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Transgenic *Babesia bovis* lacking 6-Cys sexual-stage genes as the foundation for non-transmissible live vaccines against bovine babesiosis

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ABSTRACT

Babesia bovis, a tick-borne apicomplexan parasite responsible for bovine babesiosis has a complex life cycle including sexual development in its *Rhipicephalus microplus* vector. Understanding the molecular mechanisms involved in sexual development is essential for developing future-generation transmission blocking vaccines (TBVs) and/or non-transmissible attenuated live vaccines. The widely conserved members of the *6-Cys* gene family likely play roles in the development of sexual stages of *B. bovis*, and are candidates for developing novel TBV. The recently defined sexual markers *6-CysA* and *6-CysB* of *B. bovis* are strain-conserved and exclusively surface-expressed in tick-stage parasites. However, the high level of sequence identity among the 6-Cys A and 6-Cys B proteins (52% identity), together with similar 6-Cys domain distribution and sub-cellular localization, are suggestive of redundant function. We hypothesized that disruption of both *6-CysA* and *6-CysB* in *B. bovis* would result in unaltered ability of the parasite to invade and grow in red blood cells (RBCs), with concomitant loss of the transmission phenotype. Taking advantage of their contiguous genome localization, we generated a double gene-knockout system to disrupt a 3287 bp region encompassing both *6-CysA* and *6-CysB* genes using a single transfection plasmid. The resulting red-fluorescent *ΔAΔB 6-Cys B. bovis* transgenic parasite line was able to grow continuously in bovine RBCs *in vitro* at a similar rate to wild-type parasites, demonstrating that the *6-CysA* and *6-CysB* genes are not required for the development of blood-stage parasites. This novel gene manipulation approach will allow future experiments aimed at determining the tick-transmission phenotype of parasites lacking tick-stage genes. Parasites deficient in genes required for sexual reproduction could be the foundation for genetically-defined, non-transmissible live vaccines against bovine babesiosis. Developing a non-tick transmissible live vaccine based on attenuated parasites unable to express critical *6-Cys* genes and including a molecular vaccine marker could help reduce the burden of bovine babesiosis globally.

1. Introduction

The tick-borne apicomplexan parasite, *Babesia bovis*, is responsible for acute and persistent disease in cattle in tropical and semi-tropical regions worldwide (Bock et al., 2004; Florin-Christensen et al., 2014). The acute form of the disease is particularly insidious, causing high mortality especially when naïve cattle are infected by the parasite. Acutely infected animals can be treated with babesicidal drugs (Mosqueda et al., 2012), but this is unpractical in many endemic areas. In addition, acute disease can be prevented using live-attenuated vaccines, but these also have several drawbacks (Florin-Christensen et al., 2014; de Waal and Combrink, 2006). Two important limitations of current live vaccines include tick transmissibility and the absence of a molecular marker which might allow differentiation between vaccine and field strains. A new generation of safer, more effective and practical vaccines are therefore urgently needed.

So far, despite many years of research, sub-unit vaccines that can prevent acute babesiosis remain unavailable, due in part to the complexity of the parasite host-relationship and an incomplete understanding of protective immune mechanisms that occur following *B. bovis* infection (Jalovecka et al., 2018). While such vaccines might be available in the future, the development of improved live vaccines remains a reasonable and realistic goal. A serious problem with currently available live-attenuated *Babesia* vaccines is the potential of the parasite to revert to a virulent phenotype (Timms et al., 1990). This problem can also be potentiated by the ability of vaccine strains to be

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transmitted by ticks, where they may undergo recombination events during their sexual reproduction in the midgut of the tick vector (Berens et al., 2007). Such recombination events may involve vaccine and co-infective virulent field strains, leading to genetic changes in the vaccine parasites that may result in increased virulence. Thus, the use of such live vaccines may cause increasing population complexity and other undesirable effects that may render these vaccines ineffective in the long term. We therefore propose developing alternative live vaccines based on attenuated *B. bovis* parasites that cannot be transmitted by ticks. Achieving such a goal requires identification and deletion of genes required for development of sexual stages of the parasite and successful transmission by their tick vector. Developing new vaccines comprising non-tick-transmissible parasites is thus a critical milestone in our quest for developing improved live babesiosis vaccines.

