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The Bactericidal Effect of High Temperature Is an Essential Resistance Mechanism of Chicken Macrophage against *Brucella abortus* Infection

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Knowledge of avian host responses to brucellosis is critical to understanding how birds resist this infection; however, this mechanism is not well established. On the other hand, temperature has a major involvement in the physiology of living organisms, and cell death induced by heat is attributed to protein denaturation. This study demonstrates the direct bactericidal effect of a high temperature (41°C) on *Brucella abortus* that resulted in the gradual reduction of intracellular bacteria and inhibited bacterial growth within avian macrophage HD11 in an increasing period of time. On the other hand, this study also revealed that high temperature does not affect the rate of bacterial uptake, as confirmed by the bacterial adherence assay. No significant difference was observed in the expression of target genes between infected and uninfected cells for both temperatures. This study suggests the susceptibility of *B. abortus* to bacterial death under a high temperature with an increased period of incubation, leading to suppression of bacterial growth.

Keywords: *B. abortus*, avian macrophage, bactericidal effect, body temperature

Introduction

Innate and adaptive immune host responses are critical into the understanding of how certain species resist certain diseases. However, the molecular basis of *Brucella* and chicken host interaction is not well elucidated to date [1]. Innate resistance to diseases involves a complex process that could be due to an inherent multigenic trait characterized in a particular species [2]. On the other hand, temperature is highly involved in an array of intricate physiologic activities throughout evolution [3]. High body temperature has precluded the occurrence of several infectious diseases in avian species. For example, Blackleg disease in cattle is prevented in poultry owing to this temperature difference [4]. It has been demonstrated that hyperthermia can reduce the proliferation of pathogens, specifically bacteria, which can be considered a major advantage to the host. It has long been recognized that birds would respond to infectious agents with a significant increase in the core body temperature [5]. Researchers have utilized lipopolysaccharide (LPS) as the most common form of pathogen-associated molecular patterns to trigger the immune system and to investigate the dynamics of hyperthermia in this species [5]. Phagocytic activity is considered one of the most important conserved evolutionary functions. Specific receptors are present on the surface of avian macrophages essential for the uptake of bacteria as well as receptors intended to mediate activation signals for LPS-binding receptors, including mannose, Fc receptors, as well as scavenger receptors [6]. Although disease resistance to various infectious agents has been recognized as a multifaceted trait in avian species, the precise pathogenic and resistance mechanisms of avian brucellosis are not yet understood.

Several insights into the relationship of temperature with bacterial replication have been explored [3]. A number of studies relating to thermal adaptation being dependent on protein stability in relation to temperature have provided sufficient data indicating that bacterial growth is precipitously diminished upon increasing temperature [7, 8]. Optimal bacterial growth is achieved only when products of rate-determining proteins are intact. In one study, the effect of

temperature was evaluated on the functionality of proteins that are responsible for replication and concluded that a high temperature causes a significant decrease of folded or functional rate-determining protein, leading to inhibition of bacterial growth [3]. On the extreme end, the lethal denaturation temperature was also determined when bacterial proteins were denatured upon incubation with higher temperature in reference to 37°C, which is considered to be an original evolutionary temperature [3].

The goal of this study was to determine the effect of temperature directly on *Brucella abortus* and in chicken macrophages incubated at a normothermic temperature of 37°C and a higher temperature of 41°C, in relation to the bacterial invasion and intracellular replication of *B. abortus* that would likely simulate the mammalian and avian general core body temperature, respectively. In addition, we assessed the immune response by assessing the expression of chicken proinflammatory cytokines, such as IL-1β, IL-6, and IFN-α, in *B. abortus*-infected and uninfected cells through the use of quantitative RT-PCR.

