Babesia bovis BOV57, a *Theileria parva* P67 homolog, is an invasion-related, neutralization-sensitive antigen

Takahiro Ishizaki, Thillaiampalam Sivakumar, Kyoko Hayashida, Hitoshi Takemae, Bumduuren Tuvshintulga, Tserendorj Munkhjargal, Azirwan Guswanto, Ikuo Igarashi, Naoaki Yokoyama*

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan.

Corresponding author: Naoaki Yokoyama

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan.

Tel.: +81-155-49-5649; Fax: +81-155-49-5643;

E-mail address: yokoyama@obihiro.ac.jp

Abstract

Babesia bovis BOV57, which is a homolog of the *Theileria parva* vaccine candidate antigen P67, is expressed in both the tick and blood stages of the life cycle of this parasite. However, the vaccine potential of BOV57 remained to be investigated. In the present study, we generated recombinant BOV57 (rBOV57) and prepared polyclonal antibodies against rBOV57 in mice and rabbits. Indirect immunofluorescence assays conducted with the mouse anti-rBOV57 antibody demonstrated that BOV57 localized at the apical end of paired merozoites in infected bovine red blood cells, whereas the antigen was found in the parasite membrane around the apical end of intraerythrocytic single and extracellular merozoites. In an invasion-inhibition assay, the rabbit anti-rBOV57 antibody potentially inhibited RBC invasion of *B. bovis* merozoites *in vitro*. In addition, the invasion inhibition mediated by rabbit anti-rBOV57 antibody resulted in a reduced growth rate of *B. bovis* in the *in vitro* culture. These findings indicated that *B. bovis* BOV57 plays a critical role in the invasion of merozoites into red blood cells, suggesting its potential as a subunit vaccine candidate against *B. bovis* infection in cattle. Furthermore, we analyzed the genetic diversity of *bov57* gene sequences isolated from Sri Lanka, Mongolia, the Philippines, and Vietnam. The *bov57* gene sequences derived from Mongolia, the Philippines, and Vietnam were conserved, whereas

insertion and/or deletion mutations resulted in sequence diversity among the Sri Lankan samples. In summary, BOV57 is an invasion-related, neutralization-sensitive antigen encoded by the *bov57* gene, which displays higher sequence diversity than previously reported.

Keywords: *Babesia bovis*, BOV57, genetic diversity, neutralization sensitive

1. **Introduction**

In apicomplexan species, several protozoan molecules skillfully mediate parasite invasion into host cells. The molecules involved in their invasion process are often considered targets for the development of effective immune control and chemotherapeutic methods (Musoke et al., 1992; Srinivasan et al., 2014). *Babesia bovis*, which is an intraerythrocytic protozoa transmitted by ixodid ticks, infects cattle and causes a fatal bovine babesiosis characterized by fever, anemia, icterus, hemoglobinuria, neurological and respiratory syndromes, and sometimes death (Bock et al., 2004). Live attenuated vaccines have been used in some endemic countries to immunize cattle against *B*. *bovis* (Shkap et al., 2007). However, several limitations, such as contamination of the vaccine with other blood pathogens and strain-specific immunity against *B*. *bovis,* prevent the widespread use of live vaccines (Bock et al., 1992; Rogers et al., 1988). Although the use of subunit vaccines to immunize cattle against *B*. *bovis* might overcome these constraints, such vaccines were not developed until now (Brown et al., 2006). At present, several parasite antigens are known to be involved in the invasion of *B*. *bovis* merozoites into red blood cells (RBCs) (Yokoyama et al., 2006).

The glycosyl-phosphatidylinositol-anchored merozoite surface antigens (MSA), including MSA-1 and the MSA-2 family that consists of MSA-2c, MSA-2a1, MSA-2a2, and

MSA-2b are associated with the attachment of merozoites to bovine RBCs, as well as with the gliding motility of *B. bovis* merozoites (Asada et al., 2012; Florin-Christensen et al., 2002; Suarez et al., 2000). After the attachment of merozoites, the formation of tight junctions and the subsequent invasion process are mediated by secretory antigens, such as rhoptry-associated protein (RAP)-1, apical membrane protein (AMA)-1, and thrombospondin-related anonymous protein (TRAP) (Gaffer et al., 2004a,b; Yokoyama et al., 2000). Finally, the spherical body proteins are considered to play a critical role in the internalization of *B. bovis* merozoites (Dowling et al., 1996; Hines et al., 1995a). Although immune sera against some of these parasite molecules effectively inhibited *B. bovis* growth *in vitro* (Gaffer et al., 2004a,b; Suarez et al., 2000; Yokoyama et al., 2000), the cattle experiments failed to yield expected results (Antonio Alvarez et al., 2010; Hines et al., 1995b). Additionally, the antigenic variation of some of the candidate antigens, in particular the MSAs, is a stumbling block for vaccine development (Berens et al., 2005; Leroith et al., 2005; Liyanagunawardena et al., 2016; Sivakumar et al., 2013; Tattiyapong et al., 2016). Therefore, vaccine development against *B. bovis* will benefit from studies that identify additional invasion-related protozoan molecules.

Analyses of the *B. bovis* genome detected a novel gene that was homologous to the *Theileria parva p67* gene (Freeman et al., 2010). The antigen P67, encoded by the *p67* gene,

has been well studied as a potential candidate for the development of subunit vaccine against East coast fever caused by *T. parva* (Brayton et al., 2007; Musoke et al., 1992, 2005). A recent study found that the P67-homologous antigen in *B. bovis*, also known as BOV57, is expressed in both the blood and tick stages of *B. bovis* (Freeman et al., 2010). Since P67 and its homologous antigen in *Theileria annulata*, SPAG-1, offered protection against clinical disease induced by *T. parva* and *T. annulata* infections, respectively (Boulter et al., 1994, 1999; Musoke et al., 1992), it would be interesting to investigate BOV57 as a neutralization-sensitive antigen.

In the present study, we produced recombinant BOV57 (rBOV57) and used it to immunize mice and rabbits to obtain polyclonal antibodies. The mouse and rabbit anti-rBOV57 antibodies were then used to localize the native BOV57 antigen in *B. bovis* merozoites and to analyze the neutralization sensitivity of the antigen, respectively. Additionally, *bov57* gene sequences were isolated from *B. bovis*-positive blood DNA samples sourced from cattle populations reared in different countries in Asia, to analyze the genetic diversity of this gene.

2. Materials and Methods

2.1. In vitro *culture of B. bovis and cDNA synthesis*

Babesia bovis (Texas strain) was cultured *in vitro* with purified bovine RBCs in serum-free GIT medium (WAKO Pure Chemical Industrial, Ltd, Osaka, Japan), as described previously (Bork et al. 2005). A SuperScript III Reverse Transcriptase kit (ThermoFisher Scientific K.K., Yokohama, Japan) was employed to synthesis the cDNA pool derived from the total RNAs that had been extracted from the *in vitro* culture of *B. bovis* using TRI reagent (Sigma, Tokyo, Japan), according to the manufacturer's instructions.

2.2. Expression and purification of recombinant BOV57

The amino acid sequence translated from the *bov57* gene of *B. bovis* strain T2Bo (GenBank accession number XM_001610647) was analyzed using SignalP 4.0 (Petersen et al., 2011) to predict the signal peptide. Then, a pair of forward (5′-AAAGAATTCTTCGATTTGGGTGAATGGTCACATGATGCG-3′, the *EcoR*I restriction site is underlined) and reverse (5′-AAACTCGAGTTACCACCTGTAAATGTGTGGATGGTG-3′, the *Xho*I restriction site is underlined) primers was used to amplify the truncated *bov57* gene from the cDNA pool,

excluding the signal peptide region. The PCR product was digested with the corresponding restriction enzymes, and then cloned into a similarly digested expression vector, pGEX4T-1 (GE Healthcare, Uppsala, Sweden). Subsequently, the plasmid vector was transformed into *Escherichia coli* BL21, and then expressed as a glutathione S-transferase (GST)-fusion recombinant protein (GST-rBOV57), as described previously (Tattiyapong et al., 2016). After GST-rBOV57 had been purified, the GST-tag was excised using Thrombin (GE Healthcare) to obtain the recombinant BOV57 (rBOV57) (Tattiyapong et al., 2016).