The life cycle of *B. bovis* includes sexual reproduction in the midgut of the tick vector (its definitive host), but this remains incompletely understood, particularly at the molecular level. It appears that certain differential environmental conditions occurring in the tick midgut favor the development of sexual forms of the parasites that are present in the blood-stage parasites that are ingested by the tick during blood feeding. The morphological changes observed in sexual forms can also be correlated with molecular changes (Alzan et al., 2016; Hussein et al., 2017) including the differential expression of a subset of genes required for sexual development and reproduction. Male and female sexual forms of the parasite (gametes) then fuse to form zygotes that invade the epithelial cells of the tick midgut. Zygotes gain access to the hemolymph as kinetes that invade the ovary and the eggs of the next generation of ticks (referred to as transovarial transmission) and is a hallmark feature of *Babesia* spp. parasites (Jalovecka et al., 2018). Development of sexual stage parasites likely includes the coordinated expression of members of the *B. bovis 6-Cys* gene family (Silva et al., 2011; Alzan et al., 2016). Additionally, the expression of the *6-Cys A* and *6-Cys B* genes on the surface of sexual-stage parasites (Alzan et al., 2016) correlates with expression of the *hap2* gene, which may be involved in regulation of the development of sexual-stage parasites (Hussein et al., 2017). Because the highly related *6-Cys A* and *6-Cys B* genes are expressed in the midgut tick stages, but not in blood stages, they have been defined as markers of sexual-stage parasites in *B. bovis* (Alzan et al., 2016).

An essential requirement for the implementation of a vaccination strategy based on non-transmissible attenuated *B. bovis* parasites is that the genes targeted for deletion are not essential for the development of blood-stage parasites, since elimination of such essential genes would impede the isolation of the mutated organisms. In this study, we describe the simultaneous double-deletion of a region of the genome containing both copies of the *6-Cys* genes (*6-Cys A* and *6-Cys B*) of *B. bovis*. These two genes were selected because, in addition to their identification as sexual-stage markers, they are expressed on the surface of sexual-stage parasites (Alzan et al., 2016). Based on research performed in related parasites (van Dijk et al., 2001, 2010 and Tonkin et al., 2013), the 6-Cys proteins may be involved in sexual stage fusion and zygote formation (Alzan et al., 2016; Hussein et al., 2017), but other functions, such as recognition, attachment and invasion of tick cells needed for parasite proliferation, motility, differentiation are also possible. Given the contiguous genomic location of these two genes with almost identical length, two 6-Cys domains, high sequence identity, and pattern of expression in blood and tick stages (Alzan et al., 2016), there is a risk that mutation of a single *6-Cys* gene might still result in a transmissible parasite, due to functional redundancy. Ideally, it would be best to test transmissibility using parasites lacking both genes. Furthermore, since the timing of expression of these two genes is similar, and both might be required in at least two key tick stages (sexual stages and kinetes), it is likely that *6-Cys A* and *6-Cys B* mutations may result in total abrogation of transmission (Alzan et al., 2016). At the same time, *6-Cys A* and *6-Cys B* deleted parasites should still be able to develop in red blood cells (RBCs) allowing them to be propagated as a vaccine strain. In addition, such transfected parasites contain a unique exogenous gene, stably incorporated in their genome, which could be used for simplified parasite identification; a feature that would be useful as a molecular marker to discriminate vaccine from field strains. In this study, we describe the feasibility of the production of double *6-Cys A* and *6-Cys B* deleted *B. bovis* parasites which maintain the ability to grow in RBCs *in vitro*. These mutated parasites will be ideal tools for testing whether mutation of both *6-Cys A* and *6-Cys B* results in the production of non-transmissible *B. bovis* parasites that could be used as future, next-generation vaccines.

2. Materials and methods

2.1. Parasites

The *B. bovis* Texas T3Bo strain was propagated in microaerophilic stationary phase (MASP) culture as previously described (Alzan et al., 2016).

2.2. Double gene knockout [DKO] plasmid construct for B. bovis 6-CysA and 6-CysB

Plasmid *p6Cys A-B-dko* was designed for simultaneous knock-out of the contiguous *B. bovis* genes *6-Cys A* and 6-Cys *B* located on chromosome 2 (Figs. 1a and 2 a,b). Plasmid *p6Cys A-B-dko* was constructed to express a red fluorescent protein-blasticidin [rfp-bsd] fusion protein under the control of the *ef-1α* promoter, and is schematized in Fig. 2c. The *B. bovis ef-1α-B* promoter region (Suarez et al., 2006), followed by the *rfp-bsd* gene and the terminator 3′ region from the *B. bovis msa-1* gene (3′- *MSA*) were synthetized (GenScript, Piscataway, USA), and cloned at the unique *Hind*III cloning site in the *puc57* plasmid vector. M13 forward (5′gta aaa cga cgg cca g-3′) and M13 reverse (5′cag gaa aca gct atg ac-3′) primers present in the 5′ and 3′ multiple cloning site of the puc57 plasmid vector were used to amplify the *ef-1α* promoter, *rfp-bsd* fused gene and 3′- *MSA* sequences by PCR. This PCR fragment was purified and digested with *Hind*III for cloning into the *Hind*III site of plasmid *pBluescript* used as a backbone for the construction of the transfection plasmid *p6Cys A-B-dko.*

