Different invasion efficiencies of *Brucella abortus* wild-type and mutants in RAW 264.7 and THP-1 phagocytic cells and HeLa non-phagocytic cells

Soojin Shim¹, Young Bin Im¹, Myunghwan Jung², Woo Bin Park¹, Han Sang Yoo¹,*

¹Department of Infectious Diseases, College of Veterinary Medicine, Seoul National University, Seoul 08826, Korea
²Department of Microbiology, Research Institute of Life Sciences, Gyeongsang National University School of Medicine, Jinju 52828, Korea

(Received: April 3, 2018; Revised: April 26, 2018; Accepted: April 30, 2018)

**Abstract:** Brucellosis is one of the common zoonoses caused by *Brucella abortus* (*B. abortus*). However, little has been reported on factors affecting invasion of *B. abortus* into host cells. To investigate cell-type dependent invasion of *B. abortus*, phagocytic RAW 264.7 and THP-1 cells and non-phagocytic HeLa cells were infected with wild-type and mutant *B. abortus*, and their invasion efficiencies were compared. The invasion efficiencies of the strains were cell-type dependent. Wild-type *B. abortus* invasion efficiency was greater in phagocytic cells than in epithelial cells. The results also indicated that there are different factors involved in the invasion of *B. abortus* into phagocytic cells.

**Keywords:** *Brucella abortus*, cell invasion, mutants, non-phagocytes, phagocytes

*Brucella abortus* (*B. abortus*) is a facultative intracellular bacterium and is the causative agent of brucellosis that mainly causes abortion and infertility in domestic animals, undulant fever severe symptoms in humans, and serious economic impacts on related industries [4, 8, 15]. The pathogen eludes initial immune recognition through Toll-like receptors (TLRs) and modified virulence factors in the host [1]. The characteristics of intracellular parasitism of the bacteria have been regarded as serious problems in the bacterial infection. Therefore, understanding of the interaction between bacterium and host cells has been required to elucidation of the infectious process.

Brucellosis has been received attention since eradication of the disease is difficult due to its intracellular survival in various types of permissive cells such as macrophages, dendritic cells, neutrophils, trophoblasts, endothelial cells and epithelial cells, etc [5, 10, 12]. Over the years, researches on the function of *Brucellae* genes have been proceeded and genes related in *B. abortus* invasion have also been reported [6, 9, 11, 12]. The mutants of bvrR and bvrS in *B. abortus* showed less efficiency than the wild-type strain in macrophages and HeLa cells, and both mutants failed to replicate within phagocytic or non-phagocytic cells [13]. However, exact difference of *B. abortus* invasion into the professional phagocytes and non-professional phagocytes are still remained to be resolved [10]. In our previous study, *B. abortus* mutants were generated using transposon mutagenesis and general characteristics of the mutants were investigated [3, 9]. Then, as the first step of the understanding of infection at early stage, invasion efficacy of *B. abortus* mutants was investigated in phagocytic RAW 264.7 and THP-1 cells, and non-phagocytic HeLa cells and compared based on the efficacy of wild-type.

Mutant strains of *B. abortus* were generated from *B. abortus* 1119-3 strain by electroporation using the EZ-TnSTM transposome complex (Epicentre R Biotechnologies, USA) and insertion of the transposon was confirmed by PCR and general characteristics of the mutants were reported [3]. The bacterium was cultured in *Brucella* broth or agar (Difco, USA), and kanamycin (30 µg/mL) was added into the media for culture of *B. abortus* mutant strains. All procedures were approved by Seoul National University Institutional Biosafety Committee (SNUIBC-R160314-1-1).

Two phagocytic, RAW 264.7 (murine leukemic monocyte cell line) and THP-1 cells (human leukemic monocyte cell line) and one non-phagocytic, HeLa cells (human epithelial cell line) were obtained from the Korea Cell Line Bank (Korea). Two phagocytic cells were grown at 37°C in humidified atmosphere with 5% CO₂ in Roswell Park Memorial Institute medium (RPMI 1640; Gibco, USA) containing 10% fetal bovine serum (FBS) and Antibiotic–Antimycotic (Gibco). RAW 264.7 and differentiated THP-1 cells (2 × 10⁵ cells/mL) were inoculated with *B. abortus* wild-type and mutants at a multiplicity of infection (MOI) of 100:1. THP-1 cells were

*Corresponding author
Tel: +82-2-880-1263, Fax: +82-2-874-2738
E-mail: yoohs@snu.ac.kr
differently differentiated into macrophages by stimulation with phorbol-12-myristate-13-acetate (50 ng/mL; Sigma, USA) for 72 h. After washing with RPMI 1640 medium, the cells were further incubated with 5% FBS-RPMI 1640 medium without antibiotics for 24 h before the experiments. HeLa cells were grown at 37°C in humidified air with 5% CO₂ in Dulbecco’s Modified Eagle medium (Gibco) with 10% FBS and Antimicrototic. Also, HeLa cells of 3 x 10⁵ cells/mL were inoculated with the bacterial strains at MOI of 100:1.

