NOTE *Parasitology*

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

Masahiro YAMASAKI[1)]*, Yoshihiro TSUBOI[2)], Yusuke TANIYAMA[2)], Naohiro UCHIDA[1)], Reeko SATO[1)], Kensuke NAKAMURA[3)], Hiroshi OHTA[2)] and Mitsuyoshi TAKIGUCHI[2)]

[1)]*Laboratory of Veterinary Small Animal Internal Medicine, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka, Iwate 020–8550, Japan*
[2)]*Laboratory of Veterinary Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060–0818, Japan*
[3)]*Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060–0818, Japan*

(Received 18 January 2016/Accepted 14 April 2016/Published online in J-STAGE 28 April 2016)

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 quantitative 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

doi: 10.1292/jvms.16-0027; *J. Vet. Med. Sci.* 78(8): 1355–1360, 2016

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

*CORRESPONDENCE TO: YAMASAKI, M., Laboratory of Veterinary Small Animal Internal Medicine, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka, Iwate 020–8550, Japan. e-mail: masayama@iwate-u.ac.jp

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 *BgHSP90* 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 BigDye 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 *HSP90* gene from *Babesia* and *Theileria* parasites. This gene included an open reading frame of 2,148 bp, and the encoded polypeptide was comprised of 716 amino acid residues with a predicted size of 82.8 kDa, as determined using a computer-based molecular weight calculator. The accession number of *BgHSP90* in DDBJ database is LC064030. The comparison of the coding sequence (CDS) of *HSP90* gene was performed using GENETYX-MAC ver. 11.2 (Genetyx Co., Tokyo, Japan). The CDS of the *HSP90* gene from *B. gibsoni* was compared with that of *B. bovis*, *Babesia* sp. BQ1/Lintan (GQ397856), *T. parva*, *E. tenella* (AF042329) and *Toxoplasma gondii* (AY344115), and found to have 78.9, 80.9, 74.1, 71.7 and 69.2% identity, respectively. The predicted amino acid sequence of HSP90 from *B. gibsoni* was also compared with that of *B. bovis*, *Babesia* sp. BQ1/Lintan, *T. parva*, *E. tenella* and *T. gondii*, and found to show 89.0, 89.7, 81.6, 72.0 and 70.4% identity, respectively. Among the amino acid sequences of *HSP90* from *Babesia* parasites, amino acids from positions 1 to 9, and 231 to 267 were characteristic for each species; the remaining parts were almost the same among those *Babesia* parasites (Fig. 1). In the present study, *BgHSP90* had higher identity with *HSP90* from *B. bovis* and *Babesia* sp. BQ1/Lintan than that from other protozoan, suggesting that *HSP90* gene would be well conserved among *Babesia* parasites.

The *BgHSP90* gene was amplified from both genomic DNA and cDNA by PCR. The amplified sequence using BgHSP90F6 and BgHSP90R3 primers from genomic DNA contained 1,038 bp, and that from cDNA contained 918 bp. Moreover, the amplified sequence using BgHSP90F2 and BgHSP90R2 primers from genomic DNA contained 1,449 bp, and that from cDNA contained 1,192 bp. The sequence of *BgHSP90* gene from genomic DNA was 2,610 bp long, while that from cDNA was 2,233 bp long. These results showed that the *BgHSP90* gene analyzed in the present study included two introns at positions 75 to 195 and 1,471 to 1,728 bp. Khan *et al.* [7] identified and characterized two novel isoforms of *HSP90* from *B. orientalis* encoding *HSP90-A* (KF379584) and *HSP90-B* (KF379584). One intron was detected in the *HSP90-A* gene, although the *HSP90-B* gene had no intron. The role of introns in *HSP90* gene of *Babesia* parasites is still unknown.