Materials and Methods

Bacteria and Cell Cultures

Standard wild-type strains were derived from *B. abortus* 544 (ATCC 23448), a smooth, virulent *B. abortus* biovar 1 strain. The *B. abortus* strain was cultivated in Brucella broth or on Brucella agar. Bacteria were grown at 37°C with vigorous shaking until they reached the stationary phase. HD11 cells, an avian macrophage cell line established according to a previously reported method [9], were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Gibco, USA) at 37°C and 41°C at 5% CO_2 atmosphere.

Bactericidal Analysis

Bacteria were diluted with PBS to a concentration of 2×10^4 colony forming units (CFU)/ml, and incubated at 37°C or 41°C for 0, 4, 24, 48, and 72 h. Each diluent was plated onto Brucella agar and incubated for 2 days at 37°C. The bacterial survival rates were expressed as the percentage of the survival of the sample incubated at 41°C relative to the control that was incubated at 37°C and set at 100% [10].

Cytotoxicity Assay

The cytotoxic effect of temperature at 37°C and 41°C was evaluated by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Furthermore, to evaluate the cytotoxicity of temperature with infection in HD11, overnight culture at 37°C and 41°C in 96-well plates at 2×10^4 cells/well were infected with *B. abortus* at a multiplicity of infection (MOI) of 10, and then centrifuged at 150 ×*g* for 10 min at room temperature. The infected cells were washed once with PBS and then incubated at 37°C and 41°C with DMEM containing 10% (v/v) FBS and gentamicin (30 μg/ml) for 30 min to kill any remaining extracellular bacteria, and then incubated at 37°C and 41°C in 5% CO_2 for 4, 24, 48, and 72 h. At the end of each incubation, the cytotoxic effect was evaluated using MTT assay [11]. The viability rate of infected HD11 was expressed as the percentage of the absorbance of the sample relative to uninfected cells incubated at 37°C at 0 h, which was set at 100%.

Determination of Uptake and Survival of *B. abortus* in Chicken Macrophages

HD11 cells, overnight culture in 96-well plates at 2×10^4 cells/well, were infected with *B. abortus*. The bacteria were then deposited onto the cells at a MOI of 100, centrifuged at 150 ×*g* for 10 min at room temperature, and incubated at 37°C or 41°C in 5% CO_2 for 0, 15, and 30 min. The infected cells were washed once with PBS and then incubated at 37°C or 41°C in DMEM containing 10% FBS and gentamicin (30 μg/ml) for 30 min. The cells were washed twice with PBS and lysed with distilled water. For test of intracellular growth efficiency, bacterial infection was done as described above for bacterial internalization. Infected cells were incubated for 2, 24, and 48 h with DMEM containing 10% FBS and gentamicin (30 μg/ml). The same protocol for washing, lysis, and plating was conducted to analyze the efficiency of bacterial internalization as conducted according to a previously reported method [12].

Bacterial Adherence Assay

HD11 cells were cultured in 12-well plates in 18 mm glass coverslips, incubated overnight at 37°C or 41°C. Cells were treated with a final concentration of 1.25 μg/ml cytochalasin D by adding 2.5 μl (500 μg/ml) to inhibit bacterial internalization 40 min prior to infection. Cells were infected with an MOI of 10 for 30 min, fixed with 4% paraformaldehyde and incubated at 37°C for 1 h, then permeabilized with 0.1% Triton X-100 at 22°C for 10 min followed by incubation with blocking buffer (2% goat serum in PBS) for 30 min. A series of further incubation with anti-*B. abortus* polyclonal rabbit serum in blocking buffer and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma-Aldrich, USA) in blocking buffer at 37°C for 1 h were conducted. The coverslips were washed and mounted with a fluorescent solution. A laser scanning microscope (FV1000; Olympus, Japan) was used to capture the fluorescence images. One hundred macrophages were chosen randomly and adhered bacteria were counted [10].

