Molecular cloning, phylogenetic analysis and heat shock response of *Babesia gibsoni* heat shock protein 90

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**ABSTRACT.** The *Babesia gibsoni* heat shock protein 90 (*BgHSP90*) gene was cloned and sequenced. The length of the gene was 2,610 bp with two introns. This gene was amplified from cDNA corresponding to full length coding sequence (CDS) with an open reading frame of 2,148 bp. A phylogenetic analysis of the CDS of HSP90 gene showed that *B. gibsoni* was most closely related to *B. bovis* and *Babesia* sp. BQ1/Lintan and lies within a phylogenetic cluster of protozoa. Moreover, mRNA transcription profile for *BgHSP90* exposed to high temperature were examined by qualitative real-time reverse transcription-polymerase chain reaction. *BgHSP90* levels were elevated when the parasites were incubated at 43°C for 1 hr.

**KEY WORDS:** *Babesia gibsoni*, heat shock protein 90, phylogenetic analysis

Heat shock protein 90 (HSP90) is a 90 kDa HSP and one of the molecular chaperones responsible for managing protein folding of diverse sets of proteins, including regulatory kinases and numerous other proteins [25]. Based on the function of HSP90, HSP90 of pathogens might play important roles in the pathogens’ survival and proliferation within the host. An 82 kDa protein of the HSP90 family has recently been identified in many protozoan parasites, such as *Leishmania donovani*, *Trypanosoma cruzi*, *Toxoplasma gondii*, *Plasmodium falciparum*, *Eimeria tenella* and *E. acervulina* [1, 2, 4, 11, 21]. Several studies demonstrated that this HSP90 molecule is associated with the entry of parasite into the host cells [1, 15]. In addition, experimental evidence suggested that this molecule, localized both in cytosol and nucleus, is an essential component for stage differentiation and intracellular growth inside the host cells of many protozoans [9, 10, 14, 15, 21]. Higher class eukaryotes contain two different HSP90 isoforms, which are encoded by two different genes [8]. Human HSP90-α and HSP90-β isoforms contain 86% base pair homology [6, 16]. Both isoforms are able to form homodimers and higher order structures [13]. Recently, two HSP90 isoforms were also identified in *Babesia orientalis* and *Theileria annulata* [7, 12], and one HSP90 was identified in *B. bovis*, *Babesia* sp. BQ1/Lintan and *T. parva* [3, 5, 17]. At the current moment, the role and function of Hsp90 of those *Babesia* and *Theileria* parasites have not been well elucidated. *Babesia gibsoni* is a protozoan parasite that infects dogs and causes canine babesiosis. Canine babesiosis is a worldwide disease of hemolytic anemia and thrombocytopenia. There have been no reports regarding the HSP90 of *B. gibsoni*. In the present study, molecular cloning of the HSP90 gene of *B. gibsoni* and its phylogenetic analysis in relation to other protozoan parasites, bacteria and mammals were performed. Additionally, we investigated the change in gene transcription for HSP90 of *B. gibsoni* after exposing to high temperatures as a first step to understand the function of this molecule.

The *B. gibsoni* used in the present study had been maintained in cultures for several years [24]. To prepare dog RBCs and sera for a culture, three beagle dogs were used. The dogs used had body weight of 8–12 kg and were 2–3 years old. Regarding the experimental protocols for animal care and handling, the investigators adhered to the guidelines of Hokkaido University, which basically conform to those of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The present study was approved by the Committee for Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University (approval number: 1022).

Genomic DNA [22] and total RNA [23] of *B. gibsoni* were extracted as described previously [22]. To avoid the contamination of genomic DNA, genomic DNA was digested on the column using the RNase-free DNase set (QIAGEN, Valencia, CA, U.S.A.) during total RNA extraction. cDNA was synthesized from the total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Tokyo, Japan) according to the manufacturer’s instructions. The PCR
primers used for the amplification of the partial \( HSP90 \) gene of \( B. \ gibsoni \) were designed based on sequences conserved among the \( HSP90 \) gene of \( B. \ bovis \) (AF136649) and \( T. \ parva \) (M57386). The primers used in the present study are listed in Table 1. These primers had a degeneracy to allow amplification of different bases. Genomic DNA and cDNA in reaction mixtures were prepared according to the manufacturer’s protocol (Ex-Taq polymerase; Takara, Tokyo, Japan), and then, it was amplified for 35 cycles (denaturation for 1 min at 95°C, annealing for 1 min at 55°C and extension for 1 min at 72°C) followed by the final extension for 5 min at 72°C in a Veriti™ 96 Well Thermal Cycler (Applied Biosystems).