Table 1. Oligonucleotides for the analysis of the *Babesia gibsoni HSP90* gene

Name	Sequences	Tm (°C)[d)]
BgHSP90F1	5′-ggt gts ggt ttc tac tca gc-3′	64
BgHSP90F2	5′-cgt tga agg tca act cga at-3′	58
BgHSP90F3	5′-caa ggt aag tca cag gat ct-3′	58
BgHSP90F4	5′-gcc aac cgc aac aag atc gc-3′	64
BgHSP90F5	5′-agt ggg aga tgc tca aca agc-3′	64
BgHSP90F6	5′-tag ctg ctc agg aga cct ac-3′	62
BgHSP90R1[a)]	5′-ttg ctg aac tgc tag aac t-3′	64
BgHSP90R2[a)]	5′-ytt agt caa ctt cyt cca tyt t-3′	60
BgHSP90R3[a)]	5′-tcc cag tcg ttg cac agg ttc-3′	66
BgHSP90-5′[b)]	5′-cca cgc ttc aac atg tca cca gat tcg-3′	82
BgHSP90-3′[c)]	5′-ctt cgt gac aac agc ttc ggg agc-3′	76

a) Antisense primers. b) Primer for 5′-RACE. c) Primer for 3′-RACE. d) Melting temperature.

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 [TA12105], XM_948193 [TA10720], XM_948749 [TA06470]), *P. falciparum* 3D7 (NC_004317), *P. knowlesi* (XM_002259147), *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* subsp. enterocolitica 8081 (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_175761 [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

