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Full Length Research Paper

Molecular cloning, sequencing and recombinant expression of a putative tick protective antigen from three ixodid ticks

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The *4D8* gene was recently discovered in *Ixodes scapularis* and identified as a tick protective antigen. Vaccination using recombinant 4D8 from *I. scapularis* showed a significant reduction against *I. scapularis* tick infestation in a sheep model. This protein is expressed in both salivary gland and gut tissues, and is thought to be conserved in ixodid tick species. The objective of this study was to provide evidence of the presence of 4D8 and investigate its sequence homology in three *Rhipicephalus* tick species from Africa. The gene encoding this tick protective antigen in *Rhipicephalus appendiculatus*, *Rhipicephalus decoloratus* and *Rhipicephalus microplus* ticks was amplified, cloned, sequenced and expressed as a recombinant protein. The amino acid sequences between these three ticks species was found to be conserved with an identity of 96 to 98%. Recombinant 4D8 from the three tick species was expressed as a His-Tag fusion protein in *Escherichia coli* and the affinity-purified recombinant protein separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), then analyzed in immuno-blot analysis with anti-His-Tag antibody. A unique strong band of the predicted molecular weight of 17 kDa appeared, suggesting presence of a protein corresponding to 4D8. These results confirm the presence of a 4D8 homologue in *Rhipicephalus* tick stocks from East Africa and further support the hypothesis that it is conserved in different tick species. This conservation among different tick species may suggest that it could potentially be an antigen in subunit vaccines for the control of multiple tick species.

Key words: Ticks, vaccine, 4D8, conserved, antigen.

INTRODUCTION

Ticks transmit a wide variety of pathogenic microorganisms affecting livestock, humans and companion animals. These include protozoa, rickettsiae, spirochaetes and viruses, making them the leading vector, compared to any other arthropod (Jongejan and Uielenberg, 2004). For many years, tick and tick borne diseases have been controlled by application of acaricides, either by dipping, spraying or as pour-ons. This control method has become unsustainable for a variety of reasons, such as high cost of acaricide application, selection of acaricide resistant ticks, concerns of environmental contamination with toxic residues, and the potential risk to human health (George, 2000). In small holder dairy farms, the shift towards intensive management with cattle confined to zero grazing units where the only vector contact possibility is through tick infested fodder, has helped reduce tick infestation and incidence of tick borne diseases (Gitau et al.,2000a, b; Maloo et al., 2001, Jongejan and Uielenberg, 2004). Use of tick pathogens and predators is a potential alternative method that remains largely unexploited (Samish and Rehacek, 1999).

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Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PCR,** polymerase chain reaction.

Alternative tick control measures to induce host resistance through vaccination against ticks have been suggested and tested. It is envisaged that effective immunization of livestock against ticks could significantly reduce the use of acaricides (Opdebeeck, 1994; Willadsen, 1999). Currently there are only two comercialIly available anti-tick vaccines marketed as Tick*GARD*$^{®Plus}$ and Gavac^{TM}, for the control of *Rhipicephalus microplus*. These vaccines are based on a recombinant glycoprotein named Bm86 present on the tick midgut cells of *R. microplus* expressed in *Escherichia coli* and *Pichia pastoris* for Tick*GARD*$^{®Plus}$ and Gavac^{TM}, respectively (Willadsen et al., 1989; Willadsen et al., 1995; Canales et al., 1997). These Bm86 based vaccines have been shown to confer protective immunity against *R. microplus, Rhipicephalus decoloratus* and *Rhipicephalus annulatus* infestation in cattle (Fragoso et al., 1998; de Vos et al., 2001; Odongo et al., 2007). A major constraint in the development of vaccines is the identification of antigens that elicit protective immune responses and their expression as recombinant antigens (Willadsen, 2004). Recently, cDNA expression library immunization (ELI), followed by sequence analysis was used to identify candidate tick vaccine antigens (Almazan et al., 2003). ELI was first reported in *Mycoplasma pulmonis* (Barry et al., 1995), and since then has been used in unicellular and multicellular pathogens as well as viruses (Manoutcharian et al., 1998; Alberti et al., 1998; Brayton et al., 1998; Melby et al., 2000; Smooker et al., 2000; Singh et al., 2002). ELI was used to identify three potentially protective tick antigens in *Ixodes scapularis* ticks whose effects were evaluated by vaccination in mice model (Almazan et al., 2003).

Gene knockdown studies using RNAi have shown that 4D8 plays an important role in modulating tick feeding, oviposition, spermatogonia development and reducing potency of acquiring rhickettsiae *Anaplasma phagocytophilum* and *Anaplasma marginale* (de la Fuente et al., 2006a, b; Kocan et al., 2007). Proteins containing domains and post translational modification sites found in proteins of regulatory functions have been found to be interacting with 4D8 in *R. microplus*, suggesting existence of a regulatory role (de la Fuente et al., 2008). 4D8 expression has also been shown to be up regulated or down regulated in response to infection. The expression varies with pathogen, tick tissue and tick species, suggesting a role in the innate immune system of ticks (Zivkovic et al., 2010).

