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Identification of *Babesia bovis* MSA-1 functionally constraint regions capable of binding to bovine erythrocytes

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ABSTRACT

Merozoite surface antigen-1 is a glycoprotein expressed by *Babesia bovis* and is considered a vaccine candidate given that antibodies against it are able to partially block *in vitro* invasion of bovine erythrocytes. Despite this, no study to date has confirmed the target cell binding properties of the full MSA-1 or its fragments. This research has thus been focused on identifying protein regions playing a role in erythrocyte attachment, based on genetic diversity and natural selection analysis. Two regions under functional constraint (nucleotides 134–428 and 464–629) having a preponderance of negatively-selected signals were identified *in silico*. Three non-overlapping peptides derived from functionally constraint regions (42422 (^{39}PEGSFYDDMSKFYGAVGSFD^{58}), 42424 (^{91}NALIKNNPMIRPDLFNATIV^{110}) and 42426 (^{150}TDIVEEDREKAVEYFKKHVY^{169})) were able to specifically bind to a sialoglycoprotein located on the bovine erythrocyte surface as confirmed by sensitive and specific peptide-cell interaction competition assays using both enzymatically treated and untreated red blood cells. Interestingly, it was predicted that peptides 42422 and 42426 have a helical structure and conserved motifs in all strain/isolates. These findings provide evidence, for the first time, related to *B. bovis* MSA-1 short regions used by the parasite in erythrocyte binding which could be predicted using natural selection analysis. Future work focused on evaluating these peptides' antigenic ability during natural infection, and their ability to induce protection in immunisation assays are needed to confirm their usefulness as synthetic vaccine candidates.

1. Introduction

Babesiosis is one of the most significant tick-borne veterinary diseases, mainly affecting wild and domestic animals, and occasionally humans, worldwide (Gray, 2006; Jacob et al., 2020; OIE, 2022). *Babesia bovis* and *Babesia bigemina* are the main haemoprotozoa belonging to the phylum Apicomplexa causing bovine babesiosis in tropical and subtropical regions (Bock et al., 2004; Chauvin et al., 2009; Suarez and Noh, 2011), *B. bovis* being particularly associated with a negative impact on the livestock industry since it often causes high morbimortality rates,

Abbreviations: Mrz, Merozoite; MSA, merozoite surface antigens; VMSA, Variable merozoite surface antigen; Spz, Sporozoite; GPI, Glycosylphosphatidylinositol; HABPs, High Activity Binding Peptides; Ab, Antibodies; MHC, Major Histocompatibility Complex; aa, amino acid; NCBI, National Center for Biotechnology Information; GWAS, Genome-wide association study.

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loss of dairy and meat products, high investment in control measures and international livestock trade conflicts (Bock et al., 2004; Suarez and Noh, 2011).

Integrated control methods focused on eliminating the vector or the parasite have been considered to decrease the disease's incidence and frequency; however, this strategy involves many limitations, given ticks' increased resistance to acaricides (Andreotti et al., 2011; Faza et al., 2013; Higa Lde et al., 2016), the parasite's developed resistance to chemically-based drugs due to their indiscriminate use at sublethal doses (Mosqueda et al., 2012; Vial and Gorenflot, 2006) and there are significant adverse effects regarding animal-derived products treated with these drugs (de Waal and Combrink, 2006; Florin-Christensen et al., 2014; Yusuf, 2017). On the other hand, chemotherapy of sick animals as the only control strategy is not suitable for extensive livestock farming since its success depends on early diagnosis and timely treatment, which increases the cost due to the continuous control that must be done (Vial and Gorenflot, 2006); immunisation with *B. bovis* attenuated parasites or recombinant proteins is thus considered the best alternative for controlling babesiosis in endemic areas (De Vos and Bock, 2000; Suarez et al., 2019). Despite this, several vaccines based on whole parasite proteins have induced partial protection in immunised cattle (Hines et al., 1995; Hope et al., 2005) thus highlighting the fact that the molecular mechanism by which *Babesia* spp. parasites infect target cells should be properly understood; other strategies focused on searching for and selecting suitable vaccine candidates are thus needed.

