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The Enigma of Identifying New Cattle Tick Vaccine Antigens

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Abstract

Several reviews have summarised cattle tick *Rhipicephalus (Boophilus) microplus* vaccine candidate discoveries by comparing efficacies and localisation characteristics. However, few have re-analysed all the reported proteins using modern bioinformatics tools. Bm86 was developed as a successful vaccine in the 1980s; however, global efficacies vary from 45 to 100%. Subsequent vaccines, including four published patents, were discovered by targeting enzymes important for blood digestion and/or metabolism or by targeting genes shown to disrupt tick survival following RNA interference experiments. This chapter analyses published vaccine candidates using InterPro, BLASTP, SignalP, TMHMM and PredGPI tools to confirm whether each reported protein is likely to be secreted, membrane associated or intracellular. Conversely, these proteins are considered as 'exposed', 'exposed' and 'concealed' or 'concealed', respectively. Bm86 was always described as a 'concealed' antigen; however, the protein has a confirmed signal peptide and GPI anchor which suggests it is anchored to the cell membrane and exposed on the surface of gut cells. It is the only tick vaccine with a GPI anchor. Secreted vaccine candidates appear to have promise and exhibit higher efficacies if delivered with an 'intracellular'/'concealed' antigen. Improvements in tick genomics and bovine immunomic resources will assist to identify robust new cattle tick vaccines.

Keywords: cattle tick, vaccines, bioinformatics, Bm86, review, *Rhipicephalus microplus*

1. Introduction

Cattle ticks (*Rhipicephalus (Boophilus) microplus*) and the diseases they carry affect almost 80% of the world's population of domestic cattle at an economic burden approximately \$US 25–30 billion per annum [1]. The *R. (B.) microplus* taxonomic status is based upon *Cytochrome c oxidase I (COX1)* mitochondrial gene sequencing. There are three clades of *R. (B.) microplus*, plus *R. (B.) australis* and *R. (B.) annulatus* which are monophyletic with a different *R. (B.) microplus* clade [2, 3]. A recent study expanded this analysis and showed that *R. (B.) australis* is most similar to a large *R. (B.) microplus* clade (A) which has worldwide distribution, whereas *R. (B.) annulatus* is similar to *R. (B.) microplus* clade B predominantly from China [4]. An additional *R. (B.) microplus* clade C consists of Malaysian and Indian isolates [3]. Separation of species from several continents using morphological characters was not consistent with the above COI sequence clades and suggested that in some regions there exists a mixture of both *R. (B.) microplus* and *R. (B.) australis* [4]. It has been noted that more crossing studies need to be undertaken using geographically diverse wild strains

and preferably not ‘inbred’ colony isolates of *R. (B.) microplus* before conclusions on clades and species relationships can be confirmed. Publications and sequences reviewed here are most likely to be from different *R. (B.) microplus* clades and *R. (B.) australis* but will be referred to collectively as *Rhipicephalus microplus*.

Regardless of the above seemingly complicated taxonomic status, the treatment of cattle tick infestations is either addressed by vaccination using Bm86-based vaccines: TickGARD^{PLUS} (now discontinued) or GAVACTM and most commonly through the application of chemical acaricides [5]. Bm86 vaccines have diverse efficacies reported worldwide (45–100%), but in a few isolated countries, the vaccines have worked well apart from the need for multiple annual boosts to achieve adequate efficacies [1, 5, 6]. Ticks are also quite capable of developing resistance to acaricides; thus vaccine research continues globally [7] to identify conserved and immunogenic alternatives to Bm86.

The first notion that tick guts could be the source of viable tick vaccines was reported in 1979 [8] where native tick gut and organ extracts protected guinea pigs and cattle from *Dermacentor andersoni* ticks. The authors also suggested that this vaccine would affect tick feeding and reproduction and would be ideal for ‘*Boophilus microplus*’ as all tick stages feed on the same host [8]. A gut protein named Bm86 was discovered in the 1980s as a protective antigen isolated from *R. microplus* in Australia [9]. The most notable characteristics at this time was the presence of epidermal growth factor (EGF) domains which are highly conserved extracellular domains associated with membrane-bound or secreted proteins (<https://www.ebi.ac.uk/interpro/entry/IPR000742>).