To determine the nucleotide sequence of 5′-end and 3′-end of \( B. \ bovis \) and \( T. \ parva \) among the \( HSP90 \) gene of different \( Babesia \) species, \( HSP90 \) mRNA, the Rapid Amplification of cDNA Ends (RACE) method was performed using SMART™ RACE cDNA Amplification Kit (Clontech Laboratory, Mountain View, CA, U.S.A.) according to the manufacturer’s instructions. The primers for the RACE method (BgHSP90-5′ and BgHSP90-3′) were designed based on the analyzed nucleotide sequence (Table 1). Each reaction product was examined by electrophoresis on a 1.5% agarose gel to confirm that it was a single product and directly utilized for the sequencing analysis. The nucleotide sequence of the amplification products was determined by an Applied Biosystems 3130 genetic analyzer (Applied Biosystems) using the ABI PRISM Big-Dye Terminator v 3.1 Cycle-Sequencing kit (Applied Biosystems) [22]. The primers for the amplification were also utilized for the sequencing analysis. The nucleotide sequence analyzed was confirmed as the \( HSP90 \) gene from \( B. \ gibsoni \) (BgHSP90) through BLAST search, because that showed the high identity with \( T. \ gondii \) (AF038559) and \( B. \ orientalis \) (KF379584) and \( HSP90-B \) gene, although the \( HSP90-B \) gene of mammal parasites is still unknown. A phylogenetic tree was inferred using ClustalX ver. 2.1 by the neighbor-joining method [20]. In addition to \( B. \ gibsoni \), CDS of the \( HSP90 \) gene from GenBank database for \( B. \ bovis \) (B. bovis T2Bo (XM_001611817), Babesia sp. BQ1/Lintan, B. orientalis (KF379584 [clone 14a], KF379585 [clone 14c]), \( T. \ parva \), \( T. \ annulata \) strain Ankara (XM_947380 [TA1201], XM_948193 [TA10720], XM_948749 [TA06430]), \( P. \ falicatunum \) 3D7 (NC_004317), \( P. \ knowlesi \) (XM_00259147), \( E. \ tenella \), \( E. \ acervulina \) (AY459429), \( T. \ gondii \), \( Cryptosporidium parvum \) (AF038559) and \( C. \ parvum \) Iowa II (XM_626924) was used in the phylogenetic analysis. To estimate the genetic distance from other species, CDS of the \( htpG \) genes from bacteria, such as \( Yersinia enterocolitica \) subspp. enterocolitica 8011 (NC_008800) and \( Bordetella pertussis \) CS (NC_017223), and mRNA of the \( HSP90 \) genes from vertebrates, such as \( Homo sapiens \) (NM_001017963 [HSP90-α], NM_003299 [HSP90-β]), \( Sus scrofa \) (NM_213973 [HSP90-α], NM_214103 [HSP90-β]), \( Mus musculus \) (NM_010480 [HSP90-α], NM_011631 [HSP90-β]) and \( Rattus norvegicus \) (NM_157561 [HSP90-α], NM_001012197 [HSP90-β]) were also included in the analysis. To estimate the genetic distance from other species, the \( HSP70 \) gene of \( B. \ gibsoni \) (AB083510) was also included as an outgroup. A phylogenetic analysis of the \( HSP90 \) gene showed that \( HSP90-α \) and \( -β \) isoforms from mammals made