Proteins located on the parasite membrane surface are considered the best vaccine candidates since an immune response against them should be able to inhibit initial contact and reorientation, two essential steps in merozoite (Mrz) invasion of erythrocytes (Yokoyama et al., 2006). Previous studies have shown that *Babesia* spp. parasites express several MSAs belonging to the variable merozoite surface antigen (VMSA) family (MSA-1, MSA-2a1, MSA-2a2, MSA-2b and MSA-2c (Goff et al., 1988; Hines et al., 1989; Nie et al., 2020; Suarez et al., 2000) which are variable amongst *B. bovis* isolate sequences worldwide (Altangerel et al., 2012; Berens et al., 2005; Borgonio et al., 2008; Florin-Christensen et al., 2002; Leroith et al., 2005; Molad et al., 2014; Nagano et al., 2013; Simking et al., 2013; Sivakumar et al., 2013; Suarez et al., 2000; Tattiyapong et al., 2014) and some of them playing an important role in Mrz and sporozoite (Spz) invasion of erythrocytes (Mosqueda et al., 2002a; Mosqueda et al., 2002b; Suarez et al., 2000; Wilkowsky et al., 2003). It has also been reported that *B. bovis* MSA-1 is the most polymorphic VMSA member, whilst MSA-2 has moderate to low polymorphism worldwide (Dominguez et al., 2010).

MSA-1 is expressed as a 42 kDa glycoprotein on *B. bovis* mature Mrz surface which is encoded by a single gene (Suarez et al., 2000). This protein contains a C-terminal glycosylphosphatidylinositol (GPI) anchor, like that of surface proteins from other organisms. Interestingly, MSA-1 is used as a survival strategy by *B. bovis* parasites, given its genetically and antigenically difference amongst *B. bovis* isolates sequences from diverse regions worldwide (Carcy et al., 2006). Interestingly, *B. bovis* MSA-1 has been considered a vaccine candidate given that antibodies against this protein affect Mrz invasion of bovine erythrocytes *in vivo* and are able to neutralise Mrz infectivity *in vitro* (Hines et al., 1995; Hines et al., 1992; Mosqueda et al., 2002b; Suarez et al., 2000). Despite this, recombinant *B. bovis* MSA-1 immunisation has not conferred complete protection against challenge by the virulent *B. bovis* strain (Antonio Alvarez et al., 2010; Hines et al., 1995) suggesting that the immune response might be directed towards regions that are either not involved in protein-cell interaction or that are polymorphic.

It has been reported that short regions (known as high activity binding peptides (HABPs)) derived from *Plasmodium falciparum* proteins having an important function in target cell interaction are the best candidates for inducing protective antibodies (Ab) after they have been modified to fit into the major histocompatibility complex (MHC) (Patarroyo et al., 2011). Such regions have been predicted in *Plasmodium vivax*-derived proteins according to their evolution patterns and residues under negative selection, this being a promising approach to understanding parasite proteins' functional role during erythrocyte invasion. Despite this, this functional approach has been poorly explored regarding *B. bovis* (Suarez and Noh, 2011) and this research was therefore proposed to elucidate the regions under functional constraint governing *B. bovis* MSA-1 binding to bovine erythrocytes.

2. Materials and methods

2.1. Functional constraint analysis

The partial *B. bovis T2Bo msa-1* nucleotide sequence (BBOV_I003060) was used as reference for identifying *msa-1* gene homologous sequences in *Babesia* species (*Babesia orientalis* (NCBI accession number: MN308083), *B. bovis* (*msa-2a1* (PiroplasmaDB accesion number: BBOV_I003010), *msa-2a2* (BBOV_I003000), *msa-2b* (BBOV_I002990) and *msa-2c* (BBOV_I003020)) and *B. bovis* isolate sequences (NCBI accession numbers: AB612244, AB612245, AB612246, AB612247, AB612248, AB612249, AB612250, AB612251, AB763993, AB763994, AB763995, AB763996, AB763997, AB763998, AB763999, AB764000, EF640942, EF640943, EF640944, EF640945, EF640946, EF640947, EF640948, EF640949, EF640950, EF640951, EF640952, EF640953, EF640954, LC099095, LC099096, LC099097, LC099098 and LC099099) and strain sequences (NCBI accession numbers: AF275908 (Mo7), AF275909 (SP2), AF275910 (R1A) and AF275911 (T2B)). On the other hand, a protein BLAST using the *B. bovis* MSA-1 aa reference sequence was done to find divergent sequences in the non-redundant protein sequences database, excluding the organism *B. bovis* (taxid: 5865) and using the BLOSUM45 substitution matrix. A significant percentage of identity was further ascertained using the HHpred server (Zimmermann et al., 2018) which uses multiple alignment and pairwise comparison of profile hidden Markov models for finding remote protein similarity. PROMALS3D (Pei et al., 2008) was used for aligning the deduced aa sequences and the codon alignment was performed with TranslatorX (Abascal et al., 2010).