Bm86 is also a glycosylphosphatidylinositol (GPI)-anchored protein and as such is modified post-translationally [10]. It has been proposed that Bm86 is secreted and anchored to gut digestive cells through its C terminus [11]. Using immunogold labelling Bm86 was found to be located on the microvilli of gut digest cells [12]. The immune response induced by Bm86 was hypothesised to be mediated through host complement and anti-Bm86 antibodies which damage the tick gut surface affecting egg viability [13, 14]. However, the actual function of this tick protein has never been determined. Nonetheless, the early successes of Bm86 vaccines such as TickGARD^{PLUS} in Australia and GAVACTM in Cuba provided researchers with the necessary fervour to identify alternative vaccine candidates to potentially be either ‘broad spectrum’ (i.e. cross protective for different tick species) or with a longer duration of immunity compared to Bm86-based vaccines.

2. Methods

Previously reviewed antigen types were summarised as ‘secreted’, ‘intracellular’ or ‘membrane associated’ [1]. In this review, each antigen was analysed *in silico* to confirm previously described localisations. Each ORF was submitted to InterPro to determine if the candidate antigen had domains or motifs representative of conserved protein families including the predicted GO Terms associated with ‘biological process’, ‘molecular function’ and ‘cellular component’ (<https://www.ebi.ac.uk/interpro/>) [15]. InterPro also predicts the presence of signal peptides and transmembrane helices; however these were examined separately using the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>) [16, 17] and the TMHMM server v. 2 (<http://www.cbs.dtu.dk/services/TMHMM/>). GPI anchor predictions were undertaken using PredGPI (<http://gpcr.biocomp.unibo.it/predgpi/pred.htm>) [18]. The BLASTP server was employed to confirm published sequence identities (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). This analysis was limited to vaccine candidates reported as screened against *R. microplus* ticks in cattle challenge trials.

3. Results and discussion

Table 1 summarises BLASTP and InterPro analyses of published *R. microplus* recombinant vaccines which have cattle (or other ruminant) trial data. **Table 2** summarises localisations of these vaccine candidates analysed through SignalP, TMHMM and PredGPI and provides known trial data, references and patents (if applicable).

3.1 Secreted antigens

Most tested antigens are predicted to be secreted with no membrane-associated moieties (transmembrane helices or GPI anchors) (**Table 2**). The idea of selecting secreted proteins may have been cultivated to identify putative antigens that are more immunogenic in comparison to Bm86 and therefore boosted by natural tick challenge. The latter is usually associated with the injection of proteins by tick salivary glands. Studies have also shown that tick gut proteins also elicit host antibody responses; however perhaps gut protein-based vaccines are less immunogenic, that is, Bm86, which requires multiple annual boosts.

Two secreted proteins were also isolated from salivary gland and gut fractions similarly to how Bm86 was originally derived: 5' **nucleotidase** [19] and **Bm91 angiotensin converting enzyme-like** protein [20, 21]. However, neither demonstrated notable vaccine efficacies to warrant further development (**Table 2**).

In other studies, successful vaccine candidates were identified in other tick species, that is, *Ixodes ricinus* (sheep tick) **Ferritin-2** at 96% efficacy [22]. The researchers subsequently mined the *R. microplus* (BmGI) database for a *R. microplus* IrFerritin-2 homologue [22, 23], and RmFerritin-2 was patented at 64% vaccination efficacy [24]. Ferritin-2 was discovered in the sheep tick when studying iron homeostasis and it was found to be required for optimal tick feeding. In addition, unlike other tick ferritins, it was found to be unique without functional orthologs in vertebrate hosts [25].