2.3. Anti-rBOV57 polyclonal antibodies

Three six-week-old BALB/c mice (Clea, Tokyo, Japan) were subcutaneously injected with 50 μg of the rBOV57 antigen emulsified in a Titer Max Gold adjuvant (TiterMax USA Inc., Norcross, GA, U.S.A), three times at intervals of 10 days. The immune sera were collected on day 14 from the third immunization.

The production of rabbit anti-rBOV57 antibody and the subsequent purification of total IgG were contracted to an antibody production service (Japan Bio Serum, Hiroshima, Japan).

2.4. Immunofluorescence antibody test (IFAT)

Thin RBC smears were prepared from the *in vitro* culture of *B*. *bovis* and were then fixed with 50% acetone-methanol. A double IFAT was conducted using the mouse anti-rBOV57 antibody, together with rabbit anti-rRAP-1 or anti-rMSA-2c IgG (Terkawi et al., 2011) as primary antibodies, and subsequently goat anti-mouse IgG alexa fluor 488 and goat anti-rabbit IgG alexa fluor 594 (ThermoFisher) as secondary antibodies, as described previously (Terkawi et al., 2013).

2.5. Western blot analysis

For the extraction of total protein, 20 µl of *B*. *bovis*-infected RBCs and non-infected RBCs were lysed with 50 µl of 0.2% Triton X-100 in PBS containing a protease-inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) at 4°C for 30 min. The supernatant collected after centrifugation at 15,000 rpm for 30 min at 4°C was suspended in an equal volume of 2× SDS sample buffer (Nacalai Tesque), and then boiled at 95°C for 5 min. The samples (approximately 50 µg of the total extracted protein) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel. Then proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane, and subjected to western blot analysis using the mouse anti-rBOV57 antibody (1:250 dilution) as the primary antibody, and horseradish peroxidase-conjugated sheep anti-mouse IgG (1:2000;

GE healthcare) as secondary antibody, as described previously (Yokoyama et al., 2000).

2.6. Invasion-inhibition and growth-inhibition assays

An invasion-inhibition assay was conducted essentially as described previously (Ishizaki et al., 2016). Briefly, an *in vitro* culture of *B*. *bovis* was incubated on ice for 2 hr, and then, free merozoites liberated from the infected RBCs were purified using a Percoll gradient method. The *B*. *bovis* merozoites were then incubated with uninfected bovine RBCs in culture medium containing rabbit anti-rBOV57 IgG (0.25, 0.5 and 1 mg/ml), rabbit anti-rRAP-1 IgG (1 mg/ml) (Terkawi et al., 2011) or control rabbit IgG (1 mg/ml) (Terkawi et al., 2013). The percent of parasitemia in the cultures was determined after a 60-min incubation.

A growth-inhibition assay was performed as described previously, with minor modifications (Terkawi et al., 2013). Briefly, *B*. *bovis*-infected RBCs were diluted with uninfected bovine RBCs to achieve 1% parasitemia with a 10% packed volume, and were then incubated with culture medium containing 1 mg/ml of the rabbit anti-rBOV57 IgG or control rabbit IgG. The culture medium containing the rabbit anti-rBOV57 IgG or control rabbit IgG was replaced every day, and parasite growth was monitored by microscopic examination of the Giemsa-stained RBC smears.

2.7. PCR amplification and sequencing of the bov57 gene from B. bovis-positive bovine DNA samples, and phylogenetic analysis

B. bovis-positive archived blood-DNA samples that had been sourced from cattle in Sri Lanka (n=12, Sivakumar et al., 2013), Mongolia (n=8, Altangerel et al., 2012), the Philippines (n=3, Ybañez et al., 2013) and Vietnam (n=4, Yokoyama et al., 2015), were used to amplify the *bov57* gene using a nested PCR assay. The Sri Lankan DNA samples were extracted from blood samples collected in four districts, namely the Nuwara Eliya (n=1), Polonnaruwa (n=4), Ampara (n=4), and Jaffna (n=1), while the Mongolian DNA samples were sourced from cattle in five districts, Binder (n=3), Bogd (n=1), Dadal (n=1), Jargaltkhan (n=2), and Undurkhan (n=1). The DNA samples from the Philippines were prepared from blood samples collected in two municipalities, Naga (n=2) and Ginatilan (n=1), in Cebu Island, while all four Vietnamese DNA samples were from the Hue province. The first round of the nested PCR assay was conducted using an outer forward primer (5′-TTTCGTCGTAGCTTCACTGTGGTA-3′) located 293-bp upstream from the start codon and an outer reverse primer (5′-GGCCATACTTTGCATCTCTCGT-3′) located 388-bp downstream from the stop codon of the *bov57* gene, while the full length *bov57* gene was amplified by the nested round of the PCR assay using an inner forward primer

(5′-ATGGCATTTGCAAAGTTGTCTATTTTGTTTACGTTT-3′) and a reverse primer (5′-TTACCACCTGTAAATGTGTGGATGGTG-3′). For the first round of the PCR assay, 1 µl of DNA sample was added into a reaction mixture containing 25 µl of 2× PCR buffer (ToYoBo, Osaka, Japan), 10 µl of 2 mM dNTPs (ToYoBo), 1.5 µl of 10 µM outer forward and reverse primers, 1 µl of KOD FX Neo DNA polymerase (ToYoBo), and 10 µl of double-distilled water. After an initial denaturation step at 94°C for 2 min, the reaction mixture was subjected to 35 cycles each contained a denaturation step at 98°C for 10 sec and a combined annealing and elongation step at 68°C for 2.5 min. After the first round, 1 µl of the PCR product was transferred to a new PCR tube containing a reaction mix similar to that of the first round, except that the PCR primers were replaced with the inner forward and reverse primers. The cycling conditions for the second round of PCR were similar to the first round, except that the duration of the annealing and extension step was reduced to 1.5 min. The PCR amplicons were then gel-extracted, ligated to a pCR-Blunt II TOPO vector (Invitrogen, Carlsbad, CA, USA), and sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

The *bov57* gene sequences generated in the present study were initially analyzed by a basic local alignment tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and then by a MatGAT software programme to determine the identity and similarity scores (Campanella et

al., 2003). In addition, newly-generated *bov57* sequences and its homologous gene sequences that had been previously submitted to the GenBank database, were used to construct a phylogenetic tree. Briefly, the gene sequences were aligned using a MAFFT software program (Katoh et al., 2002) available online (http://mafft.cbrc.jp/alignment/server/) and then analyzed by the MEGA (version 6.06) software program (Tamura et al., 2013) to predict the best fitting model. The General Time Reversible model (Nei and Kumar, 2000) had the lowest AIC score and was employed to construct a maximum likelihood phylogeny with 1000 bootstrap replicates using the MEGA.

