Expression and Immunogenicity of Recombinant Immunoreactive Surface Protein 2 of Anaplasma phagocytophilum

Qiang Yu,a,b Chuang-fu Chen,b Qiang Chen,a and Li-juan Zhanga

Department of Rickettsiology, National Institute of Communicable Disease Control and Prevention, China CDC, Beijing, People’s Republic of China,a and Shihezi University, Shihezi, Xinjiang Province, People’s Republic of Chinab

Anaplasma phagocytophilum, an obligate intracellular bacterium, is the etiological agent of the emerging tick-borne disease human granulocytic anaplasmosis (HGA) (11, 12). The disease has been reported with increasing frequency in the United States and several European and Asian countries since the first case was reported in the United States in 1994 (3, 7, 9, 12, 15, 18). A. phagocytophilum DNA has been detected in ticks collected in Israel, Japan, China, and elsewhere (8, 14, 16). HGA was first reported in China in 2008, and the first HGA outbreak was noteworthy because it caused nosocomial infection (20). Thereafter, Zhang et al. (22) conducted serial retrospective laboratory surveys in Jiangsu, Henan, Zhejiang, Anhui, Shandong, Yunnan, Hainan, and Xinjiang Provinces and Tianjin and Beijing Cities during 2007 and 2008. These researchers confirmed the prevalence of anaplasmosis in Yiyuan County, Shandong Province, in 2007. Subsequently, Zhang et al. (21) conducted seroepidemiological investigations of high-risk people engaged in agriculture and animal husbandry within eight districts and counties of Tianjin City; their results showed that 8.8% of the human serum samples tested were positive for A. phagocytophilum antibodies. Furthermore, a broad active surveillance of tick vectors and host animals revealed that both were commonly infected with A. phagocytophilum in many parts of China (8, 19). Consequently, the Health Ministry of the People’s Republic of China issued “Guidelines for prevention and control of human granulocytic anaplasmosis” in 2008 and “Urgent information on further prevention and control of anaplasmosis” in 2009. However, the greatest challenge posed by this emerging zoonotic rickettsiosis infection is rapid and reliable diagnosis during the early stage of illness. As for other rickettsioses, the indirect immunofluorescent assay (IFA) proposed by WHO has been used as the gold-standard method for diagnosis of HGA. However, it requires a specific, expensive fluorescence microscope and acute-and convalescent-phase serum samples whose collection needs several weeks (4, 6). Nested PCR and real-time PCR are the best tests for the early diagnosis of HGA, but these methods require an expensive thermocycler. Therefore, a simple and rapid test for A. phagocytophilum is urgently needed. It is reported that the A. phagocytophilum major surface protein 2 (MSP2) is antigenic and is unique to this bacterium (5, 13). In this study, an immunologically important fragment (432 bp long) of MSP2 was selected, cloned, expressed, purified, and tested for natural antigenicity and specificity. MATERIALS AND METHODS

Bacterial strains and sera. A. phagocytophilum strain Webster (GenBank sequence accession number AY164491.1, which was kindly provided by J. S. Dumler, Medical College of Johns Hopkins University), was cultivated in HL-60 cells. A. phagocytophilum DNA was extracted by using a QIAamp blood and tissue kit (Qiagen, Hilden, Germany). Rabbit antiserum against A. phagocytophilum was prepared by immunization with A. phagocytophilum strain Webster in our laboratory. Sera of patients infected with Orientia tsutsugamushi, Rickettsia typhi, and Rickettsia helongi‐
genesis were collected from patients hospitalized in Beijing University First Hospital and Laizhou First People’s hospital and patients in the recovery period from Chengmai County, Hainan province, respectively, during 2008 to 2010. These patients were diagnosed by nested PCR, real‐time PCR, serological test, and culture isolation. Healthy human sera were collected from research staff in our laboratory. The reference rabbit sera for 10 members of the order Rickettsiales (Coxiella burnetii, Orientia tsutsugamushi strains Kato, Karp, TA763, and TH187, Rickettsia helongi‐
genesis, Bartonella quintana, Bartonella bacilliformis, Rickettsia mooseri, and Ehrlichia chaffeensis, kindly provided by Didier Raoult at the WHO Collaborating Centre for Rickettsial Reference and Research, Marseille, France) were generated by immunizing male rabbits with purified and 4% Formalin killed bacteria of the species mentioned above.