Metalloproteases were targeted as vaccine candidates as these proteins were considered crucial for the maintenance of blood meal-related functions in other tick species [26, 27]. After an examination of five *R. microplus* metalloprotease GenBank sequences (AAZ39657.1-AAZ39661.1; Untulan et al., 2005, *unpublished*), it was found that Bmi-MP4 (AAZ39660.1) was expressed in female organs and male ticks and exhibited potential antigenic properties in comparison to other *R. microplus* metalloproteases [28]. A Bmi-MP4 metalloprotease vaccination study in Brazil yielded 60% efficacy as reported in 2015 [29], with no patent published (**Table 2**). A different Brazil-based study identified an unrelated **metalloprotease** Rm239/Sequence 82 (31% identity with Bmi-MP4, data not shown) as a component of a cocktail vaccine of four proteins achieving 73% protection in a tick challenge trial [30]. These proteins were identified through a salivary gland transcriptome study; thus in this instance the researchers were targeting secreted salivary proteins. Interestingly, the proteins selected were highly up-regulated in male ticks found on tick susceptible cattle which were not known to induce antibodies in naturally infected bovines [30]. Note that these two metalloproteases (Bmi-MP4 and Rm239/Sequence 82) and the Bm91 angiotensin converting enzyme-like protein described above all possess the GO:0008237 pertaining to 'metallopeptidase activity' (**Table 1**). As metalloproteases are members of a large protein family [31], this may lead to differences between strains or clades of *R. microplus* causing variable vaccination responses. Metalloproteases have been considered as vaccine candidates for other parasite species such as hookworm and human amebiasis, but no commercial products have emerged [32, 33].

Antigen description	GenBank accession/ BLASTP hit	InterPro analysis	GO term predictions (InterPro) ³		
			Biological process	Molecular function	Cellular component
Secreted					
Angiotensin converting enzyme-like (Bm91)	AAB04998.1	Family peptidase M2, peptidyl-dipeptidase A	GO:0006508 proteolysis	GO:0008237 metallopeptidase and peptidyl-dipeptidase activity GO:0008241	GO:0016020 membrane
¹ Extra-cellular matrix protein (Rm39)	No significant hit	No significant hit	—	—	—
Ferritin-2	CK190528 ²	Ferritin homologous superfamily	GO:0006826 iron ion transport GO:0006879 cellular iron ion homeostasis	GO:0008199 ferric iron binding	—
¹ Immunoglobulin G-binding protein C (Rm76)	AAB68803.1	GM2-AP, lipid-recognition domain superfamily	GO:0006689 ganglioside catabolic process	GO:0008047 enzyme activator activity	—
Metalloprotease Bmi-MP4	AAZ39660.1	Metallopeptidase homologous superfamily	GO:0006508 proteolysis	GO:0008237 metallopeptidase	—
¹ Metalloprotease (Rm239)	BAF43574.1		—	GO:0008237 metallopeptidase	—
5' Nucleotidase	AAB38963.1	5'-Nucleotidase/apyrase	GO:0009166 nucleotide catabolic process	GO:0000166 nucleotide binding GO:0016787 hydrolase activity GO:0016788 hydrolase activity-ester bonds GO:0046872 metal ion binding	—
¹ Proteinase inhibitor domain (Rm180)	XM_011553087.1	Pancreatic trypsin inhibitor Kunitz domain superfamily	—	GO:0004867 serine-type endopeptidase inhibitor activity	—
'SILK'	No significant hit	No significant hit	—	—	—
Membrane associated					

Antigen description	GenBank accession/ BLASTP hit	InterPro analysis	GO term predictions (InterPro) ³		
			Biological process	Molecular function	Cellular component
Aquaporin	AIT69684.1	Aquaporin-like	GO:0055085 transmembrane transport	GO:0015267 channel activity	GO:0016020 membrane
Bm86/Bm95	M29321	EGF-like domains	—	—	—
Intracellular					
60S acidic ribosomal protein P0	AGQ49465.1	Ribosomal protein L10P	GO:0042254 ribosome biogenesis	—	GO:0005622 intracellular
Glutathione S-transferase <i>Haemaphysalis longicornis</i>	AAQ74441.1	GST, Mu class homologous superfamily	GO:0008152 metabolic process	GO:0004364 GST activity GO:0005515 protein binding	—
Subolesin and akirin chimeras	ABZ89745.1 AGI44632.1	Akirin protein family	—	—	—
Trypsin inhibitor 1-BmTI-6	P83606.2 CK186726 ²	Pancreatic trypsin inhibitor Kunitz domain superfamily	—	GO:0004867 serine-type endopeptidase inhibitor activity	—
Vitellin	AAA92143.1	Lipovitellin-phosvitin complex, lipid transport protein	GO:0006869 lipid transport	GO:0005319 lipid transporter activity	—