2.8. Statistical analyses

The invasion-inhibition rates and parasitemia values were analyzed by a two-tailed Student's *t* test using Excel software. *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Localization of BOV57 in B. bovis merozoites

In agreement with a previous study (Freeman et al., 2010), western blot analysis and IFAT using the mouse anti-rBOV57 antibody confirmed the expression of native BOV57 in *B*. *bovis* merozoites. On western blot analysis using the *B*. *bovis* lysate, a 57-kDa band was evident confirming the reactivity of mouse anti-rBOV57 antibody to BOV57 (Fig. 1). In a double IFAT using the anti-rBOV57 antibody with anti-rRAP-1 or anti-rMSA-2c IgG, BOV57 preferentially localized at the apical end in the paired merozoites in infected RBCs, whereas the antigen was distributed on the surface around the apical end of the intraerythrocytic single and extracellular merozoites (Fig. 2). Furthermore, BOV57 did not co-localize with RAP-1 (Fig. 2A). The pattern of BOV57 localization also differed from that of MSA-2c, which was distributed on the entire surface of intraerythrocytic paired and single merozoites, as well as extracellular merozoites (Fig. 2B).

3.2. Invasion inhibition and growth inhibition induced by anti-rBOV57 antibodies

A RBC invasion-inhibition assay was conducted using rabbit anti-rBOV57 IgG to examine whether BOV57 is a neutralization-sensitive antigen. Free merozoites isolated from

the *in vitro* culture of *B*. *bovis* were mixed with non-infected bovine RBCs and incubated in medium containing rabbit anti-rBOV57, anti-rRAP-1, or control IgG. The results showed that the rabbit anti-rBOV57 IgG at concentrations of 0.25, 0.5, and 1 mg/ml significantly inhibited parasite invasion ($P < 0.05$) with inhibition rates of 29.07 ± 7.52%, 38.84 ± 6.27%, and 51.40 ± 8.72%, respectively. By contrast, the inhibition rate of 1 mg of rabbit anti-rRAP-1 IgG (positive control) was 29.02 ± 5.43%, compared with the invasion rate in the control culture with control rabbit IgG (Fig. 3A). These results indicated that the anti-rBOV57 IgG could inhibit parasite invasion in a dose-dependent manner.

Subsequently, a growth-inhibition assay was performed to confirm whether the inhibitory effect of anti-rBOV57 IgG on parasite invasion resulted in growth inhibition of *B*. *bovis*. The findings showed that 1 mg of anti-rBOV57 IgG significantly reduced the growth of *B*. *bovis* on days 1–3 of cultivation ($P < 0.05$) (Fig. 3B). The rates of growth inhibition induced by anti-rBOV57 IgG, as calculated based on the parasitemia reduction compared to the cultures containing control rabbit IgG, on days 1, 2, and 3 of cultivation were 16.37 ± 3.39%, 35.41 ± 8.32%, and 79.77 ± 1.76%, respectively.

3.3. Genetic diversity of the bov57 *gene*

The *bov57* gene sequences were isolated from 12, eight, three, and four *B*.

bovis-positive DNA samples sourced from cattle bred in Sri Lanka, Mongolia, the Philippines, and Vietnam, respectively. After removing the primer-binding regions, all of the sequences from Mongolia (GenBank accession numbers: LC228461–LC228468), the Philippines (LC228469–LC228471), and Vietnam (LC228472–LC228475) were 1491 bp in length, as expected. However, among the 12 *bov57* sequences (LC228449–LC228460) from Sri Lankan DNA samples, seven (LC228450–LC228452, LC228454, LC228456, LC228458, and LC228460), four (LC228449, LC228455, LC228457, and LC228459), and one (LC228453) of the sequences were 1488 bp, 1494 bp, and 1491 bp in length, respectively. The length of the multiple alignment generated using the newly-obtained gene sequences was 1494 bp. Eleven (LC228449–LC228452 and LC228454–LC228460) of the 12 Sri Lankan *bov57* sequences contained an insert of three nucleotides (CGA) between positions 1413 and 1415 of the alignment, resulting in addition and substitution of amino acids histidine and glutamic acid (Fig. 4). Seven (LC228450–LC228452, LC228454, LC228456, LC228458, and LC228460) of these 11 sequences were characterized by a deletion of six nucleotides (A/CTCCTG/A) between positions 218 and 223, resulting in a deletion of amino acids aspartic acid/alanine/threonine and proline (Fig. 4). The overall scores for identity and similarity shared among the newly-generated nucleotide and the deduced amino acid sequences were 94.0–99.9% and 95.4–100%, respectively. On a per country basis, the lowest

identity (95%) and similarity scores (96.4%) were observed among Sri Lankan *bov57* sequences as compared to the sequences from Mongolia (99.7 and 99.6, respectively), the Philippine (96.9 and 98.2%, respectively), and Vietnam (97.7 and 99.0%, respectively) (Fig. S1). In the phylogeny, *bov57* sequences clustered together and formed a sister clade to the common ancestor of *B*. *bigemina* and *B*. *ovata* sequences (Fig. 5). The monophyletic clade formed by *bov57* sequences contained two subclades, one of which was formed by 10 (LC228449–LC228452 and LC228454–LC228459) of the 11 Sri Lankan sequences that possessed an insertion and/or deletion compared with the rest of the *bov57* sequences (Fig. 5).

4. Discussion

The P67 of *T. parva* and its homologous antigen SPAG-1 in *T. annulata*, which are exclusively expressed in their sporozoite stage, have been identified as potential candidate antigens for the development of subunit vaccines (Boulter et al., 1994, 1999; Musoke et al., 1992). Previous research identified a *p67* homolog in the *B. bovis* genome and demonstrated that is was expressed in both the blood and tick stages (Brayton et al., 2007; Freeman et al., 2010). However, the potential of the P67 homolog in *B. bovis* (BOV57) as a vaccine candidate against parasite infection remained to be investigated. Thus, the aim of this study was to characterize BOV57 as a neutralization-sensitive antigen in the context of subunit vaccine development.

The localization of BOV57 in *B. bovis* was notably different between the parasite stages. BOV57 localized at the apical end of intracellular dividing merozoites, whereas the antigen was distributed on the membrane around the apical end in the extracellular merozoites as well as in the intracellular single form, which might represent newly-invaded merozoites. These observations may infer that although BOV57 is localized in the apical end during asexual reproduction within infected RBCs, the antigen might be translocated to the membrane around the apical end in extracellular merozoites, suggesting that BOV57 may

play an important role in the RBC invasion of merozoites. However, the pattern of BOV57 localization differed clearly from those of MSA-2c and RAP-1, indicating that the function of this antigen might be different from those of MSA-2c and RAP-1.

The findings from the RBC invasion-inhibition assay showed that RBC invasion of merozoites was significantly inhibited by anti-rBOV57 antibodies, demonstrating that BOV57 is an invasion-related, neutralization-sensitive molecule of *B. bovis*. Consistent with the findings of the invasion-inhibition assay, lower parasitemia values were observed in *B. bovis* cultures that contained the anti-rBOV57 antibodies, compared with the control cultures. These findings collectively indicated that BOV57 is a potential candidate antigen for the development of subunit vaccines. A multicomponent vaccine against *Babesia* parasites should preferably include merozoite as well as the sporozoite antigens (Brown and Palmer, 1999). BOV57 of *B. bovis* is expressed in the merozoite and kinate stages. Future studies should therefore investigate whether BOV57 is expressed in sporozoites and whether RBC entry of sporozoites is mediated by BOV57. If so, subunit vaccines based on BOV57 would target both the merozoite and sporozoite stages of *B. bovis*, and thereby offer improved protection against bovine babesiosis.