However, the precise mechanism by which 4D8 acts, remains unknown. 4D8 is hypothesized to be a conserved antigen in Ixodid ticks, with orthologs in other eukaryotes, and its classification as tick akirin suggests evolutionary conservation throughout the metazoan (de la Fuente et al., 2006a; Galindo et al., 2009; Macqueen and Johnston, 2009). The 4D8 mosquito (*Aedes albopictus*) ortholog has been expressed in yeast then tested in an immunization trial against other mosquitoes, sandfly and *I. scapularis*. Feeding on immune serum reduced survival of *Anopheles atroparvus* and *Culex pipiens,* while also reducing sand fly fecundity and survival. Vaccination of sheep with recombinant *A. albopictus* 4D8 ortholog inhibited adult *I. scapularis* infestations and reduced tick weight (Canales et al., 2009).

The only two commercially available anti-tick vaccines are based on the same antigen, making the identification and testing of additional tick antigens as potential anti-tick vaccine candidates important. The *4D8* gene is hypothesized to be conserved among ixodid ticks, suggesting possibility of employing it in a broad spectrum anti-tick vaccine formulation. We present herein data supporting the existence of 4D8 homologs in *R. microplus*, *R. decoloratus* and *Rhipicephalus appendiculatus* African tick stocks, its cloning, sequence analysis and expression as a recombinant protein.

MATERIALS AND METHODS

Ticks used in this study

R. decoloratus ticks were obtained from Rusinga Island in Western Kenya in the 1980s, while *R. microplus* was from Zanzibar and both were subsequently maintained at the International Livestock Research Institute (ILRI) tick unit in an incubator at 28°C, with 80% relative humidity. *R. appendiculatus* used was the Muguga stock which was originally isolated from the Kiambu region of the central highlands of Kenya in the 1960s and maintained in the East African Veterinary Research Organization-Kenya Agricultural Research Institute (EAVRO-KARI) Muguga, Kenya, and subsequently in ILRI, under an incubator at 24°C, with 80% relative humidity. The stock has been maintained on cattle and rabbits according to methods described by Bailey (1960). For all the three tick species, whole guts were dissected from approximately 400 semi-engorged female ticks, and total RNA isolated from the pooled guts using RNA extraction buffer containing phenol and guanidine isothyocyanate (Xie and Rothblum, 1991). Total RNA was used in amplify reverse transcription polymerase chain reaction (RT-PCR) to amplify first strand cDNA using Access reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's instruction.

PCR amplification, cloning and sequencing of 4D8 homologs

All primers used in this study were obtained commercially from Bioneer (Daejeon, Korea) and are indicated in Table 1. Restriction endonuclease sites for *Bam*HI and *Hind*III were incorporated in the 5' end of some PCR primers to facilitate cloning of the PCR product into the expression plasmid vector. The 4D8 primers for *R. microplus* and *R. appendiculatus* were designed from the nucleotide sequence available at GeneBank accession numbers ABA62330 and ABA62331, respectively. Using first strand cDNA template, PCR was performed at 94°C for 1 min, then followed by 30 cycles of a sequence of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, with a final step of 72°C for 9 min, including a control reaction where primers were excluded. All products were electrophoresed on 1.2% agarose gel for a DNA molecular weight marker (100 bp DNA ladder, Promega). Amplified fragments were resin purified (Wizard® *Plus* SV Minipreps, Promega) and cloned into pGEM-T vector (Promega) for sequencing both strands, using Applied Biosystems 3730 DNA analyzer capillary sequencer (Rosenblum et al., 1997). The chromatogram output data was analyzed by GeneMapper software(Applied Biosystems, CA, USA) and multiple sequence alignments were performed using ClustalW

Table 1. Oligonucleotide primer pairs used in this study.

Primer ID	Sequence 5'- 3'	Comment
BO4D8+*Bam*HI	*CGC $^{+}$GGA TCC ATG GCT TGY GCR ACA TTA AAG CGR	Degenerate forward primer with *Bam*HI restriction site
BO4D8+*Hind*III	*CGC $^{+}$AAG CTT TTA YGA CAA ATA GCT KGG MGT RGC	Degenerate reverse primer with *Hind*III restriction site
RA4D8+*Bam*HI	*CGC $^{+}$GGA TCC ATG GCT TGT GCG ACA TTA AAG CGG	*R. appendiculatus* forward primer with *Bam*HI restriction site
RA4D8+*Hind*III	*CGC $^{+}$AAG CTT TTA CGA CAA ATA GCT GGG CGT AGC	*R. appendiculatus* reverse primer with *Hind*III restriction site
pGEM-T (forward)	TAA TAC GAC TCA CTA TAG GG	pGEM-T universal primers
pGEM-T(reverse)	TAT TTA GGT GAC ACT ATA G	
pQE 30 (forward)	GGA GAA ATT AAC TAT GAG AGG	pQE 30 universal primers
pQE 30 (reverse)	GTT CTG AGG TCA TTA CTG G	

*The trinucleotide anchor sequence (CGC) and $^{+}$restriction sequence are shown underlined.

(Thompson et al., 1994). Basic local alignment search tool (BLAST) program suite (Altschul et al., 1990) searches were performed against the non-redundant database at National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/blast) using the translated protein sequence to confirm sequence similarity. Data on percent identity were obtained and used to determine level of conservation between the sequenced clones and other 4D8 homologs.

