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Original article

Subolesin gene structure and mRNA isoform diversity in South African *R. microplus* ticks: Relevance for understanding subolesin-based tick vaccines

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ABSTRACT

Designing a universal vaccine against ticks, capable of protecting a wide range of species, has long been an appealing goal. One antigen that has been proposed for a universal tick vaccine is Subolesin. Despite its intracellular and mostly nuclear location, this antigen has seen some success in bovine vaccine trials. The mechanism behind the observed efficacy remains elusive and may be due to various isoforms being produced in tick cells. By means of RNA sequencing and mapping to the annotated genome of *R. microplus*, this study confirms the presence of a single *subolesin* gene along with four distinct transcripts, resulting in three protein variants. However, none of the putative protein variants have extracellular location signals or known functional motifs. Furthermore, this study offers insights into the antigenic diversity of Subolesin isoforms and their expression across multiple life stages in *R. microplus* ticks from South Africa. This study also raise the question regarding the contrast between Subolesin's nuclear location, function, and its observed efficacy in bovine vaccine trials as an antigen accessible to the host immune system. Future studies evaluating antisera cross-reactivity with other tick proteins is therefore essential to fully understand subolesin as a protective antigen.

1. Introduction

Chemical acaricides have long formed the cornerstone of tick control. However, the exclusive long-term use of acaricides is not feasible, since ticks quickly develop resistance against chemical acaricides after their introduction due to their short generational times in conjunction with incorrect use of acaricides (Obaid et al., 2022). Moreover, chemical acaricides negatively impact the environment and are therefore unsustainable as the exclusive control measure for ticks (Santos et al., 2018). Although several complementary measures that combine the use of biological control, growth regulators, cultural methods and ingested medications have been investigated (Showler and Saelao, 2022), one of the most promising and widely investigated candidates remains the use of vaccines targeting ticks.

The development of tick vaccines is ongoing and many antigens have been evaluated in bovine trials as listed and review in numerous papers (Abbas et al., 2023; Bishop et al., 2023). One of these antigens is the tick Subolesin. Several RNA interference studies demonstrated that Subolesin is crucial for tick reproduction, survival, and overall function (de la Fuente et al., 2006a; Kocan et al., 2007; Nandy et al., 2023; Nijhof et al., 2007). Subolesin was evaluated in vaccine trials, with some success (Almazán et al., 2010; Almazán et al., 2012; de la Fuente et al., 2010;; Schetters, 2014). Typically, tick vaccine antigens elicit protection against ticks by inducing a humoral immune response in the host, where after antigen specific antibodies can recognise and interfere with accessible tick proteins. These include extracellular proteins exposed to the host immune system as secreted proteins (in saliva) or through expression on tick cell membranes (Nuttall et al., 2006). However, since Subolesin is an intracellular protein (Antunes et al., 2014; Trentelman et al., 2019) proposed to function in the nucleus (Artigas-Jerónimo et al., 2018), the manner through which immunological protection against tick infestations is achieved remains controversial.

One possible explanation could be that a different variant of Subolesin, which is exposed to the host immune system, is produced. A time-efficient and high throughput way of investigating this is by using RNA sequencing data (Gao et al., 2017). This approach has several other advantages: (1) analysing transcriptome data generated by RNA-sequencing does not require a reference genome (Wang et al., 2009). This is especially beneficial in ticks, since the most complete *R. microplus* genome assembly is still only partially annotated and has

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highly repetitive content (Jia et al., 2020); and (2) RNA-sequencing data generates more complex transcriptome data that can provide insights into transcript splicing isoforms as well as their expression levels and diversity across geographical areas (Ozsolak and Milos, 2011).

To date, the *subolesin* gene structure has only been elucidated in *I. scapularis* (Naranjo et al., 2013), with preliminary studies predicting the exon/intron organization in other tick species (de la Fuente et al., 2006b; Hu et al., 2014). Elucidating the gene structure and transcriptional regulatory sequences of *subolesin* in *R. microplus* could offer important insights into whether there could be more than one copy of the *subolesin* gene or alternative splicing resulting in alternative Subolesin variants (e.g. mRNA/protein isoforms). Therefore, this study used transcriptome data from field collected *R. microplus* ticks as well as a laboratory reared strain to determine on both a gene and transcript level whether an alternative *subolesin* variant(s) are expressed that is accessible to the immune system e.g. secreted or exposed on a cell membrane, which could explain the observed efficacy in bovine vaccine trials.

A significant challenge to vaccine efficacy is antigenic diversity within and between tick populations (Parizi et al., 2023). Although the amino acid sequence of Subolesin is highly conserved, there is diversity between genera. Antigenic diversity can significantly influence the recognition of epitopes, with a single amino acid change having the potential to abrogate recognition (Rojas, 2022). Furthermore, amino acid changes can alter the sequence and structure (also conformation) of proteins, thereby potentially impacting both continuous and discontinuous epitopes (Prabantu et al., 2020). Therefore, evaluating the antigenic diversity of Subolesin, especially in precited epitope regions, is an important step in predicting possible cross-protective response(s). In this study, we used RNA sequencing to analyse the mRNA expression and diversity of *subolesin* in *R. microplus* field samples from South Africa (Mpumalanga) and a laboratory-reared strain, comparing them to global sequences.

2. Materials and methods

2.1. Tick collection and tick dissection

Engorged or partially engorged adult female *R. microplus* ticks were collected from cattle at five communal dip stations in the Mnisi area, a closed community in Mpumalanga where cattle movement is restricted due to the presence of highly infectious FMD (foot-and-mouth disease), especially in buffalo, and the frequent contact between livestock and wildlife in the area(Supplementary Figure S1). Tick collection was performed according to the terms and provisions of the National Environmental Management: Biodiversity Act (Act of 2004, permit number: 2787NT (12/11/1/1). Dissections were performed with the help of Dr C. Stutzer and Miss. M. Archary. Briefly, ticks were washed in SABAX Pour Saline (0.9 %) solution before dissection. All internal organs were gently removed from the exoskeleton. The organs were rinsed in SABAX Pour Saline (0.9 %) solution, and the individual organs (salivary gland, ovary, and gut) were collected and immediately placed in TRI Reagent® (Catalogue number: T3809–25ML, Sigma-Aldrich, USA) and stored at −80 °C. Ticks (partially and fully engorged) were randomly selected for each dip station and a minimum of tissues from ten ticks were pooled per dip station.

