Recombinant protein Bd37 protected gerbils against heterologous challenges with isolates of *Babesia divergens* polymorphic for the *bd37* gene

K. HADJ-KADDOUR[1]*, B. CARCY[1], A. VALLET[1], S. RANDAZZO[1], S. DELBECQ[1], J. KLEUSKENS[2], T. SCHETTERS[1,2], A. GORENFLOT[1] *and* E. PRECIGOUT[1]†

[1] *Laboratoire Biologie Cellulaire and Moléculaire, ERT 1038 'Vaccination anti-parasitaire', UFR Pharmacie, Université Montpellier I, 15 Avenue Charles Flahault, B.P. 14491, 34093 Montpellier Cedex 5, France*
[2] *Department of Parasitology, Intervet International B.V., Boxmeer, The Netherlands*
† *Deceased April 2006*

(*Received 25 May 2006; revised 26 July 2006; accepted 28 July 2006; first published online 13 October 2006*)

SUMMARY

The Bd37gene encoding for a glycosyl-phosphatidyl-inositol anchored protein of *Babesia divergens* displays genetic polymorphisms among isolates. Five major polymorphic groups (clades) were shown by PCR-RFLP among different *B. divergens* isolates. Each group has been characterized according to a reference Bd37 gene (Rouen87, W8843, Y5, 6303E and 1705B). Recombinant (GST fusion) protein (Bd37r) expressed from the Bd37 gene, was used as antigen in a saponin-based formulation and was able to protect gerbils, after 2 injections at low dose vaccine concentration (1 μg per dose), against a virulent challenge with the *B. divergens* Rouen87 isolate. In spite of polymorphism of *Bd37* gene, Bd37r induced complete immunoprotection against challenges with each of the 5 reference isolate groups defined by PCR-RFLP.

Key words: Babesiosis, recombinant protein, heterologous vaccine, *Babesia divergens*.

INTRODUCTION

Babesia divergens, an intra-erythrocytic protozoan parasite, transmitted by the tick *Ixodes ricinus*, is the most pathogenic *Babesia* species affecting cattle in Europe (Beattie *et al*. 2002; Yang *et al*. 2005). Moreover, several cases of *B. divergens* infection have been described in splenectomized human patients (Gorenflot *et al*. 1998). Moreover, several human cases reported to date in the United States, from Missouri (Herwaldt *et al*. 1996), Kentucky (Beattie *et al*. 2002) and Washington State (Herwaldt *et al*. 2004), were consistent with the diagnosis of *B. divergens*. As for all *Babesia* species, *B. divergens* parasites proliferate in the erythrocytes of their hosts leading to severe anaemia or death for the infected animals (Kuttler, 1988; Goethert and Telford, 2003; Holman *et al*. 2005). Therefore, *B. divergens* infection outcomes (mortality, reduction in meat and milk production) generate important economic losses in the cattle breeding industry (Gray and Harte, 1985). In terms of immunological response to *B. divergens* infection, the data available in Europe originates mainly from cattle (Christensson, 1987, 1989; L'Hostis *et al*. 1997). When bovine animals survive infection with *B. divergens*, they develop an immune response that protects them against subsequent challenges (Joyner and Davies, 1967). The mechanisms of such an immune protection against *B. divergens* are not well understood. However, passive transfer experiments showed clearly that antibodies can play a major role in the prevention of *B. divergens* bovine babesiosis (Ben Musa and Dawoud, 2004; Precigout *et al*. 2004). Presumably, these antibodies block essential processes by binding to critical epitopes on sporozoites or merozoites prior to host cell invasion and/or to infected erythrocytes implicated in the mediation of opsonization.

The only vaccines currently available against *B. divergens* are live vaccine; they provide effective protection but have the usual restricting features of such vaccines. Nevertheless, some protective immunity against homologous strain challenge was also observed when culture supernatants of *B. bovis*, *B. bigemina* and *B. divergens* that contained different antigens were tested (Brown *et al*. 2006). These exoantigens consisted of proteins with molecular masses between 23–40 kDa (*B. bovis*), 23–143 kDa (*B. bigemina*) and 25–75 kDa (*B. divergens*) (Schetters and Montenegro-James, 1995). By all means, in order for a vaccine candidate to progress towards bovine trials, its formulation against *B. divergens* babesiosis would need to show a consistent level of protection

* Corresponding author: Laboratoire Biologie Cellulaire and Moléculaire, ERT 1038 'Vaccination anti-parasitaire' UFR Pharmacie, Université Montpellier I, 15 Avenue Charles Flahault, B.P. 14491, 34093 Montpellier Cedex 5, France. Tel: +04 67 66 81 20. Fax: +04 67 66 81 29. E-mail: kamel.hadj-kaddour@univ-montp1.fr

Parasitology (2007), **134**, 187–196.
doi:10.1017/S0031182006001399 Printed in the United Kingdom

in the laboratory model for *B. divergens*: the gerbil *Meriones unguiculatus* (Lewis and Williams, 1979). The first step was achieved by demonstrating that gerbils and cattle can be protected against heterologous *B. divergens* challenges using supernatants of *in vitro* cultures of the parasite (Precigout *et al.* 1991; Valentin *et al.* 1993). Among the major exoantigens of *B. divergens* recognized by sera of recovered animals, it appeared that a 37 kDa molecule was probably involved in the protective efficacy of *in vitro* culture supernatants (Gorenflot *et al.* 1990; Carcy *et al.* 1995; Precigout *et al.* 2004). This Bd37 molecule is a membrane-anchored protein. It is localized at the surface of *B. divergens* merozoites, and anchored in the parasite membrane via a glycosylphosphatidyl-inositol moiety. After phospholipase cleavage of this GPI-anchor, Bd37 is secreted in the culture medium or in the host plasma (Delbecq *et al.* 2002). Moreover, a monoclonal antibody directed against Bd37, F4-2F8-INT is able to protect gerbils in passive transfer experiments against *B. divergens* infections (Precigout *et al.* 2004). With recent knowledge on the biochemical characteristics and immunoprotective potentiality of Bd37 against *B. divergens* babesiosis, a new step in the formulation of a vaccine against *B. divergens* babesiosis became feasible through development of a recombinant vaccine.

In the present work, we demonstrated a genetic polymorphism in the Bd37 gene among isolates of *B. divergens*. According to PCR-RFLP results, we could discriminate 5 major groups (clades) among all the Bd37 isolates under study. The protein Bd37 was produced as a recombinant fusion protein tagged with glutathione-S-transferase (GST) and was tested as a recombinant vaccine against *B. divergens* heterologous infections. Subsequently, we demonstrated that this recombinant protein could confer complete immunoprotection against different *B. divergens* isolates.