Gene-wide methods were used for evaluating natural selection on the *msa-1* locus. Polymorphism pattern and divergence were evaluated by McDonald–Kreitman test (Egea et al., 2008). The relationship between the amount of non-synonymous substitutions per non-synonymous site and the amount of synonymous substitutions per synonymous site (d_N/d_S) was calculated with the Nei-Gojobori method, modified by Jukes-Cantor correction, using MEGA X (Kumar et al., 2018); the Z test was used for evaluating significance. The d_N/d_S ratio was calculated in a sliding window along *msa-1* alignment. Natural selection was evaluated by site-specific methods available on the Datamonkey web server (Delport et al., 2010). Potential recombination points were detected by the GARD algorithm, whilst negatively and positively selected codons were identified by maximum likelihood (FEL, SLAC (Kosakovsky Pond and Frost, 2005), FUBAR (Murrell et al., 2013)) and Bayesian (MEME (Murrell et al., 2012)) methods, considering a < 0.1 p-value (FEL, SLAC, and MEME) and > 0.9 posterior probability (FUBAR).

2.2. B. bovis MSA-1 peptide synthesis and radiolabelling

Twenty residue-long, non-overlapping peptides encoded by gene regions under functional constraint were searched along the *B. bovis* MSA-1 amino acid sequence since these are the basic characteristics for selecting suitable vaccine candidates (Patarroyo et al., 2020; Patarroyo et al., 2011). Therefore, five peptides from T2Bo strain *B. bovis* MSA-1 (coded as 42422 (^{39}PEGSFYDDMSKFYGAVGSFD^{58}), 42423 (^{62}LYSVLSANFKAAKMDDQKVKD^{81}), 42424 (^{91}NALIKNNPMIRPDLFNATIV^{110}), 42425 (^{113}FSTKNDEEKFNAIFDSIK^{130}) and 42426 (^{150}TDIVEEDREKAVEYFKKHVY^{169}) were synthesised by the tert-butoxy carbonyl (t-Boc) strategy, using solid-phase synthesis methodology (Houghten, 1985). A tyrosine residue was added to those lacking one in their native sequences to enable radiolabelling. These molecules were purified by

reversed-phase high-performance liquid chromatography (RP-HPLC) and analysed by MALDI-TOF mass spectrometry (Bruker Daltonics) after having been cleaved by low–high hydrogen fluoride technique. Radiolabelling involved taking 1 mg/mL from each peptide diluted in 4-(2-hydroxyethyl)− 1-piperazineethanesulphonic acid buffered saline (HBS) for 15 min, using 3 μL $Na^{125}I$ (100 mCi/mL; ARC) and 15 μL chloramine T (2.75 mg/mL); the reaction was halted with 15 μL sodium metabisulphite (2.25 mg/mL). Each peptide was purified by size exclusion chromatography using Sephadex G-10 columns (Pharmacia); single sample's radioactivity was measured/quantified using a gamma counter (Packard Cobra II).

2.3. Animal handling and blood collection/preparation

A blood sample (20 mL) from a clinically healthy Normande 18-month-old bull was obtained in sodium citrate tubes, following a previously described protocol (Cuy-Chaparro et al., 2021) and the guidelines for the care of large animals established by U.D.C.A's ethics committee (regulated by agreement No. 285/2008, chapter VII). The sera and leucocyte layers were removed by washing the collected sample thrice with HBS (1:1 ratio), spinning at 1600 xg for 5 min for each run. Erythrocytes (60% solution) were treated with trypsin (1 mg/mL), chymotrypsin (1 mg/mL) and neuraminidase (100 μU/mL) at 37 °C for 1 h 30 min, washed three times using 250 xg for 3 min and then used in binding assays.

2.4. Radiolabelled peptide binding assays

Binding assays were done in triplicate. Briefly, B. bovis MSA-1 radiolabelled peptides (in 20–200 nM increasing concentrations) were incubated with bovine erythrocytes (7.5×10^7 normal cells) for 2 h at room temperature in the absence (total binding) or presence (nonspecific binding) of the same non-radiolabelled peptide (20 μM) at 200 μL final volume. A similar binding assay was done incubating enzymatically-treated cells, as previously described (Cuy-Chaparro et al., 2021). Cells were washed twice with HBS and radiolabelled peptide bound to erythrocytes was quantified by gamma counter. Peptides (HABPs) having strong bovine erythrocyte binding activity were those having a specific binding curve greater than or equal to 0.01 (1%) (0.010pmol bound peptide/pmol added peptide) using the Ka.r = [b]/[l] equation, where Ka is the association constant, r the receptor sites, b bound ligand and l free ligand. The [b]/[l] ratio is binding activity, represented by the slope (m) of the specific binding curve (Cuy-Chaparro et al., 2021).