¹Four proteins conform a cocktail vaccination [30]; see **Table 2** for vaccine efficacies.

²Sourced from the BmGI database [23].

³Nil predictions denoted by a dash.

Table 1.

Reported *Rhipicephalus (Boophilus) microplus* antigens with published vaccine challenge efficacies¹ analysed using InterPro (GO terms, domains, protein family identification) including relevant GenBank accessions².

Antigen description	Published efficacy ²	Signal P ³	TMHMM ³	PredGPI ³	References and patents ⁴
Secreted					
Angiotensin converting enzyme-like (Bm91)	7% reduction egg viability	Secreted	—	—	[20, 21]
‘Extracellular matrix protein’ Rm39/Sequence81 ¹	~73% in mix of four proteins	Unknown	—	—	[30, 37] ⁴
Ferritin-2	64%	Secreted	—	—	[22–24] ⁴
Immunoglobulin G-binding protein C Rm76/Sequence76 ¹	~73% in mix of four proteins	¹ Incomplete ORF (likely secreted)	—	—	[30, 37] ⁴
Metalloprotease Bmi-MP4	60%	Secreted	—	—	[29, 74]
Metalloprotease Rm239/Sequence82 ¹	~73% in mix of four proteins	¹ Incomplete ORF (likely secreted)	—	—	[30, 37] ⁴
5’ Nucleotidase	No protection	Secreted	—	Weakly probable	[19]
Proteinase inhibitor domain Rm180/Sequence79 ¹	~73% in mix of four proteins	¹ Incomplete ORF (likely secreted)	—	—	[30, 37] ⁴
‘SILK’	62%	Secreted	—	—	[38, 39]
Membrane associated					
Aquaporin	73%	—	Four transmembrane helices	—	[40] ⁴ , [41]
Bm86/Bm95	45–100%	Secreted	—	Highly probable	[53] ⁴ , [75, 76]
Intracellular					
60S acidic ribosomal protein P0—peptide	96%	—	—	—	[55, 56] ⁴ , [77]
Glutathione S-transferase <i>Haemaphysalis longicornis</i>	57%	—	—	—	[60]
Subolesin and akirin chimeras	83% (deer) 60–75%	—	—	—	[39, 53] ⁴ , [76]
Trypsin inhibitor 1-BmTI-6	32%	—	—	—	[23, 67]
Vitellin	Native protein 68%, recombinant 0% (sheep)	—	—	—	[70]

¹Four proteins conform a cocktail vaccination with ‘Rm’ names [30] and ‘sequence’ names from corresponding patent [37].

²Efficacy from cattle tick challenge trial unless otherwise stated in parentheses.

³Nil predictions denoted by a dash.

⁴Denotes published patent record.

Table 2.

Reported *Rhipicephalus (Boophilus) microplus* antigens with published vaccine challenge efficacies analysed using SignalP (secretion), TMHMM (transmembrane helices) and PredGPI (GPI anchor) including relevant references and patents.

The second protein in the above-described cocktail with Rm239/Sequence 82 metalloproteinase was Rm180/Sequence 79 which has a **proteinase inhibitor** domain (IPR002223: pancreatic trypsin inhibitor Kunitz domain) similar to a **trypsin inhibitor** on the ‘intracellular’ list (**Tables 1** and **2**), also tested in Brazil. Rm180/Sequence 79 in contrast is likely to have a signal peptide based on its top BLAST hit, and this new proteinase inhibitor does not appear to have any homology with known tick proteins (data not shown). Trypsin inhibitors are serine protease inhibitors potentially involved with tick blood meal digestion through the inhibition of trypsin (a serine protease which hydrolyses proteins).