MATERIALS AND METHODS

Animals and Babesia divergens *isolates*

Gerbils (*Meriones unguiculatus*) (13–14 weeks old) were obtained from the Centre d'Elevage René Janvier (CERJ, Saint-Genest, France). The gerbils were fed *ad libitum*.

B. divergens isolates used in this work are shown in Table 1. The *B. divergens* Rouen87 isolate was obtained from a naturally infected human (Gorenflot *et al.* 1991). All bovine *B. divergens* isolates were collected by Dr M. L'Hostis (Ecole Nationale Vétérinaire de Nantes, France) except Weybridge 8843 (W8843), Munich87 and Y5 which originated from the UK, Germany and Ireland, respectively.

These isolates were maintained either in gerbils by syringe blood passage, twice weekly (21) or in long-term *in vitro* culture (Gorenflot *et al.* 1991). Briefly, *in vitro* cultures were set up using human erythrocytes at 5% haematocrit in buffered RPMI 1640 supplemented with 10% human serum. The *in vitro* cultures were maintained at 37 °C under a 5% CO_2 atmosphere. *B. divergens* cultures reaching between 15 and 20% parasitaemia were washed twice in cold Tris-buffered saline (TBS) and the pellets were stored at −80 °C for later use in DNA extraction.

Blood group O human erythrocytes and human serum were obtained from the Centre Régional de Transfusion Sanguine, Montpellier (France).

Nucleotide sequence Accession numbers

The nucleotide sequence data reported in this paper are available at GenBank, EMBL, and DDBJ databases under Accession number AJ422214 for Rouen87 Bd37 cDNA, AJ509155 for W8843 Bd37 cDNA, AJ507803 for 6303E Bd37 cDNA, AJ509156 for Y5 Bd37 cDNA and AJ509157 for 1705B Bd37 cDNA.

PCR assays and RFLP analysis

For the PCR assay (Precigout *et al.* 1991), 100 ng of genomic DNA obtained from each isolate were amplified. Sense oligonucleotide primer Bd37-9 (5′-ATG AAA ACC AGT AAG ATT CTC AAC AC-3′) and antisense oligonucleotide primer Bd37-10 (5′- GCC GTA TAG CAA ATC CAT CAT-3′) were used to amplify the Bd37 gene of all *B. divergens* isolates. Briefly, genomic DNA was added to a 50 μl PCR reaction containing 500 μM of each deoxyribonucleotide, 0·5 U of DynaZyme II recombinant DNA polymerase (FinnZymes Oy, Espoo, Finland) and 1 μM of Bd37-9 and Bd37-10 primers in the reaction buffer provided by the manufacturer (1·5 mM $MgCl_2$). Amplification was done using a programmable thermocycler (MJ Research Inc., Watertown, MA), with a 3-step cycling programme (30 cycles): 1 min denaturation (94 °C), 1 min annealing (55 °C) and 1 min extension (72 °C). The amplification products were then visualized on an ethidium bromide-stained 0·8% Seakem agarose gel (FMC Bioproducts, Rockland, USA).

For the restriction fragment length polymorphism (RFLP) analysis of the Bd37-9/Bd37-10 amplification products, 25% of each amplification product was digested for 3 h, separately with 10 U of BglII and Rsa I endonucleases in their appropriate buffer. The digestion fragments were visualized on an ethidium bromide-stained 2% Seakem agarose gel (FMC Bioproducts, Rockland, USA).

Cloning of the different Bd37 genes

The Bd37 protein from the 1705A and Y5 *B. divergens* isolates were cloned as previously described for

https://doi.org/10.1017/S0031182006001399 Published online by Cambridge University Press

Table 1. Geographical origins of the different *Babesia divergens* isolates used in PCR-RFLP analysis

B. divergens isolates	Geographical origin		Host
	Country	County (Region)	
Munich87	Germany	Baviere	Bovine
W8843	Great Britain	England	Bovine
Y5	Ireland		Bovine
Rouen87	France	Seine-Maritime (Haute-Normandie)	Human
0904A	France	Ariège (Midi-Pyrénées)	Bovine
1505B	France	Cantal (Auvergne)	Bovine
1705A	France	Charente-Maritime (Poitou-Charentes)	Bovine
2101B	France	Côte d'Or (Bourgogne)	Bovine
2906A	France	Finistère (Bretagne)	Bovine
3601B	France	Indre (Centre)	Bovine
6111A	France	Orne (Basse-Normandie)	Bovine
6204B	France	Pas-de-Calais (Nord-Pas-de-Calais)	Bovine
6303E	France	Puy-de-Dôme (Auvergne)	Bovine
7107B	France	Saône-et-Loire (Bourgogne)	Bovine

the Bd37 protein from W8843 and 6303E *B. divergens* isolates (Precigout *et al*. 2004). Briefly, genomic DNA from 1705A and Y5 isolates were obtained by extraction of *in vitro* cultivated parasites using a NucleoSpin Blood Genomic DNA Purification kit (Macherey-Nagel, France). The Bd37 gene from 1705A and Y5 isolates were PCR-amplified using primers derived from the Rouen Bd37 cDNA sequence: sense primer Bd37-Up, including an ATG initiation codon (5′-CAA GTT CTT TCT AAG TAC G*AT G*AA AAC CAG TA-3′) and antisense primer Bd37-Low, including a TAG stop codon (5′-ACA AAT CCA AAA AGC TAC A*TA G*CT GTC CAC T-3′). After gel purification, the PCR products were cloned into a pGEM-T plasmid vector (Promega, France). Then, the ligation products were used to transform JM109 competent cells (Promega, France). Recombinant plasmids were screened for appropriate insert size and then sequenced by Genome express (Meylan, France).

ClustalW software (http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl) was used to generate the multiple alignments. Then, the obtained alignments were copied into Boxshade 3.1 (http://www.ch.embnet.org/software/BOX_form.html) to produce a visual output of the alignments.

