Microbial Pathogenesis 52 (2012) 267–277

Contents lists available at SciVerse ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Deep sequencing-based expression transcriptional profiling changes during *Brucella* infection

Qianhong Liu [a,b,1], Wenyu Han [a,*], Changjiang Sun [a], Liang Zhou [a], Limin Ma [a], Liancheng Lei [a], Shouqing Yan [a], Shanshan Liu [a], Chongtao Du [a], Xin Feng [a]

[a] *College of Animal Science and Veterinary Medicine, Jilin University, Xi'an Road 5333, Changchun 130062, PR China*
[b] *College of Jilin Agricultural Technology, Jilin 132101, PR China*

ARTICLE INFO

Article history:
Received 12 November 2011
Received in revised form
27 January 2012
Accepted 2 February 2012
Available online 8 February 2012

Keywords:
Deep sequencing-based expression
Transcription
Infection
Brucella

ABSTRACT

Brucellosis is a worldwide zoonotic infectious disease that has significant economic effects on animal production and human health. The host macrophage – *Brucella* interaction is critical to the establishment of infections. Thus, the kinetic transcriptional profile of gene expression in macrophages infected with the *Brucella melitensis* strain 16M was investigated in the current study using a technology based on deep sequencing. The total RNA was extracted from macrophages 0, 4, and 24 h post-infection. Data analysis showed that in the gene ontology term, the expression of genes in the endoplasmic reticulum, lysosomes, as well as those involved in programmed cell death and apoptosis significantly changed during the first 24 h post-infection. Pathway enrichment analysis indicated that the genes in the apoptosis pathway, NOD-like receptor signaling pathway, Fc gamma R-mediated phagocytosis, lysosome pathway, p53 signaling pathway, and protein processing in the endoplasmic reticulum significantly changed during the first 24 h post-infection. The B-cell receptor and toll-like receptor signaling pathways were also significantly changed 24 h post-infection compared with those 4 h post-infection. The results of the current study can contribute to an improved understanding of the manner by which host cell responses may be manipulated to prevent *Brucella* infection.

1. Introduction

Brucellae are facultative intracellular pathogens that cause brucellosis in a broad range of hosts, including humans [1]. *Brucellae* cause a serious debilitating disease in humans, as well as abortion and sterility in domestic animals. Therefore, *Brucellae* play a significant role in biosecurity and community health worldwide as a potential bioterrorism threat [1].

Brucellae act as an intracellular pathogen that has the ability to proliferate within professional and nonprofessional phagocytic host cells. However, host infection depends not only on the pathogenicity of the host but also on its defense mechanisms. Macrophages constitute the first line of defense of the innate immune response against invading microorganisms [1]. Most studies have focused on pathogens [2–7], and only a few have focused on hosts [8–10].

Digital gene expression (DGE) is a tag-based transcriptome sequencing approach in which short raw tags are generated by a combination of polyA isolation and endonucleases. One short fragment (21 bp) is produced per transcript. The transcripts are then aligned to a reference, and the number of fragments per annotated gene is counted. The expression level of virtually all genes in a sample is measured by counting the number of individual mRNA molecules produced from each gene. The DGE protocol is very suitable for comparative gene expression studies because it enables direct transcript profiling without compromise and potential bias. Consequently, a sensitive and accurate profiling of the transcriptome that closely resembles cell biology is facilitated [11,12]. However, the DGE technology has not been used to analyze the transcriptome profiles of macrophages in post-*Brucella* infection.

In the current study, the DGE technology was used to analyze the gene expression profiles of mouse macrophages during a virulent *Brucella melitensis* strain 16M infection. The aim was to analyze the transcriptional profiling changes during *Brucella* infection. The results lay the foundation for discovering genes that are possibly critical to the defense against *Brucella* infection.

---

[*] Corresponding author.
E-mail addresses: qianhongliu@126.com (Q. Liu), hanwy@jlu.edu.cn (W. Han).
[1] Tel./fax: +186 431 8783 6001.

0882-4010/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micpath.2012.02.001

2. Materials and methods

2.1. Bacterial strain and cell culture

The *B. melitensis* strain 16M was obtained from the laboratory of Wang Xinglong and confirmed by polymerase chain reaction (PCR) [13]. *B. melitensis* 16M was grown either on tryptic soy agar (TSA) plates at 37 °C or in broth [1].

The mouse macrophage cell line RAW264.7 was propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. The cells were passaged every 3 days to 5 days and discarded after the 15th passage. RAW264.7 at a low passage was used to seed the wells of a 24-well plate at 2.5×10^5 cells/well in 0.5 mL of DMEM.