Expression of His-Tag recombinant 4D8 protein

Bacterial recombinant 4D8 was expressed as a C-terminal fusion protein with 6x histidine tag (His-Tag) using the pQE 30 expression system (Qiagen, Hilden, Germany). A DNA fragment encoding the full-length 4D8 from the three tick species was amplified from gut cDNA using Expand^{TM} High Fidelity DNA polymerase (Roche Diagnostics, Mannheim, Germany). The DNA fragment was cloned into pQE 30 expression vector and expressed in *E. coli* strain AD494 (DE3) and JM109 (DE3) sourced from Stratagene (CA, USA). Briefly, an overnight bacterial culture in 2xYT media with 50 μg/ml ampicilin was diluted 1:10 in fresh media, grown to log phase at 37°C and recombinant protein induced with 1 mM isopropyl-β-D-thio-galactoside (IPTG) for 4 h. Recombinant 4D8 was affinity purified by batch purification using Ni-NTA agarose (Qiagen). The resin was washed twice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM Imidazole, pH 8.0) and recombinant protein eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, 0.05% Tween 20 pH 8.0). Crude and purified recombinant proteins were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

Cloning and sequence analysis of tick 4D8

PCR primers generated a fragment of approximately 500 bp for all the three tick species under study (Figure 1, lanes 1 to 4). Although initially degenerate primers were used to amplify 4D8 homologs from the three tick species, species-specific primers yielded better PCR product for the *R. appendiculatus*. The negative control yielded no amplified product (Figure 1, lane 5). A total of 27 recombinant plasmids from different colonies (representing 8, 9 and 10 clones of *R. decoloratus, R. microplus* and *R. appendiculatus*, respectively) were sequenced on both strands using pGEM-T vector primers. The intragroup DNA sequence identity varied between 99 to 100% for *R. appendiculatus*, 94 to 100% for *R. decoloratus* and 98 to 99% for *R. microplus* (Table 2). Intragroup amino acid sequence identity on the other hand was 98 to 100% for *R. appendiculatus* and 96 to 100% for both *R. decoloratus* and *R. microplus*, respectively (Figures 2a to c). The consensus amino acid sequences of the sequenced 4D8 homologs were also aligned with 4D8 homologous sequences from other ixodid tick species (*Amblyomma americanum*, *Dermacentor marginatus*, *Dermacentor variabilis*, *Hyalomma marginatum*, *Haemaphysalis punctata*, *Haemaphysalis qinghaiensis*, *Ixodes ricinus*, *I. scapularis and Rhipicephalus sanguineus*) and the *A. albopictus* mosquito orthologous sequence available in GeneBank. Inter-species sequence diversity was not observed with the sequence identity of 83 to 100% (Figure 3). The *R. decoloratus*, *R. microplus* and *R. appendiculatus* 4D8 homologs had an identity of 96 to 98% between themselves. The sequences when compared against ixodid ticks of other species on the other hand were 84 to 98%, 83 to 98% and 84 to 100%, respectively, as shown in Table 3. The sequence diversity was greater when

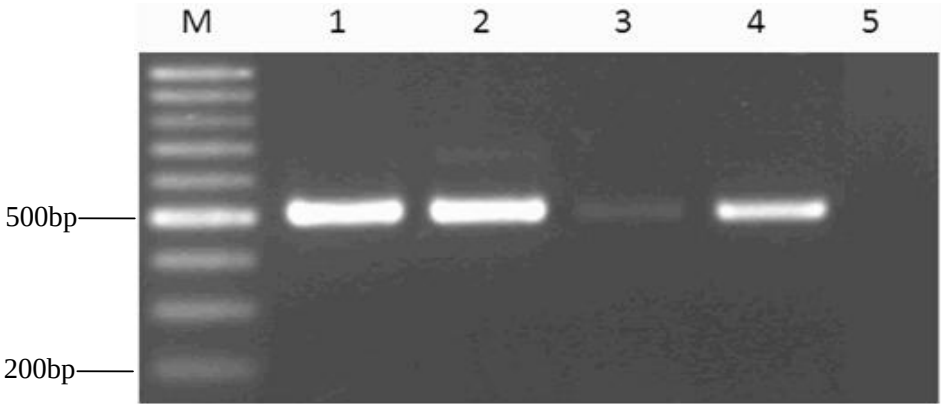

Figure 1. PCR amplification of 4D8 homologs resolved in 1.2% agarose gel. The 100 bp DNA molecular size marker (New England Biolabs, MA, USA) was run in lane marked M while lanes 1 and 2 are PCR products amplified using degenerate primers from *R. decoloratus* and *R. microplus* cDNA, respectively. Lanes 3 and 4, PCR products amplified from *R. appendiculatus* cDNA using degenerate and specific primers, respectively. Lane 5, negative control. The 500 bp line corresponds to the position of the expected PCR products.

Table 2. 4D8 DNA and amino acid sequence alignment identity scores for *R. appendiculatus*, *R. decoloratus* and *R. microplus*.