Expression of GST-Bd37 from B. divergens *Rouen 87 and GST-Bd35 from* B. divergens *W8843*

Rouen87 Bd37 or the W8843 Bd35 genes, inserted into pGEX plasmids (Amersham, France), were used to transform *Escherichia coli* cells (Epicurian Coli® SURE®2 Supercompetent cells, Stratagene, Amsterdam, The Netherlands). Bacterial cells containing GST-Bd37 or GST-Bd35 were grown overnight in LB-ampicillin medium at 37 °C with shaking. Overnight cultures were 10-fold diluted with fresh medium and grown for 1 h. Then, expression of the Bd37 or Bd35 proteins was induced with a 0·1 mM IPTG (Isopropyl-beta-D-thiogalactopyranoside) for 3 h at 37 °C. Then, the cells were spun down (15 000 ***g***, 5 min) and the pellet was suspended in PBS with 1% Triton X-100, disrupted by sonication (5 min, 4 °C), and spun down at 15 000 ***g***, 5 min, 4 °C. The fusion proteins were affinity-purified from the supernatant using glutathione-conjugated agarose beads (Sigma, France) for 15 min at room temperature. The beads were centrifuged (500 ***g***, 2 min) and washed 3 times with PBS. Then, the fusion proteins were eluted with 5 mM reduced glutathione in 50 mM Tris-HCl, pH 8·0.

The purity of the recombinant proteins was assessed by SDS-PAGE electrophoresis followed by Coomassie Blue staining. The protein concentrations of GST-Bd37 and GST-Bd35 were estimated using the Coomassie Protein Assay Reagent (Pierce, France). Briefly, 10 μl of the Bd37 or Bd35 samples were diluted with 490 μl of Tris-HCl (50 mM, pH 8·0). Then, 500 μl of Coomassie Reagent were added to each sample and mixed during 30 sec. For each sample, the absorbance was measured on a spectrophotometer set to 595 nm. A standard curve, with a working range between 1 and 25 μg/ml of bovine serum albumin was prepared using the same protocol. This standard curve was used to determine the protein concentration of each Bd37 or Bd35 sample.

Vaccination and challenge procedure

Two weeks before beginning the vaccination trial, the gerbils were randomly distributed into cages (10 gerbils per cage). One hour before the 2 vaccine injections, adjuvant Quil-A saponin (Superfos

https://doi.org/10.1017/S0031182006001399 Published online by Cambridge University Press

Biosector a/s, Vedbaek, Denmark) was added to the different vaccine doses at a final concentration of 70 μg/ml. The first and second immunizations were performed on days 0 and 21, respectively. On day 42, the gerbils were challenged by intraperitoneal injection of 10^6 parasitized gerbil erythrocytes resuspended in 100 μl of RPMI 1640 with 10% serum.

Three experiments were done, in order to analyse the protection induced by different *B. divergens* recombinant proteins, the dose effect protection of Bd37r, and the heterologous challenge after vaccination with Bd37r. Firstly, the vaccines consist of 25 μg of Bd37r, or 25 μg of Bd35r, or 12·5 μg of each protein. Secondly, the dose effect was performed using a different quantity of Bd37r (0·01 μg to 25 μg). Thirdly, 25 μg of Bd37r were used to vaccinate 5 groups of gerbils which were challenged with Rouen87, W8843, 1705A, 6303E or Y5 strain.

After the virulent challenge, the survival rate of vaccinated gerbils and placebo controls were recorded daily and dead animals were removed from the cages. The packed cell volume (PCV) and the parasitaemia (percentage of parasitized red blood cells) were determined daily. The different cages of animals were divided into 2 groups of 5 animals each. For each group, PCV and parasitaemia of 5 animals were determined on the first day, and on the second day PCV and parasitaemia from the other 5 animals were determined. The same procedure was maintained on the following days. PCV was determined from a sample of venous blood collected directly in a capillary by orbita puncture. The parasitaemia was estimated on blood smears stained by Diff-Quick (Dade S.A., France) by counting 2000 erythrocytes.

On days 0 and 21 (just before the first and second vaccine injections, respectively) and day 42 (just before the virulent challenge), a blood sample was collected from the animals of each group. The serum samples of each group were pooled and the antibody titre was determined with an indirect immunofluorescence assay on blood smears prepared with parasitized erythrocytes from *B. divergens* Rouen87 *in vitro* culture. The slides were air-dried and erythrocytes fixed in acetone : methanol (80 : 20) for 5 min at −20 °C. All the dilutions and washings were made in PBS 1% milk. The titre of a plasma sample was expressed as the highest dilution at which immunofluorescence against parasites was still detectable. Sera were applied as the first antibody on the fixed smears and incubated for 1 h at 37 °C. After 3 washings, FITC-conjugated goat anti-mouse IgG solution (SIGMA, USA) was applied (1/250 dilution) as a secondary antibody and incubated for 1 h at RT. After 3 washings, the glass slides were mounted by adding Immuno Fluore Mounting medium (ICN, France) and covered with a glass cover-slip.

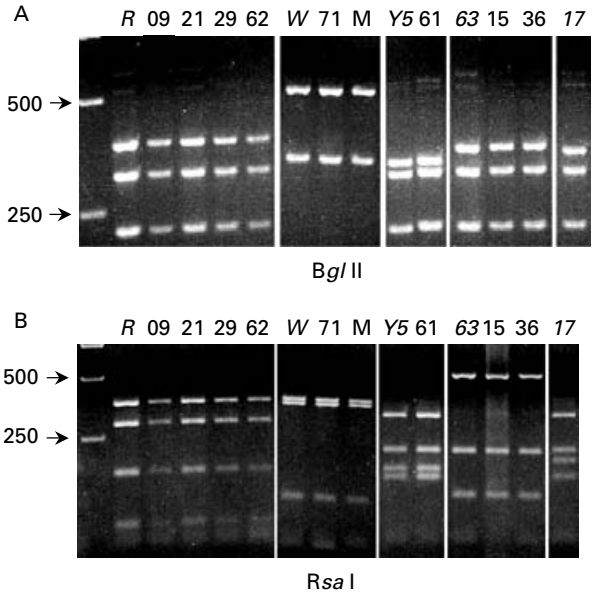

Fig. 1. PCR assays were done on the *Babesia divergens* isolates and RFLP analysis was performed with B*gl*II (A) or R*sa*I (B). The PCR-RFLP analysis discriminated 5 groups among the 14 isolates (Group Rouen87 with **R**ouen87, **09**04A, **21**01B, **29**06A and **62**04B; Group W8843 with **W**8843, **71**07B and **M**unich87; Group Y5 with **Y5** and **61**11A; Group 6303E with **63**03E, **15**05B and **36**01B; Group 1705A with **17**05A).