A 985 bp of the 5′ flanking region (designated “A Flanking”, Fig. 2c) and a 894 bp 3′ flanking region (designated “B-Flanking”, Fig. 2c) of the 6-Cys gene locus were amplified from *B. bovis* genomic DNA using the forward primer 5′ *6-CysA-F*: [5′- **gaa ttc** tcc ata cgt caa cga gta acc ggg-3′] and reverse primer-5′ *6-CysA- R* [5′- **gaa ttc** tat tga gtt ctg cgc att ta -3′], and forward primer 3′*-6-CysB-F* [5′- **ctc gag** ccg aga att tga atc ata tag ttg c -3′] and reverse primers 3′*-6-Cys B-R* [5′- **ctc gag** cca tat gcc ata ggg tat aca gc -3′] respectively. The primers contain either *Eco*RI (5′ Flanking region) or *Xho*I (3′ Flanking region) sites (in bold) to facilitate cloning into the transfection plasmid. The location of the primers, the regions amplified, and the organization of the transfection plasmid is represented in Fig. 2. The so constructed plasmid, designated as *p6Cys A-B-dk,* was fully sequenced (GenBank accession number MH057540).

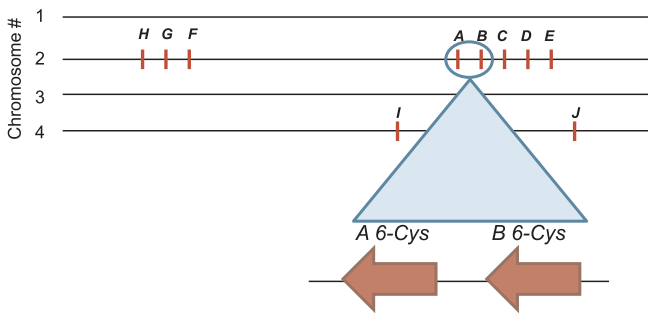

Fig. 1. Schematic representation of the *6-Cys* family in *B. bovis*. The contiguous targeted genes *6-Cys A* and *B* are shown magnified in the lower part of the figure.

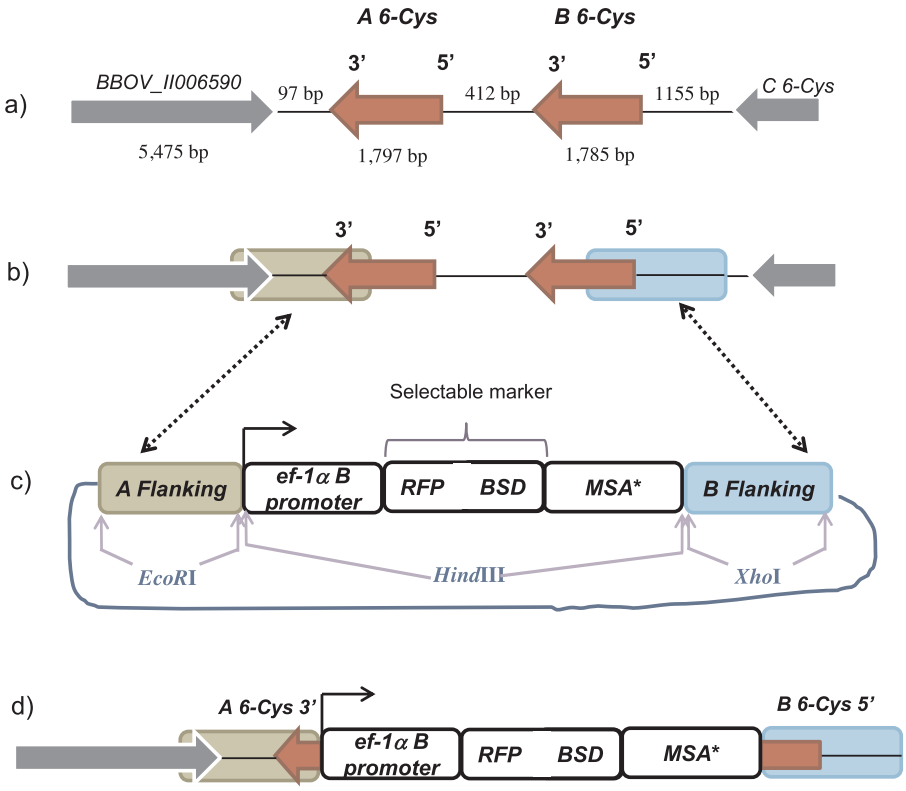

Fig. 2. Schematic representation of: **a)** Arrangement of the *6-Cys A* and *B* genes on chromosome 2 of *B. bovis*. The lengths (bp) of the open reading frames and their intergenic regions are indicated. The gene orientations are indicated by arrows. **b)** Location and design of the 5′ and 3′ flanking regions used in the plasmid construct in order to facilitate homologous recombination events leading at specific KO of the *6-Cys A* and *B* genes. **c)** Schematic representation of the plasmid construct *p6-Cys A-Bdko*. The 5′ and 3′ flanking regions for homologous recombination, the *ef-1α* B promotor, the *rfp-bsd* selectable marker and the 3′ *msa-1* regions for termination of transcription and translation are indicated. The locations of the restriction sites used for cloning (*Eco*RI, *Hind*III and *Xho*I) are indicated. **d)** Partial structure of the *6-Cys* locus in the transfected line *ΔAΔB 6-Cys B. bovis* showing the location of the transfected *rfp-bsd* gene and its regulatory regions.

