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Characterization of culture supernatant proteins from *Brucella abortus* and its protection effects against murine brucellosis

Jin Ju Lee[a,b,1], Jeong Ju Lim[b,1], Dae Geun Kim[b], Hannah Leah Simborio[b], Dong Hyeok Kim[b], Alisha Wehdnesday Bernardo Reyes[b], WonGi Min[b], Hu Jang Lee[b], Dong Hee Kim[c], Hong Hee Chang[d], Suk Kim[b,d,*]

[a] *Animal and Plant Quarantine Agency, Anyang, Gyeonggi-do 430-757, Republic of Korea*
[b] *College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea*
[c] *School of Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea*
[d] *Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea*

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ABSTRACT

In this study, we characterized the secreted proteins of *Brucella abortus* into the enriched media under the bacterial laboratory growth condition and investigated the pathogenic importance of culture supernatant (CS) proteins to *B. abortus* infection. CS proteins from stationary phase were concentrated and analyzed using 2D electrophoresis. In MALDI TOF/TOF analysis, more than 27 proteins including CuZn SOD, Dps, Tat, OMPs, Adh, LivF, Tuf, SucC, GroEL and DnaK were identified. Cytotoxic effects of CS proteins were found to increase in a dose-dependent manner in RAW 264.7 cells. Upon *B. abortus* challenge into phagocytes, however, CS proteins pre-treated cells exhibited lower bacterial uptake and intracellular replication compared to untreated cells. Immunization with CS proteins induced a strong humoral and cell mediated immune responses and exhibited significant higher degree of protection against virulence of *B. abortus* infection compared to mice immunized with Brucella broth protein (BBP). Taken together, these results indicate that *B. abortus* secreted a number of soluble immunogenic proteins under laboratory culture condition, which can promote antibody production resulted in enhancing host defense against to subsequently bacterial infection. Moreover, further analysis of CS proteins may help to understand the pathogenic mechanism of *B. abortus* infection and host–pathogen interaction.

1. Introduction

Brucellosis leads to sterility and abortion in animals and an undulant fever associated with severe fatigue in humans including endocarditis, osteoarthritis, and neurological damage [1]. *Brucella* spp. are uptaken by phagocytes within the host and establish a replicative niche within macrophages and dendritic cells [2]. Although killed *Brucella* are engulfed by macrophages, they fail to associate with the ER and are rapidly degraded, suggesting that

* Corresponding author at: College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea.
Tel.: +82 55 772 2359; fax: +82 55 772 2349.
E-mail address: kimsuk@gnu.ac.kr (S. Kim).
[1] These authors contributed equally to this work.

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de novo protein expression and/or secretion is required for bacterial trafficking during infection [3]. The *Brucella* type IV secretion system (T4SS), known as VirB system, is required during internalization by macropinocytosis and for fusion with the endoplasmic reticulum (ER) to establish the replicative niche [1,2]. The *Brucella virB* mutants lost the ability to affect the endosomal pathway that docks with ER and were unable to survive within macrophages and mice [4].

A recent study revealed that the cytotoxic effect of rough *Brucella* mutants against macrophages depends on the expression of the T4SS [5]. Interestingly, several intracellular survival-related proteins, including VjbR, DnaK, HtrA, Omp25 and GntR, were down-regulated in the *virB* mutant. These and other findings suggested that *Brucella* spp. secrete virulence factors through the T4SS [6]. Although reporter fusions to N-terminal portions of several *Brucella* proteins were secreted in an apparently VirB-dependent manner, it was subsequently shown that the addition of a Sec secretory tag to the YopP reporter was sufficient to promote VirB-dependent secretion [7,8]. Therefore, proteins secreted into the periplasm by the Sec-pathway might be non-specifically secreted via VirB. More recently, two proteins of the VjbR regulon of *Brucella*, VceA and VceC were found to be translocated into macrophages in a T4SS-dependent manner, and secreted into the cytosol of infected macrophages in a Sec-independent, VirB-dependent manner [9]. The C-terminus of these proteins in *Brucella suis* contains a C-terminal secretion motif seen in proteins secreted by the *Agrobacterium tumefaciens* VirB system, although this signal is not present in proteins of all *Brucella* species due to a frame shift [9].