Tick	AA\DNA	RA1	RA11	RA12	RA13	RA17	RA18	RA2	RA20	RA21	RA7
R. appendiculatus	RA1	100	99	100	99	99	99	99	99	99	99
	RA11	100	100	99	99	99	99	99	99	99	99
	RA12	100	100	100	99	99	99	99	99	99	99
	RA13	99	99	99	100	99	99	99	99	99	99
	RA17	99	99	99	98	100	99	99	99	99	99
	RA18	100	100	100	99	99	100	99	99	99	99
	RA2	100	100	100	99	99	100	100	100	99	99
	RA20	100	100	100	99	99	100	100	100	99	99
	RA21	99	99	99	98	98	99	99	99	100	99
	RA7	99	99	99	98	98	99	99	99	98	100

	AA\DNA	RD11	RD18	RD21	RD22	RD23	RD7	RD8	RD9
R. decoloratus	RD11	100	99	99	99	98	94	98	98
	RD18	98	100	99	99	98	95	99	99
	RD21	98	100	100	99	98	95	99	99
	RD22	98	100	100	100	98	95	99	99
	RD23	97	98	98	98	100	95	98	99
	RD7	96	97	97	97	96	100	95	95
	RD8	98	100	100	100	98	97	100	99
	RD9	98	100	100	100	98	97	100	100

	AA\DNA	RM11	RM18	RM19	RM22	RM23	RM24	RM5	RM6	RM8
R. microplus	RM11	100	99	99	98	98	98	99	99	98
	RM18	98	100	99	99	99	98	99	99	98
	RM19	97	98	100	98	99	99	99	98	99
	RM22	99	98	96	100	99	98	98	98	98
	RM23	100	98	97	99	100	99	99	98	99
	RM24	100	98	97	99	100	100	99	98	99
	RM5	99	98	96	98	99	99	100	98	98
	RM6	98	97	96	98	98	98	98	100	98
	RM8	99	98	96	98	99	99	98	98	100

DNA alignment scores are shown in red while amino acid alignment scores are shown in black.

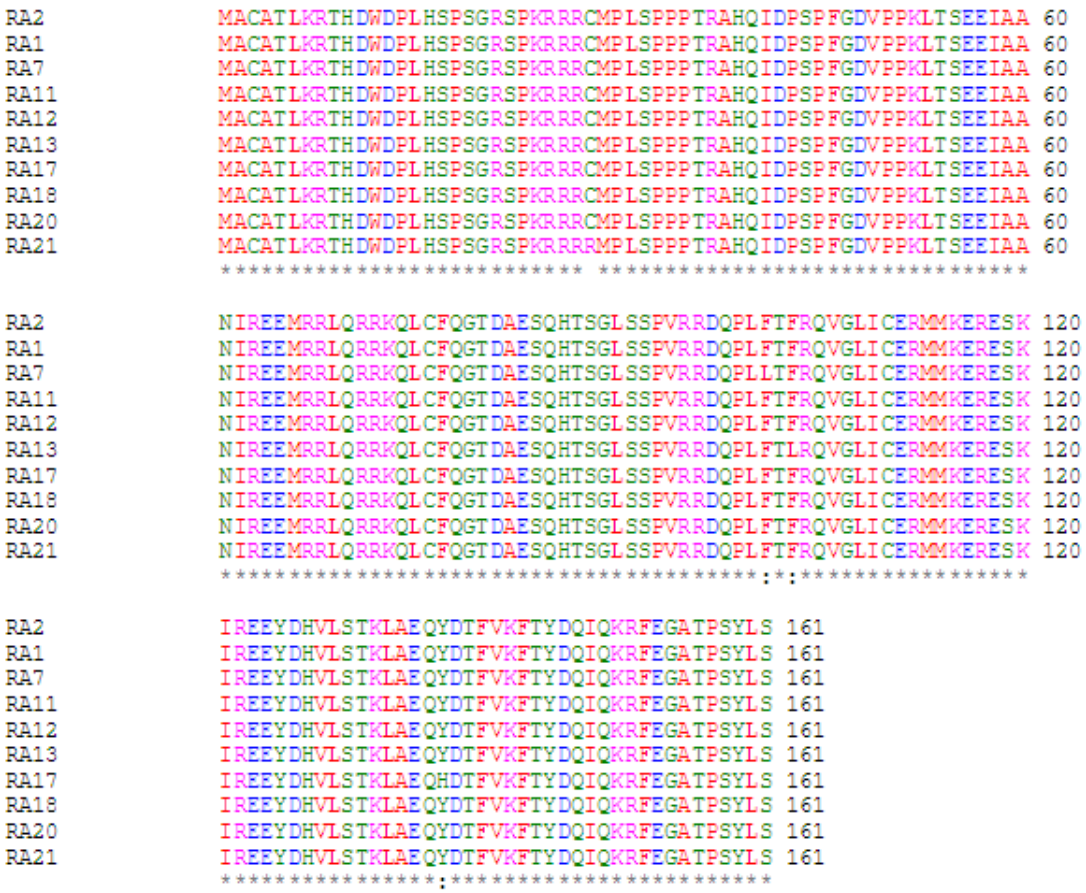

Figure 2a. Predicted amino acid sequence alignment of *R. appendiculatus* 4D8 clones. *****Identical, **:**conserved substitution.

compared to the mosquito ortholog exhibiting identity of between 48 and 59% at the amino acid level (Table 3).