All animal experiments took place in an approved animal house (classify in Number A34585) and procedures were performed under personal licenses 00841 and 004990 (under the licensing authority: 'Ministère de l'Agriculture et de la Pêche').

RESULTS

Genetic polymorphism in the Bd37 gene evidenced by PCR-RFLP

Fourteen isolates were chosen for their representative geographical origin in France or in Europe (Table 1). All the *B. divergens* isolates were isolated from bovine hosts except the Rouen87 *B. divergens* isolate. Rouen87 is our reference isolate and it was previously demonstrated that *in vitro* culture supernatants from this isolate were able to confer heterologous protection against *B. divergens* babesiosis (Precigout *et al.* 1991).

PCR amplifications were performed using the Bd37-9 and Bd37-10 primers, on genomic DNA extracted from the 14 isolates maintained *in vitro*. The Bd37-9 and Bd37-10 primers allowed amplification of a 900 bp fragment in all the *B. divergens* tested (data not shown). For all the isolates, this fragment was systematically digested with *Bgl*II or *Rsa*I restriction enzymes (Fig. 1A and B, respectively). With these 2 enzymes, we were able to discriminate 5 groups displaying genetic polymorphism inside the Bd37 gene and, whatever the enzyme used

https://doi.org/10.1017/S0031182006001399 Published online by Cambridge University Press

we found always the same genes inside a given group. Each of the 5 clades was named according to a reference Bd37 gene. Group Rouen87 is composed with the Bd37 genes from the *B. divergens* isolates Rouen87, 0904A, 2101B, 2906A, 6204B. We were able to put together W8843, 7107B and Munich Bd37 genes in group W8843. GroupY5 contained the Bd37 genes amplified from the Y5 and 6111A *B. divergens* isolates. Bd37 genes from isolates 6303E, 1505B and 3601B composed the fourth group called 6303E. The last group was named after its only representative, the Bd37 gene from the 1705A *B. divergens* isolate.

The Bd37 gene from 1705A, Y5, W8843 and 6303E *B. divergens* isolates were PCR-amplified using primers derived from the Bd37 gene from Rouen87. No significant homologies with already described proteins were obtained when sequence comparisons were done with BLAST against non-redundant databases. The Bd37 proteins are conserved among the 5 reference isolates as shown by the ClustalW multiple alignments (Fig. 2). Nevertheless, sequence polymorphism was observed in the N-terminal part, just after the predicted cleavage site of the N-terminal hydrophobic signal peptide (V^{23} and S^{24} for the 1705B Bd37 protein and between G^{18} and F^{19} for the others).

Bd37r and Bd35r are two different recombinant proteins, able to confer complete protection

Bd37r and Bd35r are recombinant proteins, tagged with glutathione-S-transferase, corresponding to the Bd37 genes obtained from Rouen87 and W8843 *B. divergens* isolates, respectively. These proteins were tested for their capacity to confer immunoprotection against *B. divergens* Rouen87 infection (Table 2).

When gerbils were vaccinated twice with 25 μg of Bd37r or Bd35r, they were fully protected against a *B. divergens* Rouen87 challenge. Nevertheless, a strong difference was observed in the capacity of Bd37r or Bd35r to induce antibodies directed against Bd37 from the challenge isolate. The titres were 1 : 32 768 on day 21 and 1 : 131 072 on day 42 for gerbils vaccinated with Bd37r. On the same days, the titres were 4 times less (1 : 4096 and 1 : 32 768, respectively) for gerbils vaccinated with Bd35r. When a mixture of Bd37r and Bd35r (12·5 μg of Bd37r and 12·5 μg of Bd35r) was used, the protection against *B. divergens* Rouen87 was also complete (100% survival rate). The titres were 1 : 8192 on day 21 and 1 : 65 356 on day 42. As already shown (Gorenflot *et al.* 1990), an *in vitro* culture supernatant of *B. divergens* Rouen87 (PCS) was able to confer complete immunoprotection against a homologous challenge (antibody titres: 1 : 16 384 on day 21 and 1 : 32 768 on day 42). No protection (0% survival rate) was observed with unparasitized *in vitro* culture supernatant (UPCS) or with 25 μg GST used as controls.

Following our results on immunoprotection and antibody titres, we mainly focused on the Bd37r and we performed a dose-effect vaccination assay using 0·01–25 μg of the recombinant protein (Table 3). We used parasitized culture supernatant (PCS) from *B. divergens* Rouen87 as our positive control. Negative controls were UPCS and GST (10 μg per injection), as previously mentioned. Two injections of 25, 10, 5, 2, 1 or 0·1 μg of Bd37r conferred complete immunoprotection of gerbils against *B. divergens* Rouen87 challenge. Whatever the dose between 25 μg and 1 μg of Bd37r, the same antibody titres were observed (1 : 16 384 or 1 : 32 768 on day 21 and 1 : 131 072 on day 42). With 2 injections of 0·1 μg of Bd37r, there were low antibody titres on day 21 (1 : 4096) and day 42 (1 : 32 768). Nevertheless, this decrease had no effect on immunoprotection and clinical outcomes (decrease in haematocrit and presence of parasite in gerbil blood). Moreover, even though vaccination with 0·01 μg of Bd37r gave a humoral response (antibody titres: 1 : 1024 on day 21 and 1 : 16 384 on day 42), it did not confer complete immunoprotection. Among the gerbils vaccinated with 0·01 μg of Bd37r, 20% presented with clinical symptoms and 10% died. All gerbils vaccinated with *B. divergens* Rouen87 PCS survived the challenge and all the ones from negative control batches (UPCS and 10 μg GST) died.

