infected hosts. Int J Mol Sci 17: 659.
6. International Office of Epizootics. Biological Standards Commission, International Office of Epizootics. International Committee. (2008) Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees. Office international des épizooties. 1092-1106.Avaliable: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.04_BRUCELLOSIS.pdf. Accessed 22 November 2017.
7. Zhu L, Feng Y, Zhang G, Jiang H, Zhang Z, Wang N, Ding J, Suo X (2016) *Brucella suis* strain 2 vaccine is safe and protective against heterologous *Brucella* spp. infections. Vaccine 34: 395-400.
8. Dorneles EM, Lima GK, Teixeira-Carvalho A, Araújo MS, Martins-Filho OA, Sriranganathan N, Al Qublan H, Heinemann MB, Lage AP (2015) Immune response of calves

vaccinated with *Brucella abortus* S19 or RB51 and revaccinated with RB51. PloS one 10: e0136696.

9. Sakai S, Kauffman KD, Sallin MA, Sharpe AH, Young HA, Ganusov VV, Barber DL (2016) CD4 T cell-derived IFN-γ plays a minimal role in control of pulmonary *Mycobacterium tuberculosis* infection and must be actively repressed by PD-1 to prevent lethal disease. PLoS Pathog 12: e1005667.
10. Clifford V, He Y, Zufferey C, Connell T, Curtis N (2015) Interferon gamma release assays for monitoring the response to treatment for tuberculosis: A systematic review. Tuberculosis (Edinb) 95: 639-650.
11. Boehm U, Klamp T, Groot M, Howard JC (1997) Cellular responses to interferon-gamma. Annu Rev Immunol 15: 749-795.
12. Nansen A, Christensen JP, Marker O, Thomsen AR (1997) Sensitization to lipopolysaccharide in mice with asymptomatic viral infection: role of T cell-dependent production of interferon-gamma. J Infect Dis 176: 151-157.
13. Fejér G, Szalay K, Gyory I, Fejes M, Kúsz E, Nedieanu S, Páli T, Schmidt T, Siklódi B, Lázár G Sr, Duda, E (2005) Adenovirus infection dramatically augments lipopolysaccharide-induced TNF production and sensitizes to lethal shock. J Immunol 175: 1498-1506.
14. Kalis C, Gumenscheimer M, Freudenberg N, Tchaptchet S, Fejer G, Heit A, Akira S, Galanos C, Freudenberg MA (2005) Requirement for TLR9 in the immunomodulatory activity of *Propionibacterium acnes*. J Immunol 174: 4295-4300.
15. Brandão AP, Oliveira FS, Carvalho NB, Vieira LQ, Azevedo V, Macedo GC, Oliveira SC (2012) Host susceptibility to *Brucella abortus* infection is more pronounced in IFN-γ knockout than IL-12/β2-microglobulin double-deficient mice. Cli Dev Immunol 2012: 589494.
16. Barquero-Calvo E, Chaves-Olarte E, Weiss DS, Guzmán-Verri C, Chacón-Diaz C, Rucavado A, Moriyón I, Moreno E (2007) *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. PLoS One 2: e631.
17. Vitry MA, Hanot Mambres D, De Trez C, Akira S, Ryffel B, Letesson JJ, Muraille E (2014) Humoral immunity and CD4+ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*. J Immunol 192: 3740-3752.
18. Ducrotoy MJ, Conde-Álvarez R, Blasco JM, Moriyón I (2016) A review of the basis of the immunological diagnosis of ruminant brucellosis. Vet Immunol Immunopathol 171: 81-102.
19. Enright FM (1990) Animal brucellosis. In Nielsen K, Duncan Jr, editors. Boston: CRC Press. 301-320.

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