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# Up-regulated expression of spherical body protein 2 truncated copy 11 in *Babesia bovis* is associated with reduced cytoadhesion to vascular endothelial cells

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## ABSTRACT

The factors involved in gain or loss of virulence in *Babesia bovis* are unknown. Spherical body protein 2 truncated copy 11 (*sbp2t11*) transcripts in *B. bovis* were recently reported to be a marker of attenuation for *B. bovis* strains. Increased cytoadhesion of *B. bovis*-infected red blood cells (iRBC) to vascular endothelial cells is associated with severe disease outcomes and an indicator of parasite virulence. Here, we created a stable *B. bovis* transfected line over-expressing *sbp2t11* to determine whether up-regulation of *sbp2t11* is associated with changes in cytoadhesion. This line was designated sbp2t11up and five *B. bovis* clonal lines were derived from the sbp2t11up line by limiting dilution for characterisation. We compared the ability of iRBCs from the sbp2t11up line and its five derivative clonal lines to adhere to bovine brain endothelial cells, using an in vitro cytoadhesion assay. The same lines were selected for in vitro cytoadhesion and the levels of *sbp2t11* transcripts in each selected line were quantified. Our results demonstrate that up-regulation of *sbp2t11* is accompanied by a statistically significant reduction in cytoadhesion. Confirmed up-regulation of *sbp2t11* in *B. bovis* concomitant with the reduction of iRBC in vitro cytoadhesion to bovine brain endothelial cell is consistent with our previous finding that up-regulation of *sbp2t11* is an attenuation marker in *B. bovis* and suggests the involvement of *sbp2t11* transcription in *B. bovis* virulence.

## 1. Introduction

Bovine babesiosis caused by *Babesia bovis* is a tick-borne haemoparasitic disease with more than 1.2 billion cattle around the world exposed to this disease (Bock et al., 2004). *Babesia bovis* is transmitted by ixodid ticks, primarily *Rhipicephalus microplus*. There are important variations of virulence among distinct *B. bovis* strain phenotypes. Highly virulent strains can cause acute infections characterised by hyperthermia, decreased hematocrit, and neurovirulence signs such as ataxia, paddling, and inappetence (Nevils et al., 2000). These neurological signs are likely due to the adhesion of *B. bovis* infected red blood cells (iRBC) to host endothelial cells, resulting in the sequestration of iRBC within cerebral capillaries (Wright, 1972; Hutchings et al., 2007; Sondgeroth et al., 2013) and induction of endothelial inflammation (Clark, 1998). In contrast, attenuated strains, derived from the virulent parental strains, do not induce neurovirulence in inoculated young calves, and significantly lower levels of sequestration, hyperthermia and hematocrit are common (Sondgeroth et al., 2013; Florin-Christensen et al., 2014). Such attenuated strains are usually derived from virulent strains that undergo a series of rapid passages (usually 22–28) in splenectomised calves (Callow, 1979; Florin-Christensen et al., 2014). Young calves, up to 1 year old,

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vaccinated with attenuated strains are usually able to survive without chemotherapeutic intervention, but that is not the case for adult cattle (Suarez and Noh, 2011; Florin-Christensen et al., 2014). Although live vaccines are used routinely in endemic countries for prophylaxis, those are costly and cumbersome to produce, may be lethal to adult cattle, require cold chain storage during transportation, and have a high risk of virulence reversion and contamination (Musoke et al., 1996). Better-defined live or subunit vaccines would be ideal substitutes for the current vaccines. Developing novel control strategies for bovine babesiosis requires a better understanding of the factors involved in the host-parasite relationships and the molecular mechanisms underlying virulence.

Factors involved in attenuation and/or increased virulence in *B. bovis* remain essentially unknown. Attenuation after repeated calf passages reduced genome diversity in *B. bovis* (Lau et al., 2011). Additionally, phenotype-specific gene regulatory differences were found between virulent and attenuated strains in *B. bovis* (Pedroni et al., 2013). Interestingly, members of a *Babesia*-specific gene family, spherical body proteins (SBP) have been shown to be transcriptionally up-regulated exclusively in the attenuated derivatives (Pedroni et al., 2013; Gallego-Lopez et al., 2018).

Among *sbp* genes, *sbp2t11* (sbp2 truncated copy 11) showed consistent up-regulation in multiple attenuated strains compared with their virulent parental strains, and was recently confirmed as an attenuation marker for *B. bovis* (Gallego-Lopez et al., 2018). In addition, *sbp2t11* transcript levels were correlated with SBP2t11 expression in both Texas (Tx) attenuated and virulent strains of *B. bovis*. Furthermore, *sbp2t11* is differentially expressed in the blood stages of the parasite but is undetectable in the kinete stage (Gallego-Lopez et al., 2018). Additionally, SBP2t11 is expressed as a 30 kDa full-length protein that is cleaved to a 17 kDa protein by the recognition of a PEXEL-like motif (PLM) (Pellé et al., 2015; Gallego-Lopez et al., 2018).

This study analysed and phenotypically characterised a virulent *B. bovis* strain containing an extra copy of stably-transfected *sbp2t11* that mimics the over-expression of this gene as consistently observed in the attenuated *B. bovis* strains. We hypothesised that natural up-regulation of *sbp2t11* is an attenuation marker and is associated with cytoadhesion reduction in vitro. Our results are consistent with the hypothesis that up-regulation of *sbp2t11* significantly reduces cytoadhesion of *B. bovis* iRBC to bovine brain endothelial cells (BBEC). Overall, the data further defines the involvement of *sbp2t11* transcription in *B. bovis* virulence.

## 2. Materials and methods

### 2.1. In vitro propagation of B. bovis culture

Various *B. bovis* strains were maintained as in vitro microaerophilous stationary phase (MASP) blood cultures at 10% hematocrit, using bovine RBC as previously described (Levy and Ristic, 1980). These strains are virulent (Vir) parental field isolates and their respective attenuated (Att) derivatives. Animals use as blood donors for the maintenance of in vitro cultures of *B. bovis* was approved by the Institutional Animal Care and Use Committee (protocol 2013–66, University of Idaho, USA).

### 2.2. Genomic and complimentary DNA synthesis

An in vitro *B. bovis* culture with approximately 25% parasitised erythrocytes (PPE) was used for the isolation of genomic DNA (gDNA) using a Puregene Blood Core Kit C (Qiagen, MD, USA). Briefly, cells were centrifuged at 1048*g* for 10 min at 4 °C and washed with cold PBS. The pellet was frozen at −80 °C and stored overnight to lyse RBC and release merozoites. The gDNA isolation procedure was followed as reported previously (Gallego-Lopez et al., 2018).

For the generation of cDNA, in vitro *B. bovis* cultures at 25 PPE were used for Trizol RNA isolation (Ambion, MA, USA). *Babesia bovis* cultures were centrifuged and the pellets suspended in 3 ml of Trizol. Total RNA was isolated as indicated by the manufacturer (J.T. Baker, PA, USA), suspended in 25 µl of diethylpyrocarbonate-treated (DEPC), and treated with Turbo DNA free-DNase (Ambion). Reverse transcriptase-PCR (RT-PCR) was carried out for cDNA synthesis using random hexamers in the RT reaction (Ambion).

### 2.3. Quantitative PCR (qPCR)

qPCR of *sbp2t11* in wild type (wt) $Tx_{Vir}$, $Tx_{Att}$ and stably transfected lines was carried out as previously described (Pedroni et al., 2013). Briefly, SybrGreen supermix (Bio-Rad, CA, USA), *sbp2t1*1-specific primers and 70 ng of cDNA were used for the qPCR. The conditions were: one cycle at 95 °C for 10 min; 39 cycles of 95 °C for 10 s, 57.5 °C for 30 s, 72 °C for 30 s and finally one cycle at 72 °C for 5 min and 55 °C for 1 min. Amplicons were cloned and sequenced. Three replicates per strain were used. To normalise the reactions, amplification of gene BBOV_II004820 (GenBank) which encodes for a single copy the topoisomerase II gene, was set up using the same template source. The transcript level of this gene remains the same in the virulent and attenuated *B. bovis* strain pairs (Pedroni et al., 2013). Final data are represented as the cycle threshold ratio (CT ratio) rather than the actual CT values, where the lower the ratio value, the higher the expression. (Supplementary Table S1 lists all the primer sequences for the qPCR).