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Cloning, expression, and purification of the recombinant MSP2 protein. The fragment from amino acid (aa) 15 to aa 147 of MSP2 of *A. phagocytophilum* was used to generate the gene of the MSP2 fragment from amino acid (aa) 15 to aa 147 of MSP2 of *A. phagocytophilum*. The primers were synthesized by Shanghai Sangon Company. PCR amplification was carried out with an MJ Research PTC-100 Peltier thermal cycler under the following conditions: 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, and a final extension for 10 min at 72°C. The amplified 432-bp PCR products were purified and digested with Ncol and Xhol (TaKaRa Biotechnology Co., Ltd., Dalian, China) and then ligated into pET30a (+) (Novagen, Madison, WI) at 16°C overnight. The ligation products were transformed into *Escherichia coli* DE5s, positive transformants with the appropriate insert were screened on medium supplemented with 50 μl/ml kanamycin, and the recombinant plasmids were identified by PCR amplification with the msp2-F and msp2-R primers and DNA sequencing. The recombinant plasmids were transformed into *E. coli* BL21(DE3) and induced for expressing with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 7 h. Cells were centrifuged (5,000 rpm for 10 min) at 4°C and resuspended in 50 mM phosphate buffer (pH 7.4). The cells were lysed by sonication, and the cellular debris was removed by centrifugation at 8,000 rpm for 15 min at 4°C. The water-soluble fraction containing all potential hexa-His tag proteins was filtered (0.2 μm filter), and purification of the MSP2 protein was performed by using Ni-NTA (nitrilotriacetic acid) His-bind resin as described by the manufacturer (Novagen, Madison, WI).

**SDS-PAGE and mass spectrometry analysis.** SDS-PAGE analysis was performed with the DYY-5 protein electrophoresis system (12 by 8 by 0.75 cm; Beijing Kesheng) with 5% stacking gels and 10% separating gels. Electrophoresis was conducted at a constant voltage of 100 V for approximately 120 min. The gels were stained with Coomassie blue R, the expected protein bands were excised from the gels and then digested by trypsin, and the mass profiles of the peptides were determined using a 4700 proteomics analyzer (ABI, United States) to identify the recombinant MSP2 protein.

**Immunoreactivity of the recombinant MSP2 protein.** The immunoreactivity of the recombinant MSP2 protein was identified by Western immunoblot assay as described by Sambrook et al. (17a). Briefly, the recombinant MSP2 proteins were separated by PAGE, as mentioned above, and then transferred to a pure nitrocellulose sheet using an electroblotting cell (Bio–Rad, Hercules, CA). The sheet was cut into individual membrane strips, and each strip was immersed in 5% nonfat milk–Tris-buffered saline (TBS) in a small container for 1 h at room temperature and then incubated with rabbit antiserum against *A. phagocytophilum* diluted 1:20 at 37°C for 1 h. A batch of sera, including mixed nonimmunized-rabbit sera diluted 1:20, human HGA-positive sera diluted 1:100, healthy human serum diluted 1:50, human scrub typhus sera diluted 1:50, human endemic typhus sera diluted 1:50, human spotted fever sera diluted 1:50, and 5% nonfat milk–TBS, were also used as negative controls. The strips were incubated with peroxidase-conjugated affinity-purified anti-human IgG(H+L) and anti-rabbit IgG(H+L) sera (Sigma, St. Louis, MO) at a 1:10,000 dilution. The peroxidase-positive bands were detected by immersing the sheets in diaminobenzidine (DAB) substrate solution (Tiangen Biotechnology Co., Ltd., Beijing, China).