Intracellular Bacterial Staining

Intracellular bacterial staining was conducted with slight modification [10]. HD11 cells incubated at 37°C or 41°C overnight were infected for 1 h at 37°C or 41°C, washed with medium, and further incubated in DMEM containing 10% FBS and gentamicin (30 μg/ml) for 24 and 48 h for both temperatures. Fixation,

permeabilization, and blocking protocols were conducted as with the bacterial adherence assay. Fluorescence images were processed with FV10-ASW Viewer 3.1 software. One hundred macrophages were chosen randomly and intracellular bacteria were counted. The total number of intracellular bacteria in 100 random macrophages incubated at 37°C was set at 100%.

RNA Extraction and cDNA Synthesis

HD11 cells at 2 × 10^6 cells/well in 6-well culture plates were cultured overnight at 37°C or 41°C at 5% CO_2. The cells were infected with *B. abortus* at a MOI of 10, centrifuged at 150 ×*g* for 10 min at room temperature, and incubated at 37°C or 41°C in 5% CO_2 for 30 min. The infected cells were washed once with PBS and then incubated at 37°C or 41°C at 5% CO_2 in DMEM containing 10% FBS and gentamicin (30 μg/ml) at indicated times (12, 24, and 48 h) prior to total RNA harvest. Total RNA was extracted from HD11 cells with the use of RiboEx reagent (Geneall, South Korea) and a commercially available reagent RNeasy Mini kit (Qiagen, Germany) [13]. To remove any contaminating genomic DNA, the sample was treated with RNase-free DNase I (Qiagen, Korea). Purified RNA was eluted in 15 μl of RNase-free water and stored at −70°C. Total RNA was measured using a spectrophotometer (Optizen, Korea). Single stranded cDNA was synthesized from 1−2 μg of total RNA using a Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Real-Time RT-PCR

Real-time RT-PCR was performed in duplicate using a CFX96 real-time RT-PCR system (Bio-Rad, USA) with SYBR Green (Bioneer, Korea). Expression levels of target genes were determined using the following primers: IL-1β forward 5′-TGGGCATCAAGGGCTACA-3′, reverse 5′-TCGGGTTGGTTGGTGATG-3′; IFN-α forward 5′-GAC ATCCTTCAGCATCTCTTCA-3′, reverse 5′-AGGCGCTGTAAT CGTTGTCT-3′ [14]; and IL-6 forward 5′-GCGAGAACAGCA TGGAGATG-3′, reverse 5′-GTAGGTCTGAAAGGCGAACAG-3′ [15]. Gene expression levels were quantified using $\Delta\Delta C_t$ [16]. A melting curve was obtained at the end of each run to rule out the presence of primer dimers. β-Actin (primers, forward 5′-CAC AGATCATGTTTGAGACCTT-3′ and reverse 5′-CATCACAAT ACCAGTGGTACG-3′) was used as the reference gene to normalize the relative expression levels of individual transcripts with the use of Bio-Rad CFX software.

Statistical Analysis

Gathered data were statistically analyzed using Student's *t*-test or one-way ANOVA, using Instat software (GraphPad, USA), followed by Dunnet's multiple comparison test. The mRNA levels were analyzed using Mann-Whitney, Kruskal-Wallis, and Dunn's multiple comparison tests. Data were considered statistically significant at $p < 0.05$. Data were expressed as the mean ± standard error (SE).

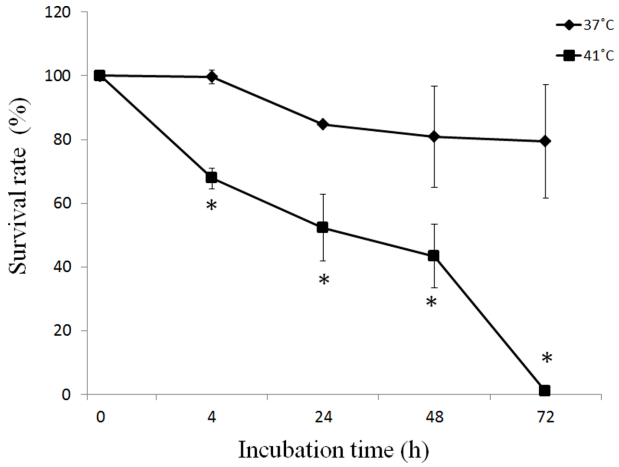

Fig. 1. Determination of the effect of temperature on *B. abortus* and HD11 cells.