Expression and immuno-blot analysis of two tick 4D8 homologs

The complete *4D8* gene was expressed as a His-Tag recombinant protein in *E. coli*. Crude lysates and affinity purified recombinant protein were separated by SDS-PAGE and exposed to immuno-blot analysis with anti-His Tag antibody (Figure 4). A unique band of the predicted molecular weight of 17 kDa appeared for both the crude lysates and purified recombinant protein, suggesting that the protein corresponded to 4D8. The figure shows 12% SDS PAGE and immuno-blot analysis of both crude lysate and purified His-Tag protein. Lane 1 is crude lysate from uninduced bacterial culture. Lane 2 to 4 are *R. decoloratus*, *R. microplus* and *R. appendiculatus* His-Tag protein from IPTG induced bacterial cultures crude lysates, respectively. Lane 5 to 7 are immuno-blot analysis of *R. decoloratus*, *R. microplus* and *R. appendiculatus* His-Tag protein from IPTG induced bacterial cultures crude lysates, respectively probed with anti-His-Tag antibody. Lane 8 shows affinity purified recombinant 4D8. Immuno-blot analysis of purified His-Tag protein elution products 2 and 3 from *R. decoloratus* are in lanes 9 and 10, respectively. Corresponding elution products 2 and 3 from *R. appendiculatus* are in lanes 11 and 12, respectively. The high range Rainbow® (GE Healthcare Amersham, Buckinghamshire, UK) molecular size marker is indicated in kDa against lane 1.

DISCUSSION

The observed high intra and inter-species sequence identity confirms that 4D8 is conserved in these three Rhipicephaline ticks of East Africa. Comparison of these sequences with Genebank sourced tick 4D8 homologs revealed high sequence homology, confirming that *4D8* is a conserved gene among Ixodid ticks. Cross-reactive antibodies against r4D8 orthologs of *I. scapularis* and *A. albopictus,* with amino acid sequence identity of 48%, have previously been shown by western blots confirming presence of conserved epitopes (Canales et al., 2009).

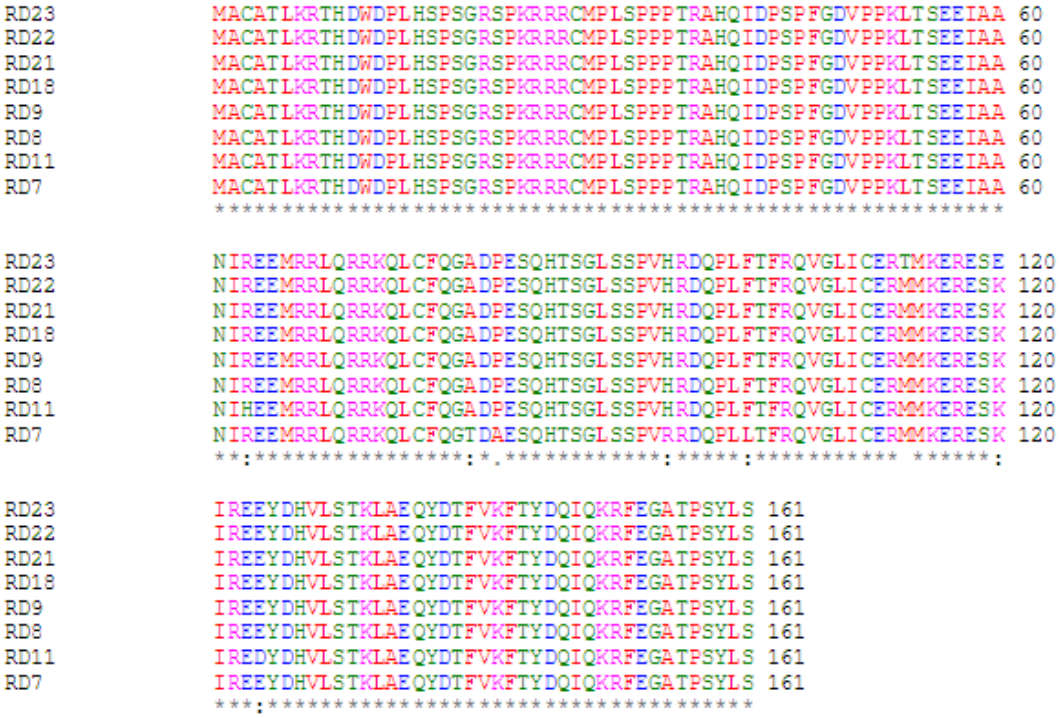

Figure 2b. Predicted amino acid sequence alignment of *R. decoloratus* 4D8 clones. *****Identical, :conserved substitution.

This suggests existence of conserved epitopes among ixodid ticks which exhibited a higher sequence similarity (83 to 100%). A tick 4D8 based vaccine would therefore potentially elicit protective immune response in immunized hosts against Ixodid ticks and other arthropod disease vectors bearing 4D8 orthologs, with relatively similar sequences, thus acting as a good candidate for developing a universal vaccine for the control of tick vector species. The reported reduction of *A. marginale* and *A. phagocytophilum* transmission due to 4D8 silencing (dela Fuente et al., 2006b) suggests a potential advantage of impairing tick-borne diseases (TBD) transmission by utilizing 4D8 as a tick vaccine antigen.