2.2. RNA isolation and RNA-Sequencing

Total RNA was extracted using a modified TRI Reagent® (Catalogue number: T3809–25ML, Sigma-Aldrich, USA) protocol followed by purification using the RNeasy® Mini Kit (Catalogue number: 74,104, Qiagen, Germany). A minimum of 2 μg of total RNA was precipitated using 1/10 vol of sodium acetate (3 M, pH 5.2) and 2.5 vol of absolute ethanol (v/v). Precipitated samples were shipped to Macrogen for RNA sequencing. Briefly, Library preparation was performed using the TruSeq® Stranded mRNA Kit (Catalogue number: 20,020,594, Illumina, USA), which was then sequenced using the NovaSeq 6000 sequencing instrument (Catalogue number: 20,012,850, Illumina, USA). A minimum of 80 million read-pairs per sample was obtained (Supplementary Table S1)

2.3. RNA-Sequencing data quality control and trimming

The quality of the RNA-seq data confirmed using FastQC v0.11.7 (Andrews, 2010). Low-quality reads, Illumina primers and adapter sequences, and the first 11 nucleotides from each read were removed using Trimmomatic v0.36 (Bolger et al., 2014). Once the RNA-seq data was imported into CLC Genomics Workbench (Qiagen, v.23.0.4), reads were trimmed once again using the default parameters.

2.4. De novo transcriptome assembly

The draft genome assembly used in this study (accession number GCF_013339725.1) was created using sequencing data from *R. microplus* ticks from China (Jia et al., 2020). Therefore, to exclude variance due to geographical location, it was necessary to create a South African representative reference against which national variants can be called. A *de novo* assembly with all available for *R. microplus* RNA-sequencing data from the Mnisi area (two timepoints, 3 years apart) and KwaZulu-Natal was prepared (in-house database with sequencing data from >25 locations). Tissue-specific RNA sequencing data from a laboratory-reared *R. microplus* tick (larvae, nymphs and adult life stages) samples (ClinVet Pty Ltd., South Africa) was also included in this study. The *de novo* assembly was created using the *de novo* assembly tool (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=De_Novo_Assembly.html) in CLC Genomics Workbench (Qiagen, v.23.0.4).

2.5. Annotation of subolesin gene, mRNA species and protein variants

The gene location of *subolesin* was determined through BLAST with *the I. ricinus subolesin* sequence (accession number AGI44593.1) against the *R. microplus* genome assembly (accession number GCF_013339725.1) (Jia et al., 2020), which corresponded with the annotated gene location (LOC119175104). The gene features were manually annotated using consensus sequences obtained from literature and IUPAC (Supplementary Table S2). Furthermore, several online prediction tools were used at default settings to predict gene and mRNA features, including TSSW (Solovyev et al., 2010), Nsite (Shahmuradov and Solovyev, 2015) and POLYAH (Salamov and Solovyev, 1997).

Protein domains and features were predicted using several online prediction tools, namely, InterPro v.95.0. (Paysan-Lafosse et al., 2023), SignalP-6.0 (v.6.0.) (Teufel et al., 2022), TMHMM-2.0 (Krogh et al., 2001) and DeepTMHMM (v.1.0.24.) (Jeppe et al., 2022). The secondary structure of each protein variant was predicted using the secondary structure prediction tool in CLC Genomics Workbench (Qiagen, v.23.0.4) (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Secondary_structure_prediction.html). Protein variant 3D structures were created using ColabFold v.1.5.3. (Mirdita et al., 2022) with default parameters.

2.6. Consensus sequence of subolesin RNA species

To obtain a South African *R. microplus* reference sequence for each *subolesin* mature mRNA species, allowing RNA sequencing data to be mapped for national variant detection, a consensus sequence was created. This was achieved by conducting BLASTn (Altschul et al., 1990) using the NCBI-listed sequence of the corresponding mRNA species as the query sequence and the in-house South African *de novo* transcriptome assembly as the database. BLAST parameters were adjusted until the entire query sequence was represented in the BLAST results and the corresponding reads were extracted and used to make a consensus

sequence for each mRNA species. Introns were removed from pre-mRNA sequences (X1–4) to construct mature mRNAs encoding the 3 protein variants and these were used in further analyses.

2.7. Comparison of the expression of subolesin in South African tick populations

The differences in expression between the various mature *subolesin* mRNA species were analyzed using an expression browser (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Create_Expression_Browser.html) created in CLC Genomics Workbench (Qiagen, v.23.0.4) . Samples were normalized and TMM-adjusted log CPM (counts per million) values were calculated for each sample with default settings. The log CPM threshold was determined based on (Law et al., 2018) to exclude mRNA species with <10 read counts and calculated as follows:

$$logCPM\ cutoff = 10 \div median\ library\ size\ (million)$$

The logCPM cutoff value was set as 0.1, meaning that mRNA species values above 0.1 were considered expressed (Supplementary Table S1). Sashimi plots (Katz et al., 2015) were created using Integrative Genomics Viewer (IGV, v.2.16.2) (Robinson et al., 2011) with default parameters.

2.8. Variant detection workflow

The variant detection workflow consisted of extracting RNA sequencing reads from each sample that mapped exclusively to the *subolesin* gene region, before the extracted reads were mapped to the appropriate mRNA species' consensus sequence. Variants were detected using the fixed ploidy variant detection tool (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Fixed_Ploidy_Variant_Detection.html) with the required variant probability set at 90 % and a ploidy of 2 in CLC Genomics Workbench (Qiagen, v.23.0.4).

2.9. Amino acid sequence alignment of subolesin protein sequences

Subolesin amino acid sequences from NCBI (Sayers et al., 2022) were aligned using the MAFFT v7 server (Katoh et al., 2017) with default settings, except for the gap penalty which was increased to 5.0. The alignment was visualised in CLC Genomics Workbench (Qiagen, v.23.0.4). Epitope sequences used in this study is shown in Table 2.

3. Results

3.1. South African de novo transcript assembly

A total of 2656,875,685 forward and 2604,598,983 reverse reads were used in the *de novo* assembly (Supplementary Table S1).

3.2. Annotation of the R. microplus subolesin gene and RNA species

The *R. microplus subolesin* gene (NC_051171.1) occurs on chromosome 7 and is approximately 60,903 nucleotides in length, with five predicted exons and four introns (Fig. 1). This finding contrasts with the gene sequence determined for *I. scapularis subolesin* (Supplementary Figure S2). To confirm the expression of the exons, Sashimi plots indicating the read coverage per exon of the *subolesin* gene were constructed (Supplementary Figure S3) and shown to confirm the above findings.

For the *R. microplus* gene, no TATA boxes were found but two putative arthropod initiation factor sequences (INRs) ([TAG]-C-A-[GT]-T) were identified in the 5′ UTR (Fig. 1). An arthropod initiation factor sequence (INR) was found upstream (−48) of the exon 1 TSS of the X1 mRNA (Fig. 2). Another arthropod INR was found just before exon 2 at position +263, which corresponds to the proposed transcription start site for the X2 mRNA species. Additionally, two more isoforms were identified, bringing the total to four putative pre-mRNA species (X1 - X4, Fig. 2). Only the first pre-mRNA species contains five exons (X1), whereas the other three isoforms (X2-X4) each contain four exons (Fig. 2).