Bactericidal effect of temperature at specified time points. Bacterial viability was assessed through CFU counting on culture plates, and the rate of bacterial viability was compared with the zero time point at 37°C (Control). Data represent the mean ± SD of triplicate samples from three identical experiments. Statistically significant difference relative to control is indicated by an asterisk (*, $p < 0.05$).

Results

Bactericidal and Cytotoxicity Assays

Evaluation of bacterial viability at 37°C and 41°C resulted in a significant difference in bacterial viability at the 0, 4, 24, 48, and 72 h time points. Incubation at 41°C markedly resulted in decreased bacterial CFU count with viability of 67.84%, 52%, 43.41%, and 1% at 4, 24, 48, and 72 h, respectively, as opposed to incubation at 37°C with viability of 99.74%, 84.72%, 80.84%, and 79.49% at 4, 24, 48, and 72 h, respectively ($p < 0.05$) (Fig. 1). On the other hand, evaluation on the cytotoxicity of temperature on HD11 was found to have no difference between incubation at 37°C and 41°C in both uninfected (Fig. 2A) and infected cells (Fig. 2B).

Determination of Uptake and Survival of *B. abortus* in Chicken Macrophages

The results indicated that *B. abortus* invasion at 41°C was not significantly different at 0, 15, and 30 min post-infection (Fig. 3A). In the intracellular survival assay, intracellular replication was significantly reduced by 51% and 59% at 24 and 48 h, respectively, while no bacterial growth was observed at 72 h incubation (Fig. 3B). The intracellular bacterial load was significantly decreased according to the

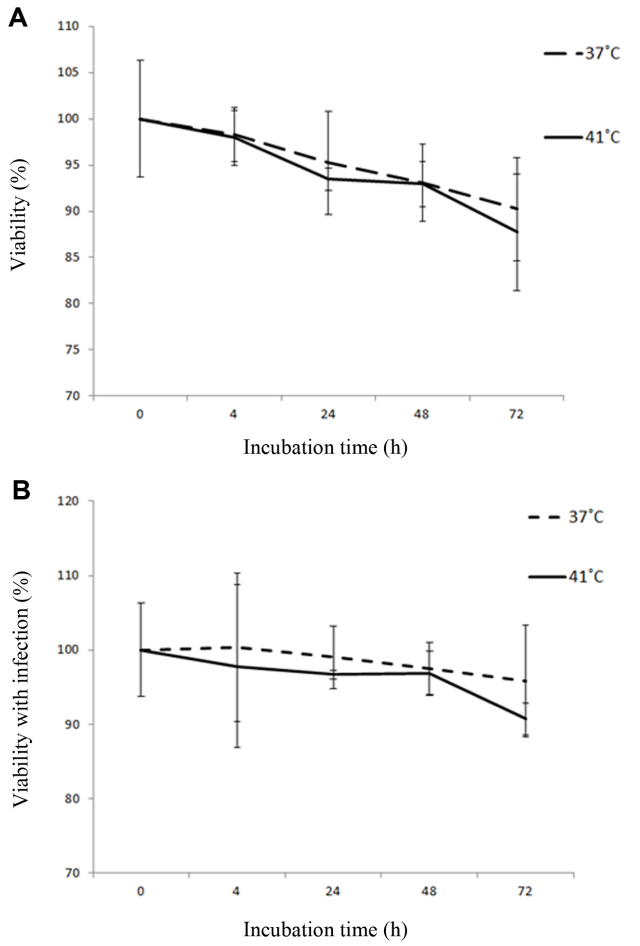

Fig. 2. Determination of the effect of temperature on HD11.
(**A**) Viability of HD11 upon incubation at 0, 4, 24, 48, and 72 h. (**B**) Viability of HD11 after infection with *B. abortus* at 0, 4, 24, 48, and 72 h using the MTT assay. Data represent the mean ± SD of triplicate samples from three identical experiments. Statistically significant difference relative to control (37°C) is indicated by asterisk (*, $p < 0.05$).