The third protein within the cocktail was Rm76/Sequence 76 (also secreted) which is an **immunoglobulin G (IgG)-binding protein C** possessing domain IPR036846 ganglioside GM2 activator associated with lipid recognition function (**Table 1**). The top BLASTP hit for this protein is AAB68803.1 *Rhipicephalus appendiculatus* IgG-binding protein C at 88% identity. Tick immunoglobulin-binding proteins have been examined previously in several other tick species including *R. appendiculatus*, *Rhipicephalus haemaphysaloides* and *Ixodes scapularis* [34–36] and are thought to function as tick defences against host antibodies. Rm 239/Sequence 82 (metalloprotease) and Rm76/Sequence 76 (IgG-binding C) were shown to be the most immunogenic proteins in the cocktail vaccine based on antibody titres, predicted T cell epitopes and antibody boosting during tick challenge [30]. The fourth protein in this cocktail Rm39/Sequence 81 did not return any significant hits using BLAST or InterPro thus could not be examined using the parameters in the tables. The vaccine cocktail consisting of the trypsin inhibitor (Sequence 79), IgG-binding protein C (Sequence 76), metalloprotease (Sequence 82) and the unknown protein (Sequence 81) has been patented [37]. All sequences were published in the associated patent [37] without signal peptide regions.

‘SILK’ protein was predicted from an expressed sequence tag (EST) library prepared from male *R. microplus* ticks in response to *Anaplasma marginale* infection, and it was thought to be similar to arachnid flagelliform silk proteins [38]. However, no significant hits of the *R. microplus* EST to a ‘SILK protein’ sequence could be confirmed in this study. The protein has not been exploited further as an anti-tick or anti-*Anaplasma* transmission vaccine; however, at 62% efficacy [39] perhaps further study is warranted. No patent has been published.

3.2 Membrane-bound antigens

Apart from Bm86, the only other published antigen with a membrane association was **aquaporin**. Aquaporin does not have a GPI anchor as Bm86 but has four transmembrane helices predicted by TMHMM (**Table 2**). A reported 73% trial efficacy has been published and the data patented [40, 41]. The protein was identified in tick gut transcriptome studies and predictably functions as a water-conducting channel. An aquaporin was previously suggested as vaccine candidate for the human blood fluke *Schistosoma japonicum* with six predicted immunogenic epitopes and an integral membrane structure [42]. No further testing has been reported which is common for many human vaccine candidates. Perhaps the tick aquaporin vaccine will inspire further investigations of similar orthologs in human parasite infections.

Bm86 is thus the only protein with a confirmed GPI anchor that has been examined as a tick vaccine candidate. GPI-anchored proteins are conserved in eukaryotes and are luminal secretory cargo proteins with several functions across mammals and parasites [10, 43]. Notably, the *R. microplus* 5’ **nucleotidase** (listed as a ‘secreted protein’) was predicted to have a ‘weakly probable GPI anchor’, and it is known that mammalian 5’ nucleotidases possess GPI anchors [10]. In terms

of vaccine candidates, GPI-anchored proteins have been investigated in several parasite species such as *Leishmania amazonensis* [44], *Plasmodium falciparum* [45], *Schistosoma mansoni* [46], *Theileria annulata* [47] and *Babesia bovis* [48] and have appeared to be associated with host invasion. In mammals, certain GPI-anchored proteins are cytokines with complement regulation functions [10]. Further studies pertaining to the discovery of tick salivary or gut proteins with GPI anchors have not been reported.

3.3 Intracellular antigens

Although Bm86 is cited as a ‘concealed antigen’ [49, 50], it appears to be a combination of ‘exposed’ and ‘concealed’ based on localisation predictions including a signal peptide (**Table 2**). Antigens in the ‘intracellular’ category do not have predicted signal peptides, GPI anchors or transmembrane helices and thus perhaps should be considered as truly ‘concealed’. Several intracellular antigens have been investigated as tick vaccine antigens; however, results have been variable and seemingly dependent on delivery mechanisms as host antibodies need to target the protein that resides intracellularly.