2.5. B. bovis MSA-1 secondary structure and B-cell epitope prediction

MSA-1 sequences viewed in the National Center for Biotechnology Information's (NCBI) multiple sequence alignment viewer were aligned using the Clustal Omega server (Sievers and Higgins, 2021) and submitted to EMBOSS-Cons server (Madeira et al., 2019) to reduce them into one unique sequence that best representing the MSA-1 protein. PROTEUS2 (Montgomerie et al., 2008), ProteinPredict (Rost et al., 2004), RaptorX (Kallberg et al., 2014) and Jpred (Cole et al., 2008) bioinformatics tools were used for individual secondary structure predictions for the consensus sequence. BebiPred 2.0 (Jespersen et al., 2017), 0.5 threshold value, was used for B-cell epitope prediction with the MSA1 consensus sequence.

3. Results

3.1. B. bovis MSA-1 contain regions under functional constraint

The *B. bovis msa-1* gene had a mean 0.25 *p*-distance ranging from 0.001 to 0.35. The maximum *p*-distance between a *B. bovis* isolate and the respective homologue sequence was 0.55 (*msa-2a1* and *msa-2a2*), 0.56 (*B. orientalis*) and 0.57 (*msa-2b* and *msa-2c*). Despite these differences, profile-profile comparison confirmed a significant percentage of identity (0.99) amongst *B. bovis msa-1, msa-2a1, msa-2a2, msa-2b, msa-2c* genes and *B. orientalis msa-1* and a shared MSA-2c domain (PF12238) which involves most of the gene sequence (Fig. 1). McDonald–Kreitman test neutrality indices indicated greater non-synonymous polymorphism than expected under selective neutrality for comparing *B. bovis msa-1* and *msa-2a2, msa-2b and msa-2c* (Table 1). However, the d_N/d_S ratio for the entire gene showed significantly fewer non-synonymous than synonymous substitutions [$d_N/d_S < 1$] when *B. orientalis msa-1* and *B. bovis msa-2c* comparisons were made (Table 1).

Natural selection was also evaluated by comparing *B. bovis msa-1* alignments with those of different homologues in a d_N/d_S sliding window. This analysis showed that *msa-2a1, msa-2a2* and *msa-2b* had two regions (around nucleotides 180–300 and 630–750) having an evolutionary rate higher than the rest of the gene (Supplementary figure 1); these sequences were therefore excluded from further analysis. *B. bovis msa-1* gene natural selection action was thus evaluated in both an alignment using intra-species *B. bovis* sequences and in an alignment with *B. orientalis msa-1* and *B. bovis msa-2c* using a d_N/d_S ratio sliding window (Supplementary figure 1). Despite these two sequences having great divergences from *B. bovis msa-1*, they had a very similar evolutionary pattern, thus making selection analysis more robust. Two regions within *B. bovis* and between homologues located on nucleotides 134–428 and 464–629 had < 1 d_N/d_S values (Fig. 1). Forty-six negatively selected codons and 32 positively selected ones were found within *B. bovis* sequences (65 and 33 when including homologues) and 73 and 44 were common for both alignments. Interestingly, 62% (45/73) of negatively selected codons and 41% (18/44) of positively selected ones were located in regions having < 1 d_N/d_S values. Moreover, considering

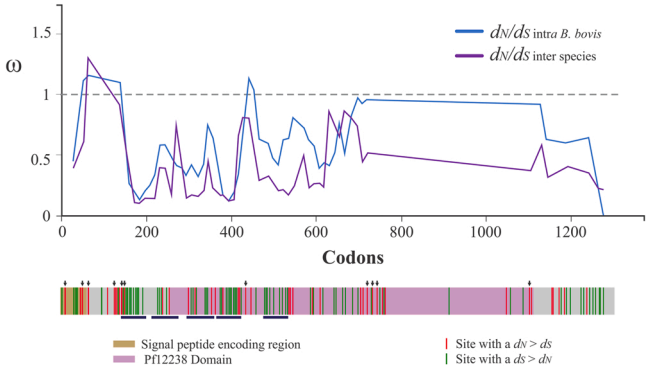

Fig. 1. Natural selection action along the *msa-1* locus. Sliding window showing ω rate (y-axis) within (intra) *B. bovis msa-1* isolate sequences and between (inter) *B. bovis msa-1, msa-2c*, and *B. orientalis msa-1* sequences) plotted against nucleotide position (x-axis). The gene diagram at the bottom shows the signal peptide (orange), the PF12238 domain (purple), the negatively (green) and positively (red) selected sites, and the selected peptide's location (horizontal black bars). Arrows indicates inter species positively selected sites.

Table 1
Natural selection tests for *B. bovis msa-1* locus. McDonald–Kreitman (MK) neutrality index (NI), considering pairwise comparison amongst *B. bovis* and phylogenetically related species. d_N: nonsynonymous substitutions. d_S: synonymous substitutions. d_N/d_S: the d_N/d_S ratio per site. * : indicates statistical significance.