Having characterized BOV57 as an invasion-related molecule, we analyzed genetic variation among *bov57* gene sequences. A previous study involving a limited number of

bov57 sequences, suggested that *bov57* was a conserved gene (Freeman et al., 2010). In the present study, although the sequences derived from Mongolia, the Philippines, and Vietnam were conserved, the Sri Lankan *bov57* gene sequences were relatively diverse. However, the present study analyzed only a few sequences from Sri Lanka, Mongolia, the Philippines, and Vietnam. Thus, the future research works should be toned to investigate the genetic diversity of *bov57* using large numbers of sequences from different geographical regions within *B. bovis* endemic countries. The genetic diversity of genes that results in antigenicity variations of surface antigens is considered to be a survival strategy of *B. bovis*. However, the genetic diversity often negatively influences the efforts to develop subunit vaccines based on surface antigens. In the present study, most of the Sri Lankan *bov57* gene sequences were characterized by insertion and/or deletion mutations. The insertion, deletion, and single nucleotide polymorphisms among *bov57* nucleotide sequences resulted in insertion, deletion, and substitution of amino acid residues. Moreover, the *bov57* sequences detected with insertion and/or deletion formed a subclade in the phylogeny. However, it is not clear whether the genetic diversity of *bov57* gene sequences results in antigenicity variations. In *T. parva*, the *p67* gene was demonstrated to be conserved in cattle but not in buffalo (Nene et al., 1996, 1999). However, high genetic diversity of this gene was evident in cattle when infected with the buffalo-derived *T. parva*, the causative agent of Corridor disease in cattle (Sibeko et al.,

2010). Similarly, genetic variations were also reported among *T. annulata spag-1* gene sequences (Williamson et al., 1989). However, P67 and SPAG-1 were found to induce cross-protective immune responses against *T. annulata* and *T. parva* infections, respectively, in cattle (Hall et al., 2000), suggesting that the genetic diversity of these antigens may not restrict the use of these antigens as vaccines (McKeever, 2009). Therefore, studies to investigate the effect of genetic diversity on the immunity against BOV57 might shed additional light on the implications of using this antigen as a vaccine.

In summary, the present study found evidence to suggest that BOV57 antigen is invasion-related and neutralization sensitive. Further studies to determine the role of BOV57 in sporozoites and to investigate the invasion inhibition induced by bovine anti-rBOV57 antibodies are of paramount importance. Additionally, the effects of genetic diversity of the *bov57* gene on the immunity against BOV57 should also be addressed in future research.

Acknowledgements

We thank Ms. Hiroko Yamamoto at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, for her excellent assistance. We are also thankful to Dr. Seekkuge Susil Priyantha Silva (Veterinary Research Institute, Sri Lanka), Dr. Badgar Battsetseg (Institute of Veterinary Medicine, Mongolia), Dr. Adrian Patalinghug Ybanez (University of the Philippines Cebu, the Philippines), and Dr. Dinh Thi Bich Lan (Hue University, Vietnam) for kindly providing field DNA samples. This study was supported by grants from JSPS KAKENHI (grant numbers: 16H05033, 15K14862, and 26257417), from Open Partnership Joint Projects of JSPS Bilateral Joint Research Projects, and from AMED/JICAm Science and Technology Research Partnership for Sustainable Development (SATREPS).

Appendix A. Supplementary data

Supplementary fig. 1

References

Altangerel, K., Sivakumar, T., Battsetseg, B., Battur, B., Ueno, A., Igarashi, I., Yokoyama, N., 2012. Phylogenetic relationships of Mongolian *Babesia bovis* isolates based on the merozoite surface antigen (MSA)-1, MSA-2b, and MSA-2c genes. Vet. Parasitol. 184, 309–316.

Antonio Alvarez, J., Lopez, U., Rojas, C., Borgonio, V.M., Sanchez, V., Castañeda, R., Vargas, P., Figueroa, J.V., 2010. Immunization of *Bos taurus* steers with *Babesia bovis* recombinant antigens MSA-1, MSA-2c and 12D3. Transbound. Emerg. Dis. 57, 87–90.

Asada, M., Goto, Y., Yahata, K., Yokoyama, N., Kawai, S., Inoue, N., Kaneko, O., Kawazu, S., 2012. Gliding motility of *Babesia bovis* merozoites visualized by time-lapse video microscopy. PLoS One. 7, e35227.

Berens, S.J., Brayton, K.A., Molloy, J.B., Bock, R.E., Lew, A.E., McElwain, T.F., 2005. Merozoite surface antigen 2 proteins of *Babesia bovis* vaccine breakthrough isolates contain a unique hypervariable region composed of degenerate repeats. Infect. Immun. 73, 7180–7189.

Bock, R.E., de Vos, A.J., Kingston, T.G., Shiels, I.A., Dalgliesh, R.J., 1992. Investigations of breakdowns in protection provided by living *Babesia bovis* vaccine. Vet. Parasitol. 43, 45–56.

Bock, R.E., Jackson, L., de Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129 (Suppl.), S247–S269.

Bork, S., Okamura, M., Matsuo, T., Kumar, S., Yokoyama, N., Igarashi, I., 2005. Host serum modifies the drug susceptibility of *Babesia bovis* in vitro. Parasitology 130, 489–492.

Boulter, N., Brown, D., Wilkie, G., Williamson, S., Kirvar, E., Knight, P., Glass, E., Campbell, J., Morzaria, S., Nene, V., Musoke, A., d'Oliveira, C., Gubbels, M.J., Jongejan, F., Hall, R., 1999. Evaluation of recombinant sporozoite antigen SPAG-1 as a vaccine candidate against *Theileria annulata* by the use of different delivery systems. Trop. Med. Int. Health 4, A71–A77.

Boulter, N., Knight, P.A., Hunt, P.D., Hennessey, E.S., Katzer, F., Tait, A., Williamson, S., Brown, D., Baylis, H.A., Hall, R., 1994. *Theileria annulata* sporozoite surface antigen (SPAG-1) contains neutralizing determinants in the C terminus. Parasite Immunol 16, 97–104.

Brayton, K.A., Lau, A.O., Herndon, D.R., Hannick, L., Kappmeyer, L.S., Berens, S.J., Bidwell, S.L., Brown, W.C., Crabtree, J., Fadrosh, D., Feldblum, T., Forberger, H.A., Haas, B.J., Howell, J.M., Khouri, H., Koo, H., Mann, D.J., Norimine, J., Paulsen, I.T., Radune, D., Ren, Q., Smith, R.K., Jr, Suarez, C.E., White, O., Wortman, J.R., Knowles, D.P., Jr, McElwain, T.F., Nene, V.M., 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS Pathog. 3, 1401–1413.

Brown, W.C., Norimine, J., Goff, W.L., Suarez, C.E., McElwain, T.F., 2006. Prospects for recombinant vaccines against *Babesia bovis* and related parasites. Parasite Immunol. 28,

315–327.

Brown, W.C., Palmer, G.H., 1999. Designing blood-stage vaccines against *Babesia bovis* and *B. bigemina*. Parasitol. Today 15, 275–281.

Campanella, J.J., Bitincka, L., Smalley, J., 2003. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinf. 4, 29.

Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16, 10881-10890.

Dowling, S.C., Perryman, L.E., Jasmer, D.P., 1996. A *Babesia bovis* 225-kilodalton spherical body protein: localization to the cytoplasmic face of infected erythrocytes after merozoite invasion. Infect. Immun. 64, 2618–2626.

Florin-Christensen, M., Suarez, C.E., Hines, S.A., Palmer, G.H., Brown, W.C., McElwain, T.F., 2002. The *Babesia bovis* merozoite surface antigen 2 locus contains four tandemly arranged and expressed genes encoding immunologically distinct proteins. Infect. Immun. 70, 3566–3575.

Freeman, J.M., Kappmeyer, L.S., Ueti, M.W., McElwain, T.F., Baszler, T.V., Echaide, I., Nene, V.M., Knowles, D.M., 2010. A *Babesia bovis* gene syntenic to *Theileria parva* p67 is expressed in blood and tick stage parasites. Vet. Parasitol. 173, 211–218.

Gaffer, F.R., Yatsuda, A.P., Franssen, F.F., de Vries, E.A., 2004a. *Babesia bovis* merozoite protein with a domain architecture highly similar to the thrombosondin-related anonymous protein (TRAP) present in *Plasmodium* sporozoites. Mol. Biochem. Parasitol. 136, 25–34.