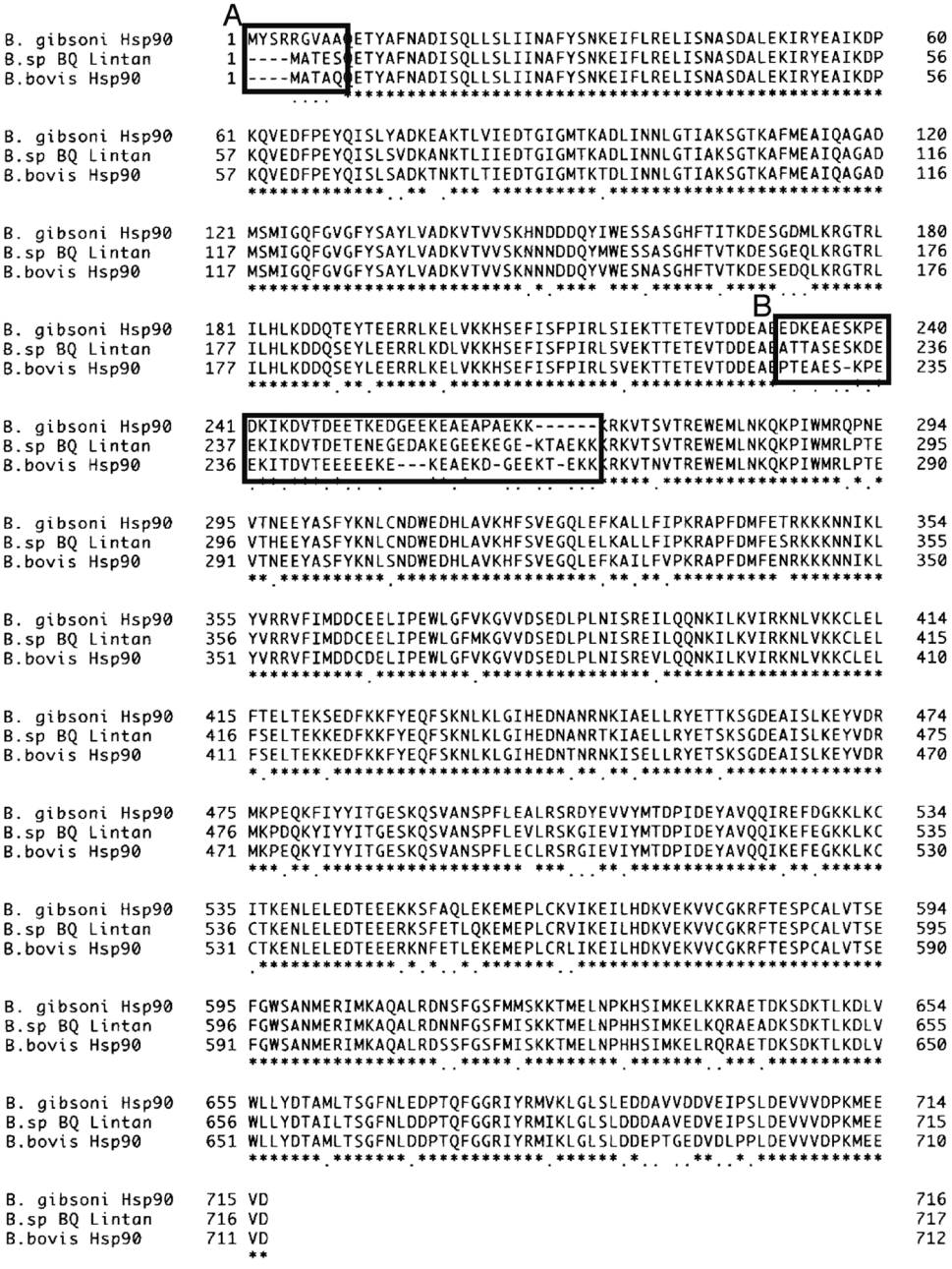

Fig. 1. Alignment of the predicted amino acid sequence of *Babesia gibsoni heat shock protein 90* (*BgHSP90*) with *HSP90* sequences of *Babesia* sp. BQ1/Lintan and *Babesia bovis*. Region A (from positions 1 to 9 amino acids) and B (from 231 to 267) are characteristic sequences for each species. Identical residues are marked by asterisk (*). Additions and gaps in the sequences are indicated by dashes (−).