### 2.4. Stable transfection of B. bovis $Tx_{Vir}$

*Sbp2t11* is an 816 bp gene. A 1210 bp amplicon was amplified from *B. bovis* $Tx_{Vir}$ gDNA including 316 bp downstream of its stop codon using specific primers with SacII restriction enzyme sequences added at 5′ and 3′ of the *sbp2t11* (Supplementary Table S1). This amplicon was cloned in the pCR®4-TOPO® vector (Invitrogen, MA, USA) and sequenced for verification. The extended *sbp2t11* amplicon was cleaved with SacII restriction enzyme (Invitrogen) and subcloned in-frame into a dephosphorylated bidirectional promoter transfection vector (Laughery et al., 2014). The transfection vector uses a constitutively active elongation factor-1α (ef-1α) bidirectional promoter with two independent expression sites, 'A' and 'B'. The *sbp2t11* gene was ligated into the 'B' expression site while the red fluorescent protein-blasticidin resistant fusion (rfp-bsdr) selectable marker was cloned into the 'A' expression site of the transfection vector. The two expression sites are separated by a 1.4 Kb intergenic region (IG). Confirmation of correct construction was carried out by sequencing (GenBank accession numbers MH238439 and MH238440). For transfections, wt $Tx_{Vir}$ was grown in long-term MASP culture at 10% hematocrit, using bovine RBC as previously described (Levy and Ristic, 1980), until 30 PPE was reached. Parasites were transformed by electroporation as previously described (Suarez and McElwain, 2009) in three independent transfections. Stable transfection controls include pBluescript plasmid (pBS) and the pEf-msa-1-Bm86ep-gfp-bsd vector (Laughery et al., 2014). The stably transfected line developed with this last plasmid was previously denoted Tf-Bm86ep-gfp-bsd (Laughery et al., 2014). Post-transfection analysis of successful integration of synthetic *sbp2t11* was carried out as previously described (Suarez and McElwain, 2009). Transfected parasites were grown in the presence of 4 µg/ml of blasticidin for selection purposes 4 h post-transfection and PPE was monitored daily. The transfected *B. bovis* was evaluated when PPE reached three and blasticidin concentration was increased to 6 µg/ml. We designated this stably transfected line sbp2t11up.

### 2.5. Clonal lines by limited dilution

The sbp2t11up transfected line was grown in long-term MASP culture at 10% hematocrit using bovine RBC as previously described (Levy and Ristic, 1980) in 4–6 µg/ml of blasticidin. When plasmid integration into the *B. bovis* genome was confirmed, a transfected clonal line was derived by limited dilution. Briefly, transfected iRBC at ~5 PPE were used to quantify the number of iRBC/µl, using a hemacytometer and orange acridine-stained coverslips. Later, adequate dilutions of the iRBC were prepared in growth media to deliver 0.25 iRBC into each well of four 96-well plates at 10% hematocrit. The cells were incubated at 2% $O_2$ and 3% $CO_2$. The media were changed every 48 h. The wells were examined for initial evidence of parasite growth from day 7 to day 22 by PCR using a hemo klein Taq polymerase (New England Biolabs, Beverly, MA, USA) and the rap primers (Supplementary Table S1). One microliter of each sample was lysed in 11 µl of diethylpyrocarbonate-treated (DEPC) water and heated at 72 °C for 10 min. Then, 8.25 µl of this sample were mixed with 4.25 µl of 1× hemo klein Taq, 10 mm NTPs, 20 µm of each primer. PCR conditions were 95 °C for 3 min for an initial cycle which was followed by 40 cycles of 95 °C 20 s, 60 °C 30 s, 68 °C for 120 s and then 68 °C 10 min. Clones were split 1:2 once PPE reached three. Positive wells were transferred to a 48-well plate. Clone characterisation was conducted via RT-PCR, qPCR, immunoblot, fluorescent microscopy and Southern blot analyses.

### 2.6. Genetic analysis of the sbp2t11up transfected line

Parasite gDNA was isolated from sbp2t11up stably transfected line and $Tx_{Vir/Att}$ wt cell lines for sequencing of PCR integration amplicons and Southern hybridisation analyses. PCR amplification was conducted using primer sets rfp, bsdr, *sbp2t11*, efa, f8, r6, 1210r, Topoisomerase II and EfUps (Supplementary Table S1). EfUps is a fragment of the glutamyl tRNA synthetase gene (GenBank accession number BB0V_IV010640) which is adjacent to the ef-1α promoter gene in the *Babesia* genome. The resulting PCR products were confirmed by gel electrophoresis, cloned into a pCR 4 Topo Vector (Invitrogen) and sequenced using standard techniques.

For Southern hybridisation analysis, total DNA from *B. bovis* parasites was digested with BglII restriction enzyme, electrophoresed, transferred to ZetaProbe nylon membranes, and hybridised as previously described (Suarez and McElwain, 2009). Digoxigenin (DIG)-labelled probes representing (i) 425 bp of the intergenic region of ef-1α promoter (efa), (ii) 1060 bp fragment of the selection marker, rfp-bsdr and (iii) bsdr, selection marker and EfUps were prepared by PCR amplification using a PCR Dig-Probe Synthesis kit as recommended by the manufacturer (Boehringer–Roche, Germany). The rfp-bsdr probe was prepared with the primers rfp-F and bsd-R, the efa probe was prepared with the primers efa-F and efa-R and finally the 'bsdr- EfUps probe was prepared with the primers bsd-F and EfUps (Supplementary Table S1).

### 2.7. Growth curve

To compare the growth rates of the different strains, in vitro cultures of stably transfected parasites and wt *B. bovis* were evaluated daily by PPE over a 4 day period, beginning at 0.5 PPE. Diff-Quick stained blood smears (RAL, France) were prepared in triplicate and read daily to determine PPE values.

### 2.8. In vitro cytoadhesion assay

BBEC were cultured to 70% confluence following previously published specifications (O'Connor et al., 1999). At passage 5, BBECs were seeded in 6-well plates at a density of approximately $1.25 \times 10^5$ cells/cm$^2$ in endothelial cell growth medium (Cell Application, Inc., San Diego, CA, USA) on coverslips (Thermanox, USA) previously coated with an attachment factor (Cell Application, Inc.). Four days later, the medium was removed, and parasite cultures at 1% packed-cell volume and 10 PPE were added to the endothelial cells. The plates were incubated at 37 °C under MASP conditions for 90 min with constant agitation to suspend the RBC. At the end of the incubation period, non-cytoadhering cells were removed with six washes using Hanks balanced salt solution (HBSS). The cells were stained with Diff-Quick (RAL, France) and the number of iRBC adhering to 100 endothelial cells was determined for each well and the results expressed as the number of iRBC/100 endothelial cells (O'Connor et al., 1999; O'Connor and Allred, 2000). Each experiment was repeated in triplicate, and within each experiment triplicate samples were read. To avoid reader bias, the slides were coded and read blind. *Babesia bovis* cytoadhesive clone CD7 (O'Connor et al., 1999) and *Babesia bigemina* were the positive and negative controls, respectively. The HIGST (Haemaphysalis longicornis glutathione-S-transferase) clone was used as additional negative control (Oldiges et al., 2016) because it is a clonal line transfected with plasmid pMSASignal-HlGST-GFP-BSD containing an identical bidirectional promoter as the sbp2t11up transfected line, and is integrated into the ef-1α locus in an identical fashion to the sbp2t11up clonal lines. The HIGST clone expresses the *H. longicornis* glutathione-S-transferase fused to merozoite surface antigen-1 (MSA-1). These three parasite lines have been amplified in vitro since their isolation.

### 2.9. In vitro selection of BBMVEC binding parasite lines

Six-well tissue culture plates were seeded with bovine brain microvascular endothelial cells (BBMVEC, Cell Application, Inc.) at a density of approximately 1.25 x10$^5$ cells / cm$^2$ in endothelial cell growth medium (Cell Application, Inc.) on coverslips (Thermanox) previously coated with an attachment factor (Cell Application, Inc.). Four days later, the medium was removed, and parasite cultures at 2.5% packed-cell volume and 10% RBC in parasite growth medium were added to the endothelial cells. The plates were incubated at 37 °C under MASP conditions for 90 min, stirring every 15 min to resuspend the RBC. At the end of the incubation period, non-cytoadhering cells were removed by three washes with HBSS (Cell Application, Inc.) and fresh uninfected RBC (uRBC), in parasite growth medium at 10%, were added to each well. After overnight incubation, the RBC suspension was removed and the parasites were cultured under MASP conditions until they reached detectable levels (O'Connor et al., 1999).