Gaffer, F.R., Yatsuda, A.P., Franssen, F.F., de Vries, E.A., 2004b. Erythrocyte invasion by *Babesia bovis* merozoites is inhibited by polyclonal antisera directed against peptides derived from a homologue of *Plasmodium falciparum* apical membrane antigen 1. Infect. Immun. 72, 2947–2955.

Hall, R., Boulter, N.R., Brown, C.G., Wilkie, G., Kirvar, E., Nene, V., Musoke, A.J., Glass, E.J., Morzaria, S.P., 2000. Reciprocal cross-protection induced by sporozoite antigens SPAG-1 from *Theileria annulata* and p67 from *Theileria parva*. Parasite Immunol. 22, 223–230.

Hines, S.A., Palmer, G.H., Brown, W.C., McElwain, T.F., Suarez, C.E., Vidotto, O., Rice-Ficht, A.C., 1995a. Genetic and antigenic characterizeation of *Babesia bovis* merozoite sphere body protein Bb-1. Mol. Biochem. Parasitol. 69, 149–159.

Hines, S.A., Palmer, G.H., Jasmer, D.P., Goff, W.L., McElwain, T.F., 1995b. Immunization of cattle with recombinant *Babesia bovis* merozoite surface antigen-1. Infect. Immun. 63, 349–352.

Ishizaki, T., Sivakumar, T., Hayashida, K., Tuvshintulga, B., Igarashi, I., Yokoyama, N., 2016. RBC invasion and invasion-inhibition assays using free merozoites isolated after cold

treatment of *Babesia bovis* in vitro culture. Exp. Parasitol. 166, 10–15.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066.

Leroith, T., Brayton, K.A., Molloy, J.B., Bock, R.E., Hines, S.A., Lew, A.E., McElwain, T.F., 2005. Sequence variation and immunologic cross-reactivity among *Babesia bovis* merozoite surface antigen 1 proteins from vaccine strains and vaccine breakthrough isolates. Infect. Immun. 73, 5388–5394.

Liyanagunawardena, N., Sivakumar, T., Kothalawala, H., Silva, S.S., Battsetseg, B., Lan, D.T., Inoue, N., Igarashi, I., Yokoyama, N., 2016. Type-specific PCR assays for *Babesia bovis msa-1* genotypes in Asia: Revisiting the genetic diversity in Sri Lanka, Mongolia, and Vietnam. Infect. Genet. Evol. 37, 64–69.

McKeever, D.J., 2009. Bovine immunity – a driver for diversity in *Theileria* parasites? Trends Parasitol. 25, 269–276.

Musoke, A., Morzaria, S., Nkonge, C., Jones, E., Nene, V., 1992. A recombinant sporozoite surface antigen of *Theileria parva* induces protection in cattle. Proc. Natl. Acad. Sci. USA 89, 514–518.

Musoke, A., Rowlands, J., Nene, V., Nyanjui, J., Katende J., Spooner, P., Mwaura, S.,

Odongo, D., Nkonge, C., Mbogo, S., Bishop, R., Morzaria, S., 2005. Subunit vaccine based on the p67 major surface protein of *Theileria parva* sporozoites reduces severity of infection derived from field tick challenge. Vaccine 23, 3084–3095.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Nene, V., Gobright, E., Bishop, R., Morzaria, S., Musoke, A., 1999. Linear peptide specificity of bovine antibody responses to p67 of *Theileria parva* and sequence diversity of sporozoite-neutralizing epitopes: implications for a vaccine. Infect. Immun. 67, 1261–1266.

Nene, V., Musoke, A., Gobright, E., Morzaria, S., 1996. Conservation of the sporozoite p67 vaccine antigen in cattle-derived *Theileria parva* stocks with different cross-immunity profiles. Infect. Immun. 64, 2056–2061.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

Rogers, R.J., Dimmock, C.K., de Vos, A.J., Rodwell, B.J., 1988. Bovine leucosis virus contamination of a vaccine produced *in vivo* against bovine babesiosis and anaplasmosis. Aust. Vet. J. 65, 285–287.

Shkap, V., de Vos, A.J., Zweygarth, E., Jongejan, F., 2007. Attenuated vaccines for tropical theileriosis, babesiosis, and heartwater: the continuing necessity. Trends. Parasitol. 23,

420–426.

Sibeko, K.P., Geysen, D., Oosthuizen, M.C., Matthee, C.A., Troskie, M., Potgieter, F.T., Coetzer, J.A., Collins, N.E., 2010. Four p67 alleles identified in South African *Theileria parva* field samples. Vet. Parasitol. 167, 244–254.

Sivakumar, T., Okubo, K., Igarashi, I., de Silva, WK., Kothalawala, H., Silva, SS., Vimalakumar, SC., Meewewa, AS., Yokoyama, N., 2013. Genetic diversity of merozoite surface antigens in *Babesia bovis* detected from Sri Lankan cattle. Infect. Genet. Evol. 19, 134–140.

Srinivasan, P., Ekanem, E., Diouf, A., Tonkin, ML., Miura, K., Boulanger, MJ., Long, CA., Narum, DL., Miller, LH., 2014. Immunization with a functional protein complex required for erythrocyte invasion protects against lethal malaria. Proc. Natl. Acad. Sci. USA 111, 10311–10316.

Suarez, C.E., Florin-Christensen, M., Hines, S.A., Palmer, G.H., Brown, W.C., McElwain, T.F., 2000. Characterization of allelic variation in the *Babesia bovis* merozoite surface antigen 1 (MSA-1) locus and identification of a cross reactive inhibition-sensitive MSA-1 epitope. Infect. Immun. 68, 6865–6870.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Terkawi, M.A., Huyen, N.X,, Wibowo, P.E., Seuseu, F.J., Aboulaila, M., Ueno, A., Goo, Y.K., Yokoyama, N., Xuan, X., Igarashi, I., 2011. Spherical body protein 4 is a new serological antigen for global detection of *Babesia bovis* infection in cattle. Clin. Vaccine Immunol. 18, 337–342.

Terkawi, M.A., Ratthanophart, J., Salama, A., AbouLaila, M., Asada, M., Ueno, A., Alhasan, H., Guswanto, A., Masatani, T., Yokoyama, N., Nishikawa, Y., Xuan, X., Igarashi, I., 2013. Molecular characterization of a new *Babesia bovis* thrombospondin-related anonymous protein (BbTRAP2). PLoS One 8, e83305.

Tattiyapong, M., Sivakumar, T., Takemae, H., Simking, P., Jittapalapong, S., Igarashi, I., Yokoyama, N., 2016. Genetic diversity and antigenicity variation of *Babesia bovis* merozoite surface antigen-1 (MSA-1) in Thailand. Infect. Genet. Evol. 41, 255–261.

Williamson, S., Tait, A., Brown, D., Walker, A., Beck, P., Shiels, B., Fletcher, J., Hall, R., 1989. *Theileria annulata* sporozoite surface antigen expressed in *Escherichia coli* elicits neutralizing antibody. Proc. Natl. Acad. Sci. USA 86, 4639–4643.

Ybanez, AP., Sivakumar, T., Ybanez, RH., Vincoy, MR., Tingson, JA., Perez, ZO., Gabotero, SR., Buchomo, LP., Inoue, N., Matsumoto, K., Inokuma, H., Yokoyama, N., 2013. Molecular survey of bovine vector-borne pathogens in Cebu, Philippines. Vet. Parasitol. 196, 13–20.

Yokoyama, N., Okamura, M., Igarashi, I., 2006. Erythrocytes invasion by *Babesia* parasites: current advances in the elucidation of the molecular interactions between the protozoan

ligands and host receptors in the invasion stage. Vet. Parasitol. 138, 22–32.