The first three exons of the putative X1, X3 and X4 pre-mRNA species are identical, with a different fourth exon (Fig. 2). Furthermore, the first exon of the X2 mRNA species is a combination of a part of the first and second exon of the X1 mRNA species. The rest of the X2 mRNA variant's exons are identical to that of the X1 mRNA species. A Kozak sequence was found at the same nucleotide position in all pre-mRNA species. Lastly, a polyadenylation signal (Poly-A) was found in the last exon of each of these mRNA species. Of the four putative pre-mRNAs, three unique putative protein variants (named P1 - P3) were identified as translatable (Fig. 3A–C).

Both X1 and X2 putative pre-mRNAs encode the same putative protein variant (P1), which is 161 amino acids in length (Fig. 3A). The X3 pre-mRNA encodes a second putative variant (P2) of 143 amino acids, whereas the X4 pre-mRNA encodes a third putative variant (P3) of 135 amino acids (Fig. 3B and C). Both TMHMM-2.0 and DeepTMHMM predicted no transmembrane regions for any of the *subolesin* variants, suggesting that all three proteins are intracellular. This prediction is

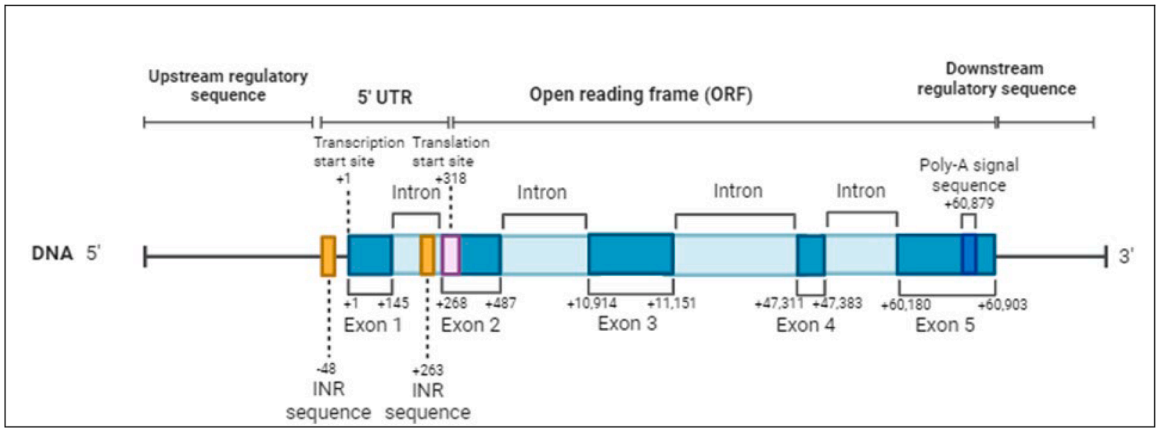


Fig. 1. Schematic representation of the *R. microplus subolesin* gene structure. Different sections of the *R. microplus subolesin* gene are indicated, including the 5′ untranslated region (5′UTR) and the open reading frame (ORF). The *subolesin* gene has five exons and four introns. Transcription initiation sequences (INR) are indicated in orange, and the translation initiation site (Kozak sequence) is indicated in pink. The polyadenylation (Poly-A) signal sequence is indicated in dark blue with its nucleotide position. Image was created with BioRender.com.