### 2.10. Generation of anti-SBP2t11 antiserum

Polyclonal anti-serum (anti-SBP2t11) was produced in mice. A 15 amino acid (aa) synthetic peptide corresponding to aa sequence 221–235 (N′-SGKGEHNNEVTEANK-C′) that is unique among SBP2 proteins and in the predicted *B. bovis* Tx proteome was selected for immunisation. The peptide was conjugated to keyhole limpet haemocyanin (KLH) and prepared at 100 µg per dose in PBS and adjuvant (Sigma Adjuvant System) for s.c. injection. Mice were immunised at 3 to 4 week intervals. Blood was collected from the lateral saphenous vein approximately 10 days after the second, and subsequent, immunisations to test serum antibody specificity by ELISA (data not shown). A final immunisation with 50 µg of peptide-KLH in PBS was administered i.v. into the tail vein, mice were euthanised 3 days later, blood was collected via cardiac puncture, and polyclonal sera were pooled. The Institutional Animal Care and Use Committee (Washington State University protocol

#04380, USA) approved all immunisation and blood collection procedures associated with antibody production.

### 2.11. Fixed cell IFA

Two slight variations of the same protocol (Tonkin et al., 2004) were used. For iRBC, cells in suspension were fixed with 4% (w/v) paraformaldehyde, 0.08% glutaraldehyde, in PBS, with overnight blocking in 3% (w/v) BSA. For BBEC, cells at 70% confluence on a flask/slide were washed with 1× PBS three times at room temperature (RT). The cells were incubated in fixative solution (4% w/v paraformaldehyde, 0.012% v/v glutaraldehyde, 0.12% NaOH 1 N in 1× PBS) for 10 min at RT. The cells then were washed three times with 1× PBS. In both procedures, fixed cells were permeabilised by incubation with 0.01% NP-40/1× PBS for 10 min on ice followed by a single wash in 1× PBS. BBEC were incubated with blocking solution (10% goat serum) and incubated for 1 h at RT. For both cell types, blocking was followed by 1 h of incubation with the primary antibody. Cells were washed and subsequently incubated with the secondary antibody for 1 h. After washings, anti-fade which contained DAPI (Invitrogen) was applied. An anti-von Willebrand Factor (vWF) antibody (EPSISR15 – Alexa Fluor® 488) (Abcam, USA) at 1:100 dilution was used to evaluate von Willebrand factor protein expression in BBEC. iRBC images were captured by epifluorescence, using an Olympus BX50 microscope equipped with a 100× oil immersion lens, NA 1.35. Images were subjected to deconvolution, using a theoretical point spread function, as described (Pellé et al., 2015). BBEC images were captured with a Leica TCS SP8 confocal laser-scanning microscope under an HC PL APO CS2 45× oil objective and evaluated using Leica application suite X at the Franceschi Microscopy and Imaging Center in Washington State University. Live transfected *B. bovis* merozoites were evaluated for red fluorescence via microscopy. When red fluorescent protein (RFP) expression was confirmed, expression of *sbp2t11* in transfected *B. bovis* was determined by qPCR.

For the localisation of SBP2t11 protein in $Tx_{Vir}$ and clonal non-selected and cytoadhesion selected strains, the same protocol was followed using phase-contrast, DAPI for nuclear counterstain and the mouse anti-SBP2t11. The anti-SBP2t11 serum was detected with goat anti-mouse IgG (H&L chains)-Alexafluor 488. The exposure range for all the anti-SBP2t11 samples was set to the same value for non-selected and selected cells of a given type.

### 2.12. SBP2t11 protein expression by western blot

$Tx_{Vir}$ and clone 11D *B. bovis* protein lysates were prepared and used in western blot analyses. Four mL of in vitro culture with PPE higher than 20 was washed with 1× protease inhibitor cocktail (Roche Diagnostics Corporation, USA) and centrifuged at 671*g*. The samples were sonicated for 1 min and centrifuged at 11,000*g* for 10 min. The pellets were solubilised in lysis buffer, 1× Bolt® LDS Sample Buffer (Novex Life Technologies, CA, USA), plus DTT and sonicated again at 100 Watts for 1 min. The supernatants were directly prepared in 1× LDS plus DTT. Samples were heated at 70 °C for 5 min. The protein concentration was determined by the method of bicinchoninic acid (BCA) (Pierce, Thermo Fisher Scientific, MA, USA). To investigate SBP2t11 expression, western blot hybridisation was conducted on 25 μg of total protein lysate. Samples included $Tx_{Vir}$, the clones, uRBC and the synthetic SBP2t11 peptide. The samples were separated by SDS-PAGE in Bolt 4–12% Bis Tris Plus Gels (Thermo Fisher Scientific) and transferred to a 0.2 μm nitrocellulose membrane using an iBlot Gel Transfer Device (Thermo Fisher Scientific).

Pre-immunisation and polyclonal anti-SBP2t11 mouse sera were used at 1:500 dilution. Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, H + L (Invitrogen) at 1:15,000 dilution. Monoclonal antibody (mAb) to rhoptry associated protein (RAP)-1 (BABB75) at 1:100 was included as a parasite-specific control (Madruga et al., 1996). Secondary antibody-only was run in parallel (data not shown).

### 2.13. Statistical analyses

Expression values as cycle thresholds were normalised to DNA topoisomerase II (putative) (GenBank BBOV_III004820). The final data were represented as the CT ratio where the lower the ratio value, the higher the expression. *sbp2t11* up-regulation was determined to be statistically significant if $P < 0.05$ using a Paired Student's *t*-test comparing each strain with the wt control $Tx_{Vir}$ or each strain between non-selected and cytoadhesion selected strains (Graphpad Prism v.6.0a). The growth rate was considered significantly different in comparison with the control $Tx_{Vir}$ (*) if $P < 0.05$ using a 2-way ANOVA with Bonferroni post-test analysis (Graphpad Prism v.6.0a). The number of iRBC cytoadhered to 100 endothelial cells was quantified ($n = 2300$ endothelial cells, bars are S.E.M., * means a significant difference from the wt $Tx_{Vir}$ control by using a paired Student's *t*-test, alpha 0.05 by (Graphpad Prism v.6.0a)). In vitro cytoadhesion selection of *B. bovis* strains to BBMVEC after the 4th round of cytoadhesion was considered significantly different in comparison with the control $Tx_{Vir}$ (*) if $P < 0.05$ using a two-way ANOVA with Dunnett's multiple comparison test (Graphpad Prism v.6.0a).

## 3. Results

### 3.1. Generation and characterisation of stable transfected sbp2t11up line

A stably transfected parasite line derived from the *B. bovis* $Tx_{Vir}$ strain incorporating one extra copy of the *sbp2t11* gene into the ef-1α locus of *B. bovis* under the control of the ef-1α constitutive promoter was produced by stable transfection with plasmid psbp2t11-rfp-bsd-ef (Fig. 1A–C). The stably transfected parasite line was termed sbp2t11up. Consistent with the integration of the transfected rfp-bsd gene, sbp2t11up parasites expressed RFP, while parasites transfected with the positive control plasmid pEf-msa-1-Bm86ep-gfp-bsd expressed the GFP (Fig. 1D, E). In contrast, no parasites transfected with the negative control plasmid pBS and in the presence of an inhibitory dose of blasticidin, grew. PCR (Supplementary Fig. S1), sequencing and Southern blot analyses of the transfected parasites (Supplementary Fig. S2) demonstrated the successful integration of the transfected genes in the targeted ef-1α locus of *B. bovis* of the line sbp2t11up (GenBank accession numbers MH238439 and MH238440).

### 3.2. Comparative growth rates and sbp2t11 transcript levels among parasite lines

We compared the amounts of *sbp2t11* transcript among the sbp2t11up transfected line, wt $Tx_{Vir}$ and $Tx_{Att}$ non-transfected *B. bovis* strains. Comparative qPCR analysis confirmed higher *sbp2t11* transcript levels in the $Tx_{Att}$ compared with $Tx_{Vir}$ and that the sbp2t11up transfected line expressed significantly higher levels of *sbp2t11* than that $Tx_{Vir}$ and $Tx_{Att}$ (Fig. 2). We subsequently compared the growth rate of the transfected sbp2t11up line with $Tx_{Att}$ and $Tx_{Vir}$ *B. bovis* strains. The in vitro growth rates found in the transfected line and the wt $Tx_{Vir}$ are similar (Supplementary Fig. S3). There was a statistically significant difference between the wt $Tx_{Vir}$ and $Tx_{Vir}$-derived transfected line compared with the $Tx_{Att}$, which grew at a slower rate (Supplementary Fig. S3).