**Polyclonal antiserum preparation and its natural antigenicity to the recombinant MSP2.** The animal procedure was approved by Beijing City Association of Experimental Animals. Approximately 200 mg (20 mg per point) of purified recombinant MSP2 in Freund's complete adjuvant was used to subcutaneously immunize 2.0-kg male New Zealand White rabbits (Beijing Xinglong Experimental Animal Rearing Farm, license number: SCXK2011-0006). Two weeks later, each rabbit was boosted using approximately 300 mg of recombinant MSP2 in Freund's incomplete adjuvant. Another 10 days later, approximately 500 mg of recombinant MSP2 protein without adjuvant was injected. Rabbit serum was obtained 7 days after the last boost, and the antibody titer was detected by Western immunoblot analysis based on the recombinant MSP2 protein as the antigen. The titer of antibody against the recombinant MSP2 of the rabbit serum was also tested by indirect immunofluorescence assay (IFA) using *A. phagocytophilum* as the antigen (catalog number IF1403-A, lot 102048; Focus, United States) according to the manufacturer’s instructions. The reference rabbit antisera against *A. phagocytophilum* was used as a positive control. The rabbit sera collected prior to immunization with the recombinant MSP2 were used as self-negative controls. Mixed rabbit sera prepared from 3 nonimmunized rabbits were also used as negative controls.

**Cross-reaction of the recombinant MSP2 protein.** To demonstrate the specificity or cross-reactivity of the recombinant MSP2 protein, an enzyme-linked immunosorbent assay (ELISA) was performed using the recombinant MSP2 protein as antigen. Rabbit antiseria against a total of 10 common members of the order *Rickettsiales* (*Coxiella burnetii, Orientia tsutsugamushi* strain Kat, *Orientia tsutsugamushi* strain Karp, *Orientia tsutsugamushi* strain TA763, *Orientia tsutsugamushi* strain TH187, *Rickettsia helingiensis*, *Bartonella quintana*, *Bartonella bacilliformis*, *Rickettsia mooseri* and *Ehrlichia chaffeensis*) were tested. Briefly, the experiment was conducted as follows.
approximately 50 μg of the recombinant MSP2 protein was fixed in each well of an ELISA plate, and the plate was incubated with each type of rabbit antiserum mentioned above at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, and 1:6,400 at 37°C for 1 h. After the sera were removed, the plate was washed with 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 0.01% Tween 20 (T-PBS) three times, incubated with peroxidase-conjugated affinity-purified anti-rabbit IgG (H+L) (1:10,000) (Sigma, St. Louis, MO) at room temperature for 45 min, and washed again. After the addition of a developing solution (70 mM sodium acetate, pH 6.2) containing 0.3% diaminobenzidine tetrahydrochloride and 0.03% H2O2 at room temperature and then, after 5 min, the addition of 0.1 M H2SO4 to terminate the reaction, the positive reaction level was measured with an ELISA reader (KCjunior; BIO-TeK, United States) at 490 nm.

RESULTS
Cloning, expression, and purification of the recombinant MSP2 protein. A 432-bp fragment (containing 133 amino acids) of the msp2 gene was obtained by PCR amplification with the msp2-F and msp2-R primers and A. phagocytophilum genomic DNA as the template, and the purified fragments were successfully cloned into the pET30a vector. Direct sequencing of the recombinant plasmid confirmed that the nucleotide sequence of the inserted PCR fragment was 100% identical to the msp2 gene of the A. phagocytophilum Webster strain deposited in GenBank (accession number AY164491.1). The recombinant plasmid containing the targeted fragment was transferred into BL21(DE3) E. coli, and a protein of the expected 24.3-kDa size was successfully expressed and purified at a high level (Fig. 1).

Mass spectrometry analysis of the recombinant MSP2 protein. The mass spectrometry analysis showed that the amino acid sequence of the recombinant protein was 100% homologous with that expected for the target protein (Fig. 2).

Immunoreactivity of the recombinant MSP2 protein. West-
ern blot analysis indicated that the recombinant MSP2 protein reacted specifically with human HGA-positive sera (1:128 by Western blotting versus 1:64 by IFA) and rabbit anti-
*Agrobacterium phagocytophilum* sera (1:40 by Western blotting versus 1:20 by IFA) (Fig. 3). However, it failed to react with nonimmunized-rabbit sera, healthy human sera, or sera of 3 patients with related diseases (scrub typhus, spotted fever, and endemic typhus sera) (Fig. 3).