Plasmid *p6Cys A-B-dko* was propagated in Topo10 *E. coli* competent cells (Invitrogen, Carlsbad, USA). The plasmid was purified using Qiagen Plasmid Midi Kit (Qiagen, MD, USA) following the manufacturer's protocol before transfection. Identically purified plasmid *pBluescript* plasmid (pBS) was used as a control in the transfection experiments.

2.3. *B. bovis* transfection

Plasmid *p6Cys A-B-dko* and negative control *pBluescript* were transfected into *B. bovis* T3Bo strain-infected bovine RBCs by electroporation as previously described (Suarez et al., 2006; Suarez and McElwain, 2009). Following electroporation, parasites were maintained in *in vitro* culture (Hines et al., 1989;Levy and Ristic, 1980) and selected by blasticidin as described previously (Suarez and McElwain, 2009). Cultures were monitored daily for the presence of the red-fluouresing transfected parasite using a fluorescence microscope at 60× magnification.

2.4. Genotypic analysis of transfected parasites

Genomic DNA was extracted from cultured parasites as described previously (Suarez and McElwain, 2009). Genomic DNA was analyzed for integration of the transfected sequences using PCR with primers described in Supplementary Table 1 using the strategy described in Fig. 4. The set of primers *p10 for*: 5′-TTGTGCGCTCCGTAATTCCT-3′, and *EF-Pr-R-4-HIII*: 5′-GACTACAAGCTTCATCGTTTGATT GAAATA TAA-3′ were used to demonstrate disruption at the targeted *6-Cys* gene *A* and *B* and verifying integration of the transfected sequences by homologous recombination using PCR (Supplementary Fig. 1). The resulting PCR product was cloned into TA cloning kit with PCR 2.1 vector (Invitrogen, USA), and sequenced with primers M13 F and M13Rev (Invitrogen). Primers used to verify the deletion of the *6-Cys A* gene were: *6-Cys A- F1*: 5′-ATGGCATATGGTTGTGAGACAGGA-3′ (representing *6-Cys A*-specific internal sequence) and BBOV_II006590- *p8 for*: 5′-ACCCATCGTCATCGCAAAGT-3′ (representing *6-Cys A* -specific external sequence). The primers used to verify the deletion of the *6-Cys B* gene were: *6-Cys B-R1*: 5′-GCATATATGTAGATTGACGGAAATC TAC-3′ (representing *6-Cys B*-specific internal sequence) and *IGB* [Intergenic region before gene 6-Cys *B*] for: 5′-TAATCTGTTTGCCGATGC AACACG-3′ (representing *6-Cys B*-specific external sequence). The localization of these primers is depicted in Fig. 5.

2.5. *In vitro* phenotypic analysis of gene-deleted parasites

Parasites were cultured in quintuplicate wells, starting at 0.5% parasitemia in the absence or presence of blasticidin, and maintained daily by replacing the media, as described previously (Suarez and McElwain, 2009). Percent parasitemia (ppe) was calculated daily by optical microscopy and statistical analysis was performed using a paired *t*-test.

3. Results and discussion

We targeted for deletion and replacement a 3, 287 bp region on chromosome 2 of *B. bovis* containing the *6-CysA* and *6-CysB* genes as represented in Fig. 1. The specific regions targeted for homologous recombination (Fig. 2a) were cloned in the flanking regions of the transfection plasmid *p6-Cys A-BdKO* (Fig. 2b and c). The full structure of the plasmid *p6-Cys A-Bdko*, designed for the simultaneous double deletion of *6-CysA* and *6-CysB* by homologous recombination is shown in Fig. 2c. Plasmid *p6-Cys A-Bdko* also contains the *rfp-bsd* fusion gene as a selectable marker under the regulation of the *ef-1α* promoter B (Suarez et al., 2006), and the 3′ region of the *msa-1* gene of *B. bovis* (Laughery et al., 2014). The complete sequence of the genes cloned in the transfection plasmid *p6-Cys A-BdKO* are available in GenBank (Accession number MH057540).