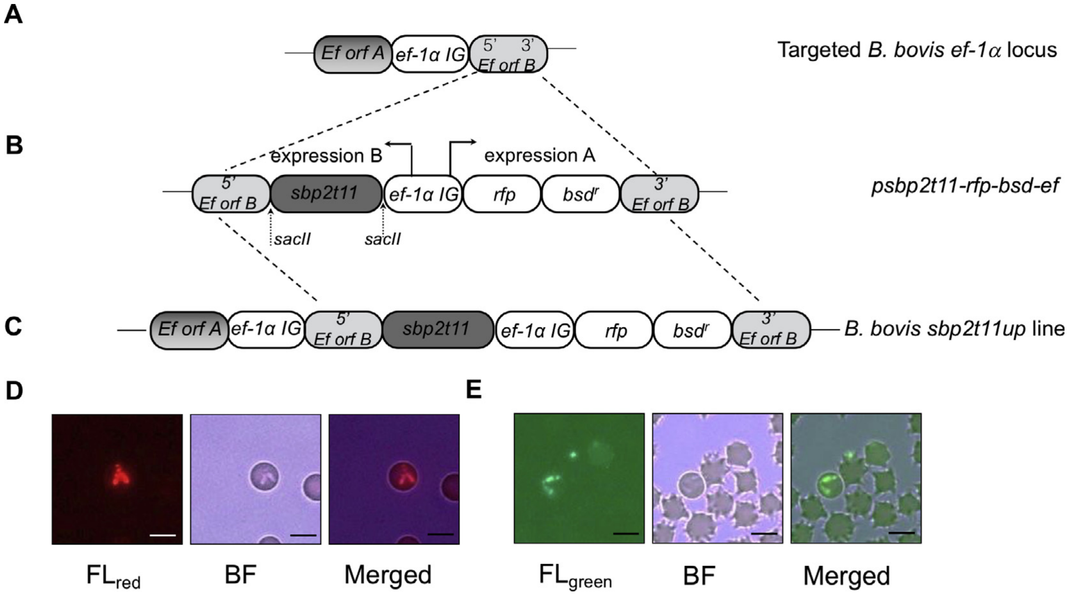

**Fig. 1.** Development of the up-regulated Spherical body protein 2 truncated copy 11 (sbp2t11up) transfected line of *Babesia bovis* (A) elongation factor-1α (ef-1α) locus in *B. bovis* genome and the lines indicate where the homologous recombination event of the transfection plasmid is designed to occur. (B) The stable transfection vector with bi-directional ef-1α promoter transcribing genes in expression sites A and B. Expression site A contains two selection markers which are genes that encode for the red fluorescent protein (RFP) fused to the blasticidin resistant (BSDR) protein. Expression site B is where the *sbp2t11* was inserted through SacII digestion. (C) The sbp2t11up transfected line. Orfs A and B represent the endogenous open reading frames of the ef-1α genes which are used for homologous recombination. Fluorescence analysis of the stably transfected $Tx_{Vir}$ lines: infected red blood cells (iRBC) examined with bright field (BF), fluorescence (FL) filter and merged images. (D) Stably transfected sbp2t11up $Tx_{Vir}$ line expressing the RFP. (E) Stably transfected Tf-Bm86ep-gfp-bsd control line $Tx_{Vir}$ expressing the GFP. Scale bar = 5 μm. IG, intergenic region.

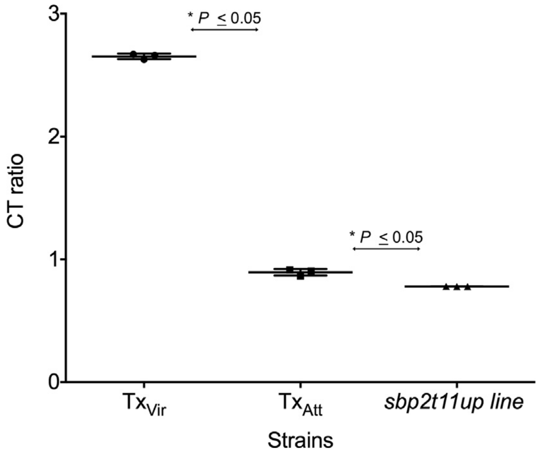

**Fig. 2.** Expression of the Spherical body protein 2 truncated copy 11 (*sbp2t11*) gene by quantitative PCR in a *Babesia bovis* transfected line and wild type (wt) controls. cDNAs were obtained from $Tx_{Vir}$ (Texas virulent strain), $Tx_{Att}$ (Texas attenuated strain) and sbp2t11up line (stably transfected sbp2t11up line). Samples were run in triplicates. The expression is considered significantly regulated (*) if $P \leq 0.05$ using a paired Student's *t*-test comparing each strain pair. Expression values as cycle thresholds were normalised to a housekeeping gene Topoisomerase II, (GenBank BBOV_III004820). The final data are represented as the cycle threshold ratio where the lower the ratio value, the higher the expression.

Therefore, the slower growth rate of the Att strain compared with the Vir strain pair is unrelated to the levels of *sbp2t11* transcripts.

### *3.3. Generation and characterisation of sbp2t11up clonal lines*

Consistent with previous data (Suarez et al., 2015), Southern blot analysis suggests the presence of at least two distinct populations of transfected parasites with a distinct pattern of transfected gene integration (Supplementary Fig. S2). Based on this observation, and using a limited dilution technique, we generated clonal lines derived from the parasite line sbp2t11up to obtain a defined transfected line containing a single integrated copy of the transfected *sbp2t11* gene. Screening of individual culture wells for parasite DNA using nested rap-1 PCR resulted in the identification of five presumptive clones (data not shown), expressing RFP, as determined by direct fluorescence. Clones 3A, 3B, 11D, 12A and 12B were screened by PCR, and integration of the sbp2t11-rfp-bsd-ef plasmid into the genome was confirmed for each one (Supplementary Fig. S4) (GenBank accession numbers MH238439 and MH238440).

We determined the patterns of integration of the transfected genes in the clonal lines, using BglII-digested DNA from the cultured parasites in Southern blots. The blots were hybridised with three DIG-labelled probes: efa, rfp-bsdr, and bsdr-EfUps (Supplementary Fig. S5). The probe efa hybridised with a single DNA fragment (~16 Kb) in the wt controls $Tx_{Vir}$ and $Tx_{Att}$, and a single DNA fragment (~23 Kb) in all the clones. The probe rfp-bsdr hybridised with one single DNA fragment of ~23 Kb in all clones but did not recognise any DNA fragment in $Tx_{Vir}$ and $Tx_{Att}$. The probe bsdr-EfUps hybridised with a single DNA fragment (~16 Kb) in the wt controls $Tx_{Vir}$ and $Tx_{Att}$; and a single DNA fragment (~23 Kb) in the psbp2t11-rfp-bsd-ef plasmid and all clones. Importantly, all clones contained a single BglII restriction fragment hybridizing with the three probes (Supplementary Fig. S5), a pattern that differs from the one obtained for the original mixed- population sbp2t11up transfected line when the same set of probes was used (Supplementary Fig. S2). Taken together, these data demonstrate that each clonal line contains a single copy of the transfected genes, integrated into the targeted ef-1α locus, and is suitable for performing further experiments aimed at defining the functional relevance of *sbpt11* expression level of on the cytoadhesive phenotype of *B. bovis*.

To verify whether expression of the *sbp2t11* gene was up-regulated in the transfected clones, we performed qPCR analysis on RNAs extracted from sbp2t11up -clones and wt $Tx_{Vir}$ and $Tx_{Att}$ *B. bovis*, as well as in the parental mixed-population sbp2t11up

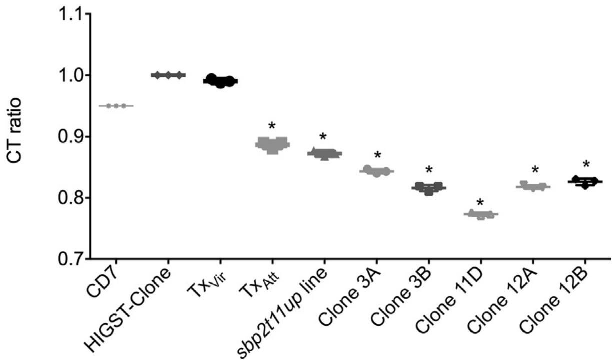

**Fig. 3.** Expression of the *sbp2t11* gene by quantitative PCR in *Babesia bovis* transfected clones and wild type (wt) controls. cDNA was obtained from $\text{Tx}_{\text{Vir}}$ (Texas virulent strain), $\text{Tx}_{\text{Att}}$ (Texas attenuated strain), CD7 (positive control for cytoadhesion), sbp2t11up line mixed population, clones 3A, 3B, 11D, 12A and 12B; HIGST (*Haemaphysalis longicornis* glutathione-S-transferase) clone was used a stably transfection control and clonal line control. The experiments were performed in triplicate. The expression was considered significantly regulated (*) if $P \leq 0.05$ using a paired *t*-test comparing each strain with the wt $\text{Tx}_{\text{Vir}}$ control. Expression values as cycle thresholds were normalised to the housekeeping gene, GeneBank BBOV_III004820, thus, the final data are represented as cycle threshold ratio where the lower the ratio value, the higher the expression.