Then, to investigate intracellular survival of *Brucella* wild-type and mutants in host cell, the gentamicin protection assay was performed using the modified method described previously [6]. Briefly, *B. abortus* strains were inoculated into those cells for one hour and the cells were washed out twice with phosphate-buffered saline (PBS) and treated with gentamicin for 2 h. After lysis of the cells with lysis buffer (diluted water with 0.01% Triton X-100), 25 µL of the cell lysate was inoculated and colony-forming unit (CFU) of each *B. abortus* strain was counted after incubation at 37°C for 48 h. Statistical significance was analyzed by Student’s t-test using SPSS (ver. 23.0; IBM, USA). Differences was set at value of p < 0.05.

*B. abortus* wild-type strain invaded with different invasion efficiency depending on cell types, phagocytic *RAW 264.7* and THP-1 cells and non-phagocytic HeLa cells (Fig. 1). The rate of intracellular survival of the wild-type was expressed as log₅₀ CFU/mL. Seven-times higher than number of bacteria were survived in RAW264.7 cells than those in THP-1 cells even though those are same phagocytic cells (Fig. 1). On the other hand, about 25 times lower number of bacteria were survived in HeLa cells than in RAW 264.7 cells.

Twenty-eight *B. abortus* mutants could invade into the three different cell types with different efficiency (Fig. 2). The level of bacterial invasion was presented as the relative percentage when the level of wild-type was regarded as 100%. In the comparison of the efficiency in the phagocytic and non-phagocytic cells, nine of them showed higher invasion efficiency in non-phagocytic cells (Fig. 2A) while ten of them were lower based on the efficiency of wild-type (Fig. 2B). With phagocytic cells, all mutants showed lower invasion efficiency in RAW264.7 cells than that of wild-type. However, in THP-1 cells, nine of mutants were higher in efficiency (Fig. 2C) and others were lower than that of wild-type (Fig. 2D).

Differences in the invasion and intracellular survival of *B. abortus* have been known according to types of cells, especially phagocytic and non-phagocytic cells [10]. Although the mechanism and factors associated with *B. abortus* invasion is not fully understood, two ways are known in the infection of *B. abortus* into macrophages; opsonized *B. abortus* through complement or Fc receptor-mediated zipper-like mechanisms, and non-opsonized *Brucellae* through cell surface lectin, fibronectin, lipid raft, and TLR4 [2, 7]. Also, rough strains of *B. abortus* mutants showed the increased macrophage uptake relative to their smooth parent strain [10]. Our mutants showed various invasion efficiency depending on cell types even though none of our mutants was rough strain [3]. However, the difference in invasion pathway into the two phagocytic cells, RAW264.7 and THP-1 cells, is not clearly known, yet. On the other hand, the mapk1 gene of the HeLa cells has shown to be associated with the bacterial invasion since invasion of *Brucellae* occurs after the mitogen activated protein kinase signaling pathway is fully activated [12]. However, it is shown that unknown receptors are involved in receptor mediated phagocytosis of *B. abortus* in non-phagocytic HeLa cells [11, 14].

As a result, all mutants showed lower efficiencies in RAW 264.7 cells than wild-type. Intracellular survival of *B. abortus* wild-type in RAW 264.7 was seven times more than into the THP-1 cells. In addition, the survival was 25 times higher in comparison with that in HeLa cells. The results indicate the different mechanism in invasion and intracellular survival of *B. abortus* depending on the cell types. The survival rates of some mutants were increased in non-phagocytic epithelial cells compared with the rates in phagocytic cells. As described above, *B. abortus* wild-type showed the lowest efficiency in the HeLa cells than these two phagocytic cells. On the other hand, some other mutants showed reverse pattern in the survival. Rough strains of *B. abortus* mutants showed the increased macrophage uptake relative to their smooth parent strain [10]. Our mutants showed various invasion efficiency depending on cell types even though none of our mutants was rough strain [3]. The results indicate that the key characteristics of these mutants had altered by transposon mutation. However, the common characteristics in these mutants were still remained to be cleared. Even though RAW264.7 cells and THP-1 cells are same phagocytic cells,
Invasion efficiency of \( B. \) abortus according to the cell types

They showed different permission according to mutants (Fig. 2D). These mutants showed faster growth rate as compared to wild-type in our previous study [9]. These characteristics might be related to \( B. \) abortus invasion into these different origin of cells.