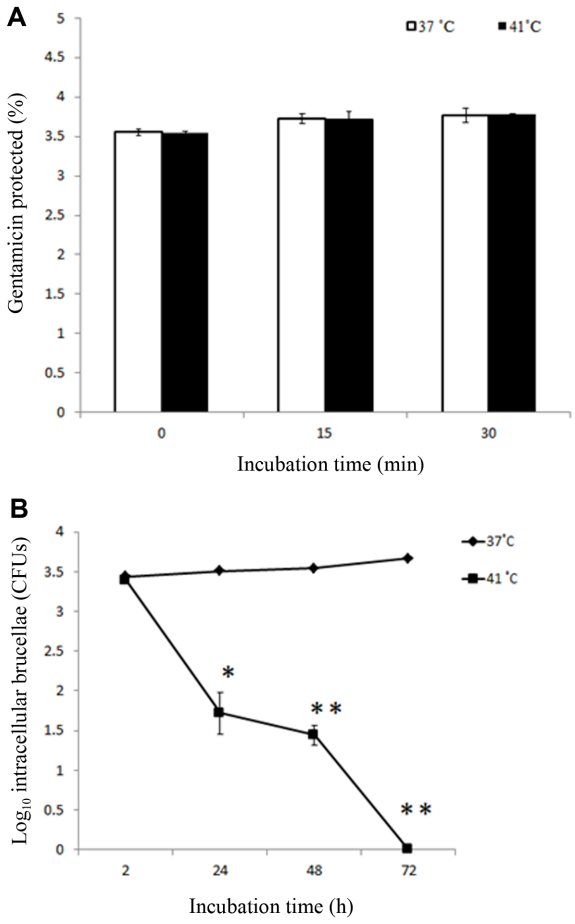

Fig. 3. Effect of temperature on bacterial uptake and intracellular survival within macrophages.
After bacterial infection, (**A**) bacterial internalization and (**B**) intracellular growth of *B. abortus* in HD11 macrophages were evaluated. Data represent the mean ± SD of triplicate samples from three identical experiments. Statistically significant difference relative to control (37°C) is indicated by asterisks (*, $p < 0.01$; **, $p < 0.001$). Values represent the means of three identical experiments performed in triplicate; error bars indicate SD.

relative bactericidal effect of temperature at 41°C. Incubation at 37°C, however, was able to retain a very slow intracellular growth.

Bacterial Adherence Assay and Intracellular Bacterial Staining

Quantification of adhered bacteria showed no difference in cells incubated at 37°C and 41°C. However, there was significant reduction in the number of intracellular bacteria in cells incubated at 41°C after 24 and 48 h incubation (Figs. 4A–4D).

Real-Time RT-PCR

Relative mRNA expression levels of IL-1β, IL-6, and IFN-α were evaluated at 37°C and 41°C, at time points 12, 24, and 48 h. This study demonstrated that there was no significant difference in the levels of expression among the cytokines in the presence or absence of *Brucella* infection. However, there was slight increase in the expression of these cytokines in cells at 41°C, particularly IL-1β at 24 h with or without infection.