2.2. Brucella invasive and intracellular survival

The details of the method used have been described elsewhere [14,15]. The RAW264.7 cells were seeded into 24-well plates at 5×10^5 cells/well, incubated in 5% CO_2 at 37 °C for 24 h, and infected with *Brucella* at a 1000 multiplicity of infection. The antibiotics were removed from the medium prior to infection. To synchronize the infection, the infected plates were centrifuged at 200× *g* for 5 min at room temperature, and incubated at 37 °C for 20 min. The infected cell monolayers were washed thrice with phosphate-buffered saline, and then overlaid with 0.5 mL of DMEM containing antibiotics at 37 °C for 0, 4, and 24 h. To assess the intracellular growth of *Brucella* 16M, the concentration of gentamicin was reduced to 20 μg/mL and ampicillin was not used 0 h post-infection. At 0, 4, and 24 h post-infection, the supernatant was discarded and the cells were lysed with 0.1% (v/v) Triton X-100. The cell lysate was vigorously pipetted to ensure cell lysis. The number of colony-forming units (CFUs) was obtained by plating a series of 1:10 dilutions on TSA plates. The percentage of bacterial uptake or invasion was calculated as the number of recovered bacteria divided by the number of bacteria inoculated onto each well. All invasion and survival assays were performed with triplicate wells, and the results are the averages of at least three separate experiments.

2.3. Macrophage cytotoxicity assay

Cells were cultured in 24-well plates in triplicate wells, and then infected with *Brucella* as described in Section 2.2. Lactate dehydrogenase (LDH) release into cell culture supernatants was detected using the CytoTox 96 nonradioactive cytotoxicity assay as previously described [15,16]. At 2, 4, 6, 12, 24, and 48 h post-infection, cell culture supernatants were collected and the LDH test was performed according to the manuscript protocol (Beijing BiYunTian Company). Cytopathic cell death was expressed as the percentage of maximum LDH released as follows: $100 \times (OD_{490}$ of infected cells $- OD_{490}$ of uninfected cells)/(OD_{490} of lysed uninfected cells $- OD_{490}$ of uninfected cells), where OD_{490} is the optical density at 490 nm [15].

2.4. RNA extraction and sample preparation for DGE

At the end of the incubation period, the cells of three independent tests were used for the RNA extraction test. The cells were removed and centrifuged at 1500 rpm for 15 min. The cell pellet and adherent cells were resuspended in TRIzol (Promega). Total RNA was isolated from TRIzol according to the manufacturer's protocol. The RNA pellet was resuspended in RNAse-free water. RNA concentrations were measured using a spectrophotometer, and their integrity was ensured by analysis on a 1.5% (w/v) agarose gel.

2.5. DGE tag preparation

Beads of Oligo(dT) were used to enrich mRNA from the total RNA, mRNA were then transformed into double-stranded cDNA via reverse transcription. The four-base recognition enzyme *Nla*III was used to digest the cDNA, and Illumina adaptor 1 was ligated. *Mme*I was used to digest at 17 bp downstream of the CATG site, and Illumina adaptor 2 was ligated at the 3' end. After 15 cycles of linear PCR amplification, 95 bp fragments were purified using 6% Tris–borate–ethylenediaminetetraacetic acid polyacrylamide gel electrophoresis. The DNA was purified and Illumina sequencing was performed. Each molecule was grown into a single-molecule cluster sequencing template via in situ amplification. Four types of nucleotides (labeled with four colors) were then added, and sequencing was performed by synthesis according to the manufacturer's protocols. Each tunnel generated millions of 35 bp raw reads. Image analysis, base calling, extraction of 17 bp tags, and tag counting were performed using the Illumina pipeline.

2.6. Gene expression annotation

To map the DGE tags of the mouse genome, virtual libraries containing all possible CATG sites, that is, the 4 bp *Nla*III recognition sites and the 17-base length sequences of the reference gene sequences were created. All clean tags were mapped into the reference sequences, and those from multiple genes were filtered. The remaining clean tags were designed as unambiguous clean tags. To monitor mapping events on both strands, both the sense and complementary antisense sequences were included in the mapping process. The number of unambiguous clean tags for each gene was calculated and then normalized to the number of transcripts per million of clean tags [12,17].

2.7. Identification of differentially expressed genes

We utilized a rigorous algorithm to identify differentially expressed genes between two samples [11]. The correlation of the number of unambiguous clean tags between two parallel libraries was statistically assessed by calculating the Pearson correlation. Assuming that we picked out R differentially expressed genes, in which S genes really showed differential expression and the other V genes were false positives. If we decided that the error ratio $Q = V/R$ must stay below a cutoff (e.g., 1%), we preset the false discovery rate (FDR) to a number not larger than 0.01 [18]. The P-value corresponded to the differential gene expression test. The FDR is used to determine the threshold of the P-value in multiple tests and analyses by manipulating the FDR value. In the current study, we used FDR $\leq$0.01 and the absolute value of the log2 Ratio $\geq$1 as the threshold to judge the significance of the gene expression difference.

2.8. Quantitative real-time PCR analysis

The RNA samples used for the quantitative PCR (qPCR) assays were. The cDNAs of the genes were amplified by reverse transcriptase PCR (RT-PCR) using the primers shown in Table 1. All PCR products were purified using a gel extraction kit (Promega). qPCRs were performed on a Light Cycler 480 (Roche) with SYBR-Green detection or using the universal probe library (Roche) when the amplification efficiencies with the SYBR-Green were less than 90%. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control. Each reaction volume was 20 μl. The amplification conditions were as follows: 95 °C for 5 min, followed by 40 cycles at 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 30 s. Each cDNA was analyzed in quadruplicate, and then the