Plasmid *p6Cys A-B-dko* was electroporated into T3Bo *B. bovis* parasites which were then immediately placed into culture and selected with inhibitory doses of blasticidin. A blasticidin-resistant parasite line expressing red fluorescence emerged 7 days after the onset of selection (Fig. 3). The blasticidin-resistant parasite line, termed *ΔAΔB 6-Cys B.*

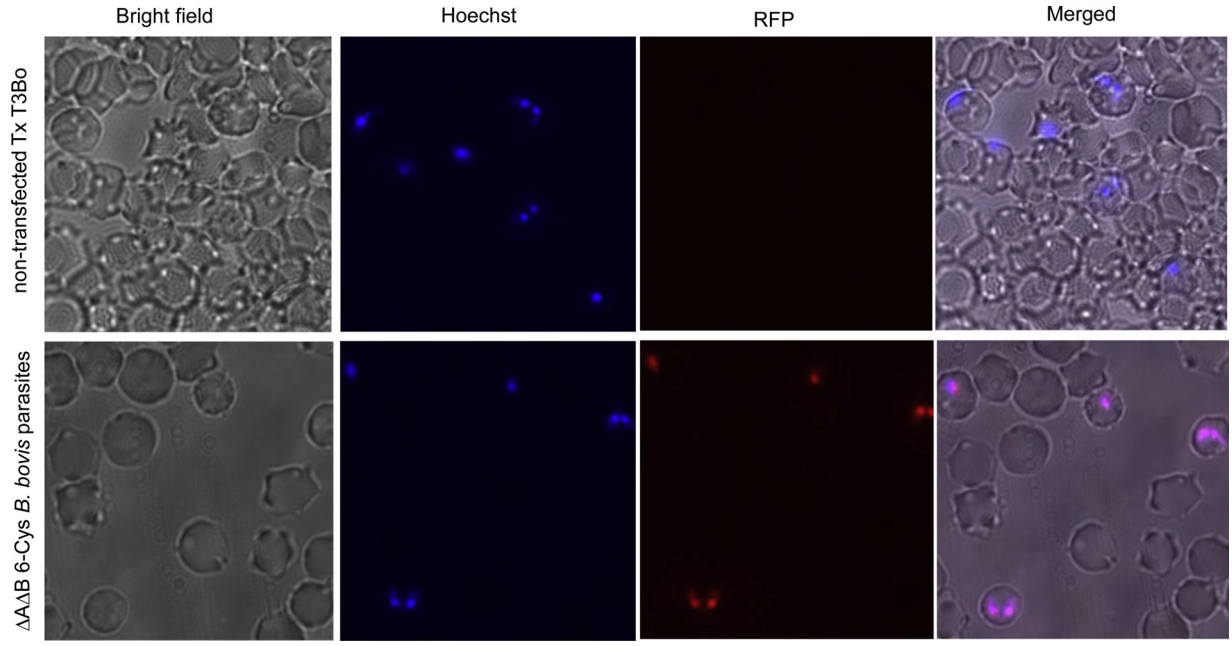

Fig. 3. Expression of red-fluorescent protein (RFP) in *B. bovis ΔAΔB 6-Cys* mutant line by fluorescence microscopy. Bright field and fluorescence (585 nm) images of control wild-type T3Bo and red-fluorescent transfected line *ΔAΔB 6-Cys B. bovis* parasites are shown. Parasites were treated with Hoechst 33342 (blue) to identify the parasite nucleus (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

bovis, was then analyzed for stable integration of the transfected genes disrupting the targeted *6-Cys A* and *6-Cys B* locus using PCR. The experimental design and a representation of the results of the PCR-integration analysis are represented in Fig. 4, and the sequences of the primers used in these reactions are shown in Table 1. No amplicons were generated when identical PCR reactions were performed on vector plasmid *pBS* or gDNA extracted from the parental T3Bo *B. bovis* strain (data not shown). Production of all these PCR amplicons (1–5, Fig. 4) was consistent with integration of the transfected *rfp-bsd* gene into the *6-Cys* gene locus as depicted in Fig. 4. More specifically, products of PCR reactions 1 and 5 confirm integration of the transfected sequences into the targeted *6-cys A* and *B* locus, since they include primer sequences (*p9-for* for PCR1 and *IGR for*, in PCR 5) that represent sequences in the *B. bovis* genome that are external to the integration sequences present in the transfection plasmid. To further confirm insertion of the transfected sequences into the targeted locus, we amplified and sequenced the region encompassed by primer *p10 for*, also external to the region targeted for insertion, and not present in the transfection plasmid, and *EF-PR-Rev4* representing sequencing internal to the transfection plasmid, represented in Supplementary Fig. 1.