Discussion

High *Brucella* antibody titers in birds indicate that natural

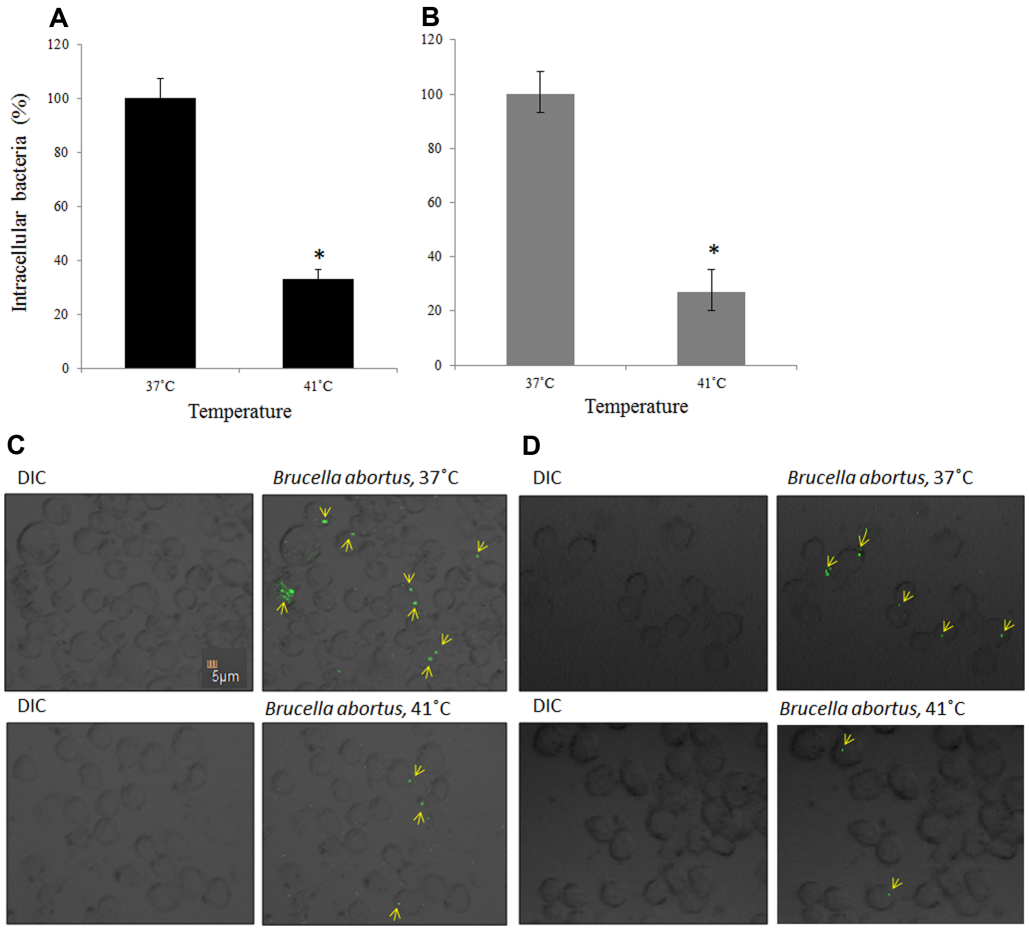

Fig. 4. Staining and quantification of intracellular bacteria under the different temperatures.
The total number of intracellular bacteria in 100 random macrophages incubated at 37°C or 41°C after 24 (**A**, **C**) and 48 h (**B**, **D**) post-infection was counted, where the bacterial number of 37°C incubation was set at 100%. Representative images of intracellular bacteria are shown in arrows. Statistically significant difference relative to control (37°C) is indicated by the asterisk (*, $p < 0.001$). Values represent means of three identical experiments performed in triplicate; error bars indicate SD.

infection can occur through exposure to the primary hosts of *Brucella*, but this exposure does not necessarily lead to chronic infection. Birds are also able to shed the organism in their droppings, making them a reservoir and potential risk for humans [17]. In this study, we utilized HD11, a chicken macrophage-like cell line that has been recognized to highly express cell surface antigens, F_c receptors, and exhibit phagocytic activity [9]. The phagocytic system is activated at the early chick embryonic life, but certain factors may influence the increased age-related resistance of embryos to *B. abortus* [18]. Interestingly, after hatching, chicks become highly resistant to *B. abortus* and are able to eliminate the infection in a matter of weeks [18].