After induction of the cultures with IPTG, a band of approximately 17 kDa was observed on Coomassie stained polyacrylamide gels. The presence of the r4D8 protein was later confirmed by immunoblotting, using anti-histidine antibody. The molecular weight closely compared to the *I. scapularis* r4D8 which is 21 kDa (reviewed by Nuttal et al., 2006). The *I. scapularis* amino acid sequence has 184 amino acids (XP_002414493) while the *Rhipicephalus* ticks in this study had 161 amino acids. The r4D8 protein in this study was therefore expected to be marginally lower than that of *I. scapularis*. *R. microplus* r4D8 was expressed at low levels, and its purification was not successful. This was attributed to sub-optimal expression in *E. coli* that could be corrected by varying expression conditions and perhaps different *E. coli* cell lines. However, the close identity of 4D8 protein sequences between *R. microplus* and *R. appendiculatus* (96%) or *R. microplus* and *R. decoloratus* (98%) suggested the homologs were identical (Table 2). The observed expressed r4D8 for all the three tick species had the same molecular size further strengthening the assumption that the homologs were identical. Failure to optimize *R. microplus* r4D8 expression was therefore tolerated. This present work describes the cloning, sequencing and expression of a putative tick vaccine antigen and confirms its conservation among Ixodid ticks. Function analysis of 4D8 by RNAi showed degeneration of salivary gland, gut and reproductive tissues (de la Fuente et al., 2006c). Making inference that this gene is present in tick salivary glands raises questions on whether it is secreted during tick feeding. Absence of a signal peptide was confirmed using signal peptide prediction tool SignalP 3.0 server (Brendtsen et al., 2004). This confirmed that 4D8 is not a secreted protein, suggesting natural boosting during repeated tick feeding to be unlikely. Although a tick 4D8 based vaccine would suggestively not have an obvious advantage against the BM86 vaccines in terms of self boosting on repeated tick feeding, it would predictably be more effective in the control of ticks and TBDs. This would be due to effect on a broad range of tick species at different life stages and impairing transmission of TBDs.

Identification and characterization of conserved antigens will facilitate the future development of a universal tick vaccine that targets multiple tick species.

```
RM24            MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAA 60
RM6             MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAA 60
RM8             MACATLKRTHDWDPLHSPSGRSPKQRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAA 60
RM5             MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAV 60
RM22            MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAA 60
RM11            MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAA 60
RM23            MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAA 60
RM19            MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAA 60
RM18            MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAHQIDPSPFGDVPPKLTSEEIAA 60
                ************************:*********************************.

RM24            NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERESK 120
RM6             NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERESK 120
RM8             NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERESK 120
RM5             NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERESK 120
RM22            NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERENK 120
RM11            NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERESK 120
RM23            NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERESK 120
RM19            NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERESK 120
RM18            NIREEMRRLQRRKQLCFQGADPESQHTSGLSSPVHRDQPLFTFRQVGLICERMMKERESK 120
                ************************************************************.*

RM24            IREEYDHVLSTKLAEQYDTFVKFTYDQIQKRFEGATPSYLS 161
RM6             IREGYDHVLSTKLAEQYDAFVKFTYDQIQKRFEGATPSYLS 161
RM8             IREEYDHVLSTKLAEQYDTFVKFTYDQIQKRFEGATPSYLS 161
RM5             IREEYDHVLSTKLAEQYDTFVKFTYDQIQKRFEGATPSYLS 161
RM22            IREEYDHVLSTKLAEQYDTFVKFTYDQIQKRFEGATPSYLS 161
RM11            IREEYDHVLSTKLAEQYDTFVKFTYDQIQKRFEGATPSYLS 161
RM23            IREEYDHVLSTKLAEQYDTFVKFTYDQIQKRFEGATPSYLS 161
RM19            IREEYDHVLSTKLAEQYDTFVKFTYDQIQKRFEGATPKLFV 161
RM18            IREEYDHVLSTKLAEQYDTFVKFTYDQIQKRFEGATPSYFV 161
                *** **************:******************. :
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Figure 2c. Predicted amino acid sequence alignment of *R. microplus* 4D8 clones. *****Identical, ˙conserved substitution, .semi conserved substitution.

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HQ              MACATLKRTHDWDPLHSPNGRSPKRRRCMPLSATP----------------------TPP 38
HP              MACATLKRTHDWDPLHSPNRRSPKRRRCMPLSVTP----------------------TPP 38
AA              MACATLKRTHDWDPLHSPNGRSPKRRRCMPFSVNPP---------------------APP 39
RM              MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSP-------------------------PP 35
RD8             MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSP-------------------------PP 35
RM6             MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSP-------------------------PP 35
RA              MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSP-------------------------PP 35
RA2             MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSP-------------------------PP 35
RS              MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSP-------------------------PP 35
DM              MACATLKRTHDWDPLHSPNGRSPKRRRCMPLSVSPP--------------------A-PP 39
DV              MACATLKRTHDWDPLHSPNGRSPKRRRCMPLSVSPP--------------------A-PP 39
IR              MACATLKRTHDWDPLHSPNGRSPKRRRCMPLSVTQA--------------------ATPP 40
IS              MACATLKRTHDWDPLHSPNGRSPKRRRCMPLSVTQA--------------------ATPP 40
HM              MACATLKRTHDWDPLHSPNGRSPKRRRCMPLSPP----------------------APP 37
AeA             MACATLKRSLDWESLNQ---RPTKRRRCHPFGSPSSNAPNSPSSSAIAAAAAAASSSNSA 57
                ********: **:.*:.   *..***** *:.                        ..

HQ              TRAHQINPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQAGAEGGTS----SGGD 93
HP              TRAHQINPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQAGAEGSPV----GGGD 93
AA              TRAHQINPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG-AECSSP----PEGC 93
RM              TRAHQIDPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG--------------- 79
RD8             TRAHQIDPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG--------------- 79
RM6             TRAHQIDPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG--------------- 79
RA              TRAHQIDPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG--------------- 79
RA2             TRAHQIDPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG--------------- 79
RS              TRAHQIDPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG--------------- 79
DM              TRAHQINPSPFGD-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG--------------- 83
DV              TRAHQINPSPFGE-VPPKLTSEEIAANIREEMRRLQRRKQLCFQG--------------- 83
IR              TRAHQINPSPFGE-VPPKLTSEEIAANIREEMRRLQRRKQLCFSSPLESGSPSVTPPAAE 99
IS              TRAHQINPSPFGE-VPPKLTSEEIAANIREEMRRLQRRKQLCFSSPLESGSPSATPPAAD 99
HM              TRAHQMNPSPFGE-VPPKMTSEEIAANIREEMRRLQRRKQLCFQR--------------- 81
AeA             MRVMEPKPSPFAEAVCPKLTPEKMAQNITEEIKRLHRRKQLTFNTGSMERMQD---SESS 114
                 *. : .****.: * **:*.*::* ** **::**:***** *.
```