Bd37r is able to induce a complete immunoprotection against heterologous challenge

Bd37r was tested for its capacity to give immunoprotection against each reference isolate from the 5 groups defined by PCR-RFLP (Table 4). As described above, these 5 reference isolates are *B. divergens* Rouen87, W8843, Y5, 6303E and 1705A. According to the dose-effect vaccination assay, we chose 1 μg of Bd37r as the dose to vaccinate gerbils. The antibody titres were either 1 : 16 384 or 1 : 32 768 on day 21 and 1 : 131 072 on day 42. After challenge with their respective isolate, all gerbils survived infection. Nevertheless, dramatic differences were observed in clinical signs developed by gerbils according to challenges with different isolates. All gerbils were protected against challenge with *B. divergens* Rouen87 and W8843 isolates and in these groups no gerbils presented parasites in the blood or a haematocrit decrease below 30%. In addition, for these 2 isolates, all control gerbils died with strong parasitaemia (>75%, data not shown) and a severe fall in haematocrit (<12%, data not shown). The results obtained after challenge with *B. divergens* 1705A were similar to those obtained with Rouen87 or W8843, except that 10% of the gerbils showed clinical signs (prostration

https://doi.org/10.1017/S0031182006001399 Published online by Cambridge University Press

Table 2. Comparison of Bd37r, Bd35r and a mixture Bd37r/Bd35r in their abilities to induce immunoprotection against *Babesia divergens* Rouen87 challenge

Vaccine[a]	Dose	Survival rate[b] (%)	Antibody titres	
			Day_{21}	Day_{42}
Bd37r	25 μg	100	1 : 32 768	1 : 131 072
Bd35r	25 μg	100	1 : 4096	1 : 32 768
Bd37r + Bd35r	12·5 μg + 12·5 μg	100	1 : 8192	1 : 65 536
GST	25 μg	0	—	—
PCS	300 μl	100	1 : 16 384	1 : 32 768
UPCS	300 μl	0	—	—

[a] Bd37r and Bd35r are GST-fusion proteins. Controls used are a recombinant GST (GST), parasitized culture supernatant (PCS) and unparasitized culture supernatant (UPCS).
[b] Percentage of gerbils surviving to the virulent challenge.

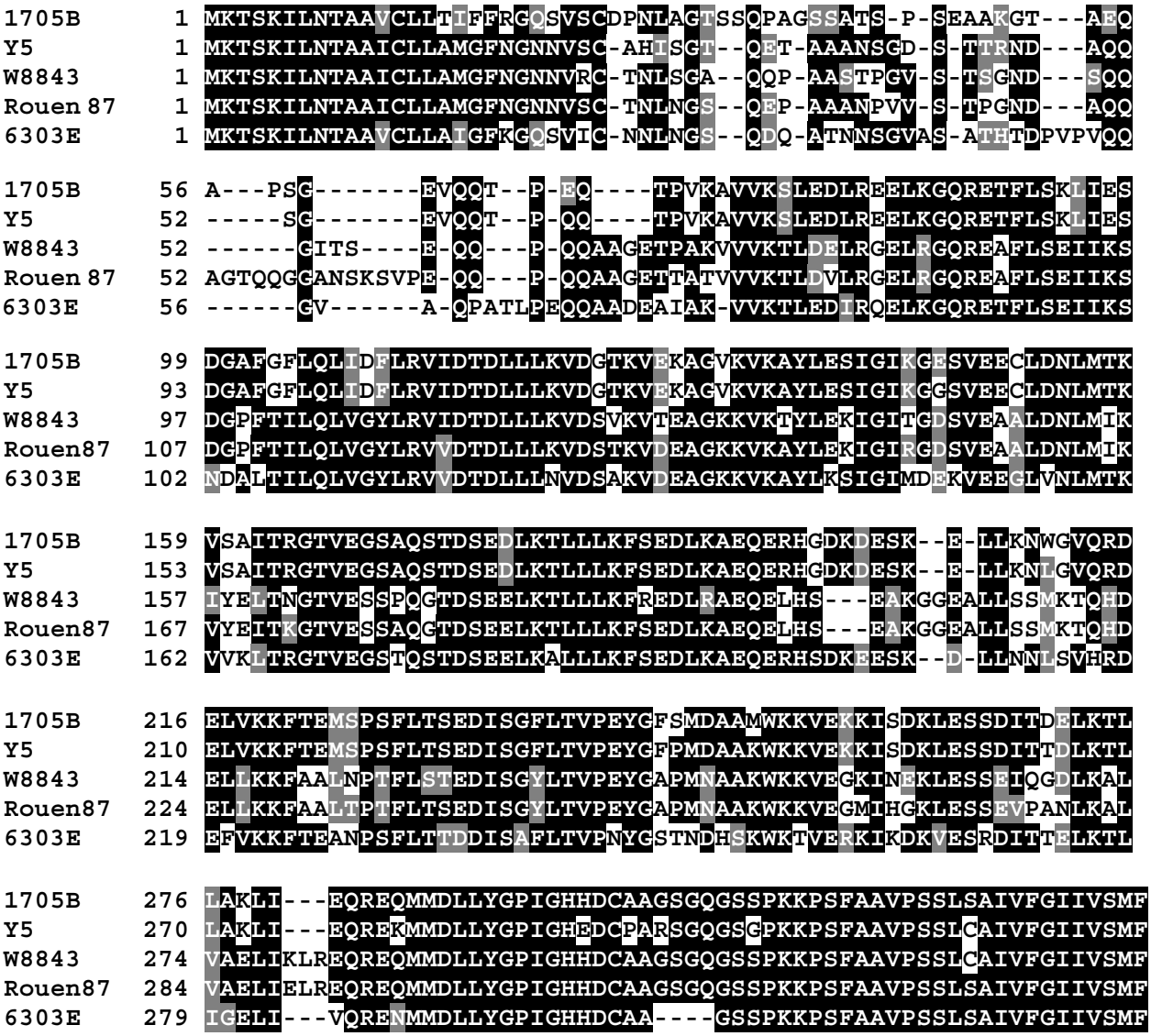

Fig. 2. ClustalW alignment and analysis of the deduced amino acid sequences of Bd37 proteins from 1705B, Y5, W8843, Rouen87 and 6303E *Babesia divergens* isolates. Black boxes represent identical amino acids that are conserved in at least 3 of the 5 sequences. Grey boxes represent similar but not identical amino acids. The sequence polymorphism is mainly observed in the N-terminal part of the Bd37 proteins. Percentage homologies between Rouen87 and 1705B, Y5, W8843, 6303E, are 79·8%, 69·9%, 85% and 59·7%, respectively.

https://doi.org/10.1017/S0031182006001399 Published online by Cambridge University Press

Table 3. Dose-effect vaccination assay with the recombinant Bd37 protein

Vaccine[a]	Dose (μg)	Survival rate[b] (%)	H<30%[c]	P+[d]	Antibody titres Day$_{21}$	Antibody titres Day$_{42}$
Bd37r	25	100	0	0	1 : 32 768	1 : 131 072
Bd37r	10	100	0	0	1 : 32 768	1 : 131 072
Bd37r	5	100	0	0	1 : 32 768	1 : 131 072
Bd37r	2	100	0	0	1 : 32 768	1 : 131 072
Bd37r	1	100	0	0	1 : 16 384	1 : 131 072
Bd37r	0·1	100	0	0	1 : 4096	1 : 32 768
Bd37r	0·01	90	20	20	1 : 1024	1 : 16 384
GST	10	0	100	100	—	—
PCS	300 μl	100	0	0	1 : 32 768	1 : 65 536
UPCS	300 μl	0	100	100	—	—

[a] Bd37r is a GST-fusion protein. Controls used are a recombinant GST (GST), parasitized culture supernatant (PCS) and unparasitized culture supernatant (UPCS).
[b] Percentage of gerbils surviving to the virulent challenge.
[c] Percentage of gerbils with a haematocrit fall below 30%.
[d] Percentage of gerbils with parasites in the blood.