Subolesin from the akirin protein family (**Table 1**) has been investigated in several tick species as a putative vaccine candidate [51] with the first *R. microplus* ORF described in GenBank as accession ABZ89745.1 (Shao et al. 2008, *unpublished*). Studies have confirmed that subolesin is involved in blood ingestion and reproduction in 2006 [52]; however, no predicted GO terms or other localisation predictions were identified in this study to confirm any of these putative functions (**Tables 1** and **2**). Subolesin was recently patented with Bm86 as a dual vaccine emulsion at a reported patented efficacy of 100% [53]. This dual vaccine is currently being testing by the CATVAC consortium in Morocco [7]. It is unknown if the varied efficacies of Bm86 will affect the activity of this dual vaccine or whether the short duration of immunity will continue to be an issue as for Bm86-based vaccines. Previously, a strong phenotypic knockdown of *Rhipicephalus sanguineus* ticks was observed using RNA interference through the silencing of subolesin and Rs86 (*R. sanguineus* Bm86 homologue) [54].

The **60S acidic ribosomal protein P0** has demonstrated 96% efficacy using a peptide fragment in cattle tick challenge trials in Cuba [55]. This is otherwise a conserved ribosomal protein, and the peptide region selected had significant sequence differences from the host ortholog. This vaccine has been patented and is under further trial testing also through CATVAC [7, 56]. Previously, gene silencing of this intracellular protein was found to be lethal to *Haemaphysalis longicornis* ticks [57]. Ubiquitin (also an intracellular protein) when silenced is also found to be lethal to *R. microplus* ticks [58] but was not found to be an effective vaccine candidate [59].

Haemaphysalis longicornis **glutathione S-transferase** (GST) showed some cross protection against *R. microplus* in a cattle trial [60]; however, further investigation as a tick vaccine candidate has not been reported. GSTs have been examined by several researchers as candidate parasite vaccines, for example, for hookworm, schistosomiasis and trichinellosis [61–63], at varying degrees of efficacy. GST proteins are considered as common ‘housekeeping’ genes forming a large protein superfamily present in eukaryotes and prokaryotes [64]. They function as detoxifying enzymes and thus in ticks may function in response to acaricides or in response to tick-borne pathogens and or stress [65, 66].

Trypsin inhibitors are serine protease inhibitors potentially involved with blood meal digestion as described above. A BmTI-6 sequence was identified in the BmGI database [23] and while native protein vaccine efficacies were high (73%), the corresponding recombinant protein efficacy was poor at 32% [67, 68] (**Table 2**).

This particular trypsin inhibitor is not predicted to be secreted (**Table 2**) thus may have a function different from gut digestion. The protein sequence reported by Andreotti et al. [67] is identical to BmTI-6 P83606.2 [69]. Alternatively, a 'secreted' trypsin Inhibitor showed promise within the cocktail vaccine described above [37]. As stated for metalloproteases, trypsin inhibitors are also members of large dynamic protein families which may circumvent host immune responses if administered as vaccines.

Vitellin was investigated as a native vaccine candidate showing some promise in sheep trials through a reduction in female ticks and their weights and a reduction in tick oviposition [70]. However, the recombinant form had no vaccine effect (**Table 2**), and no further studies were conducted. Vitellin is a high molecular weight yolk lipoglycoprotein, and in ticks and insects, it is synthesised in female fat bodies as a large precursor polypeptide—vitellogenin [70]. In insects, vitellogenin is processed into vitellin polypeptides by specific proteolytic cleavages during passage into haemolymph and/or upon receptor-mediated endocytosis by the developing oocyte [71, 72]. Tick vitellogenins are crucial for egg development and oviposition as demonstrated when silencing of three *H. longicornis* vitellogenin genes [73]. There are no reports of vitellin or vitellogenin as vaccine candidates in other species to date; however, this could be because they exist in arthropods (ticks and insects) rather than other 'pathogenic' species of parasites.