	MK		d_N/d_S			
Locus	NI	*p*-value	d_N	d_S	d_N/d_S	*p*-value
B. orientalis msa-1	0.82	0.17	0.86	1.67	0.56	0.01 *
B. bovis msa-2a1	0.85	0.31	0.93	0.76	1.23	0.17
B. bovis msa-2a2	1.53	0.01 *	0.95	0.74	1.29	0.08
B. bovis msa-2b	1.50	0.01 *	1.01	0.88	1.15	0.35
B. bovis msa-2c	1.41	0.04 *	0.89	1.60	0.56	0.02 *

only *B. bovis* isolate sequences, such regions had 66% (50%−100%) mean sequence identity and 82% (70%−100%) mean similarity, whilst there was 59% (42%−100%) identity and 74% (63%−100%) similarity considering the complete gene.

3.2. Three B. bovis MSA-1 peptides bound to bovine erythrocytes

B. bovis MSA-1 short regions (18–20 amino acid (aa) long) encoded by *msa-1* gene regions having $\omega < 0.75$ and several negatively selected intra *B. bovis* sites were selected for binding assays. Five peptides located on the *B. bovis* MSA-1 amino-terminal end (^{39}P-^{169}Y residues) were thus selected, synthesised and radiolabelled to ascertain their specific interaction with bovine erythrocytes by peptide-cell interaction competition assays. The peptides' specific binding was calculated by subtracting non-specific binding from total binding, thereby obtaining a specific binding slope plot from 0 to 0.019 (Fig. 2); peptides 42422 (^{39}PEGSFYDDMSK-FYGAVGSFD^{58}), 42424 (^{91}NALIKNNPMIRPDLFNATIV^{110}Y), and 42426 (^{150}TDIVEEDREKAVEYFKKHVY^{169}) were thus considered HABPs because they had a > 0.01 slope. Interestingly, 42422-derived 42914 (^{39}SSDPKDMGEYAGYFFFSVDG^{58}), 42424-derived 42915 (^{91}VPFRM NANIKITLINAPDLN^{100}) and 42426-derived 42916 (^{150}RKKIHEE-FEAYKDVYVEVDT^{169}) scrambled peptides did not bind to cells, thereby confirming that *B. bovis* MSA-1 peptide-binding interaction occurred dependent of aa sequence order (Supplementary figure 2). Such data supports the notion that *B. bovis* MSA-1 uses the amino acid sequences covered by these three peptides located on its amino-terminal end to interact with bovine erythrocytes, as has been reported for other apicomplexan parasites (Patarroyo et al., 2020; Patarroyo et al., 2011).

3.3. B. bovis MSA-1 binding peptides interacted with a sialoglycoprotein

B. bovis MSA-1 specific binding to a bovine erythrocyte receptor was evaluated by binding assays, using trypsin-, chymotrypsin- and neuraminidase-treated cells (Fig. 3). Analysis showed that peptide 42422 binding activity became completely reduced when erythrocytes were treated with trypsin, whilst peptide 42424 interaction with cells became reduced by 100% using chymotrypsin-treated cells. Peptide 42426 binding profile was 94% affected by trypsin treatment, 100% by chymotrypsin and 64% by neuraminidase. These findings confirmed B. bovis MSA-1 functionality and supported the notion that it contains binding regions for different sites on a protein receptor containing sialic acid residues, such as sialoglycoproteins.

3.4. MSA-1 is a highly helical protein and had B-cell epitope regions

Secondary structure *in silico* prediction (using different bioinformatics tools) stated that MSA-1 is rich in helical moieties, mainly in the regions between aa 3–20, 43–60, 78–95 and 118–145 whilst its C-terminal region is mostly unstructured (Fig. 4). Peptides 42422 and 42426 were highly helical whilst 42424 made a significant random coil contribution to the structure. Interestingly, peptide 42424 contained a potential B-cell epitope region (MIRPDL residues) whilst 42426 was predicted to be a completely B-cell epitope (Table 2 and Supplementary figure 3).