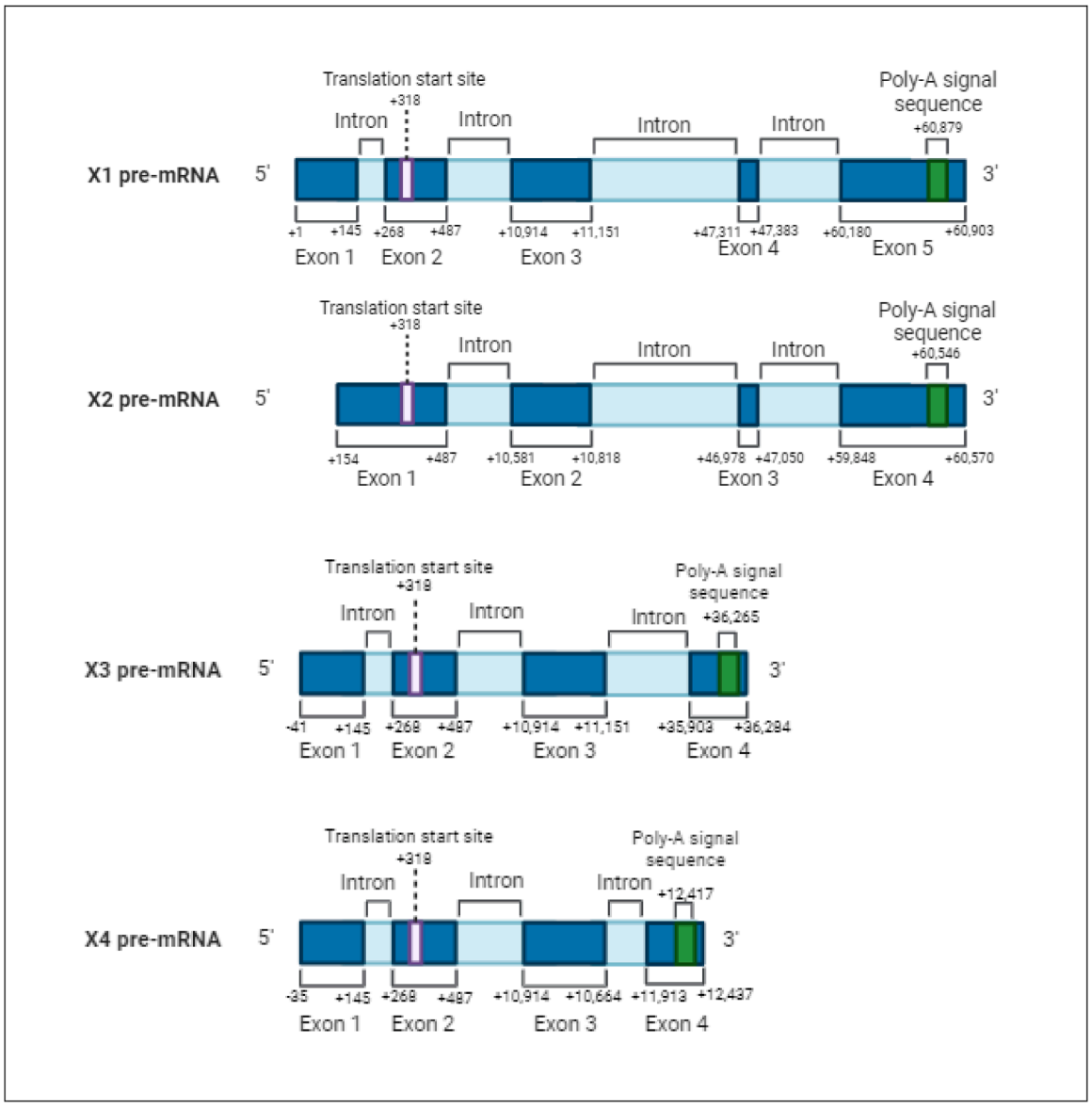


Fig. 2. Schematic representation of four putative *R. microplus subolesin* pre-mRNA species. The pre-mRNA species appear in numerical order (X1-X4) and decrease in length from X1-X4. The translation initiation site (Kozak sequence) is indicated in pink, and each mRNA species' Poly-A signal is indicated in green. Image was created with BioRender.com.

further supported by the lack of signal peptides in these proteins, as determined by SignalP-6.0 (v.6.0.). Along with intracellular location, all protein variants share a nuclear localisation signal (PKRRRC, amino acid position 23–28), as predicted by InterPro (v.9.5.0) (Fig. 3).

In regards to protein structure, a predicted disordered region (position 1–52), followed by a coil region (position 52–72), was identified for all variants using InterPro (v.9.5.0) (Fig. 3). Two additional alpha helices were predicted for each protein variant using the CLC Genomics Workbench platform. The first alpha-helix was predicted to span amino acids 55–76 and was conserved across all protein variants. In contrast, the second alpha-helix was predicted to span amino acids 103–153 for P1, while shorter helices were predicted for P2 and P3, at positions 103–138, respectively. Similarly, two alpha helices are present in the putative P1 3D predicted structure with high confidence scores (pLDDT and PAE) (Supplementary Figures S4, S5 and S6). However, beyond the alpha helix domains, the rest of the structure has very low confidence scores (both pLDDT and PAE). Therefore, no conclusions could be made regarding the possible conformation of the protein or the interactions between the potential domains. Similar results were observed for the two other putative *R. microplus* Subolesin protein variants (Supplementary Figures S4, S5 and S6).

3.3. Expression levels of subolesin in South African R. microplus populations

It is evident from Fig. 4 that the putative X1 mRNA *subolesin* species had the highest expression levels in the ClinVet strain *R. microplus* ticks and was consistently expressed across adult female and immature life stages (nymph and larvae), as well as tissues. Furthermore, the X1 mature mRNA species had higher expression values than the X2 mature mRNA species for most of the other field samples (Fig. 4) except for Utah (UTA) Gut tissues, Welverdiend (WV) and UTA Ovary tissue. However, transcription of the X3 and X4 mature mRNA species are much lower than the X1 and X2 species across all samples (tissues and life stages), with the X3 species having the lowest expression and negligible expression in the gut (SAMPCLA3_Gut) and salivary glands (SAMPUTA_SG) of two samples from Mnisi.

For further support of each pre-mRNA species, a Sashimi plot was

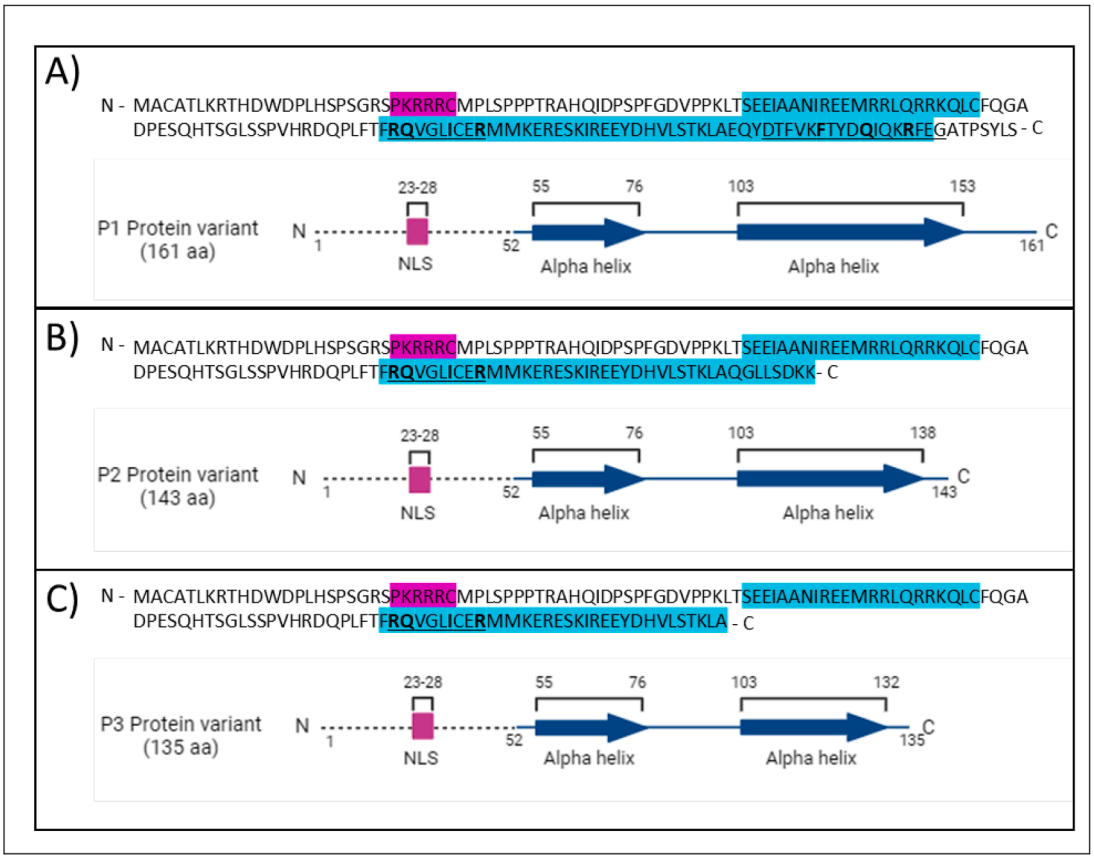


Fig. 3. Figure depicting the putative Subolesin protein variants (P1-P3) amino acid sequences and secondary structure as predicted by different platforms. The amino acid sequences and schematic representations of the **(A)** first (P1), **(B)** second (P2) and **(C)** third (P3) *R. microplus* Subolesin protein variants are shown. Nuclear localisation signal (NLS) sequences are shown in pink, with predicted alpha helices indicated in blue. Amino acid residues that form previously predicted DNA binding sites are underlined (Artigas-Jerónimo et al., 2018). In the schematic diagrams, disordered regions are indicated by dashed lines (1–52) and two alpha helices (blue arrows) are indicated. Domains were predicted by InterPro (v.9.5.0.) and CLC Genomics Workbench (v.23.0.4) and include a nuclear localisation signal (NLS) sequence and alpha helices at the amino acid positions indicated. Schematic created in BioRender.com.