Although \( B. \) abortus wild-type had a higher invasion efficiency in phagocytic cells than in non-phagocytic epithelial cells, mutants of \( B. \) abortus showed different invasion and intracellular survival rates depending on cells types. Those results suggest that mutated genes are related to \( B. \) abortus invasion and the roles of the mutated genes should be investigated in the further study. It may contribute to understanding of pathogenesis of \( B. \) abortus infection in different types of cells.

Acknowledgments

This work was supported by Korea Health Industry Development Institute (KHIDI) (No. HI16C2130) and the Brain Korea (BK) 21 PLUS program for Creative Veterinary Science Research and the Research Institute of Veterinary Science, Seoul National University, Republic of Korea.

References

1. Andersen-Nissen E, Smith KD, Strobe KL, Barrett SLR, Cookson BT, Logan SM, Aderem A. Evasion of Toll-like receptor 5 by flagellated bacteria. Proc Natl Acad Sci U S A 2005, 102, 9247-9252.
2. Arenas GN, Staskevich AS, Aballay A, Mayorga LS. Intracellular trafficking of \( Brucella \) abortus in J774 macrophages. Infect Immun 2000, 68, 4255-4263.
3. Cha SB, Rayamajhi N, Lee WJ, Shin MK, Jung MH, Shin SW, Kim JW, Yoo HS. Generation and envelope protein analysis of internalization defective \( Brucella \) abortus mutants in professional phagocytes, RAW 264.7. FEMS Immunol Med Microbiol 2012, 64, 244-254.
4. Galińska EM, Zagórski J. Brucellosis in humans – etiology, diagnostics, clinical forms. Ann Agric Environ Med 2013, 20, 233-238.
5. Gorvel JP, Moreno E. Brucella intracellular life: from invasion to intracellular replication. Vet Microbiol 2002, 90, 281-297.
6. Kim S, Watarai M, Kondo Y, Erdenebaatar J, Makino S, Shirahata T. Isolation and characterization of mini-Tn5Km2 insertion mutants of \( Brucella \) abortus deficient in internalization and intracellular growth in HeLa cells. Infect Immun 2003, 71, 3020-3027.
7. Naroeni A, Porte F. Role of cholesterol and the ganglioside GM, in entry and short-term survival of \( Brucella \) suis in murine macrophages. Infect Immun 2002, 70, 1640-1644.
8. Pappas G, Akritidis N, Bosilkovski M, Tsianos E. Brucellosis. N Engl J Med 2005, 352, 2325-2336.
9. Park WB, Im YB, Jung M, Yoo HS. Molecular characteristics of *Brucella abortus* mutants generated using EZ-Tn5™ pMOD™-3 transposon system. J Prev Vet Med 2015, 39, 144-152.

10. Pei J, Ficht TA. *Brucella abortus* rough mutants are cytopathic for macrophages in culture. Infect Immun 2004, 72, 440-450.

11. Roop RM 2nd, Gaines JM, Anderson ES, Caswell CC, Martin DW. Survival of the fittest: how *Brucella* strains adapt to their intracellular niche in the host. Med Microbiol Immunol 2009, 198, 221-238.

12. Rossetti CA, Drake KL, Adams LG. Transcriptome analysis of HeLa cells response to *Brucella melitensis* infection: a molecular approach to understand the role of the mucosal epithelium in the onset of the *Brucella* pathogenesis. Microbes Infect 2012, 14, 756-767.

13. Sola-Landa A, Pizarro-Cerdá J, Grilló MJ, Moreno E, Moriyón I, Blasco JM, Gorvel JP, López-Goñi I. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol Microbiol 1998, 29, 125-138.

14. von Bargen K, Gorvel JP, Salcedo SP. Internal affairs: investigating the *Brucella* intracellular lifestyle. FEMS Microbiol Rev 2012, 36, 533-562.

15. Young EJ. An overview of human brucellosis. Clin Infect Dis 1995, 21, 283-289.