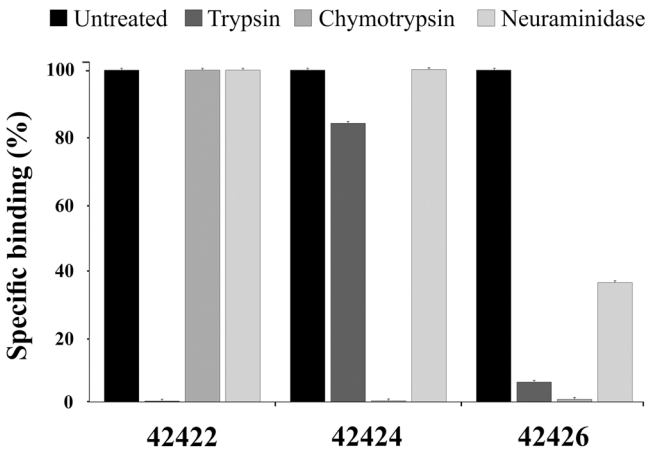

Fig. 3. : *B. bovis* MSA-1 42422, 42424 and 42426 peptides' binding to enzymatically-treated cells. The specific enzyme-treated erythrocyte binding percentage is represented by a bar. Untreated cells were used as control. Standard deviations were below 5%.

4. Discussion

B. bovis initial adhesion to erythrocytes involves VMSA family antigens; MSA-1 is of interest given that it is abundantly expressed on parasite membrane surface (Hines et al., 1995; Reduker et al., 1989) and Ab against it are able to partially block Mrz/Spz invasion *in vitro* (Suarez et al., 2000). Despite this, no study has confirmed that this protein, or fragments derived from it, play a critical role during initial contact with target cells. This work was thus aimed at reporting which *B. bovis* MSA-1 regions participate in binding to bovine erythrocytes.

Negative selection signatures and regions having functional constraint were thus predicted, since these have been used as important parameters in the search for functional regions involved in parasite-host interaction in other apicomplexan parasites (Patarroyo et al., 2020; Patarroyo et al., 2011). A *B. bovis msa-1* orthologue in *B. orientalis* having 20% aa identity was identified, similar to that for other VMSA family members (Hines et al., 1992; Suarez et al., 2000). Despite weak sequence identity, homology between *msa-1* and *msa-2* has been suggested (Amos et al., 2022; Hines et al., 1992), with *msa-1* being at least distantly related to the other VMSA genes. Analysing *B. bovis msa-1, msa-2a1, msa-2a2, msa-2b* and *msa-2c*, as well as *B. orientalis msa-1* sequences, confirmed that they had a significant similarity and shared a large domain that encompassed most of the sequence. However, *msa-2a1, msa-2a2* and *msa-2b* were excluded given that they had a different evolutionary pattern (Supplementary figure 1). Despite great divergence amongst *B. orientalis msa-1* and *B. bovis msa-2c* sequences, they had a similar evolutionary rate along the gene (Supplementary figure 1) and lacked strong positive selection. Considering that homologous genes that undergo adaptive evolution towards other functions reveal positive selection action (Long and Langley, 1993), only *B. orientalis msa-1* and *B. bovis msa-2c* sequences were used for natural selection analysis in order to identify regions having potential functional restriction and negative selection signals at the *B. bovis msa-1* locus. It is worth noting

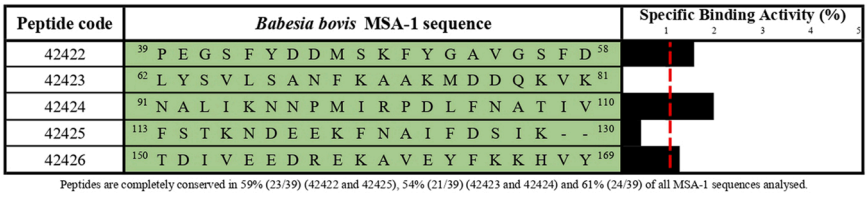

Fig. 2. *B. bovis* MSA-1 peptide bovine erythrocyte binding activity. The conserved peptide aa sequence (green) and cell binding activity percentages are shown (black bars). Peptides having ≥ 1% binding percentage (red line) were considered HABPs. Analysis was carried out in triplicate.