constructed (Supplementary Figure S3) which indicates the number of RNA sequencing reads supporting each intron/exon junction. Table 1 lists the support for each intron/exon junction, and it is evident that between the expression levels of the X1 and X2 pre-mRNA, X2 dominates. This is evidenced by the lower support for the first exon/intron junction of the X1 pre-mRNA species, which is more than two times less than the support for its other intron/exon junctions (Table 1B, E, F). Furthermore, the support for the intron that links to the unique fourth exon of the X3 pre-mRNA species is very low (Table 1 D), with some samples having no supporting RNA sequencing reads for this intron/exon junction. The same trend was observed in the case of the intron/exon junction supporting the unique fourth exon of the putative X4 pre-mRNA species (Table 1C).

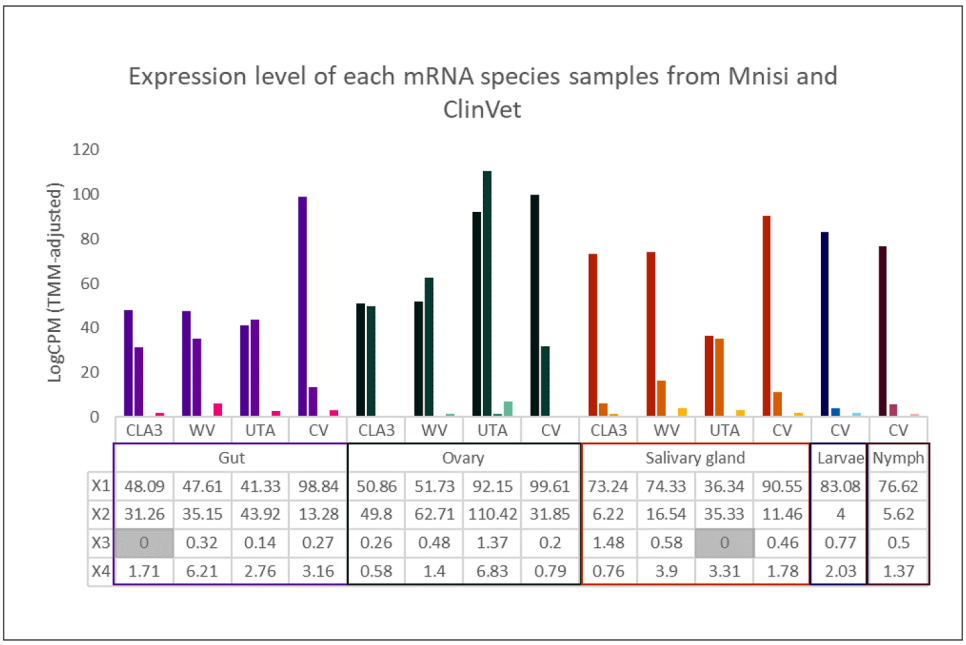
3.4. Variant detection

No variants were detected within the coding sequence (CDS) region of the X1 mRNA species, across all tissues and life stages (Supplementary Figure S7). Variants were detected outside of the CDS, but these variants did not affect the resulting protein. Furthermore, all variants detected were single nucleotide variants (SNVs) and were common across all samples, regardless of life stage, tissue, and sampling location. The same trend was observed in the case of the X2 (Supplementary Figure S8) and the X3 (Supplementary Figure S9) mRNAs, where there were no variants detected within the CDS. Furthermore, all variants detected were outside the CDS region and were either SNVs or deletions for both the X2 and X3 mRNA species. There were many more variants observed for the X2 mRNA species than the X1 and X3 mRNA species. As before these variants were not unique to any sampling location, tissue, or life stage.

Despite the X4 mRNA species having the shortest length, it had the most variants of all the mRNA species (Figure S10). Furthermore, a variant was detected in the CDS of the X4 mRNA species in the ovary tissue sample from the Clare A dip station in Mnisi (SAMPCLA3_OV) (Figure S11). The variant resulted in a non-synonymous change from thymine to cytosine at nucleotide position 639 (639 $T > C$), changing a stop codon to an arginine at amino acid position 136 that resulted in the addition of four more amino acids (i.e., ATTK) before the next stop codon. Consequently, this change leads to a new protein variant that is 140 amino acids long (Figure S11). From the current data, the influence on the predicted tertiary structure of this addition on the new Clare variant is unknown (Supplementary Figure S6).

3.5. Subolesin sequence analysis across global R. microplus data

An alignment of South African and global *R. microplus* Subolesin amino acid sequences is shown in Fig. 5. Previously, linear B cell epitopes and conformational discontinuous epitopes were predicted from the Subolesin *R. microplus* amino acid sequence from Mexico (ABA62329.1, Table 2) (Lagunes et al., 2016). These epitopes were found to be conserved across all South African Subolesin amino acid sequences (P1-P3). The linear B cell and conformational discontinuous epitopes are highly conserved across global *R. microplus* populations, except for some variants that were observed in several sequences from India (position 42) and Mexico (position 80) (Fig. 5). The other variants



	CLA3	WV	UTA	CV	CLA3	WV	UTA	CV	CLA3	WV	UTA	CV	CV	CV
	Gut				Ovary				Salivary gland				Larvae	Nymph
X1	48.09	47.61	41.33	98.84	50.86	51.73	92.15	99.61	73.24	74.33	36.34	90.55	83.08	76.62
X2	31.26	35.15	43.92	13.28	49.8	62.71	110.42	31.85	6.22	16.54	35.33	11.46	4	5.62
X3	0	0.32	0.14	0.27	0.26	0.48	1.37	0.2	1.48	0.58	0	0.46	0.77	0.5
X4	1.71	6.21	2.76	3.16	0.58	1.4	6.83	0.79	0.76	3.9	3.31	1.78	2.03	1.37

Fig. 4. Graph depicting the level of expression of the *subolesin* mRNA species (X1-X4) in different tissues/life stages in tick samples collected from Mnisi and ClinVet. The expression is split by tissue (gut, ovary, salivary gland) and life stage (larvae, nymph) and then location. The Mnisi (Mpumalanga) locations are indicated according to the names of the dip stations where the sample was collected from, namely Clare A (CLA3), Welverdiend (WV) and Utah B (UTA), and the ClinVet samples are indicated as CV. In each coloured division, the mRNA species are arranged by location from dark to light from X1-X4. The expression value of each mRNA species is indicated on the y-axis as the TMM-adjusted logCPM (counts per million), and species with logCPM values above 0.1 were considered expressed. Species below this threshold are highlighted in grey.