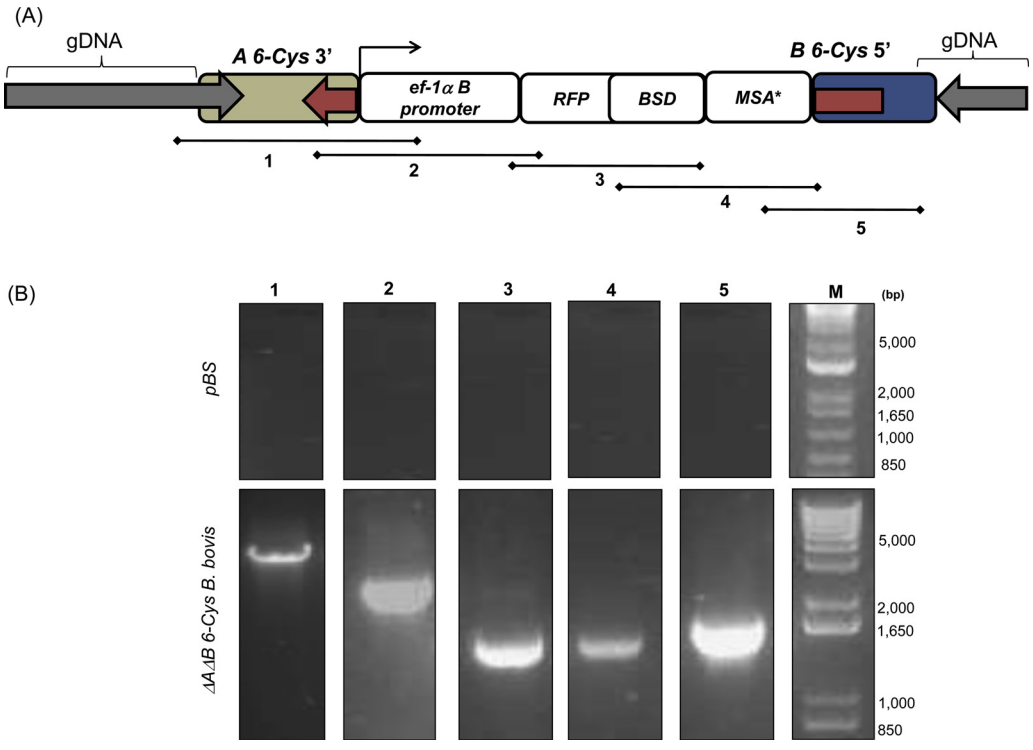

Fig. 4. A) Experimental strategy for the verification of the integration of transfected genes into the 6-Cys locus of the T3Bo *B. bovis ΔAΔB 6-Cys* mutant line using PCR on genomic DNA. The location of the PCR primers and the sizes of the predicted PCR fragments 1–5 are indicated. MSA* represents 300 bp of the 3′ region of the *msa-1* gene of *B. bovis*, and contains signals needed for termination of transcription and translation. **B)** Agarose gel analysis of the products of the PCR described in Panel A. The upper panels represent PCR amplifications performed on plasmid control *pBS* and the lower panels on genomic DNA extracted from *B. bovis ΔAΔB 6-Cys* parasites. The sizes of the PCR fragments are depicted on the right.