Table 4. Heterologous challenge after vaccination with a low dose of Bd37r protein

Vaccine[a]	Challenge strain	Survival rate[b] (%)	H<30%[c]	P+[d]	Antibody titres Day$_{21}$	Antibody titres Day$_{42}$
Bd37r	Rouen87	100	0	0	1 : 16 384	1 : 131 072
GST	Rouen87	0	100	100	—	—
Bd37r	W8843	100	0	0	1 : 32 768	1 : 131 072
GST	W8843	0	100	100	—	—
Bd37r	1705A	100	10	10	1 : 16 384	1 : 131 072
GST	1705A	0	100	100	—	—
Bd37r	6303E	100	0	70	1 : 32 768	1 : 131 072
GST	6303E	0	100	100	—	—
Bd37r	Y5	100	0	0	1 : 32 768	1 : 131 072
GST	Y5	90	90	100	—	—

[a] Bd37r is a GST-fusion protein. Controls used are a recombinant GST (GST).
[b] Percentage of gerbils surviving to the virulent challenge.
[c] Percentage of gerbils with a haematocrit fall below 30%.
[d] Percentage of gerbils with parasites in the blood.

and haemoglobinuria). In terms of protection, the results obtained with *B. divergens* 6303E pointed at a complete immunoprotection conferred by Bd37r vaccination. Nevertheless, 70% of the vaccinated gerbils were parasitized but this parasitaemia was never accompanied with a decrease in haematocrit: no gerbils presented with a decrease <30%. For *B. divergens* Y5 all gerbils also survived but the clinical and biological signs generated by Y5 in the control gerbils were totally different from all the other isolates. Only 10% of the gerbils died after the challenge but 100% of the animals were parasitized with parasitaemia reaching 20–30% (data not shown). In addition, 90% presented a decrease in haematocrit below 30% among these animals.

DISCUSSION

In previous studies on the protection of gerbils against *B. divergens* babesiosis, we demonstrated the major role played by a 37 kDa protein (Bd37) (Delbecq *et al*. 2002). At the beginning Bd37 was identified as a major component of a protective fraction derived from *in vitro* culture supernatants (Carcy *et al*. 1995). Besides, a direct association was previously shown between protection conferred by passive transfer with a monoclonal antibody directed

https://doi.org/10.1017/S0031182006001399 Published online by Cambridge University Press

against Bd37 and sequence polymorphism within the recognized epitope (Precigout *et al*. 2004). In the present work, we demonstrated that primers designed against the Bd37 gene sequence, could be used to type *B. divergens* by PCR-RFLP, allowing us to separate our different isolates into 5 distinct clades. We showed that a recombinant Bd37 protein derived from Rouen1987 was able to protect gerbils against challenges from all 5 types of *B. divergens* isolates, each bearing polymorphic Bd37 genes.

The first generation of vaccine against *Babesia sp*. consisted of parasite-derived mixtures of antigens, most of them found in culture supernatants (Timms *et al*. 1983; Montenegro-James *et al*. 1987; Gorenflot *et al*. 1990; Schetters *et al*. 1994, 2001; Schetters and Montenegro-James, 1995; Holman *et al*. 2005). This kind of vaccine showed some efficiency against a virulent challenge in the case of *B. bovis* or *B. bigemina* bovine babesiosis (Montenegro-James *et al*. 1987, 1992) and *B. canis* canine babesiosis (Schetters, 2005; Schetters *et al*. 2001). As far as *B. divergens* is concerned, culture supernatant-derived antigens when used with saponin as adjuvant proved to confer protective immunity in gerbils and bovine animals (Gorenflot *et al*. 1992). Nevertheless, the production of a large amount of *Babesia* culture supernatant requires a large supply of erythrocyte host cells as well as appropriate material and specially trained staff. These factors, along with the need to assess the genetic stability of the strain used and quality control of the released protein in the supernatant, lead to difficulties in the industrial production process. Recombinant proteins are more suitable for industrial production as vaccine, since most of the processes are highly reproducible and routinely used by pharmaceutical companies (Schetters, 1995).

Obviously, some promising proteins identified in *Babesia* culture supernatants have previously failed as recombinant vaccine candidates (Orinda *et al*. 1992; Norimine *et al*. 2003). In some cases, as for example the 19 kDa MSP-1 protein of *Plasmodium falciparum*, a recombinant protein gave good protection when emulsified with Freund's adjuvant but was inefficient when used with saponin, despite the presence of specific antibodies (Daly and Long, 1996; Kumar *et al*. 2000). In the case of Bd37, the recombinant protein was able to confer heterologous protection when combined with adjuvant and saponin. Vaccine formulations containing saponin must be preferred to the Freund's adjuvant as the latter is prohibited for veterinary or human uses.

The dose-effect assay performed on gerbils showed that small quantities of protein were sufficient to obtain maximal protection, with no extra benefits increased. In addition, antibody titre seemed to correlate with protection, since the group vaccinated with 0·01 μg of protein exhibited an antibody titre of 1 : 16 384 associated with only 90% protection, and 2 gerbils presenting with clinical babesiosis. Above a 1 : 32 768 value in antibody titre (group vaccinated with 0·1 μg), all gerbils were completely protected. Thus, we propose the use of serological analysis to assess the protective immunological status of vaccinated animals. The development of such serological tests in cattle should be a helpful tool to conduct an efficient vaccination campaign.