We compared and simulated the effect of a major physiological variation of birds over mammals, which is core body temperature, through the use of HD11 in vitro. *B. abortus* is a mesophilic bacterium that grows at 37°C optimum temperature, although this could range from 20°C to 40°C and even up to 44°C [19].

In this study, *B. abortus* was shown to be increasingly sensitive to a higher temperature over an extended period of time. In the bactericidal assay, incubation of *B. abortus* at 41°C, at 24 to 48 h time points, rendered about 50% lethality, and was absolutely bactericidal at 72 h relative to the control incubated at 37°C. Several studies, including Chen and Shakhnovich [3], have elucidated the effect of higher temperature on the viability and ability of prokaryotes to replicate. Higher temperature has a great impact on bacterial protein stability and its ability to replicate and survive. The unfolding of key proteins due to thermal changes can lead to a lethal phenotype [20]. The importance of proteins being able to maintain their native

conformation (folded state) is essential for them to be functional [21–24]. Another interesting study by Leuenberger *et al.* [25] laid out massive data on the protein thermostability of about 8,000 proteins of organisms subjected to different high temperatures, including the bacterium *Escherichia coli*, using a proteomic strategy. Their study concluded that important protein subgroups with essential functions on cells are lost owing to denaturation leading to cellular disintegration in cells subjected to high temperatures [25]. Although there are no literatures that we know of regarding *Brucella* and its thermal adaptation, these previous studies give insight into the lethal susceptibility of *B. abortus* to higher temperatures, as manifested in the bactericidal assay.

HD11 is an avian macrophage that has been routinely incubated at 41ºC as with other studies [26]. Consistent with this, our data also showed that this high temperature did not cause significant host cell death in comparison with host cells incubated at 37ºC for both infected or uninfected cells.

For internalization, there was no observed difference among all the time points for both temperatures. Thus, receptors on macrophages responsible for bacterial invasion may not be affected by the difference in temperature. To confirm the result for the rate of internalization, we conducted a bacterial adherence assay and concluded that the number of attached bacteria was not significantly different in cells incubated either at 37ºC or 41ºC. This result is consistent with one of our unpublished studies on murine macrophage RAW264.7.

The intracellular survival assay showed significant decline in the intracellular growth of *B. abortus* at 41ºC and might be attributed to the bactericidal effect of temperature. To prove this, we analyzed the percentage of intracellular bacteria through microscopy after 24 and 48 h post-infection. We determined that there was a significant reduction in the number of intracellular *Brucella* for both time points at 41ºC compared with that at 37ºC. On the other hand, even though *B. abortus* was viable up to 72 h incubation at 37ºC, infection and incubation of HD11 at this temperature did not sustain an increasing intracellular growth rate. This growth rate is not comparable to the steady growth observed in other primary mammalian cells such as RAW 264.7, reaching higher log rates and displaying a steady increased intracellular growth [27]. Additionally, cytokine expression of HD11, incubated at 37ºC and 41ºC, was not affected by *B. abortus* infection, in contrast with responses in murine macrophages. Therefore, this bacterial growth inhibition might be attributed to other factors innate to this chicken macrophage, which may require further investigation.

Taken together, given the same intracellular load upon invasion for both 37ºC and 41ºC, and higher bactericidal rate at 41ºC along an increasing period of incubation compared with 37ºC, *Brucella* the higher temperature rendered less for intracellular replication, thus suppressing bacterial growth. This study also shows that despite incubation at an ambient temperature and initially having a higher number of intracellular *Brucella*, an increasing intracellular growth is still not attained in HD11. The results of this study give clearer understanding about the direct bactericidal effect of temperature as a major factor that might confer bacterial resistance to brucellosis in avian species.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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