Polyclonal antiserum preparation and its natural antigenicity to the recombinant MSP2. The rabbit antiserum against the recombinant MSP2 protein reacted strongly with the recombinant MSP2 protein by Western blot analysis, and the highest titer of antibody was reached at 1:1,280. The titer of the polyclonal antibody against the recombinant MSP2 protein was further determined by IFA based on whole cells of *A. phagocytophilum* as the antigen, and the results revealed that the antibody titer reached 1:640 (Fig. 4). The titer of the self-negative-control serum collected prior to immunization with the recombinant MSP2 protein was <1:40 (Fig. 4).

**Cross-reaction of the recombinant MSP2 protein.** Cross-reactivity analyses between the recombinant MSP2 protein and each of 10 members of the order Rickettsiales (*C. burnetii, O. tsutsugamushi* strain Kato, strain Karp, strain TA763, and strain TH187, *R. heilongiangensis, B. quintana, B. bacilliformis, R. mooseri*, and *E. chaffeensis*) were conducted by ELISA, and cross-reaction was observed only for weak reactions between the recombinant MSP2 and anti-*C. burnetii* sera, anti-*B. quintana* sera, anti-*B. bacilliformis* sera, and anti-*E. chaffeensis* sera at dilutions of <1:100.

**DISCUSSION**

HGA, previously known as human granulocytic ehrlichiosis (HGE) (11), is an emerging tick-borne zoonotic infectious disease that is becoming an important public health problem in many countries of the world (1, 2, 4). Rapid diagnosis and prompt treat-

![FIG 3](image3.png) Recombinant MSP2 protein from this study reacted with HGA patient sera, rabbit anti-*Agrobacterium phagocytophilum* sera, and several negative-control sera. Lane M, protein size marker; lane 1, recombinant protein recognized by HGA-positive human sera (1:100); lane 2, recombinant protein reacted with healthy human sera (1:100); lane 3, recombinant protein recognized by rabbit anti-*Agrobacterium phagocytophilum* sera (1:20); lane 4, recombinant protein reacted with nonimmunized-rabbit sera (1:20); lane 5, recombinant protein reacted with human scrub typhus infection sera (1:100); lane 6, recombinant protein reacted with human Q fever sera (1:100); lane 7, recombinant protein reacted with human *R. typhi* infection sera (1:100); lane 8, recombinant protein reacted with 5% skim milk–TBS; and lane 9, negative control.

![FIG 4](image4.png) The results of IFA. N1 to N5, rabbit self control sera prior to immunization with recombinant MSP2 protein (diluted 1:40, 1:80, 1:160, 1:320, and 1:640); S1 to S5: rabbit antisera against recombinant MSP2 protein (diluted 1:40, 1:80, 1:160, 1:320, and 1:640); C1 to C5: rabbit antisera against the whole-cell antigen of *A. phagocytophilum* (diluted as 1:5, 1:10, 1:20, 1:40, and 1:80).
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...of the disease during the acute phase of illness are very important to avoid a poor prognosis. However, the clinical manifestations of HGA are nonspecific, with patients presenting with undifferentiated febrile illness resembling a viral infection. As an emerging infectious disease, HGA is widely unrecognized in clinics in China (4, 12). Underdiagnosis or misdiagnosis of HGA is common and may result in delayed or inappropriate treatment, leading to multiorgan failure and potentially fatal complications (4, 12, 17). A. phagocytophilum infection can be confirmed by nested PCR or real-time PCR detection of bacterial DNA in acute-phase blood (3, 4, 10), but this method requires laboratory staff with specialized training and expensive equipment. Isolation of A. phagocytophilum strain Kato, strain Karp, strain TA763, and strain TH187, members of the order Rickettsiales, including C. burnetii, O. tsutsugamushi strain Kato, strain Karp, strain TA763, and strain TH187, R. heilongjiangensis, B. bacilliformis, R. mooseri, and E. chaffeensis.

It is also worth noting that we found that the amount of soluble protein increases greatly when it is induced at lower IPTG concentrations and at lower temperatures.

We concluded that the recombinant MSP2 protein in the study showed excellent natural antigenicity and specificity for the MSP2 protein, results that should provide an economical source for specific and sensitive serologic tests. In particular, a simple and rapid diagnostic method, such as ELISA or the colloidal gold technique, could be automated in the diagnostic laboratory for HGA infection.

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