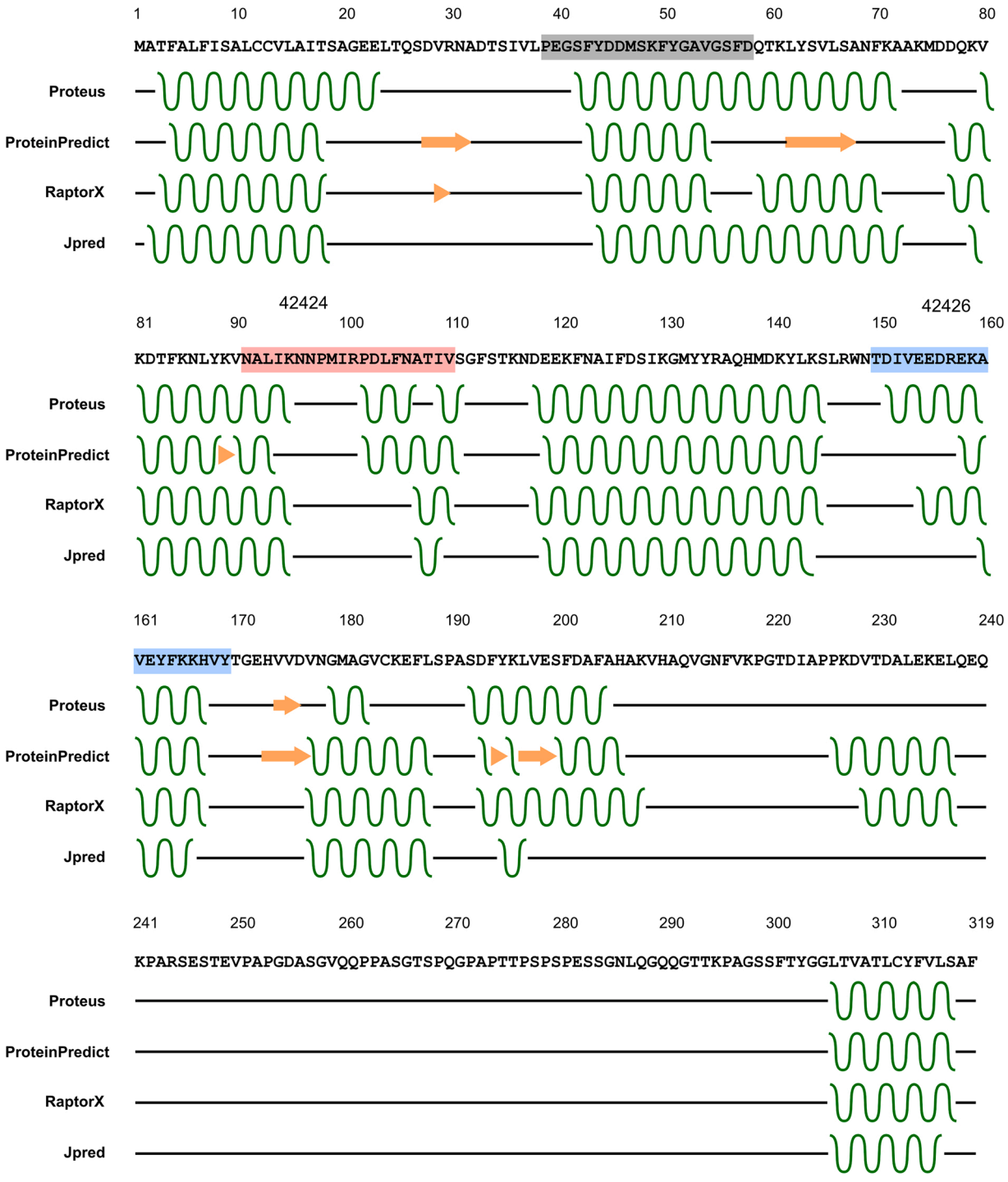

Fig. 4. MSA-1 secondary structure prediction. The figure shows the MSA-1 aa sequence, secondary structure elements (alpha helices in green and beta-sheets in orange) using different bioinformatics tools. Peptide 42420 is shown in grey, 42424 in pink and 42426 in blue.

Table 2
MSA-1 B-cell epitope prediction.

Protein region	Amino acid sequence
21–41	GEELTQSDVRNADTSIVLPEG
74–81	KMDDQKVK
99–104	MIRPDL
112–120	GFSTKNDEE
141–178	KYLKSLRWNTDIVEEDREKAVEYFKKHVYTGEHVVDVN
208–298	KVHAQVGNFVKPGTDIAPPKDVTDALEKELQEQKPARSESTEVPAPGDASGVQQPPASGTSPQGPAPTTPSPSPESSGNLQGQQGTTKPAG

that a conservative approach was used for such polymorphic locus, considering both selection signals within *B. bovis* and between homologues and most of the selected codons (especially positively selected ones) were common between the two analyses. The d_N/d_S sliding window for relationships between *B. orientalis msa-1* and *B. bovis msa-2c* genes and *B. bovis msa-1* revealed very similar natural selection patterns. Two regions had < 1 d_N/d_S values, both between homologues and within *B. bovis*, and had most of the negatively selected sites, suggesting that these regions may be functionally important in erythrocyte adhesion.