Yokoyama, N., Sivakumar, T., Tuvshintulga, B., Hayashida, K., Igarashi, I., Inoue, N., Long, P.T., Lan, D.T., 2015. Genetic variations in merozoite surface antigen genes of *Babesia bovis* detected in Vietnamese cattle and water buffaloes. Infect. Genet. Evol. 30, 288–295.

Yokoyama, N., Suthisak, B., Hirata, H., Matsuo, T., Inoue, N., Sugimoto, C., Igarashi, I., 2000. Cellular localization of *Babesia bovis* merozoite rhoptry-associated protein 1 and its erythrocyte-binding activity. Infect. Immun. 70:5822–5826.

Figure Legends

Fig. 1. Western blot analysis to confirm the expression of BOV57 in *B*. *bovis* merozoites. The lysates prepared from *B*. *bovis*-infected RBCs (lane 1) and non-infected RBCs (lane 2) were treated with mouse anti-rBOV57 antibody. Note that the *B*. *bovis* lysate reacted with the anti-rBOV57 antibody forming a specific band at approximately 57 kDa, which is the molecular weight of native BOV57, and that the immune antibody did not react with the lysate prepared from uninfected RBCs.

Fig. 2. Localization of BOV57 in *B*. *bovis* merozoites using IFAT. Mouse anti-rBOV57 antibody together with rabbit anti-rRAP-1 (panel A) or rabbit anti-rMSA-2c IgG (panel B) were used to conduct a double IFAT. In this assay, 4', 6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus of the parasite (blue fluorescence), and green fluorescence indicates the localization of BOV57. RAP-1 and MSA-2c were stained with red fluorescence. DIC indicates differential interference contrast images. Note that BOV57 localizes at the apical end of intracellular paired merozoites, whereas the antigen is distributed in the parasite membrane around the apical end of intracellular single and extracellular merozoites, and that the pattern of BOV57 localization is different from those of RAP-1 and MSA-2c.

Fig. 3. Invasion-inhibition and growth-inhibition assays. **Panel A**. Invasion-inhibition assays were conducted using 0.25, 0.5, and 1 mg/ml of rabbit anti-rBOV57 IgG, 1 mg/ml of rabbit anti-rRAP-1 IgG, or 1 mg/ml of control rabbit IgG. The invasion-inhibition rates are expressed as percentages of parasitemia reduction in comparison with the controls that were maintained with control rabbit IgG. Error bars indicate the standard deviation of three separate experiments. Asterisks indicate statistically significant inhibition rates, as compared with the control. Note that 0.25, 0.5, and 1 mg/ml of rabbit anti-rBOV57 IgG significantly inhibited merozoite invasion into RBCs. **Panel B**. Growth-inhibition assays were performed using 1 mg/ml of rabbit anti-rBOV57 (filled circles) or control rabbit IgG (open circles). Error bars indicate the standard deviation of three separate experiments. Asterisks indicate a significant reduction in parasitemia in anti-rBOV57 IgG-treated cultures compared with the parasitemia in control cultures maintained with control rabbit IgG.

Fig. 4. Multiple alignment of BOV57 amino acid sequences. The BOV57 translated amino acid sequences were aligned by Multalin software program (Corpet, 1988). The variations among the amino acid sequences were analyzed using a Mongolian BOV57 sequence as a reference. SL, MN, PL, and VI provided together with the GenBank accession numbers

denote Sri Lanka, Mongolia, the Philippines, and Vietnam, respectively. Dots denote amino acids that are identical to the reference sequence. Dash indicates deletion of an amino acid. Note that a deletion of six nucleotides among 7 Sri Lankan *bov57* gene sequences resulted in a deletion of two amino acids (aspartic acid/alanine/threonine and proline) at 73rd and 74th positions and that the addition of three nucleotides in 11 Sri Lanka sequences resulted in addition and substitution of amino acids (histidine and glutamic acid) at 471st and 472nd positions.

Fig. 5. Phylogenetic tree of the *bov57* gene sequences. A maximum likelihood phylogeny was constructed using the *bov57* gene sequences together with the homologous gene sequences of various species of *Babesia* and *Theileria*. The sequences generated in the present study are indicated by bold-face type letters. Note that 10 out of 12 *bov57* gene sequences from Sri Lanka formed a subclade within the *bov57* clade in the phylogeny.

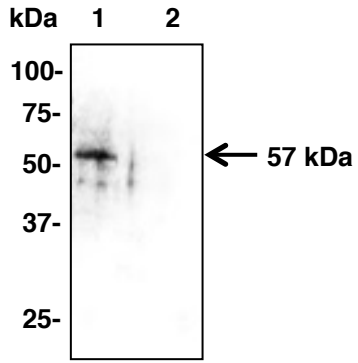

Fig. 1

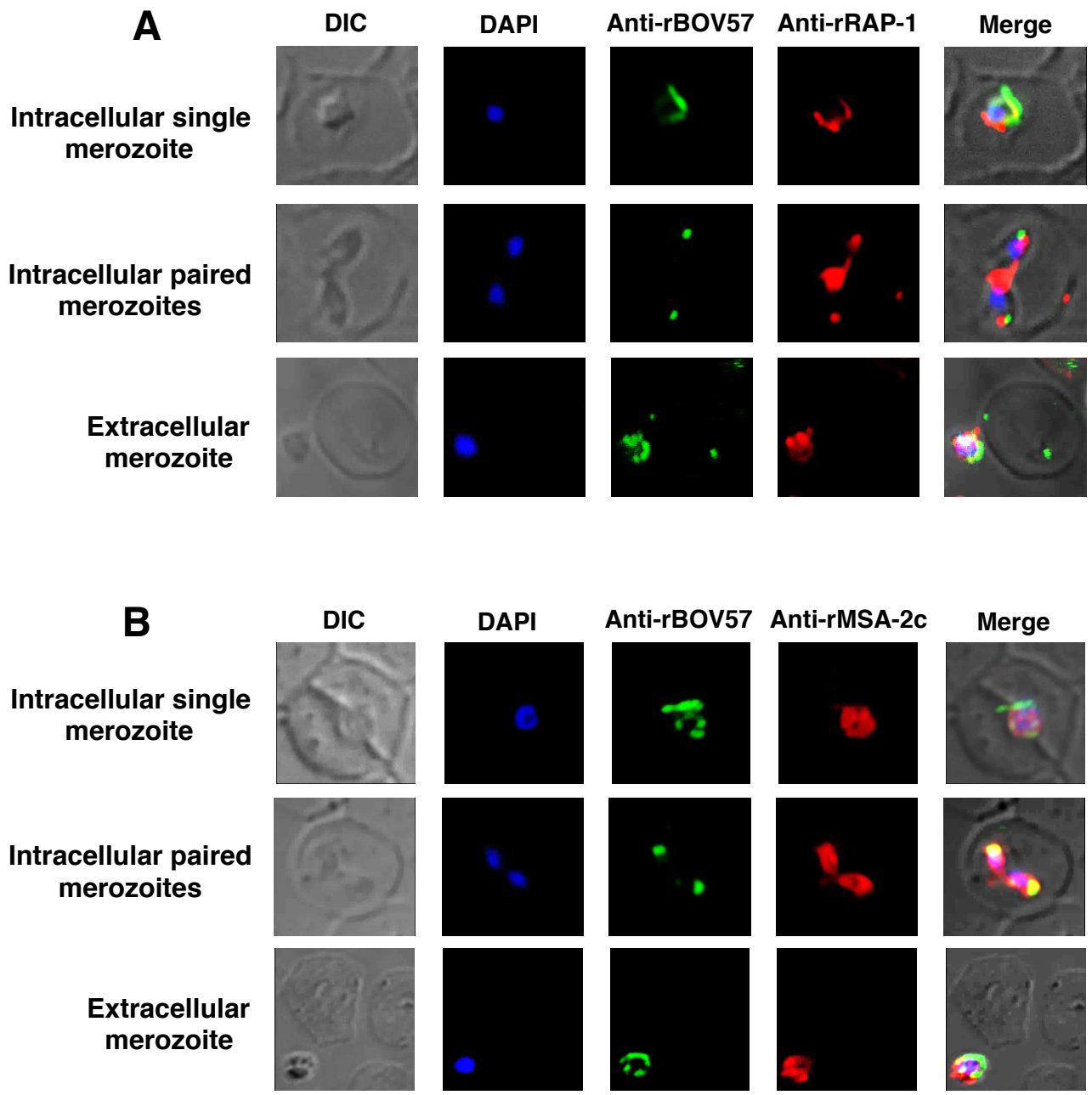
A
DIC
DAPI
Anti-rBOV57
Anti-rRAP-1
Merge
Intracellular single merozoite
Intracellular paired merozoites
Extracellular merozoite
B
DIC
DAPI
Anti-rBOV57
Anti-rMSA-2c
Merge
Intracellular single merozoite
Intracellular paired merozoites
Extracellular merozoite