Table 1
Support for each intron/exon junction as determined by Sashimi Plot. Each value represents the number of junction RNA-sequencing reads (TPM-normalised) that connect exons to each other.

RNA sequencing reads supporting each intron as indicated in Figure S3						
Sample ID	Intron A	Intron B	Intron C	Intron D	Intron E	Intron F
Rm_Gland	435	902	13	4	638	734
Rm_Gut	323	877	35	1	619	697
Rm_Larvae	454	1014	15	10	625	701
Rm_Nymph	408	887	11	7	596	684
Rm_Ovary	538	1432	14	3	1195	1352
SAMPCLA3_Gut	140	659	10	0	900	586
SAMPCLA3_Ovary	303	1566	26	6	2296	1086
SAMPCLA3_SG	125	500	2	0	765	453
SAMPUTA_Gut	62	599	30	0	851	388
SAMPUTA_Ovary	284	2156	108	0	2889	1380
SAMPUTA_SG	81	541	74	0	1208	536
SAMPWV1_Gut	147	653	107	7	698	386
SAMPWV1_Ovary	257	1862	32	21	2044	1254
SAMPWV1_SG	184	733	62	22	978	680

observed had low frequencies and did not appear in more than one sample. Therefore, these variants could be sequencing errors, and this should be confirmed in future.

As before, the differences in the linear B cell epitopes predicted by Zeb et al. (2024) are present in the *R. microplus* populations from India and Mexico (Table 2, Fig. 5) However, the last predicted epitope region covers the C-terminus (145–161 aa) of the Subolesin amino acid sequence. Since the amino acid length of the *R. microplus* Subolesin variants decrease from P1 to P3, the P2 and P3 variants do not have this terminal epitope. The most variation between *R. microplus* strains were observed in regions without organised secondary structures, whereas regions corresponding to the alpha helices were highly conserved (Supplementary Figures S4-S6).

3.6. Subolesin sequence analysis across genera

As evident from the data, Subolesin is less conserved across tick species from the genera *Rhipicephalus, Amblyomma, Dermacentor, Ixodes, Hyalomma, Haemaphysalis* and *Ornithodoros* (Fig. 6, Supplementary Figure S12). It is evident that the region encoding amino acids 154 to 170 shows considerable overlap between epitopes. This region has been shown to be recognised by IgG antibodies raised in two cattle breeds that were immunized with a cocktail of Subolesin antigens and Subolesin derived from *R. appendiculatus*. In additional studies using *R. appendiculatus, R. decoloratus* and *A. variegatum* this region was shown to be recognised by antibodies from immunisation groups (Fig. 6). It should also be noted that this region has been proposed to overlap with the linear B cell epitope region predicted in *I. scapularis* (Prudencio et al., 2010), but not with epitopes predicted in any other species. Based on the variation in amino acid sequence in epitope regions across genera,

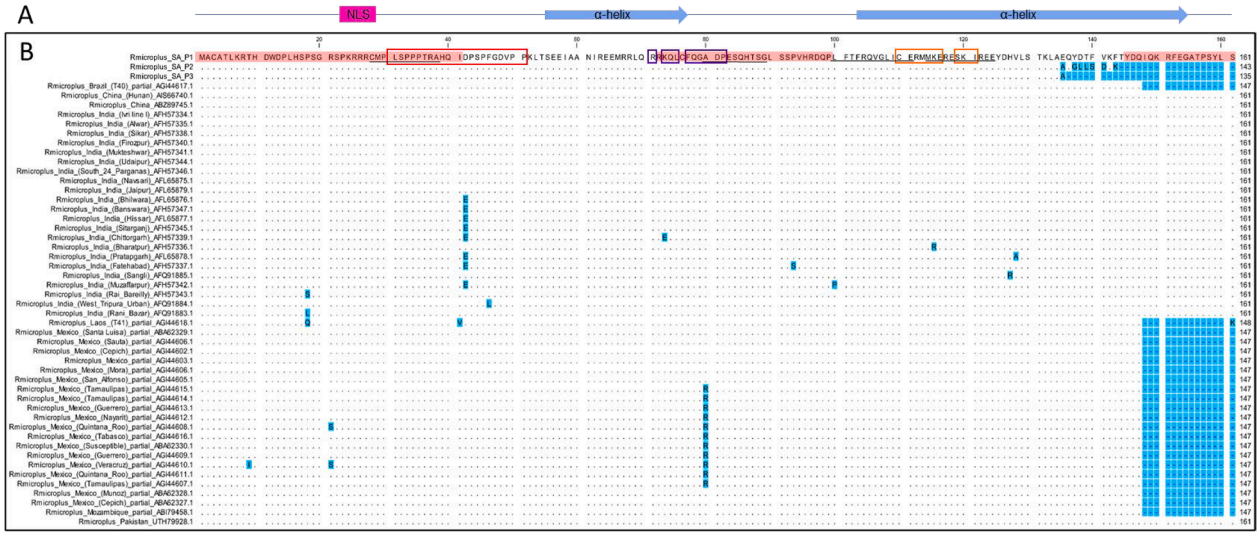


Fig. 5. Amino acid sequence alignment of Subolesin protein from *R. microplus* ticks. (A) The secondary structure of *R. microplus* Subolesin P1 is indicated at the top, with alpha helices (blue arrows) and NLS sequence (pink). **(B)** Subolesin sequences from global *R. microplus* strains were aligned to the representative South African Subolesin sequence from this study (P1). Mismatched/missing amino acids are indicated in blue. Linear B cell epitope regions predicted by Lagunes et al. (2016) are underlined, and conformational discontinuous epitopes are enclosed in red, purple and orange borders. B cell epitope regions predicted by Zeb et al. (2024) are highlighted in light red. Sequences were aligned in MAFFT (v.7.0.) using default settings, except for the gap penalty which was increased to 5.0.

future analyses of cross-reactivity across genera need to be conduct to gain insight into the protective mode of action of the subolesin antigen.