Five non-overlapping peptides derived from the *B. bovis* MSA-1 region containing the most sites having negative selection and < 0.75 omega value were thus selected for identifying which of them specifically interacted with bovine red blood cells using a sensitive and specific binding assay. It was found that three *B. bovis* MSA-1 amino-terminal derived peptides (42422, 42424 and 42426) specifically bound to bovine erythrocytes, dependent of their aa order (Fig. 2 and Supplementary Figs. 1 and 2). Despite this, it was found that peptides were variable, peptide 42422 being conserved in 59% of all sequences (23/39), peptide 42424 in 54% (21/39) and peptide 22426 in 61% of them (24/39). Interestingly, peptide 42426 was located in a hydrophobic region and contained a highly conserved YFK motif in all strain/isolates sequences previously considered to be essential for protein function (Leroith et al., 2005). These findings suggested that MSA-1 plays a functional role (despite sequence polymorphism) that could be governed by regions less than 20 aa-long, ^{51}YGA^{53} from 42422 and ^{163}YFK^{157} from 42426 being interesting motifs for evaluating its role in target cell attachment since they are completely conserved in all strain/isolate sequences. Future work focused on confirming such hypothesis is thus needed.

Several erythrocyte receptors required for apicomplexan infection have been determined by evaluating parasite/protein binding effects by pre-treating erythrocytes with proteases. Binding assays using enzyme-treated target cells were thus carried out to identify whether MSA-1 could bind to a specific receptor. It was found that trypsin treatment completely reduced peptide 42422 binding properties and chymotrypsin those of 42424, whilst peptide 42426 was completely inhibited by trypsin and chymotrypsin and 64% when using neuraminidase (Fig. 3). Such findings support the notion that the MSA-1 protein could bind to a protein receptor having sialic acid residues (probably a sialoglycoprotein), thereby agreeing with previous studies where *B. bovis in vitro* growth was significantly affected by such protease treatment (Cursino-Santos et al., 2014; Lobo, 2005). These results suggested that MSA-1 regions were able to interact with different receptor sites to ensure complete parasite adhesion, as has been confirmed for AMA-1 (Cuy--Chaparro et al., 2021), thus explaining the discrepancies found in several studies regarding protease-sensitive or -resistant *in vitro* invasion (Gaffar et al., 2003; Takabatake et al., 2007).

It has been shown that *B. bovis* MSA-1 has failed to stimulate protective immunity and there is thus a need for identifying novel regions having the potential to be B- or T-cell epitopes. Several regions on MSA-1 were thus predicted as potential B-cell epitopes (Table 2 and Fig. 4) which is consistent with this protein region's surface exposure in *in silico*-predicted MSA-1 structure (Lollier et al., 2011). The fact that peptide 42422 was a HABP (having the ^{51}YGA^{53} motif), and did not contain B-cell epitope regions, suggested that it is probably related to protein-protein or receptor-ligand interaction, as shown in other apicomplexans given that binding regions are not able to induce an immune response (Patarroyo et al., 2011). Peptide 42422 and 42426 structural conservation and selectivity landscape, along with binding properties and potential B-cell epitope regions (42426), highlighted their potential usefulness in a universal, peptide-based synthetic vaccine against *B. bovis*. Future studies evaluating peptide 42422 and 42426 ability to induce a cross-protective immune response (probably motif-mediated) could contribute towards confirming such hypothesis.

5. Conclusions

This study has highlighted natural selection analysis for predicting functional regions which are significant for protein binding. Three peptides having functional constraint and a predominance of negatively selected signals were identified for the first time in *B. bovis* MSA-1, along with their ability to interact with bovine erythrocytes *via* a protein receptor containing sialic acid residues. Future trials aimed at evaluating these peptides' antigenic capability during natural infection, and Ab ability to cross-block *B. bovis* MSA-1 binding to bovine erythrocytes and their correlation with *in vitro* invasion inhibition assays are necessary for determining their importance for inclusion when designing a multi-epitope synthetic vaccine against *B. bovis*.

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CRediT authorship contribution statement

Darwin Andrés Moreno-Pérez: Conceptualisation, Formal analysis, Writing – review & editing, Project administration. **Gabriela Arévalo-Pinzón:** Conceptualization, Methodology, Formal analysis, Project administration. **Laura Cuy-Chaparro:** Methodology, Formal analysis, Writing – original draft. **Laura Alejandra Ricaurte-Contreras:** Methodology. **Michel David Bohórquez:** Methodology, Writing – original draft, Formal analysis, Data curation. **Gabriela Arévalo-Pinzón:** Methodology, Formal analysis, Project administration. **Adriana Barreto-Santamaria:** Methodology. Methodology. **Laura Pabón:César Reyes:** Methodology, Formal analysis, Data curation, Writing – original draft. **Manuel Alfonso Patarroyo:** Writing – review & editing, Project administration, Funding acquisition. All authors have read and agreed to publishing this version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data presented in this study is available within the article and the supplementary material.

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Institutional review board statement

This study was conducted according to the guidelines for the care of large animals established by U.D.C.A's research ethics committee, using the protocol described in the minute No. 20190.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2022.109834.

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