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<Short Communication>

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

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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 also have reported [6, 9, 11, 12]. The mutants of *bvr*R and *bvr*S 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-Tn5TM 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% CO_2 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 (2×10^5 cells/mL) were inoculated with *B. abortus* wild-type and mutants at a multiplicity of infection (MOI) of 100:1. THP-1 cells were

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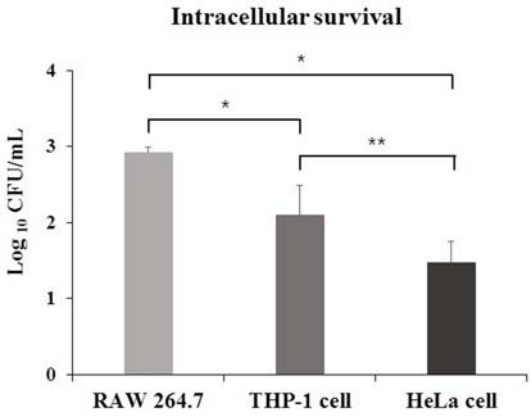

Fig. 1. Invasion efficiency of *Brucella abortus* (*B. abortus*) wild-type strain into the phagocytic cell (RAW 264.7 cells and THP-1 macrophage cells) and the non-phagocytic cell (HeLa cells). *B. abortus* wild strain type invaded into the all of three types of cells and RAW264.7 showed the highest invasion efficiency showing 7 times more than THP-1 macrophage cells. HeLa cells showed the lowest efficiency. ($^{*}p < 0.05$ and $^{**}p < 0.01$).

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% CO_2 in Dulbecco's Modified Eagle medium (Gibco) with 10% FBS and Antibiotic–Antimycotic. Also, HeLa cells of 3×10^5 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 $\log_{10}$ 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 rafts, 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 *mapk*1 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,

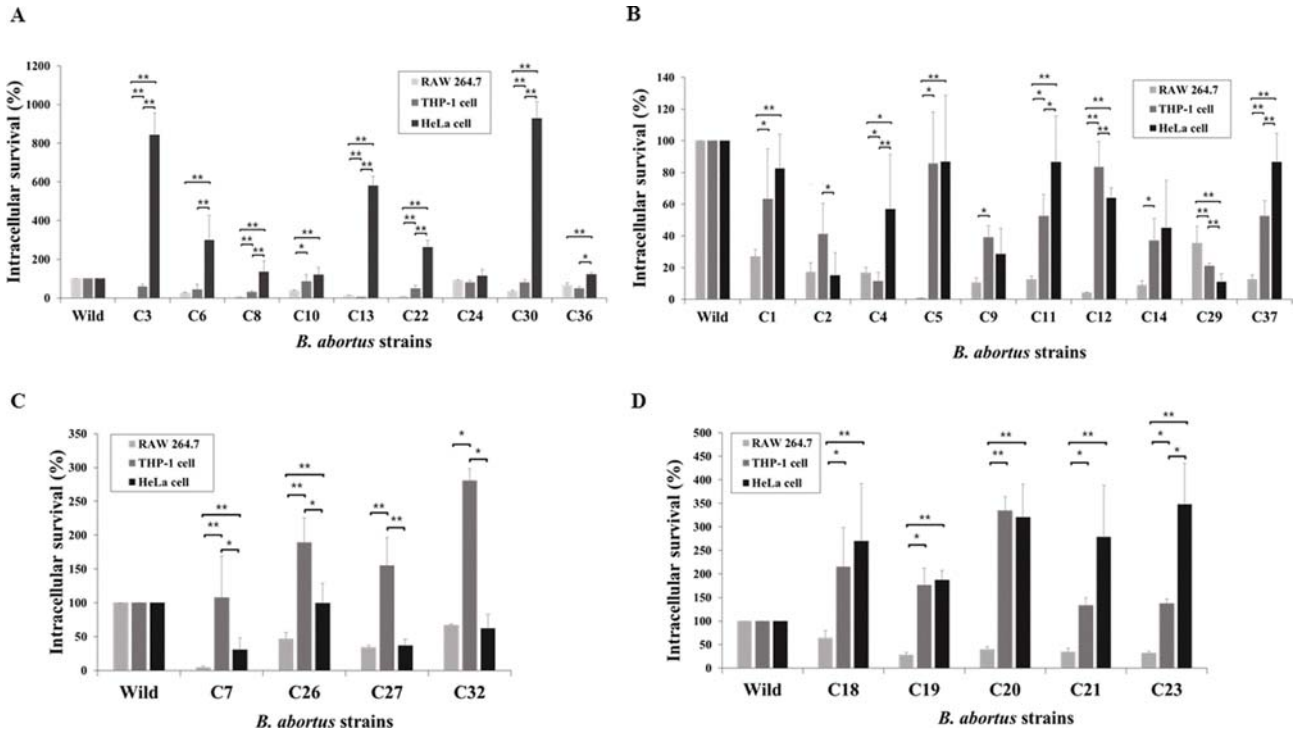

Fig. 2. Comparison of invasion efficiency of *B. abortus* mutant strains into the phagocytic cells and non-phagocytic cells. The invasion efficiency of mutants was compared in the phagocytic cells (RAW 264.7 and THP-1 macrophage cells) and non-phagocytic cells (HeLa cells). (A) The mutants that showed enhanced invasion efficiency in HeLa cells but defective invasion efficiency in macrophages than wild-type. (B) The mutants that showed defective invasion efficiency in both of HeLa cells and macrophages than wild-type. (C) The mutants that showed enhanced efficiency only in THP-1 cells than wild-type. (D) The mutants that showed enhanced efficiency in cell strains from human than wild-type. ($^{*}p < 0.05$ and $^{**}p < 0.01$).

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.

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