(Fig. 3). All five clones showed *sbp2t11* up-regulation at statistically significant levels compared with both *B. bovis* wt virulent controls (Fig. 3). We selected the HIGST clone as a negative control (Oldiges et al., 2016) because it is a clonal line transfected with plasmid pMSASignal-HIGST-GFP-BSD containing an identical bidirectional promoter as the sbp2t11up transfected line, which is also integrated into the ef-1α locus in an identical fashion as the sbp2t11up clonal lines. The HIGST clone expresses HIGST fused to MSA-1 and does not display up-regulation of the *sbp2t11* gene (Fig. 3).

To analyze whether the cloning procedure of the stably sbp2t11up transfected line affected the fitness of the transfected *B. bovis* parasites, we compared the growth rates of in vitro cultures among the wt lines, clones and a control of the transfected clonal line HIGST clone (Oldiges et al., 2016). The results demonstrated that patterns of in vitro growth between the clonal transfected lines and wt $\text{Tx}_{\text{Vir}}$ strain were not significantly different. However, and similar to what was found for the parental transfected line, the in vitro growth rates of these clonal lines were significantly different from the wt $\text{Tx}_{\text{Att}}$ (Supplementary Fig. S6). Taken together, the data are consistent with the isolation of several clonal lines of transfected parasites transcribing *sbp2t11* at elevated levels (Fig. 3). All the clones contain a single insertion of the transfected genes (Supplementary Fig. S5), and they have growth rates in in vitro culture that are indistinguishable from the wt strains (Supplementary Fig. S6). The clonal lines therefore appeared suitable for assessing the role of *sbp2t11* up-regulation in *B. bovis* iRBC in an in vitro cytoadhesion assay.

### *3.4. BBEC characterisation and in vitro cytoadhesion assay*

We evaluated whether up-regulation of the *sbp2t11* transcript was associated with changes in the cytoadhesion phenotype of *B. bovis* iRBC to host endothelial cells. We initially evaluated the expression of the vWF in the BBEC, a specific marker of endothelial cell (Wick et al., 1987). vWF is expressed intra-cytoplasmically and exported to the extracellular space (Fig. 4A). We then evaluated the in vitro cytoadhesion of iRBC from different *B. bovis* strains using BBEC (O'Connor et al., 1999; O'Connor and Allred, 2000), (Fig. 4B, C). The *B. bovis* cytoadhesive clone CD7 was used as a positive cytoadhesion control (O'Connor et al., 1999). uRBC and *B. bigemina* iRBC were negative controls (Fig. 4C). In addition, *B. bigemina* was used as a control in these experiments because it lacks a cytoadhesive phenotype, cannot be selected for cytoadhesion (O'Connor et al., 1999) and does not have a *sbp2t11*-like gene in its genome. iRBC with wt $\text{Tx}_{\text{Vir}}$ showed higher cytoadhesion to BBEC than the wt $\text{Tx}_{\text{Att}}$ (Fig. 4B, C) which correlates with the reduced in vivo cytoadhesion observed previously in this attenuated strain (Sondgeroth et al., 2013).

All the clonal lines obtained from the transfected sbp2t11up line over-expressing *sbp2t11* showed significant reduction of cytoadhesion to BBEC in comparison with the wt $\text{Tx}_{\text{Vir}}$ and $\text{Tx}_{\text{Att}}$ strains (Fig. 4B, C), despite being derived from a $\text{Tx}_{\text{Vir}}$ parental strain. However, no significant difference in cytoadhesion was detected among the distinct clonal lines (Fig. 4C). This finding was validated by the results obtained, with the levels of cytoadhesion found for the stably transfected clonal line HIGST used as a control in identical assays. The HIGST clone was produced using a $\text{Tx}_{\text{Vir}}$ parental strain under transfection, culture, and cloning conditions similar to the sbp2t11up clones, but its *sbpt11* genotype is identical to the $\text{Tx}_{\text{Vir}}$ parental strain and, in contrast to sbp2t11up, lacks an extra *sbp2t11* gene copy (Oldiges et al., 2016). The HIGST clone did not show any significant difference in cytoadhesion compared with the wt $\text{Tx}_{\text{Vir}}$ (Fig. 4C), suggesting that the in vitro culture, transfection technique, or the cloning procedure did not influence cytoadhesion. However, all sbp2t11up clones showed significantly lower cytoadhesion to BBEC in comparison with the control HIGST clone (Fig. 4C), demonstrating that over-expression of *sbp2t11*, as the only distinctive feature between the compared parasite lines, resulted in a significant reduction of cytoadhesion of iRBC to the epithelial cells, without affecting their rate of growth in in vitro cultures.

### *3.5. BBMVEC in vitro cytoadhesion selection*

To confirm low cytoadhesion levels of the clones over-expressing *sbp2t11*, all the strains evaluated in the studies were submitted to four rounds of cytoadhesion selection as reported previously (O'Connor et al., 1999). The cytoadhesion of the CD7 line increased progressively from a mean of 4.82 iRBC/100 endothelial cells before cytoadhesion selection to a mean of 571.69 iRBC/100 endothelial cells after the fourth round of selection (Figs. 4C, 5B). The wt $\text{Tx}_{\text{Vir}}$ strain increased considerably from a mean of 2.91 iRBC/100 endothelial cells before cytoadhesion selection to a mean of 547.69 iRBC/100 endothelial cells after the fourth round of selection (Figs. 4C, 5B). Cytoadhesion of all clones before selection was almost null with a mean between 0.08 and 0.34 iRBC/100 endothelial cells. Cytoadhesion of clones 3A and 3B was increased after four rounds of selection with means of 346.15 and 189.15 iRBC/100 endothelial cells, respectively, whereas cytoadhesion levels of clones 11D, 12A and 12B were not increased significantly, even after four rounds of selection (Figs. 4C, 5B). The $\text{Tx}_{\text{Att}}$ and HIGST strains were not recovered after cytoadhesion selection despite multiple attempts.

### *3.6. Up-regulation of sbp2t11 transcript is associated with a lower cytoadhesion phenotype*

We performed qPCR to evaluate the *sbp2t11* transcript level after the fourth round of cytoadhesion selection. Fig. 6 shows that cytoadhesion was increased after four rounds of selection in the wt $\text{Tx}_{\text{Vir}}$ strain and the control CD7, and the *sbp2t11* transcript was significantly down-regulated even more after selection. Interestingly clone 3A and clone 3B, which increased cytoadhesion after selection, also significantly changed the transcript level of *sbp2t11* from up-regulated to down-regulated. Clone 11D, in which cytoadhesion ability did not change after selection, also did not change its *sbp2t11* transcript levels. In addition, the levels of *sbp2t11*