4. Discussion

The prospect of developing a universal tick vaccine has long been enticing. One antigen that has been proposed for such a universal vaccine is Subolesin, a protein that was shown to be conserved across a number of tick species (Artigas-Jerónimo et al., 2018). Silencing of Subolesin expression by RNA interference was shown to impact tick fecundity (de la Fuente et al., 2006a; Kocan et al., 2007; Nandy et al., 2023; Nijhof et al., 2007). To date, all literature indicates that Subolesin is strictly an intracellular protein (Antunes et al., 2014; Trentelman et al., 2019) that functions within the nucleus (Artigas-Jerónimo et al., 2018). However, there is a paradox between using Subolesin as an antigen and its nuclear localization in tick cells, which renders this antigen inaccessible to the host immune response. Typically, the induction of antibody dependent cell mediated cytotoxicity (ADCC) requires antibodies to bind to the target protein on the surface of a cell and recruit additional immune factors to clear the infection/target (Zahavi et al., 2018). Alternatively, in vertebrates it has been demonstrated that antibodies can launch an intracellular immune response if they enter a cell while bound to the invading pathogen (McEwan et al., 2013). However, this has not been shown in ticks.

Although antibodies have been shown to escape the tick midgut and enter the tick haemolymph (Ackerman et al., 1981), the mechanism through which this trans-tissue migration occurs remains to be fully elucidated. In a single study using fluorescent microscopy of *I. scapularis* ISE6 tick cells, the authors showed that anti-actin antibodies do occur in the cytoplasm after an overnight incubation in the absence of cell membrane permeation (de la Fuente et al., 2011). Based on these results, it was proposed that antibodies can cross the plasma membrane of tick cells and bind to intracellular proteins. This statement is controversial, as there is limited evidence suggesting that some antibodies can cross the plasma membrane of cells and enter the cytoplasm and nucleus of tick cells..

Nevertheless, several vaccines using “concealed” antigens – non-secretory proteins typically hidden from the host immune system and located on the surface of tick cells or intracellularly – as opposed to antigens that are “exposed” to the host immune system, such as salivary proteins secreted during feeding, have been evaluated as tick vaccines, with only Bm86 reaching commercialization (Jonsson et al., 2000; Rodriguez et al., 1995; Willadsen et al., 1995). Dual antigens have also been evaluated, which have both secreted and concealed antigenic epitopes (e.g. truncated constructs of the 64P cement protein) whose efficacy was partially attributed to cross-reactive epitopes and not the presence of 64P in the midgut, haemolymph and salivary glands (Havlíková et al., 2009; Trimnell et al., 2005;Trimnell et al., 2002) Therefore, answering the question whether (a) there are splice variants of Subolesin that yield an exposed variant that mediate protection upon vaccination, or (b) if the response raised against Subolesin during vaccination led to a cross-reaction with a protective antigen that has never been identified, remains crucial.

This study elucidated for the first time the gene structure of *subolesin* in *R. microplus* and highlighted several key differences between this gene and the published *I. scapularis subolesin* gene (Naranjo et al., 2013), such as size and discrepancies in transcriptional regulatory sequences (transcription initiation and NF-kB binding sites). Most importantly, this study concluded that there is a single *subolesin* gene in the *R. microplus* genome. Interestingly, this study found that this *subolesin* gene may be transcribed into four mRNA species (X1-X4), which arise due to alternative transcription start sites and/or alternative splicing (Fig. 2, 3 and S3). However, these putative transcripts only yield novel three protein isoforms, since the first two mRNA variants (X1 and X2) are translated into the same protein. Significantly, all three protein variants had nuclear localisation signal sequences and may not be accessible to the host immune system, as per current evidence. Therefore, although these findings should be confirmed in future using Long-Read Sequencing (LRS) (Branton et al., 2008; Rhoads and Au, 2015), these results indicate that there is not a gene or transcript level explanation for the observed efficacy of Subolesin in bovine vaccine trials. Future studies should investigate potential cross-reactivity of anti-Subolesin antibodies with other tick proteins. This could for instance be done by performing a Western blot using polyclonal antisera raised after vaccination with recombinant full-length Subolesin.

Subolesin was determined to be expressed across all life stages of

Table 2
Amino acid sequences of the predicted epitopes of Subolesin from different tick species. The epitope type is given, along with the species it was identified in, as well as the sequence of the epitope and its position in the alignment depicted in Figure 5.

Epitope type	Species	Sequence and position	Reference
Linear B cell	*R. microplus*	28-CMPLSPPPTRA-46 85-QGADPESQHTSG-119 130-LFTFRQVGLICE-141 143-MKERESKIREE-154	(Lagunes et al., 2016)
		1-MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQI-46 77-RKQLCFQGADPESQHTSGLSSPVHRDQP-112 175-YDQIQKRFEGATPSYLS-191	(Zeb et al., 2024)
	R. appendiculatus	28-CMPLSPPPTRAHQID-47 67-IREEMRRLQRRKQLC-81 117-TSGLSSPVRRDQPLF-131 128-QPLF-131 148-ESKIREEYDHVLSTKLAEQYDTFVKFTYDQI-178 151-IREEYDHVLSTKLAEQYDTF-170	(Contreras et al., 2022)
	R. decoloratus	40-PTRAHQIDPSPFGDVP-55 117-TSGLSSPVHRDQPLF-131 126-RDQPLFTFRQVGLIC-140 143-MMKERESKIREEYDHVLSTKLAEQYDTFV-171 151-IREEYDHVLSTKLAEQYDTFVKF-173 153-EEYDHVLSTKLAEQYDTFV-171	
	A. variegatum	49-SPFGDVPPKLTSEEI-63 73-RLQRRKQLCFQGAEC-89	
Linear B cell	*A. variegatum*	80-LCFQGAECSSPEGC-105 91-SSPEGCSP-107 117-TSGLLSPVRRDQPLF-131 149-SQIREEYDHVLSTKLAEQYDTF-170 151-IREEYDHVLSTKLAEQYDTF-170 154-EYDHVLSTKLAEQYDTF-170	(Contreras et al., 2022)
	O. erraticus	9-THDWDPLHSPNGRASKRRRCV-29 13-DPLHSPNGRASKRR-26 32-CVSPSTAPARAHQMSPSPFVEVPPK-57 54-VPPKLSS-60 65-ANIREEMRRLQR-76 83-TALDSSPQNSSTDSCSGPSSPT-115 107-CSGPSSPTGASGLLSPVRRDQP-129 119-GLSPVR-125 141-ERMMKERE-148	(Manzano-Román et al., 2015)
	O. moubata	8-RTHDWDPLHSPNGRASKRRRCVPLCVSPSTAPARAHQMSPSPFVEVP-56 13-DPLHSPNGRASKRRR-27 54-VPPKLSS-60 65-ANIREEMRRLQR-76 86-DSPPQGTSGSSAD-115 92-GTSGSSADSPTGLLSPVRRDQP-129 141-ERMMKERE-148	
	I. scapularis	128-QPLFTFRQVGLICERMMKERESKIR-152 160-SAKLAEQYDT-169	(Prudencio et al., 2010)
Conformational discontinuous*	*R. microplus*	31-**LSPPPTRAHQIDPSPFGDVPPK**-57 76-**RRKQL**CF**QGADP**-112 140-**CERMMKE**RE**SKI**-151	(Lagunes et al., 2016)
	I. scapularis	1-**M**ACAT**L**K**R**T**HDW**DP**L**H**SPNGR**S**PK**…**SPF**GEV**P**P**K**…**SPTGLSP**GG**LS**-122 82-**F**SS**PLE**SGSPSAT**PP**ADCGGPASPTGLSPG**G**LLSPVR**R**-126 181-**RFEGATP**SY**L**-191	(Prudencio et al., 2010)

*Predicted epitope regions of the possible conformational discontinuous epitopes are underlined and indicated in bold.