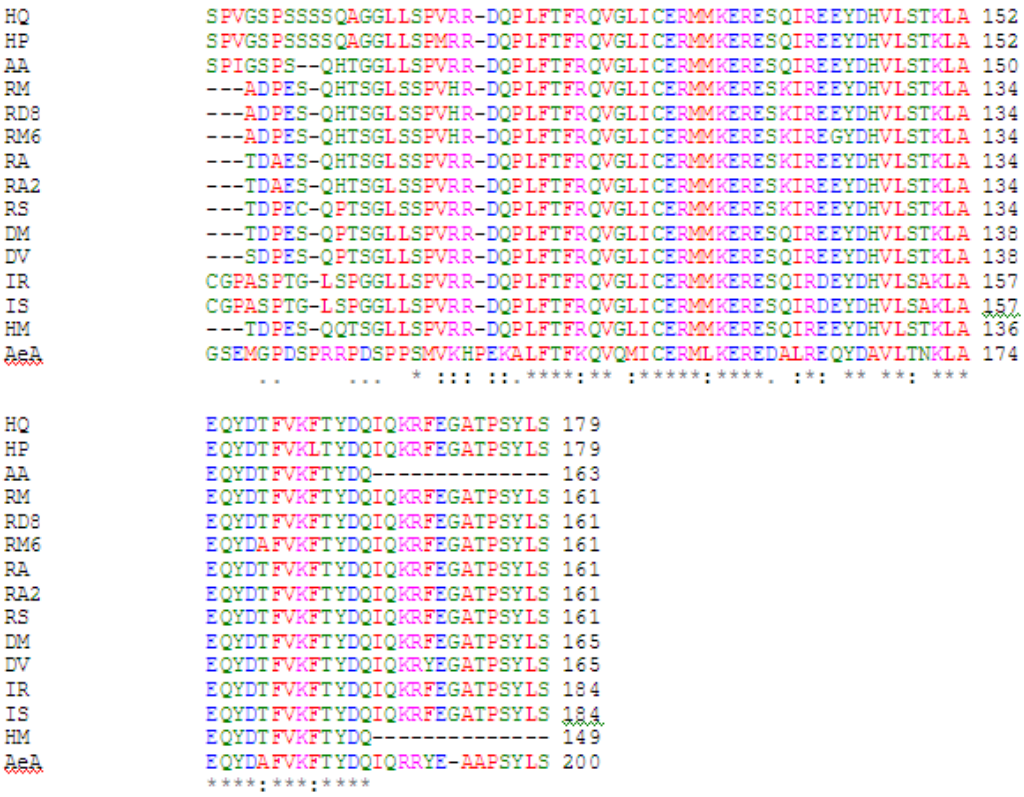

Figure 3. Multi sequence alignment of deduced homologous tick 4D8 amino acid sequences and the mosquito 4D8 orthologs.* *****Identical, :conserved substitution, .semi conserved substitution.

Table 3. Multi sequence alignment identity scores of tick 4D8 homologs and mosquito ortholog.

	AeA	AA	DM	DV	HM	HP	HQ	IR	IS	RA	RA2	RD8	RM6	RM	RS
AeA	100														
AA	51	100													
DM	53	87	100												
DV	55	87	98	100											
HM	59	92	96	96	100										
HP	49	89	91	90	89	100									
HQ	51	90	92	92	91	95	100								
IR	48	84	90	90	88	84	85	100							
IS	48	84	90	90	88	84	85	98	100						
RA	54	84	95	93	91	90	91	88	88	100					
RA2	54	84	95	93	91	90	91	88	88	100	100				
RD8	55	84	95	93	91	89	91	88	88	98	98	100			
RM6	55	83	93	92	89	88	90	87	87	96	96	98	100		
RM	57	91	94	93	92	88	90	87	87	97	97	99	98	100	
RS	55	83	96	94	91	89	91	88	88	98	98	97	96	97	100