The PCR-RFLP typing assay based on the Bd37 gene sequence allowed us to discriminate 5 groups among the 14 isolates that we tested. These isolates were from diverse geographical origins and different hosts, and all gave a detectable PCR product with our primer set. Polymorphism within the Bd37 protein was shown by cloning and sequencing of some PCR products from each group. The most variable region of Bd37 is the N-terminal part of the mature molecule, in which insertion/deletion events and amino-acid substitutions occur. Preliminary results of NMR studies on this protein revealed that the Bd37 protein is mainly constituted of α-helices and an unstructured part in the N-terminus (Yang *et al*. 2005). Besides, the most conserved region of Bd37 is also the most structured. So we can suppose this N-terminal part of Bd37 represents a highly antigenic loop that can generate most of the immune response from the infected or vaccinated animal, while the rest of the protein containing the functional site remains invisible to the humoral response. Therefore, it is possible that vaccination with recombinant Bd37 might neutralize this parasite avoidance strategy by directing the immune response against the conserved, structured part of the protein. This could explain the protection observed across isolates (sharing the conserved region).

It should be pointed out that we previously highlighted the major role of antibodies in the protection against *B. divergens* using monoclonal antibody transfer (Precigout *et al*. 2004). The mAb F4.2F8-INT binds to an epitope on Bd37 which is not highly conserved among different isolates. In particular, the isolate 63.03E bears a Bd37 protein which is not recognized by the mAb, and mAb injections did not protect gerbils against challenge with this isolate. Nevertheless, the vaccination with the Rouen1987-derived recombinant Bd37 conferred a complete protection against challenge with 63.03E. This fact would indicate the existence of protective epitopes on the F4.2F8-INT binding site and/or possibly the existence of antibodies generated against this site even in the presence of amino acid substitutions, thus recognizing divergent Bd37 proteins.

As mentioned above, the use of PCR-RFLP based on the Bd37 gene sequence made feasible a rapid molecular typing of *B. divergens* isolates. The result of vaccine experiments demonstrated that the recombinant protein Bd37 derived from Rouen 1987 was able to protect gerbils against heterologous challenge. In the case of a bovine babesiosis

https://doi.org/10.1017/S0031182006001399 Published online by Cambridge University Press

outbreak, we could rapidly identify whether this new isolate relates to one of the 5 groups and then gauge if a Bd37-based vaccination could be relevant. If the new isolate appears dramatically different from our 5 groups identified so far (i.e. if its sequences do not cluster with the known clades), and thus potentially bearing a new type of Bd37 protein, we would have to test these new parasites in vaccinated gerbils to assess the protective effect of the recombinant Rouen1987 Bd37 protein. We used a recombinant protein derived from isolate W8843 against a challenge with the Rouen1987 isolate and we obtained complete protection, as with a mixture of recombinant proteins derived from both Rouen1987 and W8843. Results presented in this study demonstrated the protective efficacy of a recombinant Bd37 protein used as vaccine against *B. divergens* in gerbils, the laboratory model of *B. divergens* babesiosis. These findings indicate that a broad vaccine efficacy against *B. divergens* might be rapidly achieved with a combined recombinant vaccine containing different Bd37 proteins, overcoming Bd37 polymorphism. Nevertheless, the protective effect remains to be demonstrated in the bovine host which is the target for commercial development of such a vaccine.

This work was supported by a grant from Intervet International B.V., Netherlands. Alexina Vallet was a Ph.D. student supported by a grant from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche and Intervet S.A. (Convention CIFRE). We thank Dr Pascal Michon for his fruitful discussions.

REFERENCES

Beattie, J. F., Michelson, M. L. and Holman, P. J. (2002). Acute babesiosis caused by *Babesia divergens* in a resident of Kentucky. *New England Journal of Medicine* **347**, 697–698.

Ben Musa, N. and Dawoud, H. A. (2004). The protective activity of serum and fractionated serum from rats against *Babesia divergens*. *Journal of the Egyptian Society of Parasitology* **34**, 407–422.

Brown, W. C., Norimine, J., Goff, W. L., Suarez, C. E. and McElwain, T. F. (2006). Prospects for recombinant vaccines against *Babesia bovis* and related parasites. *Parasite Immunology* **28**, 315–327.

Carcy, B., Precigout, E., Valentin, A., Gorenflot, A. and Schrevel, J. (1995). A 37-kilodalton glycoprotein of *Babesia divergens* is a major component of a protective fraction containing low-molecular-mass culture-derived exoantigens. *Infection and Immunity* **63**, 811–817.

Christensson, D. A. (1987). Clinical and serological response after experimental inoculation with *Babesia divergens* of newborn calves with and without maternal antibodies. *Acta Veterinaria Scandinavica* **28**, 381–392.

Christensson, D. A. (1989). Inverse age resistance to experimental *Babesia divergens* infection in cattle. *Acta Veterinaria Scandinavica* **30**, 453–464.

Daly, T. M. and Long, C. A. (1996). Influence of adjuvants on protection induced by a recombinant fusion protein against malarial infection. *Infection and Immunity* **64**, 2602–2608.

Delbecq, S., Precigout, E., Vallet, A., Carcy, B., Schetters, T. P. and Gorenflot, A. (2002). *Babesia divergens*: cloning and biochemical characterization of Bd37. *Parasitology* **125**, 305–312.

Goethert, H. K. and Telford, S. R. 3rd. (2003). Enzootic transmission of *Babesia divergens* among cottontail rabbits on Nantucket Island, Massachusetts. *American Journal of Tropical Medicine and Hygiene* **69**, 455–460.

Gorenflot, A., Brasseur, P., Precigout, E., L'Hostis, M., Marchand, A. and Schrevel, J. (1991). Cytological and immunological responses to *Babesia divergens* in different hosts: ox, gerbil, man. *Parasitology Research* **77**, 3–12.

Gorenflot, A., Moubri, K., Precigout, E., Carcy, B. and Schetters, T. P. (1998). Human babesiosis. *Annals of Tropical Medicine and Parasitology* **92**, 489–501.

Gorenflot, A., Precigout, E., Bissuel, G., Lecointre, O., Brasseur, P., Vidor, E., L'Hostis, M. and Schrevel, J. (1990). Identification of major *Babesia divergens* polypeptides that induce protection against homologous challenge in gerbils. *Infection and Immunity* **58**, 4076–4082.

Gorenflot, A., Precigout, E., Valentin, A., Bissuel, G., Carcy, B., Brasseur, P., Moreau, Y. and Schrevel, J. (1992). *Babesia divergens* vaccine. *Memorias do Instituto Oswaldo Cruz* **87** (Suppl. 3), 279–281.

Gray, J. S. and Harte, L. N. (1985). An estimation of the prevalence and economic importance of clinical babesiosis in the republic of Ireland. *Ireland Veterinary Journal* **69**, 455–460.