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

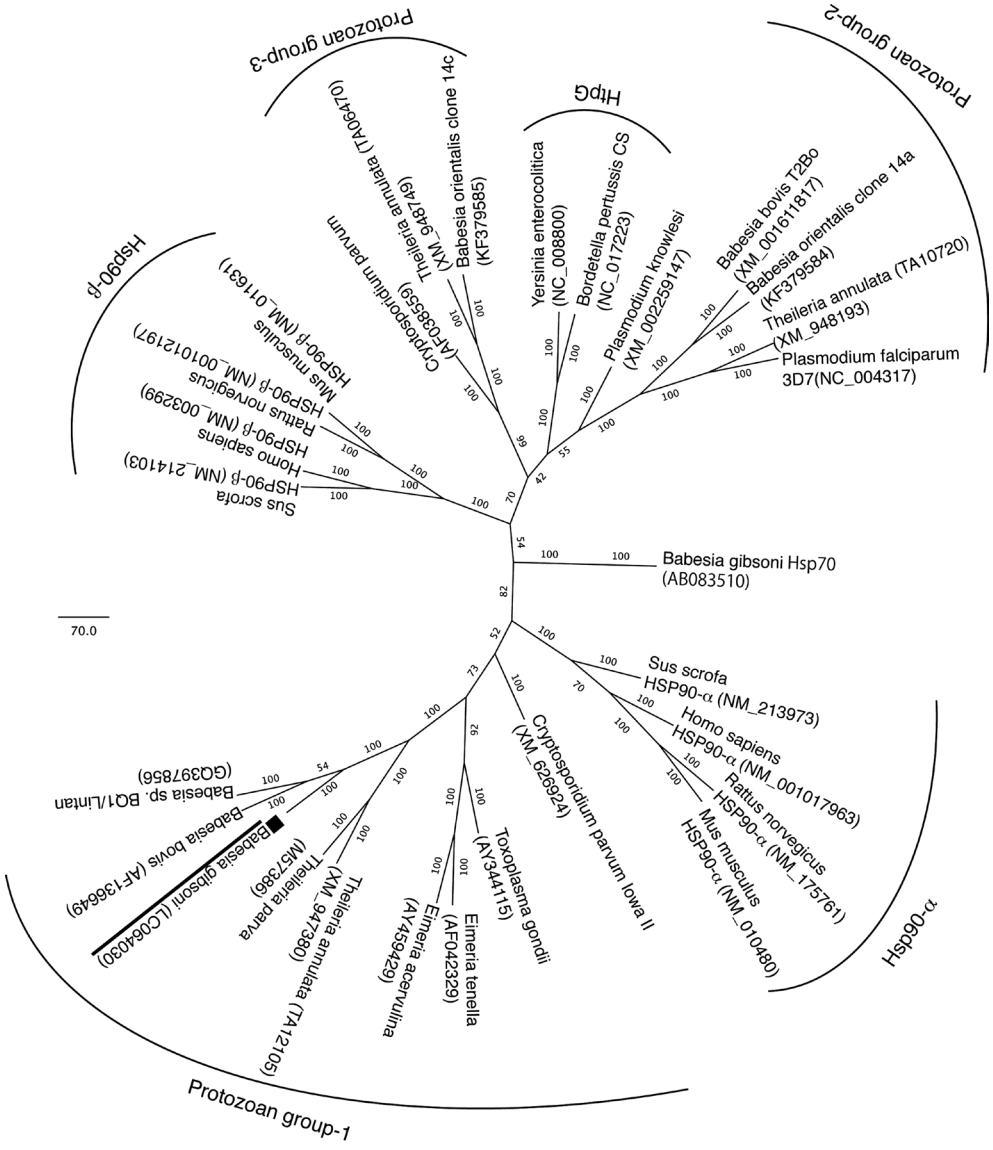

Fig. 2. Phylogenetic tree based on the full coding region of *heat shock protein 90* (*HSP90*) genes. The dendrogram was constructed using the neighbor-joining method. The numbers at the nodes indicate bootstrap support from 1,000 replications. The length of lines is proportional to the bootstrap value. The scale bar represents a 70% bootstrap value. In addition to *B. gibsoni*, complete sequences of *HSP90* gene from *B. bovis* (AF136649), *B. bovis* T2Bo (XM_001611817), *Babesia* sp. BQ1/Lintan (GQ397856), *B. orientalis* (KF379584 [clone 14a], KF379585 [clone 14c]), *T. parva* (M57386), *T. annulata* strain Ankara (XM_947380 [TA12105], XM_948193 [TA10720], XM_948749 [TA06470]), *P. falciparum* 3D7 (NC_004317), *P. knowlesi* (XM_002259147), *E. tenella* (AF042329), *E. acervulina* (AY459429), *Toxoplasma gondii* (AY344115), *Cryptosporidium parvum* (AF038559), *C. parvum* Iowa II (XM_626924), *Yersinia enterocolitica* subsp. enterocolitica 8081 (NC_008800), *Bordetella pertussis* CS (NC_017223), *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_175761 [HSP90-α], NM_001012197 [HSP90-β]) were included in the analysis. The *HSP70* gene of *B. gibsoni* (AB083510) was also included as an outgroup.

two or three *HSP90* isoforms. Therefore, *B. gibsoni* should have two or three *HSP90* isoforms.

To examine the change in the transcription of the *BgHSP90* gene in the cultured *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 *B. gibsoni* was extracted, and cDNA was synthesized. qRT-PCR was performed using the resulting cDNA as a template and a specific primer pair,

Table 2. Transcription of *BgHSP90* gene in *Babesia gibsoni* at 43°C. The real-time qRT-PCR was performed with 50 *n*g of cDNA. The copy number of the *BgHSP90* gene and 18S rRNA was calculated in the same RT-PCR run. The relative amount of the *BgHSP90* gene was also calculated by adjusting the copy number of the gene to that of 18S rRNA

Incubation temp.	38˚C	43˚C
Copy no. (/*μl*)		
BgHSP90	5,003.1 ± 789.7	15,711.1 ± 1,093.4[a)]
18S rRNA	8,113.4 ± 1,893.4	8,275.9 ± 559.1
Relative amount (*BgHSP90*/18S rRNA)	0.62 ± 0.10	1.9 ± 1.1[a)]

Data are expressed as the mean ± SD (n=3). a) Significantly ($P<0.05$) different from the value at 38°C.