Fig. 2

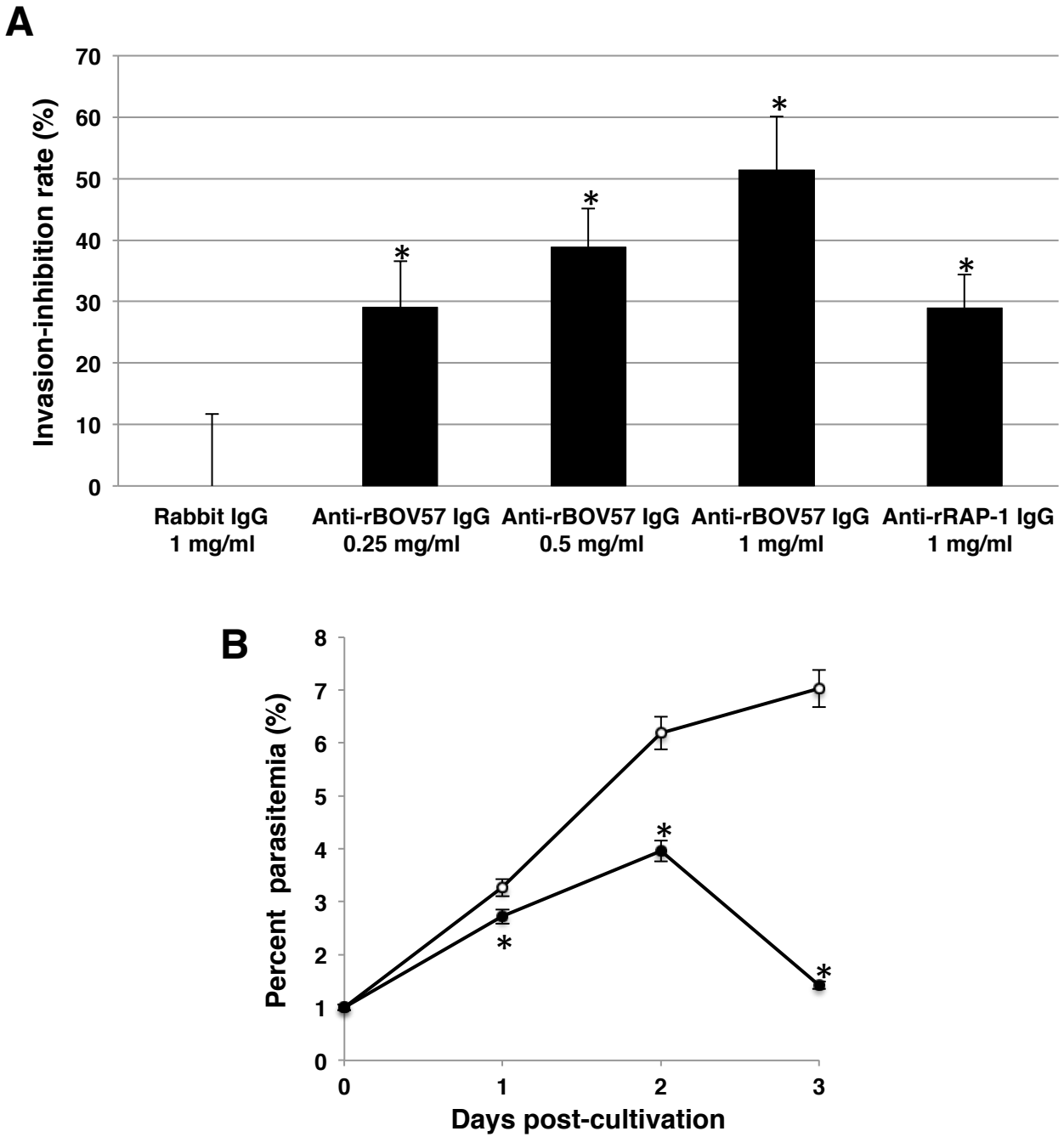
A
Invasion-inhibition rate (%)
70
60
50
40
30
20
10
0
*
*
*
*
Rabbit IgG
1 mg/ml
Anti-rBOV57 IgG
0.25 mg/ml
Anti-rBOV57 IgG
0.5 mg/ml
Anti-rBOV57 IgG
1 mg/ml
Anti-rRAP-1 IgG
1 mg/ml
B
Percent parasitemia (%)
8
7
6
5
4
3
2
1
0
*
*
*
0
1
2
3
Days post-cultivation