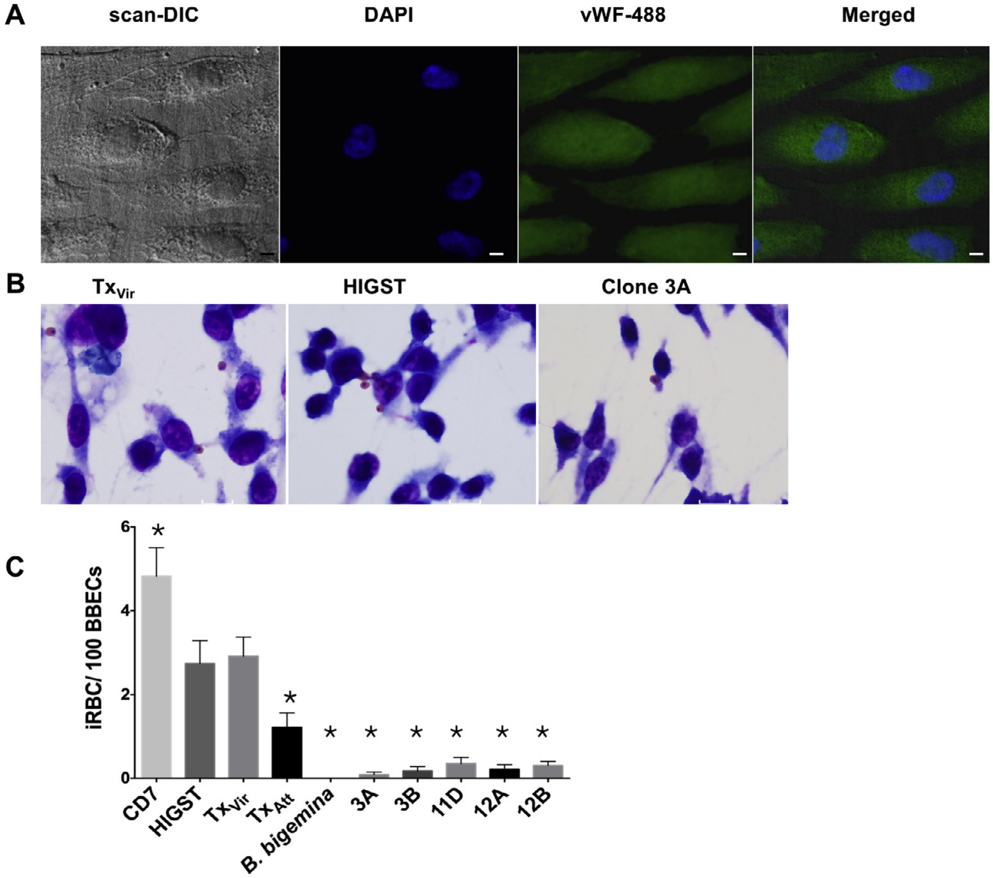

**Fig. 4.** Bovine brain endothelial cells microscopy evaluation and in vitro cytoadhesion assay of *Babesia bovis* strains to bovine brain endothelial cells. (A) Bovine brain endothelial cells fixed-cell indirect IFA to confirm identity and adherence phenotype. Bovine brain endothelial cells were visualised with scan differential interference contrast microscopy, labelled with DAPI for nucleus staining and with anti-Von Willebrand Factor conjugated to 488 as a marker for endothelial cells. anti-Von Willebrand Factor is expressed in the cytoplasm of the bovine brain endothelial cells and exported to the extracellular space. Images were obtained with a Leica TCS SP8 confocal laser-scanning microscope under an HC PL APO CS2 45× oil objective and evaluated using Leica application suite X. Lower right side scale bar = 5 μm. (B) Bovine brain endothelial cells were grown to 70% confluence and incubated for 90 min with different strains of *B. bovis*. Each experiment was performed in triplicate and each replicate was read three times. The slides were stained and read 100× oil objective. Clone 3A *(B. bovis* stably transfected sbp2t11up line- clone 3A); HIGST (*B. bovis* stably transfected *Haemaphysalis longicornis* glutathione-S-transferase clone); Tx$_{\text{Vir}}$ (wild type Texas virulent strain). (C) Quantification of an in vitro cytoadhesion assay. The number of infected red blood cells (iRBC) to 100 endothelial cells was quantified ($n = 2300$ endothelial cells). Bars indicate S.E.M. (*) if $P \leq 0.05$ by paired *t*-test between the wt Tx$_{\text{Vir}}$ control and each strain (Graphpad Prism v.6.0a); CD7 (*B. bovis* clone – positive control for cytoadhesion); *Babesia bigemina* (negative control for cytoadhesion); clones 3A, 3B, 11D, 12A, 12B (*B. bovis* stably transfected sbp2t11up- clones); HIGST (stably transfection control and clonal line control); Tx$_{\text{Att}}$ (wt Texas attenuated strain).

transcript became even more up-regulated in clones 12A and 12B after selection. Although cytoadhesion remained low in clone 12A and 12B up-regulated lines upon selection, they had similar levels of cytoadhesion compared with the non-selected lines. Taken together, the data confirm an association between *sbp2t11* up-regulation and lower cytoadhesion.

### *3.7. SBP2t11 expression was confirmed after the cytoadhesion selection*

SBP2t11 expression was confirmed by western blot in both wt Tx$_{\text{Vir}}$ and clone 11D lines after cytoadhesion selection. As reported previously, SBP2t11 was expressed as a 30 kDa full-length protein that is cleaved to a 17 kDa protein by the recognition of a PLM (Pellé et al., 2015; Gallego-Lopez et al., 2018). Protein lysates were separated in pellet and supernatant after high-speed centrifugation in order to better understand the subcellular localisation of the full-length and cleaved SBP2t11 protein. The 30 kDa full-length SBP2t11 was observed as a membrane bound protein in all strains evaluated (Fig. 7B) and was only detected in the pellets of parasite lysates. In contrast, the 17 kDa cleaved SBP2t11 protein was observed as a soluble protein in all the evaluated strains and was detected only in the supernatants (Fig. 7C).

### *3.8. SBP2t11 subcellular localisation was confirmed after the cytoadhesion selection*

We performed immunofluorescence analysis on parental and selected parasites using the anti-SBP2t11 polyclonal antibody, to test whether the correlation of decreased SBP2t11 protein expression and increased cytoadhesion phenotype holds, and whether there is any alteration in subcellular localisation based upon expression levels. The low cytoadhesive phenotype parasites (11D-4) displayed increased fluorescent signals (Fig. 8E, F) compared with the highly adhesive and selected parasite lines (Tx$_{\text{Vir}}$-4) (Fig. 8C, D). Interestingly, clonal line 3A, which became increasingly adhesive upon selection, also showed decreased levels of fluorescent signal compared with the low adhesive selected line 11D-4 and had a pattern of reactivity which reassembles that displayed by the Tx$_{\text{Vir}}$-4 line (Fig. 8B). There were no observed changes in SBP2t11 localisation based on expression levels (Fig. 8). Interestingly, the IFA results also suggest that SBP2t11, or SBP2t11-derived products of cleavage (likely the 17 kDa cleavage product containing the C-terminal of SBP2t11) are secreted into the cytoplasm of the RBC in a pattern resembling that of other previously characterised SBP proteins (Hines et al., 1995; Dowling et al., 1996; Terkawi et al., 2011; Pellé et al., 2015).

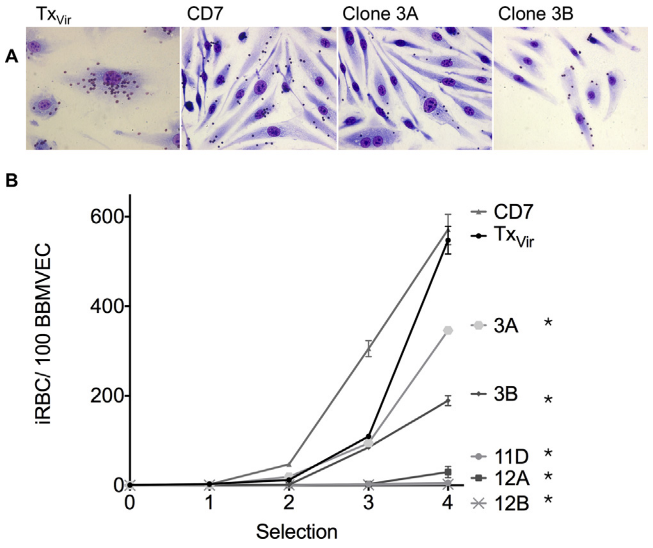

**Fig. 5.** In vitro cytoadhesion selection of *Babesia bovis* strains to bovine brain microvascular endothelial cells. (A) Bovine brain microvascular endothelial cells were grown to 70% confluence and incubated for 90 min with different strains of *B. bovis* for four rounds of cytoadhesion selection. An In vitro static cytoadhesion assay was performed in triplicate for each strain after each round of cytoadhesion selection. The slides were stained and read with a 100× oil immersion objective. Representative images after the fourth cytoadhesion selection of CD7 (positive control), $Tx_{Vir}$ (wild type (wt) Texas virulent strain), and clones 3A and 3B. (B) Quantification of an in vitro cytoadhesion assay during four rounds of cytoadhesion selection. The number of infected red blood cells (iRBC) cytoadhered to 100 endothelial cells was quantified ($n = 1300$ endothelial cells). Bars indicate S.E.M. After the fourth round of cytoadhesion, $P \leq 0.05$ was considered significantly different in comparison with the control $Tx_{Vir}$ (*) using a two-way ANOVA with Dunnetts multiple comparison test (Graphpad Prism v.6.0a). Clones 3A, 3B, 11D, 12A, 12B, *B. bovis* stably transfected sbp2t11up- clones.