BgHSP90F5-BgHSP90R3. Reaction mixtures with 50 *n*g 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 quantitiy 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.

ACKNOWLEDGMENT. This work was supported in part by a grant from the Science Research Fund of the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant number: 2458045602).

REFERENCES

1. Ahn, H. J., Kim, S. and Nam, H. W. 2003. Molecular cloning of the 82-kDa heat shock protein (HSP90) of *Toxoplasma gondii* associated with the entry into and growth in host cells. *Biochem. Biophys. Res. Commun.* **311**: 654–659. [Medline] [CrossRef]
2. Banumathy, G., Singh, V., Pavithra, S. R. and Tatu, U. 2003. Heat shock protein 90 function is essential for *Plasmodium falciparum* growth in human erythrocytes. *J. Biol. Chem.* **278**: 18336–18345. [Medline] [CrossRef]
3. Gerhards, J., Ebel, T., Dobbelaere, D. D., Morzaria, S. P., Musoke, A. J., Williams, R. O. and Lipp, J. 1994. Sequence and expression of a 90-kilodalton heat-shock protein family member of *Theileria parva*. *Mol. Biochem. Parasitol.* **68**: 235–246. [Medline] [CrossRef]
4. Graefe, S. E. B., Wiesgigl, M., Gaworski, I., Macdonald, A. and Clos, J. 2002. Inhibition of HSP90 in Trypanosoma cruzi induces a stress response but no stage differentiation. *Eukaryot. Cell* **1**: 936–943. [Medline] [CrossRef]
5. Guan, G., Chauvin, A., Yin, H., Luo, J. and Moreau, E. 2010. Course of infection by Babesia sp. BQ1 (Lintan) and B. divergens in sheep depends on the production of IFNgamma and IL10. *Parasite Immunol.* **32**: 143–152. [Medline] [CrossRef]
6. Hickey, E., Brandon, S. E., Smale, G., Lloyd, D. and Weber, L. A. 1989. Sequence and regulation of a gene encoding a human 89-kilodalton heat shock protein. *Mol. Cell. Biol.* **9**: 2615–2626. [Medline] [CrossRef]
7. Khan, M. K., He, L., Zhang, W., Wang, Y., Tao, Q., Song, Q., Sajid, M. S., Yu, Q., Hu, J., Fang, R., Hu, M., Zhou, Y. and Zhao, J. 2014. Identification of two novel HSP90 proteins in *Babesia orientalis*: molecular characterization, and computational analyses of their structure, function, antigenicity and inhibitor interaction. *Parasit Vectors* **7**: 293. [Medline] [CrossRef]
8. Langer, T. and Fasold, H. 2001. Isolation and quantification of the heat shock protein 90 alpha and beta isoforms from rat liver. *Protoplasma* **218**: 54–56. [Medline] [CrossRef]
9. Li, Q., Zhou, Y., Yao, C., Ma, X., Wang, L., Xu, W., Wang, Z. and Qiao, Z. 2009. Apoptosis caused by Hsp90 inhibitor geldanamycin in *Leishmania donovani* during promastigote-to-amastigote transformation stage. *Parasitol. Res.* **105**: 1539–1548. [Medline] [CrossRef]
10. Meyer, K. J. and Shapiro, T. A. 2013. Potent antitrypanosomal activities of heat shock protein 90 inhibitors *in vitro* and *in vivo*. *J. Infect. Dis.* **208**: 489–499. [Medline] [CrossRef]
11. Miska, K. B., Fetterer, R. H., Min, W. and Lillehoj, H. S. 2005. Heat shock protein 90 genes of two species of poultry Eimeria: expression and evolutionary analysis. *J. Parasitol.* **91**: 300–306. [Medline] [CrossRef]
12. Mohammed, S. B., Bakheit, M. A., Ernst, M., Ahmed, J. S. and Seitzer, U. 2013. Identification and characterization of *Theileria annulata* heat-shock protein 90 (HSP90) isoforms. *Transbound. Emerg. Dis.* **60** Suppl 2: 137–149. [Medline] [CrossRef]
13. Nemoto, T. and Sato, N. 1998. Oligomeric forms of the 90-kDa heat shock protein. *Biochem. J.* **330**: 989–995. [Medline]