Herwaldt, B., Persing, D. H., Precigout, E., Goff, W. L., Mathiesen, D. A., Taylor, P. W., Eberhard, M. L. and Gorenflot, A. (1996). A fatal case of babesiosis in Missouri: identification of another piroplasm that infects humans. *Annals of Internal Medicine* **124**, 643–650.

Herwaldt, B. L., de Bruyn, G., Pieniazek, N. J., Homer, M., Lofy, K. H., Slemenda, S. B., Fritsche, T. R., Persing, D. H. and Limaye, A. P. (2004). *Babesia divergens*-like infection, Washington State. *Emerging Infectious Diseases* **10**, 622–629.

Holman, P. J., Spencer, A. M., Droleskey, R. E., Goethert, H. K. and Telford, S. R. 3rd. (2005). *In vitro* cultivation of a zoonotic *Babesia sp.* isolated from eastern cottontail rabbits (*Sylvilagus floridanus*) on Nantucket Island, Massachusetts. *Journal of Clinical Microbiology* **8**, 3995–4001.

James, M. A. (1989). Application of exoantigens of *Babesia* and *Plasmodium* in vaccine development. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **83** (Suppl.), 67–72.

Joyner, L. P. and Davies, S. F. (1967). Acquired resistance to *Babesia divergens* in experimental calves. *Journal of Protozoology* **14**, 260–262.

Kumar, S., Collins, W., Egan, A., Yadava, A., Garraud, O., Blackman, M. J., Guevara Patino, J. A., Diggs, C. and Kaslow, D. C. (2000). Immunogenicity and efficacy in aotus monkeys of four recombinant *Plasmodium falciparum* vaccines in multiple adjuvant formulations based on the 19-kilodalton

C terminus of merozoite surface protein 1. *Infection and Immunity* **68**, 2215–2223.

Kuttler, K. L. (1988). World-wide impact of babesiosis. In *Babesiosis of Domestic Animals and Man* (ed. Ristic, M.), pp. 1–22. CRC Press, Inc., Boca Raton, Florida.

Lewis, D. and Williams, H. (1979). Infection of the Mongolian gerbil with the cattle piroplasm *Babesia divergens*. *Nature, London* **278**, 170–171.

L'Hostis, M., Chauvin, A., Valentin, A., Precigout, E. and Gorenflot, A. (1997). Survey of *Babesia divergens* antibody kinetics in cattle in western France. *Veterinary Research* **28**, 481–848.

Montenegro-James, S., Toro Benitez, M., Leon, E., Lopez, R. and Ristic, M. (1987). Bovine babesiosis: induction of protective immunity with culture-derived *Babesia bovis* and *Babesia bigemina* immunogens. *Parasitology Research* **74**, 142–150.

Montenegro-James, S., Toro, M., Leon, E. and Guillen, A. T. (1992). Field evaluation of an exoantigen-containing *Babesia* vaccine in Venezuela. *Memorias do Instituto Oswaldo Cruz* **87** (Suppl.) **3**, 283–288.

Norimine, J., Mosqueda, J., Suarez, C., Palmer, G. H., McElwain, T. F., Mbassa, G. and Brown, W. C. (2003). Stimulation of T-helper cell gamma interferon and immunoglobulin G responses specific for *Babesia bovis* rhoptry-associated protein 1 (RAP-1) or a RAP-1 protein lacking the carboxy-terminal repeat region is insufficient to provide protective immunity against virulent B. bovis challenge. *Infection and Immunity* **71**, 5021–5032.

Orinda, G. O., Gale, K. R., Wright, I. G. and Parrodi, F. (1992). Bovine babesiosis: failure to induce interferon gamma production in response to *Babesia bovis* antigens in cattle. *International Journal for Parasitology* **22**, 395–398.

Precigout, E., Delbecq, S., Vallet, A., Carcy, B., Camillieri, S., Hadj-Kaddour, K., Kleuskens, J., Schetters, T. and Gorenflot, A. (2004). Association between sequence polymorphism in an epitope of *Babesia divergens* Bd37 exoantigen and protection induced by passive transfer. *International Journal for Parasitology* **34**, 585–593.

Precigout, E., Gorenflot, A., Valentin, A., Bissuel, G., Carcy, B., Brasseur, P., Moreau, Y. and Schrevel, J. (1991). Analysis of immune responses of different hosts to *Babesia divergens* isolates from different geographic areas and capacity of culture-derived exoantigens to induce efficient cross-protection. *Infection and Immunity* **59**, 2799–2805.

Schetters, T. (2005). Vaccination against canine babesiosis. *Trends in Parasitology* **21**, 179–184.

Schetters, T. (1995). Vaccine development from a commercial point of view. *Veterinary Parasitology* **57**, 267–275.

Schetters, T. P., Kleuskens, J. A., Scholtes, N. C., Gorenflot, A., Moubri, K. and Vermeulen, A. N. (2001). Vaccination of dogs against heterologous *Babesia canis* infection using antigens from culture supernatants. *Veterinary Parasitology* **100**, 75–86.

Schetters, T. P., Kleuskens, J. A., Scholtes, N. C., Pasman, J. W. and Bos, H. J. (1994). Vaccination of dogs against *Babesia canis* infection using antigens from culture supernatants with emphasis on clinical babesiosis. *Veterinary Parasitology* **52**, 219–233.

Schetters, T. P. and Montenero-James, S. (1995). Vaccines against babesiosis using soluble parasite antigens. *Parasitology Today* **11**, 456–462.

Timms, P., Dalgliesh, R. J., Barry, D. N., Dimmock, C. K. and Rodwell, B. J. (1983). *Babesia bovis*: comparison of culture-derived parasites, non-living antigen and conventional vaccine in the protection of cattle against heterologous challenge. *Australian Veterinary Journal* **60**, 75–77.

Valentin, A., Precigout, E., L'Hostis, M., Carcy, B., Gorenflot, A. and Schrevel, J. (1993). Cellular and humoral immune responses induced in cattle by vaccination with *Babesia divergens* culture-derived exoantigens correlate with protection. *Infection and Immunity* **61**, 734–741.

Yang, Y. S., Delbecq, S., Strub, M. P., Lohr, F., Schetters, T., Gorenflot, A., Precigout, E. and Roumestand, C. (2005). 1H, 15N and 13C Backbone resonance assignments of the 37 kDa surface antigen protein Bd37 from *Babesia divergens*. *Journal of Biomolecular NMR* **31**, 67–68.