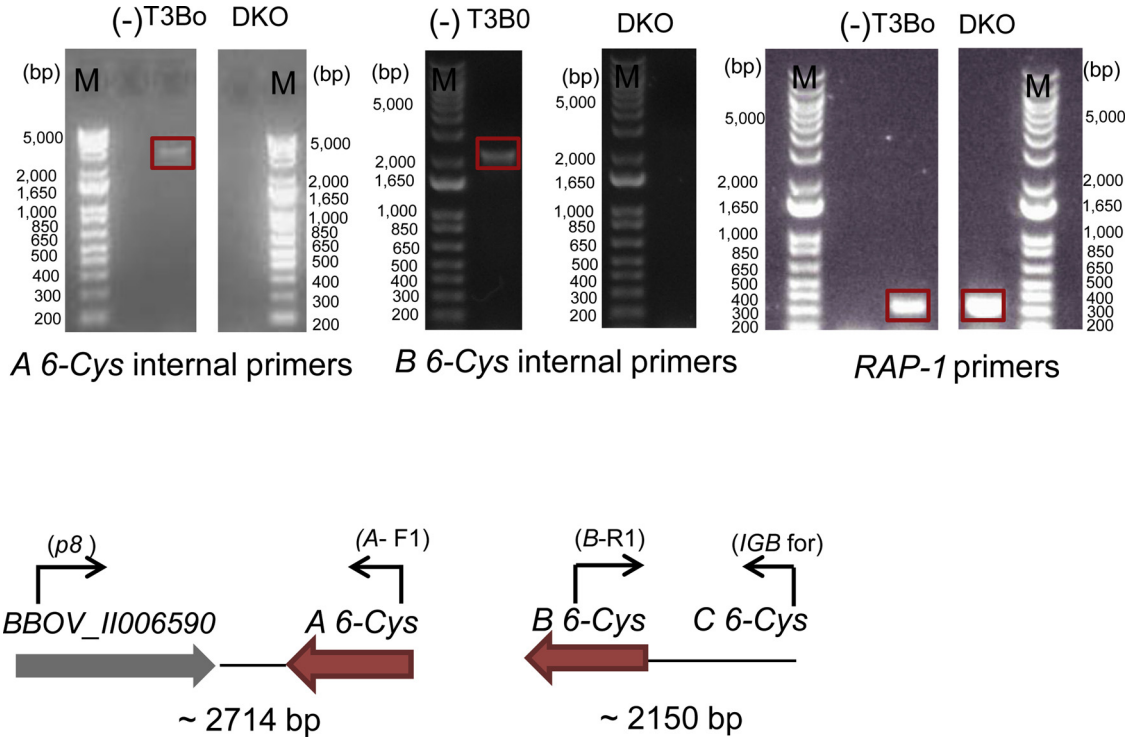

Fig. 5. PCR analysis demonstrating replacement of the *6Cys A* and *6 Cys B* genes in the *B. bovis ΔAΔB 6-Cys* parasite line (DKO) using *6-Cys A* and *6-Cys B* internal and selected external primers. The localization of the primers used in the PCR reactions is shown in the lower panel. PCR performed with *rap-1* primers was used as a positive control. The PCR amplicons are marked with red boxes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
PCR primers used to verify integration of transfected genes into the 6-Cys locus of the T3Bo *B. bovis ΔAΔB 6-Cys* mutant line. The PCR products are described in Fig. 4.

Amplicon #	Primer Pairs	Primer sequence [bp]
1	*BBOV_II006590 p9-for*	5'- taagcggctctgttcagcaa -3′
	Elongation factor promoter-R5	5'- tcgcaaagctttttcgtaaagttgcaataaattatc-3
2	*A Cys gene reverse*	5'- ctagaatctcgtaggctttagcatg -3′
	Elongation factor promoter-R5	5'- tcgcaaagctttttcgtaaagttgcaataaattatc-3
3	*Elongation factor promoter-F8*	5'-agatacaagcttgtctttataacttaataaagtaattcc-3
	Bsd gene rev	5'-gccctcccacacataaccagag-3'
4	*Rfp gene for*	5'-gtgagcaagggcgaggaggat aac-3′
	MSA 3' rev	5'-gcgaattcttatttattaatgttcc-3'
5	*MSA 3' for*	5'-gcctagctgcagttaaaaactaatggtagtgacg-
	IGR for	3' 5'-taatctgtttgccgatgcaacacg-3′

Sequence analysis of the resulting PCR product confirms the integration of the transfected sequences into the intended *6-cys A* locus by homologous recombination, as shown in Supp. Fig. 1. In addition, we confirmed deletion of the *6-Cys A* and *B* genes by performing additional PCR analysis using [1] primers *IgB* for that represent sequences in the intergenic region upstream the *6-Cys B* gene and *B–R1*, that represent sequences internal in the *6 Cys B* gene; and [2] primers *p8* that represent sequences in the gene BBOV_II006590, located downstream the *6-Cys A* gene and primer *A-F1*, that represent sequences internal in the *6- Cys* gene *A* (Fig. 5). The location of the primers and the results of the PCRs are shown in Fig. 5. The expected 2150 and 2714 bp amplicons were successfully generated only when gDNA from the wild-type T3Bo strain was used as template, but not with gDNA from the *ΔAΔB 6-Cys B. bovis* parasite line, since the *6-Cys A* and *6-Cys B* genes were removed upon successful gene KO in the later parasite line (Fig. 5). Taking together, the data represented in Fig. 4 and 5, confirms the lack of the full size *6-Cys A* and *B* genes and their replacement by the *rfp-bsd* genes and accessory sequences exactly as organized in the transfection plasmid *p6Cys A-B-dko*.

In summary, PCR and sequencing data is fully consistent with the disruption of a 3287 bp region encompassing both *6-Cys A* and *6-Cys B* genes, using a single transfection plasmid, with full-genome insertion of the promoter and selectable marker genes present in the transfection plasmid *p6Cys A-B-dko*. As previously demonstrated (Suarez et al., 2015; Alzan et al., 2017) using a similar transfection plasmid, insertion likely occurred by homologous recombination.

We then compared the rate of growth of *B. bovis* T3Bo wild-type in blasticidin-free media and *ΔAΔB 6-Cys B. bovis* parasites in media containing inhibitory doses of blasticicin *in vitro*. Both parasite lines grew at comparable rates (Fig. 6) over a period of 7 days, suggesting that disruption of 6-Cys *A* and 6-Cys *B* and expression of the *rfp-bsd* gene does not significantly ($P = 0.55$) affect the ability of the parasites to invade and replicate in bovine RBCs, despite the addition of blasticidin into the *ΔAΔB 6-Cys B. bovis* parasite cultures. The T3B wild-type parasites were unable to grow in the presence of similar doses of blasticidin in the culture media (Alzan et al., 2017). Therefore, loss of the of 6-Cys *A* and 6-Cys *B* genes in *ΔAΔB 6-Cys B. bovis* does not affect the growth of this parasite line *in vitro*, which is consistent with a role for *6-Cys A* and *6-Cys B* exclusively in tick-stage parasites.