Fig. 3

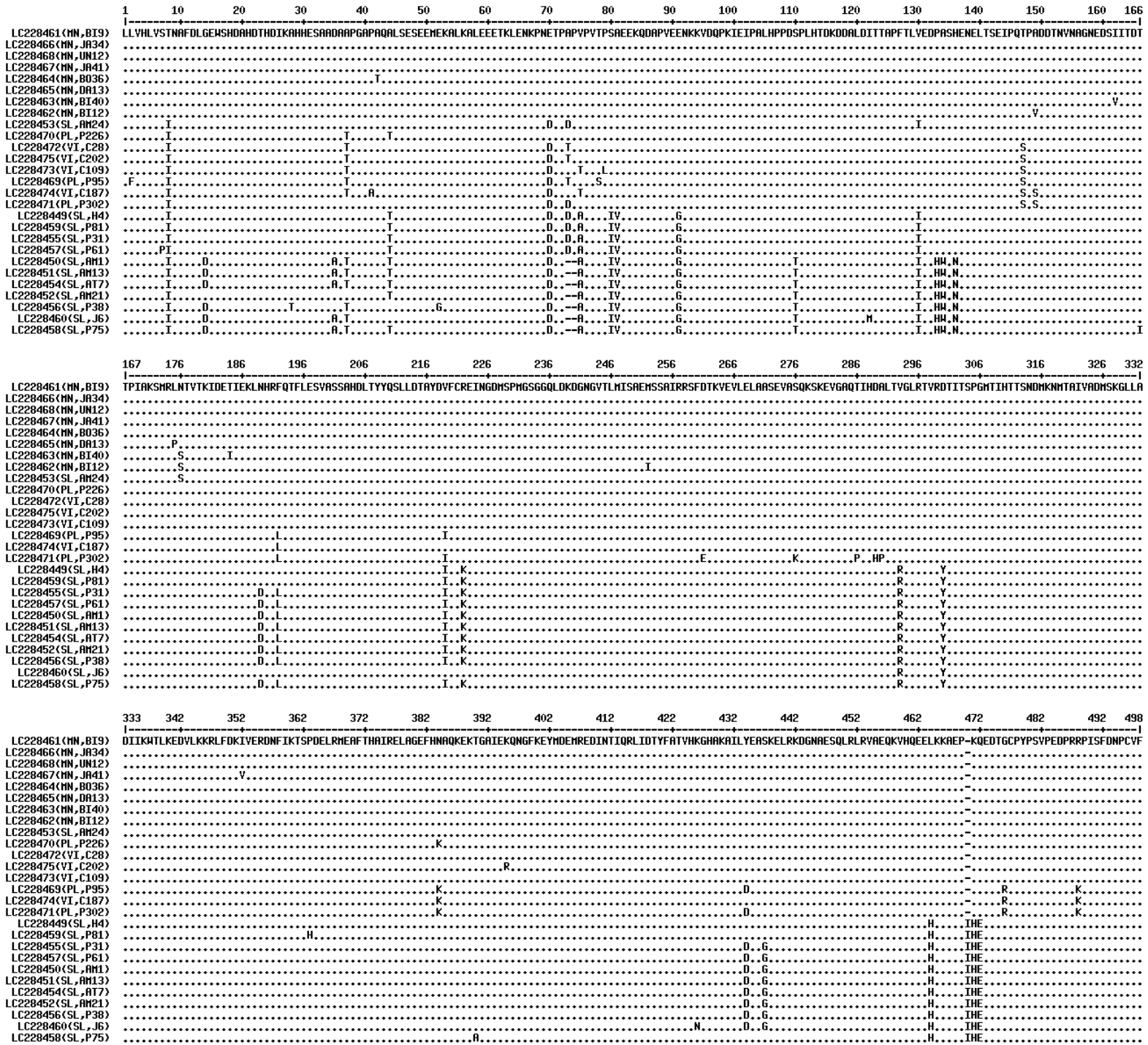

Fig. 4

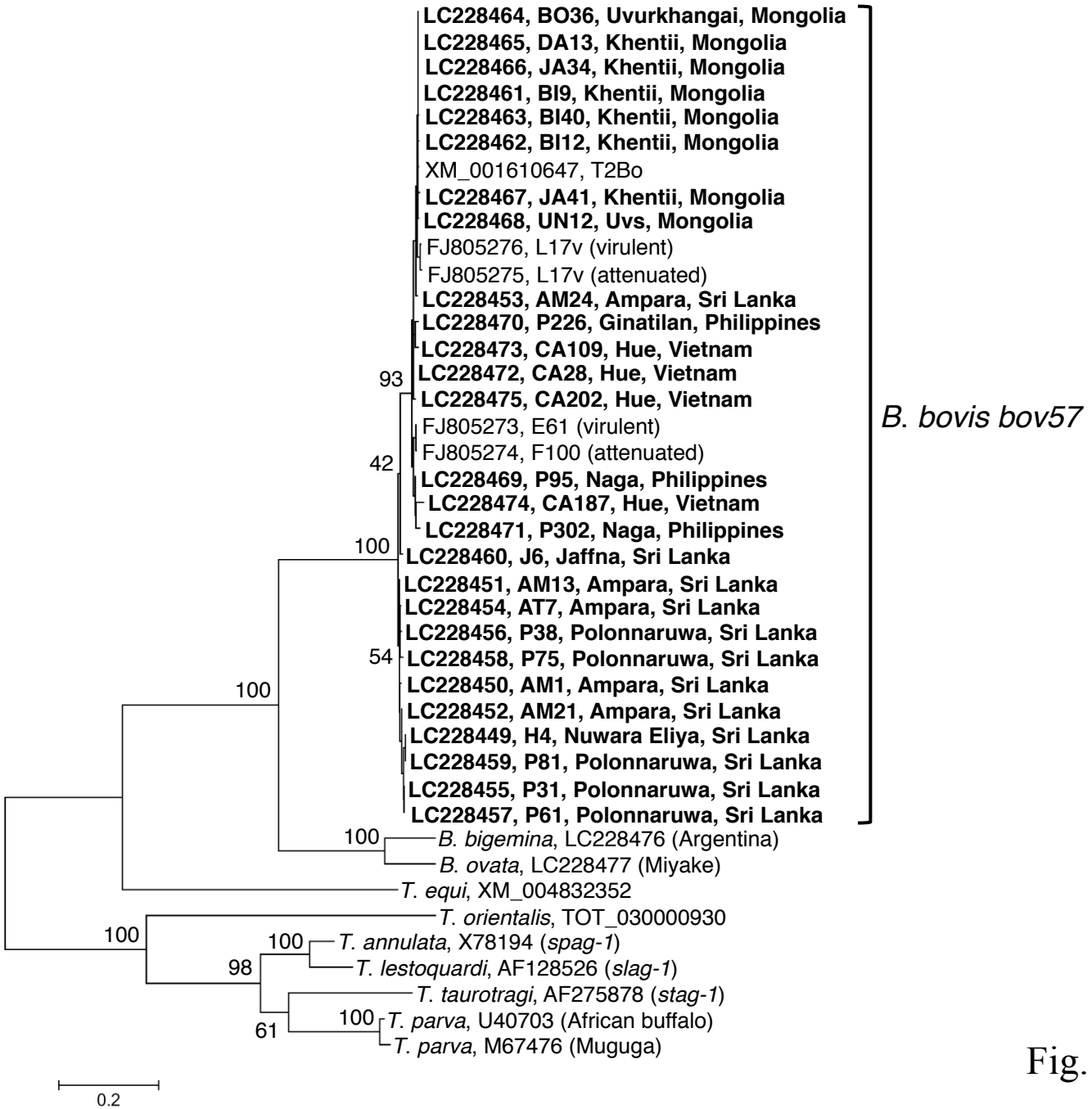
LC228464, BO36, Uvurkhangai, Mongolia
LC228465, DA13, Khentii, Mongolia
LC228466, JA34, Khentii, Mongolia
LC228461, BI9, Khentii, Mongolia
LC228463, BI40, Khentii, Mongolia
LC228462, BI12, Khentii, Mongolia
XM_001610647, T2Bo
LC228467, JA41, Khentii, Mongolia
LC228468, UN12, Uvs, Mongolia
FJ805276, L17v (virulent)
FJ805275, L17v (attenuated)
LC228453, AM24, Ampara, Sri Lanka
LC228470, P226, Ginatilan, Philippines
LC228473, CA109, Hue, Vietnam
LC228472, CA28, Hue, Vietnam
LC228475, CA202, Hue, Vietnam
FJ805273, E61 (virulent)
FJ805274, F100 (attenuated)
LC228469, P95, Naga, Philippines
LC228474, CA187, Hue, Vietnam
LC228471, P302, Naga, Philippines
LC228460, J6, Jaffna, Sri Lanka
LC228451, AM13, Ampara, Sri Lanka
LC228454, AT7, Ampara, Sri Lanka
LC228456, P38, Polonnaruwa, Sri Lanka
LC228458, P75, Polonnaruwa, Sri Lanka
LC228450, AM1, Ampara, Sri Lanka
LC228452, AM21, Ampara, Sri Lanka
LC228449, H4, Nuwara Eliya, Sri Lanka
LC228459, P81, Polonnaruwa, Sri Lanka
LC228455, P31, Polonnaruwa, Sri Lanka
LC228457, P61, Polonnaruwa, Sri Lanka
B. bovis bov57
93
42
100
54
100
100
B. bigemina, LC228476 (Argentina)
B. ovata, LC228477 (Miyake)
T. equi, XM_004832352
T. orientalis, TOT_030000930
100
100
T. annulata, X78194 (spag-1)
98
T. lestoquardi, AF128526 (slag-1)
T. taurotragi, AF275878 (stag-1)
100
T. parva, U40703 (African buffalo)
61
T. parva, M67476 (Muguga)
0.2

Fig. 5