A previously study has been reported that the difference of CS patterns in semisynthetic medium, which is to minimize the exogenous proteins from medium, between *B. abortus* wild type and its *virB* mutant were analyzed by 2DE and MALDI TOF [10]. This study showed that 16 of different spots were identified between *B. abortus* 2308 and *virB* mutant, 11 were identified by MALDI TOF analysis [10]. However, the CS patterns in enriched medium, the virulence ability and immunogenicity of *B. abortus* CS proteins are still unclear.

Herein, we characterized the secretion proteins of *B. abortus* 544 in laboratory culture condition using enriched medium. The CS proteins from *B. abortus* cultured Brucella broth were analyzed by proteomic analyses, and its biological characterizations and protection effects against a challenge with the virulent *B. abortus* were determined.

2. Materials and methods

2.1. Bacterial strain and growth condition

The standard wild-type strains were derived from *B. abortus* 544 (ATCC 23448), a smooth, virulent *B. abortus* biovar 1 strains. The cultivation of *B. abortus* strains was carried out in Brucella broth or on Brucella agar (Becton Dickinson, Sparks, MD). Bacteria were grown at 37 °C with vigorous shaking until they reached the stationary phase, and the viable counting of bacteria was assessed by plating serial dilutions on agar plates.

2.2. Cell culture

RAW 264.7 cells were grown at 37 °C in 5% CO_2 atmosphere in RPMI 1640 (Gibco, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all provided by Gibco). The cells were seeded (1×10^4 per well) in 96-well tissue culture plates and incubated for 24 h before infection for all assays.

2.3. Preparation of culture supernatant for analysis

Preparation of culture supernatant proteins was performed as described previously with some modifications [10]. Briefly, *B. abortus* cultures at the stationary phase in Brucella broth were centrifuged at 15,000 × *g* for 1 h and removed the bacterial pellet and the resultant culture supernatants were carefully collected and filter-sterilized (Sartorius stedim, UK). As a control, the same procedure was performed in uninoculated Brucella broth (BB). CS or BB was concentrated by freeze-drying, dialysis and stored in −70 °C before use. Protein extraction was performed using a modified protocol [11]. All operations were carried out on ice with ice-cold reagents, and all centrifugations were done for 20 min, 100,000 × *g*, at 4 °C. Freeze-dried CS or BB was mixed with trichloroacetic acid (final concentration 10%; Wako, Japan), incubated for 1 h and then centrifuged. The pellet was then washed with 1 ml of acetone and centrifuged twice.

2.4. Two-dimensional electrophoresis (2-DE)

The 2-DE was performed as described previously with some modifications [12]. Briefly, proteins subjected to 2-DE were mixed with rehydration buffer and immobilized pH gradient (IPG) buffer (pH 4–7, GE Healthcare, UK). The 350 μl samples containing 500 μg total proteins were rehydrated at room temperature for 12 h using 18-cm IPG strips (pH 4–7, GE Healthcare, UK). Isoelectric focusing (IEF) was conducted on a Protean IEF (Amersham Phamacia, NJ, USA) at 20 °C for 14 h. The parameters for IEF were: 500 V for 1 h, 1000 V for 1 h (gradient), 1000 V for 3 h, 10,000 V for 3 h (gradient), 10,000 V for 5 h, 50 V for 30 min and final phase of 50 V for 30 min. After IEF, the strips were equilibrated in 5 ml equilibration buffer I (50 mM Tris–HCl, 6 M urea, 30% glycerol, 2% SDS, bromphenol, 10 mg/ml DTT) for 15 min, subsequently in equilibration buffer II (50 mM Tris–HCl, 6 M urea, 30% glycerol, 2% SDS, bromophenol, 45 mg/ml iodoacetamide) for 15 min. The equilibrated strips were loaded into 12% SDS–polyacrylamide gels and the proteins were two-dimensionally separated by SDS–PAGE, followed by visualization through silver staining. Three replicates of 2-DE were carried out and the stained gel images were scanned by a GS-800 Imaging Densitometer and analyzed using the software PDQuest version 7.2.0 (Bio-Rad, CA).

2.5. Protein identification by MALDI-TOF/TOF MS analysis

The protein spots of culture supernatants or Brucella broth were compared and only CS protein spots