The investigation of intracellular vaccine candidates appears to less likely lead to a successful outcome. Perhaps some of these proteins could be delivered in dual emulsions as shown above for Bm86 and subolesin for a strong vaccination effect. It seems prudent to suggest that an intracellularly localised vaccine candidate requires a mechanism whereby host antibodies are able to access cells internally in order to be active against feeding ticks.

3.4 Other potential protein features

G protein-coupled receptors (GPCRs), also known as 'seven-(pass)-transmembrane domain receptors' are associated with many diseases and as such are the targets of several treatments. They are receptors for pheromones, hormones and neurotransmitters and could potentially be targeted as tick vaccine candidates [78]. Most literature associated with GPCR studies in ticks to date are acaricide-related and not associated with vaccines.

3.5 Protective immune response

The identification of tick vaccine candidates since the discovery of Bm86 appears to be haphazard in that selection has involved either targeting an enzyme involved with feeding or metabolism or to target a gene that showed diminished tick survival following RNA interference silencing. Neither of these approaches is directly linked to the development of a protective immune response which is fundamental for a protective vaccine. Many different experiments have been undertaken describing effective tick immune responses in different breeds of cattle including different mixtures of *Bos indicus* (naturally tick resistant) and *Bos taurus* (innately tick susceptible) cattle. These studies have also been undertaken in many different geographic regions with the use of highly divergent tick infestation protocols. The latter is particularly problematic where in some instances tick-naïve cattle cannot be sourced, and researchers treat the cattle for ticks prior to artificial tick infestations and subsequent immune studies. This topic has been reviewed in detail elsewhere and will not be repeated here [79]. The latter review summarised that there are different immune responses in tick-susceptible and tick-resistant breeds of cattle.

Perhaps different *R. microplus* tick vaccine candidates will need to be developed for different cattle breeds and crosses? Is the tick host response in tick-resistant breeds of cattle a result of superior immune function or genetic differences or a combination of both? One theory is that naïve tick-resistant breeds are readily primed with epithelial $\gamma\delta$ T cells able to respond to larval ticks, whereas susceptible breeds need to recruit these T cells to the larval bite sites [80, 81]. This immune cell recruitment phase seems to manifest in an inefficient immune response in susceptible breeds. It has been a challenge to demonstrate this phenomenon in all immune studies due to the common practise of studying previously exposed cattle in several published experiments, reviewed previously [79].

3.6 Further research

Reverse vaccinology or genome-based approaches have been reviewed elsewhere, and promise in this approach has been reported [1]. Studies have used EST and transcriptome sequence databases to mine for potential tick antigens using a variety of approaches [1, 30]. Tick genomics has only recently become possible due to the availability of new ‘long read’ sequencing technologies and a dramatic decrease in the cost of sequencing large repetitive genomes [82, 83]. Bovine-specific immunology resources are also increasing [84, 85] with earlier research relying on human models for the major histocompatibility complex predictions. In combination with new genome sequences and bovine immunomic resources, a modern approach to identify robust tick candidates could perhaps finally be developed.

4. Conclusions

Although several approaches have been examined, one way to determine the true significance of a particular antigen or protein is to examine the current-published patents associated with cattle tick (*R. microplus*) vaccines. Upon examination of all patents and publications with cattle trial data to date, there are mixed features for *R. microplus* vaccine candidates with either secreted, membrane-bound or intracellular localisations which can also be described as ‘exposed’, ‘a combination of exposed and concealed’ and ‘concealed, respectively. Intracellularly localised antigens are truly ‘concealed’ and in comparison to ‘secreted’ antigen types have highly variable outcomes. The key to identifying efficacious vaccine candidate(s) is to determine how best to stimulate a long-term protective immune response. This may also be feasible through new vaccine delivery options such as nanotechnologies or liposomes which may enhance the immunity to previously identified vaccine candidates.

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