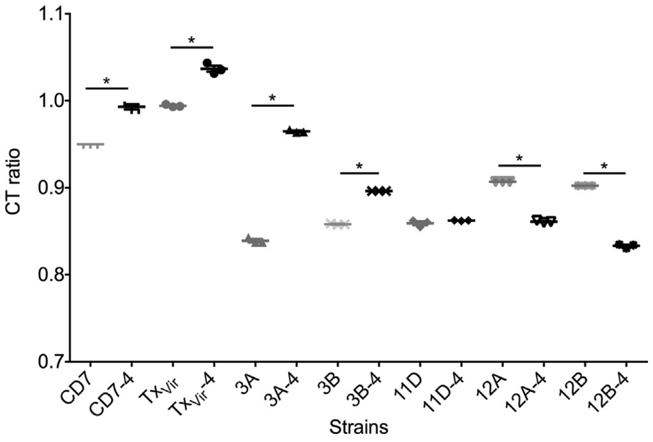

**Fig. 6.** Expression of the *sbp2t11* gene by quantitative PCR in *Babesia bovis* transfected clones and wild type) controls after the fourth round (−4) of cytoadhesion selection. cDNA obtained from Texas virulent strain ($Tx_{Vir}$), and clones 3A, 3B, 11D, 12A and 12B. CD7 was used as a control for cytoadhesion. The experiments were performed in triplicate. *sbp2t11* expression is considered significantly regulated (*) if $P \leq 0.05$ using a Paired Student's *t*-test. Comparisons were made between non-selected and cytoadhesion selected strains. Expression values as cycle thresholds were normalised to the housekeeping gene, GeneBank BBOV_III004820. The final data are represented as cycle threshold ratios where the lower the ratio value, the higher the expression.

## 4. Discussion

Despite important differences in genome size, composition, and life cycles, the apicomplexan haemoprotozoan parasites, *B. bovis* and *Plasmodium falciparum*, share phenotypic characteristics including morphological alterations on the membrane of iRBC and cytoadhesion mechanisms. While *Plasmodium* parasites typically modify the surface of the iRBC by forming knobs, *B. bovis* causes the formation of protrusions in the form of ridges on the surface of iRBC (Aikawa et al., 1985; Aikawa et al., 1997; Hutchings et al., 2007; Scudiero et al., 2018). It has been suggested that such ridges might perform a similar function as the knobs on iRBC with *P. falciparum*, mediating adherence of iRBC to the host endothelial cells (Wright, 1973; Hutchings et al., 2007). In support of this idea, virulent or low passage forms of *B. bovis* produce more ridges in vitro on the RBC membrane than the attenuated high passage strains (Aikawa et al., 1985; Hutchings et al., 2007).

Cytoadhesion in *P. falciparum* is known to be mediated by the highly variable *P. falciparum* erythrocyte membrane protein (PfEMP1). PfEMP1, encoded by the *var* multigene family, undergoes antigenic variation and is responsible for adhesion to different tissues. Therefore, the pattern of expression of PfEMP1 can influence severity of the disease. The extracellular portions of PfEMP1 can interact with different host receptors such as ICAM1, CD36, P-selectin, chondroitin sulfate A (CSA). Additionally, the intracellular C-terminal portion of PfEMP1 interacts with the Knob-associated histidine-rich protein (KAHRP) which has been implicated in the formation of knobs (Kumar et al., 2015). Importantly, disruption of genes involved in PfEMP1 trafficking causes a dramatic reduction in cytoadherence and formation of knobs (Rug et al., 2006; Kumar et al., 2015). It was recently reported that down-regulation of the expression of chaperone proteins involved in transportation of PfEMP1 may affect *var* gene regulation in severe malaria (Tonkin-Hill et al., 2018). Similarly, *sbp2t11* expression is down-regulated in *B. bovis* virulent strains compared with the attenuated strains. Finally, a correlation between the level of expression of *sbp2t11* and cytoadherence was also observed. These observations suggest that *sbp2t11* may also have a similar regulatory role in the expression of *B. bovis* molecules involved in cytoadhesion.

Sequestration due to cytoadhesion in bovine babesiosis occurs as iRBC adhere to vascular endothelial cells in vivo, resulting in concentration of iRBC in the host microvasculature (Wright, 1972,1973). This effect is mimicked by the in vitro cytoadhesion assay employed in this study (O'Connor et al., 1999). *Babesia bovis* sequestration has been proposed to result in avoidance of parasite splenic clearance while it facilitates the establishment of persistent infection (Allred, 2003). The observation that significant differences in the numbers of sequestered parasites in cerebral capillaries were detected between animals infected with $Tx_{Vir}$ and $Tx_{Att}$ strains (Sondgeroth et al., 2013), suggests that cytoadhesion is a mechanism that might be closely correlated with virulence.

Based on these observations, and the availability of genome and transcriptome comparisons (Pedroni et al., 2013), the molecular differences between virulent and attenuated strain pairs of *B. bovis* were investigated. First, we confirmed the up-regulation of *sbp2t* genes in attenuated *B. bovis* strains from different geographical regions (Gallego-Lopez et al., 2018). The observation that *sbp2t7* and *sbp2t9* up-regulation in attenuated strains was strain-specific, while *sbp2t11* was consistently up-regulated in all the evaluated attenuated strains, suggested that *sbp2t11* is an attenuation marker for *B. bovis*. In addition, the transcriptional profile evaluation of *sbp2t11* demonstrated that this gene is down-regulated in the kinete stage but is consistently expressed in blood stages (Gallego-Lopez et al., 2018). Moreover, SBP2t11 is cleaved from a 30 kDa full-length protein to a mature 17 kDa polypeptide through the recognition of a PLM, consistent with developmentally-staged release, and possible residence within the spherical body compartment (Pellé et al., 2015). However, in this study SBP2t11 appears to localise primarily in a perinuclear manner, suggesting residence primarily within the endoplasmic

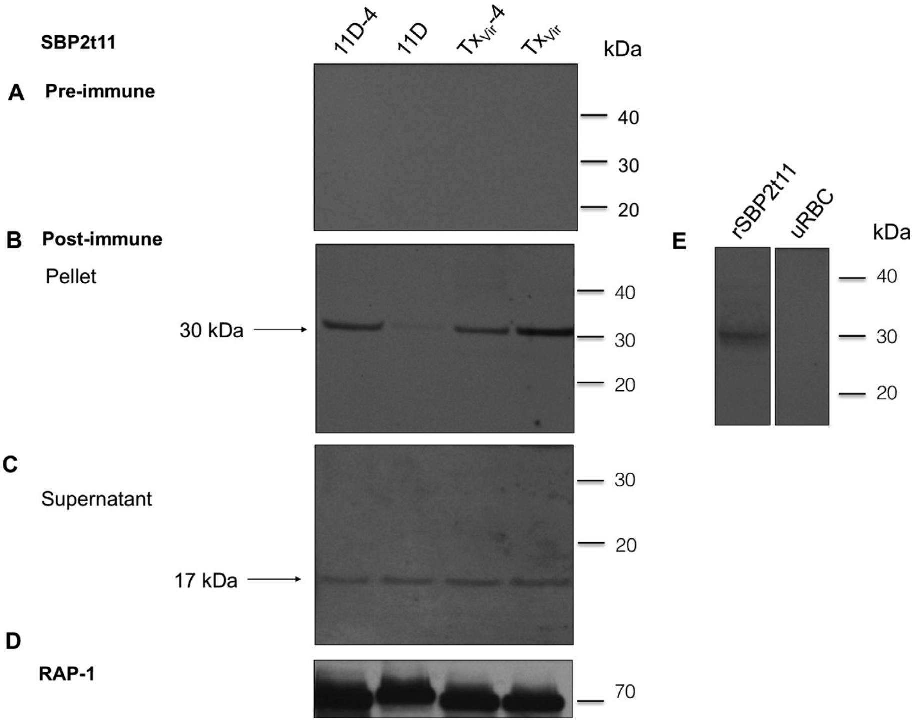

**Fig. 7.** Detection of SBP2t11 protein in non-selected and cytoadhesion selected strains of *Babesia bovis*. (A) Pre-immunisation sera from mice demonstrate a lack of non-specific binding due to mouse sera alone. (B) Post-immunisation sera from mice demonstrate that SBP2t11 full-length protein was present in the pellet of the antigen as a 30 kDa membrane-bound protein. (C) Post-immunisation sera from mice demonstrate that the 17 kDa cleaved SBP2t11 was present in the supernatant of antigen as soluble antigen protein. (D) Detection of rhoptry associated protein-1 (RAP-1) protein demonstrates equal protein loading across lanes. Antigens were loaded as follows: 11D-4, Clone 11D after cytoadhesion selection; 11D, Clone 11D; Tx$_{Vir}$-4, wild type (wt) Texas virulent strain after cytoadhesion selection; Tx$_{Vir}$, wt Tx$_{Vir}$ (E) Hybridisation of post-immunisation sera with rSBP2t11 recombinant protein as a positive control and uninfected red blood cells (uRBC) as negative controls.