[CrossRef]
14. Pallavi, R., Roy, N., Nageshan, R. K., Talukdar, P., Pavithra, S. R., Reddy, R., Venketesh, S., Kumar, R., Gupta, A. K., Singh, R. K., Yadav, S. C. and Tatu, U. 2010. Heat shock protein 90 as a drug target against protozoan infections: biochemical characterization of HSP90 from *Plasmodium falciparum* and *Trypanosoma evansi* and evaluation of its inhibitor as a candidate drug. *J. Biol. Chem.* **285**: 37964–37975. [Medline] [CrossRef]
15. Péroval, M., Péry, P. and Labbé, M. 2006. The heat shock protein 90 of *Eimeria tenella* is essential for invasion of host cell and schizont growth. *Int. J. Parasitol.* **36**: 1205–1215. [Medline] [CrossRef]
16. Rebbe, N. F., Ware, J., Bertina, R. M., Modrich, P. and Stafford, D. W. 1987. Nucleotide sequence of a cDNA for a member of the human 90-kDa heat-shock protein family. *Gene* **53**: 235–245. [Medline] [CrossRef]
17. Ruef, B. J., Ward, T. J., Oxner, C. R., Conley, P. G., Brown, W. C. and Rice-Ficht, A. C. 2000. Phylogenetic analysis with newly characterized *Babesia bovis* hsp70 and hsp90 provides strong support for paraphyly within the piroplasms. *Mol. Biochem. Parasitol.* **109**: 67–72. [Medline] [CrossRef]
18. Smirlis, D., Duszenko, M., Ruiz, A. J., Scoulica, E., Bastien, P., Fasel, N. and Soteriadou, K. 2010. Targeting essential pathways in trypanosomatids gives insights into protozoan mechanisms of cell death. *Parasit Vectors* **3**: 107. [Medline] [CrossRef]
19. Taipale, M., Jarosz, D. F. and Lindquist, S. 2010. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat. Rev. Mol. Cell Biol.* **11**: 515–528. [Medline] [CrossRef]
20. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**: 4876–4882. [Medline] [CrossRef]
21. Wiesgigl, M. and Clos, J. 2001. Heat shock protein 90 homeostasis controls stage differentiation in *Leishmania donovani*. *Mol. Biol. Cell* **12**: 3307–3316. [Medline] [CrossRef]
22. Yamasaki, M., Tajima, M., Lee, K. W., Jeong, J. R., Yamato, O. and Maede, Y. 2002. Molecular cloning and phylogenetic analysis of *Babesia gibsoni* heat shock protein 70. *Vet. Parasitol.* **110**: 123–129. [Medline] [CrossRef]
23. Yamasaki, M., Tajima, M., Yamato, O., Hwang, S. J., Ohta, H. and Maede, Y. 2008. Heat shock response of *Babesia gibsoni* heat shock protein 70. *J. Parasitol.* **94**: 119–124. [Medline] [CrossRef]
24. Yamasaki, M., Harada, E., Tamura, Y., Lim, S. Y., Ohsuga, T., Yokoyama, N., Morishita, K., Nakamura, K., Ohta, H. and Takiguchi, M. 2014. *In vitro* and *in vivo* safety and efficacy studies of amphotericin B on *Babesia gibsoni*. *Vet. Parasitol.* **205**: 424–433. [Medline] [CrossRef]
25. Young, J. C., Agashe, V. R., Siegers, K. and Hartl, F. U. 2004. Pathways of chaperone-mediated protein folding in the cytosol. *Nat. Rev. Mol. Cell Biol.* **5**: 781–791. [Medline] [CrossRef]