R. microplus ticks, which is consistent with findings in other tick species (Lu et al., 2015; Sultana et al., 2015). However, there were no notable differences between adults and other developmental stages identified in this study (Fig. 4). This expression pattern contradicts what was found in "soft" ticks (*Ornithodoros turicata)*, where *subolesin* had significantly higher expression levels in adult life stages, compared to larvae and nymphs (Sultana et al., 2015). However, there does not seem to be a conserved expression pattern for the life stages in "hard" ticks either, as it was shown that *subolesin* was highly expressed in *R. haemaphysaloides* eggs compared to other life stages (Lu et al., 2015). Whereas for *Haemaphysalis flava, subolesin* had significantly higher expression levels in the engorged adult females (Liu et al., 2016). Moreover, the expression of *subolesin* in this study was relatively conserved across tissues similar to findings in *R. haemaphysaloides*, but contrary to findings in *H. flava* (Liu et al., 2016; Lu et al., 2015). Therefore, the expression of *subolesin* seems to vary on a species level.

Since Subolesin is a highly conserved protein, the results from variant detection were unsurprising. There were no variants detected within the CDS of the X1 and X2 transcripts, coding for the full-length Subolesin protein (possibly the main functional species). This coincides with the 'knockdown rate' hypothesis, which posits that the greater the effect a gene knockout has on overall fitness, the slower the evolutionary rate (Wilson et al., 1977). Several studies have shown that knockdown of Subolesin results in tick mortality and significantly lower fertility rates, therefore, it would be possible that the *subolesin* gene evolves slowly (de la Fuente et al., 2006a, 2005, 2006c; de la Fuente et al., 2008). Furthermore, since Subolesin (tick akirin) is conserved across the metazoa, it most likely has a low propensity of gene loss (Artigas-Jerónimo et al., 2018; Krylov et al., 2003). In eukaryotes, genes with this characteristic are associated with a lower rate of sequence evolution, high expression and are more likely to have several interacting partners (Krylov et al., 2003). Therefore, considering the findings from aforementioned studies and the findings from this study, further support has been provided for *subolesin* as an essential gene for tick

Fig. 6. Amino acid sequence alignment of the Subolesin protein from different tick species. Subolesin sequences from tick species from the genera *Rhipicephalus, Amblyomma, Dermacentor, Ixodes, Hyalomma, Haemaphysalis* and *Ornithodoros* were aligned to the representative South African Subolesin sequence from this study (P1). Residues matching the P1 sequence are indicated as black dots, missing residues are indicated as blue dashes and mismatched residues are indicated as the corresponding single letter amino acid code. Linear B cell epitope regions predicted by BCEpred, Antigenic, ABCpred, and IEDB (at least three) are underlined in black, and correspond to the epitope regions contained in the Subolesin peptide vaccine evaluated in calves (Lagunes et al., 2016), and conformational discontinuous epitopes are enclosed in a red, purple and orange borders. B cell epitope regions predicted by IEDB are highlighted in light red (Zeb et al., 2024). Linear B cell epitope regions were predicted using BcePred (highlighted in dark green) and ABCPred (underlined in dark green) for *Ornithodoros* spp (Manzano-Román et al., 2015). Conformational discontinuous epitopes predicted through epitopes peptides identified from IgG raised in sera in rabbits and sheep immunized with Subolesin, including conformational discontinuous (highlighted in dark blue, pink and yellow) and linear B cell (highlighted in orange) epitopes (Prudencio et al., 2010). Epitope regions recognised by IgG from crossbred (highlighted in grey) and *Bos indicus* (light blue border) cattle immunized with SUB cocktail, and crossbred cattle (black border) and *Bos indicus* (underlined in pink) immunized with SUBra (*R. appendiculatus* Subolesin antigen) (Contreras et al., 2022). The amino acids that constitute the Q38 chimera is enclosed in a dark blue border (Contreras et al., 2022). The sequence of the epitopes used in this figure are listed in Table 2. Sequences were aligned in MAFFT (v.7.0.) using default settings, except for the gap penalty which was increased to 5.0.

survival.

In regard to tick vaccine development, a significant challenge impacting vaccine efficacy is antigenic variation, especially within epitope regions (Parizi et al., 2023). As expected, Subolesin was highly conserved within genera, with the most variation occurring between species from different genera. This could explain the significant differences observed in protection elicited after against tick species different from the tick species the antigen was derived from (Kasaija et al., 2020; Torina et al., 2014). Moreover, the epitopes that were the most conserved were those recognized in sera from vaccinated animals, and the *in silico* predicted epitopes only showed partial overlap with the epitopes recognized in sera from vaccinated animals (Contreras et al., 2022; Prudencio et al., 2010). These results indicate that current *in silico* epitope predictions are limited and could be improved by incorporating bovine specific databases (i.e., bovine specific MHCI and MHCII allele data) and immunoinformatic tools (Vasoya et al., 2021; Fisch et al., 2021).

5. Conclusion

This study demonstrated that there is a single *subolesin* gene in the *R. microplus* genome and that four possible mRNA species can be transcribed. All three predicted *R. microplus* Subolesin protein variants had nuclear localization signals, which is consistent with previous findings. Furthermore, signal peptide sequences were absent from all putative protein variants. Therefore, the paradox between Subolesin's cellular location and observed efficacy as a vaccine candidate cannot be explained on a genome or transcriptome level. Future studies should aim to conduct proteomics or targeted protein sequencing of subolesin to verify the translation of the mature mRNAs detected, using RNA sequencing, in this study. Additional investigations into potential cross-reactivity of anti-Subolesin antibodies with other tick proteins can also aid to explain the mode of protection upon vaccination with subolesin.

CRediT authorship contribution statement

Elsje Christine Rabie: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Christine Maritz-Olivier:** Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Data curation, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2025.102502.

Data availability

Data will be made available on request.

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