reticulum (ER). It is possible, however, that SBP2t11 localised in the perinuclear region is in transit to the spherical bodies. This seeming discrepancy may result from the difficulties in identifying the developmental stages of this parasite, which has resisted efforts to synchronise its development (Franssen et al., 2003). In both studies, SBP2t11 was observed in both perinuclear and vesicular compartments at differing levels, and the precise timing of movement from ER to spherical bodies is uncertain and may even depend upon slight differences in growth conditions. In addition, the IFA data reported in our study suggest that SBPt11 is secreted into the cytoplasm of the iRBC in a pattern that is similar to the previously characterised SBP1, SBP2, and SBP4 proteins (Hines et al., 1995; Dowling et al., 1996; Terkawi et al., 2011; Pellé et al., 2015). Data reported in previous and current studies shows proteolytic cleavage of SBPt11 before secretion, resulting in a 17 kDa protein, and localisation of this cleaved fragment mainly in the soluble fraction. Taken together, the data suggest that the protein localised in the iRBC cytoplasm likely corresponds with the 17 kDa cleavage product, although this requires confirmation.

In this study, we developed stably transfected clonal lines overexpressing the *sbp2t11* gene on the virulent Texas *B. bovis* strain genetic background. The in vitro characterisation of the stably transfected clonal lines confirmed that stable insertion of sequences derived from the psbp2t11-rfp-bsd-ef plasmid into the *B. bovis* genome resulted in the artificial up-regulation of *sbp2t11* without altering the ability of the parasite to invade RBC, suggesting that the levels of expression of *sbp2t11* are irrelevant to invasion phenotype.

These data indicate that the cell line sbp2t11up is an appropriate experimental tool to study the relationships between expression levels of the *sbp2t11* gene and attenuation. Herein, we found variations in the level of expression of *sbp2t11* between clones with identical patterns of integration of the transfected *sbp2t11* gene. These variations could be explained by many factors, including several epigenetic factors such as chromatin folding, promotor activation and epigenetic markers, which could be influencing the overall *sbp2t11* expression levels differentially in each clonal line (Pilbrough et al., 2009).

We used an in vitro sequestration model to test whether there is an association between *B. bovis sbp2t11* up-regulation and the reduction of in vitro cytoadhesion to host endothelial cells (O'Connor et al., 1999). We conclude from these results that *sbp2t11* up-regulation is inversely correlated with the adhesion phenotype. Thus, selection for cytoadhesion by CD7, Tx$_{Vir}$ and clones 3A and 3B resulted in enhanced levels of cytoadhesion when *sbp2t11* was down-regulated. In contrast, clones 11D, 12A and 12B, which showed low cytoadhesion even after four rounds of selection, maintained elevated *sbp2t11* transcript levels, or even up-regulated them further. Taken together, these data are in agreement with the notion that levels of *sbp2t11* gene expression are inversely correlated with cytoadhesion of the parasite. Interestingly, not all the clonal lines responded equally to the selection procedure despite having an identical pattern of insertion of the transfected genes. This observation suggests that the cytoadhesion phenotype may result from multiple interactive factors, including the distinct patterns and levels of expression of genes directly or indirectly involved with the binding of the parasites to undefined epithelial receptors. It is possible that the differences in cytoadhesion among the clonal lines are due to differential expression of distinct sets of these genes. It is also possible that, since variation in the VESA1 (Variant Erythrocyte Surface Antigen 1) cytoadhesion ligand is a stochastic process (Al-Khedery and Allred, 2006), the individual lines may have failed to generate appropriately functional ligands during the selection process. If that is the situation,

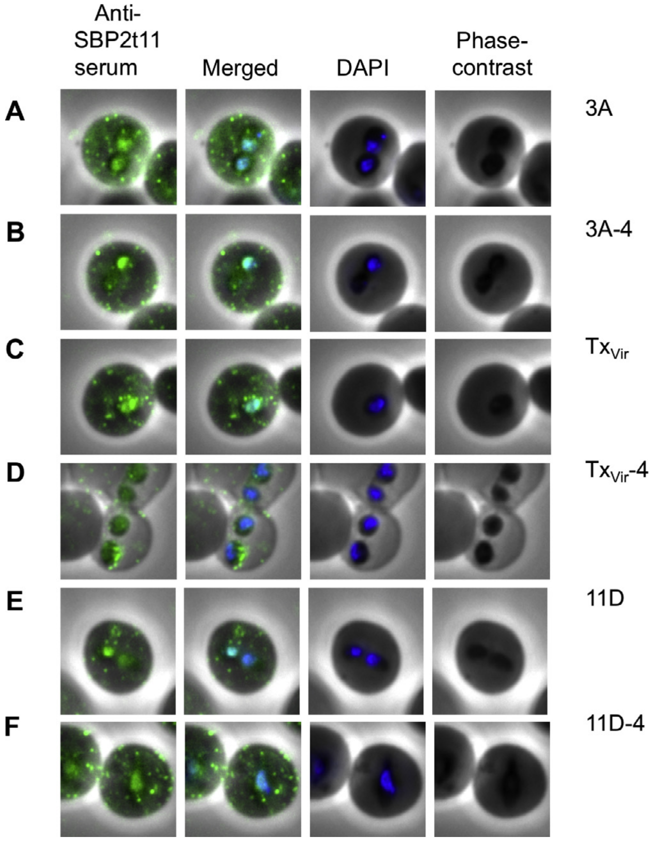

**Fig. 8.** Localisation of SBP2t11 protein in non-selected and cytoadhesion-selected strains of *Babesia bovis*. *Babesia bovis* strains were analysed by immunofluorescence using anti-SBP2t11 antiserum (green). The parasites are shown by phase-contrast only, stained with DAPI as a nuclear counter-stain (blue), or as a merge of the two labels to facilitate localisation of SBP2t11 relative to the nucleus. (A) Clone 3A. (B) Clone 3A-4, clone 3A after cytoadhesion selection. (C) Wild type Texas virulent strain ($Tx_{Vir}$). (D) $Tx_{Vir}$-4, wt $Tx_{Vir}$ after cytoadhesion selection. (E) Clone 11D. (F) Clone 11D-4, clone 11D after cytoadhesion selection. Note that in this figure the intensity of the SBP2t11 signal is deliberately enhanced to facilitate visualisation of signal location; it does not reflect accurately the relative expression levels of SBP2t11 before and after cytoadhesion selection.

then it may imply communication of binding status in the regulation of *spb2t11* transcription.

Our results are strongly suggestive that up-regulation of *sbp2t11* in the clonal lines is associated with the reduction of in vitro cytoadhesion of iRBC to BBEC, and perhaps correlates with previous observations that attenuation in *B. bovis* is directly associated with a reduction in host cell cytoadhesion in vivo (Sondgeroth et al., 2013). However, another study suggests that in vitro cytoadhesion does not directly translate to in vivo virulence (Canto et al., 2006). For this reason, it is necessary to compare the virulence phenotype of the clonal lines upon inoculation of the clones 11D, 12A and 12B into cattle, in order to determine if the up-regulation of *sbp2t11* is associated with attenuated phenotype and low cytoadhesion in vivo.

In summary, our study concludes that up-regulation of *sbp2t11* is associated with reduced cytoadhesion by attenuated *B. bovis* which corroborates our previous finding (Gallego-Lopez et al., 2018). It is possible to hypothesise that SBP2t11 is able to interact with VESA proteins or/and with proteins in the RBC cytoskeleton, including *B. bovis* secreted proteins which might be involved in the formation of the ridge structures and/or cytoadhesion. These possible protein interactions might be similar to mechanisms previously described in *Plasmodium* iRBC (Weng et al., 2014; Helms et al., 2016; Watermeyer et al., 2016). Ongoing in vivo experiments using these clonal *B. bovis* should help determine if up-regulated *sbp2t11* is directly involved in the sequestration of iRBC.

Up-regulation of *sbp2t11* transcript is a marker for attenuated *B. bovis* strains. Clones of a stably transfected line over-expressing *sbp2t11* showed significantly lower levels of cytoadhesion to bovine endothelial cells than wt controls, suggesting that up-regulation of *sbp2t11* is associated with attenuation.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.05.015.

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