| Name         | Sequences | Tm (°C) |
|--------------|-----------|---------|
| BgHSP90F1    | 5′-ggt gts gsr ttc tca gc3′ | 64      |
| BgHSP90F2    | 5′-gct tga agg tca act cga at3′ | 58      |
| BgHSP90F3    | 5′-cag ggt aag tca cag gat ct3′ | 58      |
| BgHSP90F4    | 5′-gcc aac gac aag atc gat gc3′ | 64      |
| BgHSP90F5    | 5′-agt ggg aga tgc tca aca agc-3′ | 64      |
| BgHSP90F6    | 5′-tag ctc ctc agg aga act-3′ | 62      |
| BgHSP90R1a   | 5′-tgct cag tgg tag aac ttc3′ | 64      |
| BgHSP90R2c   | 5′-ytt gcc agg ctc cgg ctt tct3′ | 60      |
| BgHSP90R3a   | 5′-tcc cag tgg tgc ctc agg ttc3′ | 66      |
| BgHSP90-5′b  | 5′-cca cgc ttc aac atg tca cca cag tgc-3′ | 82      |
| BgHSP90-3′c  | 5′-tct ggg aga tgc aag tgg aac-3′ | 76      |

a) Antisense primers. b) Primer for 5′-RACE. c) Primer for 3′-RACE. d) Melting temperature.
separate groups and that HSP90 from protozoan made three groups located outside the paraphyletic group containing HtpG from bacteria and HSP90 from mammals (Fig. 2). This result suggested that the protozoan would have several isoforms of HSP90, as reported previously [7, 12]. HSP90s of Protozoan group 1 including B. gibsoni analyzed in the present study closely related to the group of HSP90-α from mammals (Fig. 2). Therefore, BgHSP90 might have the similar role and function to that of HSP90-α. A further detailed study might be necessary to elucidate the roles and functions of BgHSP90 in the proliferation of B. gibsoni. Additionally, T. annulata has three different HSP90s in different group, and B. bovis and B. orientalis have two different HSP90s in different group, suggesting that those parasites would have

Fig. 1. Alignment of the predicted amino acid sequence of Babesia gibsoni heat shock protein 90 (BgHSP90) with HSP90 sequences of Babesia sp. BQ1/Lintan and Babesia bovis. Region A (from positions 1 to 9 amino acids) and B (from 231 to 267) are characteristic sequences for each species. Identical residues are marked by asterisk (*). Additions and gaps in the sequences are indicated by dashes (–).
two or three \textit{HSP90} isoforms. Therefore, \textit{B. gibsoni} should have two or three \textit{HSP90} isoforms.

To examine the change in the transcription of the \textit{BgHSP90} gene in the cultured \textit{B. gibsoni} after shifting temperature, we performed a quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). For exposure to high temperature, the parasites cultured under normal conditions (38°C) were divided into 2 groups. One group was incubated at 38°C for 1 hr as a control. The other group was incubated at 43°C for 1 hr. Total RNA of those \textit{B. gibsoni} was extracted, and cDNA was synthesized. qRT-PCR was performed using the resulting cDNA as a template and a specific primer pair.
BgHSP90F5-BgHSP90R3. Reaction mixtures with 50 ng of cDNA as a template were amplified with an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems) as described previously [23]. Values were expressed as raw copy numbers (per microliter of cDNA). The quantity of 18S rRNA was also measured by qRT-PCR as described previously [23]. To correct for differences in the amount of RNA, the calculated copy numbers of the BgHSP90 gene were adjusted according to the copy numbers of B. gibsoni 18S rRNA. Thus, values were also expressed as relative amounts. This experiment was conducted 3 times. Data on the relative amount or copy numbers of BgHSP90 gene, and copy number of 18S rRNA at 43°C were compared with those at 38°C. Data for each temperature were expressed as the mean ± SD (n=3). The statistical analysis was performed using a Student’s t-test. The difference between data was considered significant at P<0.05. Although, the copy number of 18S rRNA at 43°C was almost the same as that at 38°C (Table 2), both the copy number and the relative amount of the BgHSP90 gene significantly (P<0.05) increased when the temperature was shifted from 38°C to 43°C for 1 hr (Table 2). These results suggested that the expression of BgHSP90 was enhanced. In our previous study, the level of parasitemia at high temperature (42°C) was almost the same as that under normal conditions, and BgHSP70 was heat-inducible [23]. Based on the results of the present study and the previous report, it was suggested that the enhanced expression of BgHSP90 might be independent from the proliferation of the parasites and that BgHSP90 would also be heat-inducible. This is the first report concerning heat shock response of HSP90 from Babesia parasites. It is well known that HSP90 from mammals protects cells from heat and oxidative stress [18, 19]. However, the functions of BgHSP90 for the proliferation of the parasites are still unclear.

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