The *6-Cys E* gene is also differentially-expressed in tick-stage parasites, but with a pattern that differs from *6-Cys A* and *6-Cys B* (Silva et al., 2011; Alzan et al., 2016 and 2017) suggesting that these three 6-Cys proteins likely play different functional roles. Furthermore, and similar to what was found for ΔAΔB 6-Cys *B. bovis* mutants in the current study, deletion of the *6-Cys E* gene does not affect the ability of the parasites to develop in *in-vitro* culture (Alzan et al., 2017). In addition, recent work using midgut parasites and *in vitro* induced sexual forms of *B. bovis* demonstrated expression of surface exposed the 6-Cys proteins in the gamete forms, suggesting a functional role for these

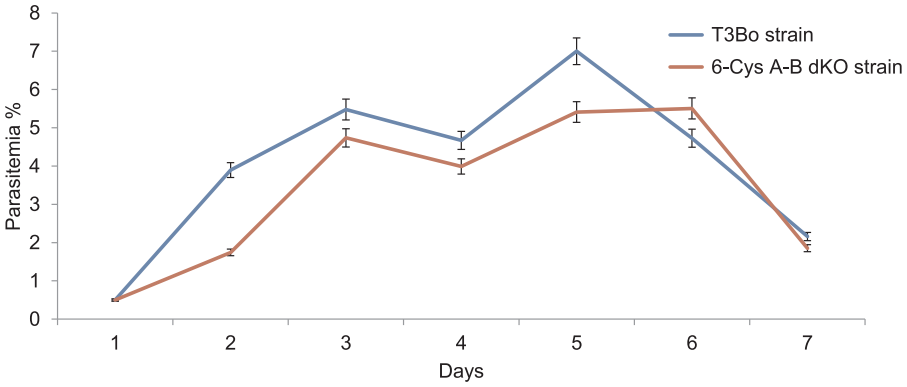

Fig. 6. Comparative *in vitro* culture growth curves among the *B. bovis* T3Bo wild-type strain (blue line) and the *ΔAΔB 6-Cys B. bovis* strain (red line). The percentage of parasitized erythrocytes (Parasitemia %) was analyzed daily for 7 days. No significant differences in growth rate between the two strains compared were found (P = 0.55) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

proteins in cell-cell interactions leading to sexual reproduction of the parasite. Based on these observations, it should now be feasible to generate triple mutant parasite lines deficient in the *6-Cys A, 6-Cys B* and *6-Cys E* genes, thus increasing the chances of abrogating transmission of the parasites included in future live vaccines.

An additional advantage of using red-fluorescent transfected ΔAΔB 6-Cys *B. bovis* parasites as vaccine components is the incorporation of a molecular marker that would facilitate their discrimination from infecting field strains in vaccinated animals, either because of their unique red fluorescent phenotype or by PCR. However, the transfected *ΔAΔB 6-Cys B. bovis* line described in this study is not clonal, and at least in theory, it is possible that it still may contain a population of non-transfected parasites. If present, non-mutated parasites might also emerge when the line is maintained either *in vitro* or *in vivo* in the absence of blasticidin, suggesting that a *ΔAΔB 6-Cys B. bovis* clonal line might be desirable for vaccine development purposes.

Future work will be aimed at generating a triple *6-Cys A*, *B* and *E* mutant parasite lines and to compare double- and triple-mutants in transmission studies. While this work may provide the foundation for a non-transmissible vaccine, it may also generate new and important information towards a better understanding of the biology of this economically-important parasite.

Overall, our data supports the feasibility of producing and testing parasites that lack *6-Cys* genes as components of a next generation, non-transmissible, live-attenuated vaccine expressing a unique, vaccine-strain specific molecular marker. In addition, our data supports the testing of the 6-Cys *A* and 6-Cys *B* proteins as components of sub-unit TBVs, aimed at interfering with parasite transmission. Novel non-transmissible live vaccines and successful TBVs against *B. bovis* should help reducing the burden of the disease globally.

4. Conclusions

Several lines of evidence confirmed the production of a mutated *B. bovis* parasite line where the *6-Cys A* and *6-Cys B* genes were removed and replaced by a transfected *RFP-BSD* expression cassette by homologous recombination. The mutated parasite line expressed the red-fluorescent protein and is able to grow continuously in bovine RBCs *in vitro*, in the presence of inhibitory doses of blasticidin, at a similar rate to wild-type parasites growing in blasticidin-free media, suggesting that the *6-Cys A* and *6-Cys B* genes are not required for the development of blood-stage parasites. The *6-Cys A* and *6-Cys B* mutated *B. bovis* parasite line can now be used to determine the functional role of these two genes in transmission of the parasite by its tick vector, and could be the foundation for the future development of genetically-defined, non-transmissible live vaccines against bovine babesiosis that contains a molecular marker for strain differentiation.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.01.006.

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