AeA, *Aedes albopictus* (accession number ACF49499); AA, *Amblyomma americanum* (accession number ABA62326); DM, *Dermacentor marginatus* (accession number ABA62333); DV, *Dermacentor variabilis* (accession number AAV67034); HM, *Hyalomma marginatum* (accession number ABA62335); HP , *Haemaphysalis punctata* (accession number ABA62336); HQ, *Haemaphysalis qinghaiensis* (accession number ACA09712); IR, *Ixodes ricinus* (accession number ABA62325); IS, *I. scapularis* (accession number XP_002414493); RA, *Rhipicephalus appendiculatus* (accession number ABA62331); RA2, *R. appendiculatus* clone 2; RD8, *R. decoloratus* clone 8; RM, *R. microplus* (accession number ABZ89745); RM6, *R. microplus* clone 6; RS, *R. sanguineus* (accession number ABA62332). DNA alignment scores are shown in red while amino acid alignment scores are shown in black.

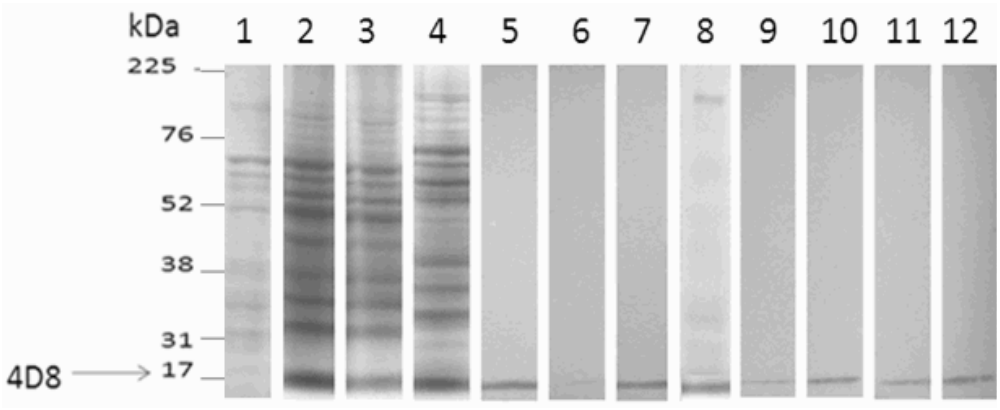

Figure 4. SDS-PAGE and immuno-blot analysis of 4D8 recombinant protein.

The 4D8 antigen represents a candidate vaccine antigen for the simultaneous control of *R. appendiculatus*, *R. microplus* and *R. decoloratus* ticks. In future, it will be interesting to test whether vaccination with r4D8 antigen affords protection from infestation against African *Rhipicephalus* ticks.

REFERENCES

Alberti E, Acosta A, Sarmiento ME, Hidalgo C, Vidal T, Fachado A, Fonte L, Izquierdo L, Infante JF, Finlay CM, Sierra G (1998). Specific cellular and humoral immune response in Balb/c mice immunised with an expression genomic library of *Trypanosoma cruzi*. Vaccine 16(6):608-612.

Almazan C, Kocan KM, Bergman DK, Garcia-Garcia JC, Blouin EF, de la, Fuente J (2003). Identification of protective antigens for the control of *Ixodes scapularis* infestations using cDNA expression library immunization. Vaccine 21(13-14):1492-1501.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol. 215(3):403-410.

Bailey KP (1960). Notes on the rearing of *Rhipicephalus appendiculatus* and their infection with *Theileria parva* for experimental transmission. Bull. Epizoot. Dis. Afr. 8:33-43.

Barry MA, Lai WC, Johnston SA (1995). Protection against mycoplasma infection using expression-library immunization. Nature 377(6550):632-635.

Brayton KA, Vogel SW, Allsopp BA (1998). Expression library immunization to identify protective antigens from *Cowdria ruminantium*. Ann. N.Y. Acad. Sci. 849:369-371.

Brendtsen JD, Nielsen H, von Heijne G, Brunak S (2004). Improved prediction of signal peptides: Signal P 3.0. J. Mol. Biol. 340(4):783-795.

Canales M, Enriquez A, Ramos E, Cabrera D, Dandie H, Soto A, Falcon V, Rodriguez M, de la, Fuente J (1997). Large-scale production in *Pichia pastoris* of the recombinant vaccine Gavac against cattle tick. Vaccine 15(4):414-422.

Canales M, Naranjo V, Almazan C, Molina R, Tsuruta SA, Szabo MP, Manzano-Roman R, Perez de la Lastra JM, Kocan, KM, Jimenez MI, Lucientes J, Villar M, de la Fuente J (2009). Conservation and immunogenicity of the mosquito ortholog of the tick-protective antigen, 4D8. Parasitol. Res. 105(1):97-111.

de la Fuente J, Blouin EF, Manzano-Roman R, Naranjo V, Almazán C, Pérez de la Lastra JM, Zivkovic Z, Massung RF, Jongejan F, Kocan KM. (2008). Differential expression of the tick protective antigen, subolesin, in *Anaplasma marginale* and *A. phagocytophilum* infected host cells. Ann N.Y Acad. Sci. 1149:27-35.

de la Fuente J, Almazan C, Blas-Machado U, Naranjo V, Mangold AJ, Blouin EF, Gortazar C, Kocan KM (2006a). The tick protective antigen, 4D8, is a conserved protein involved in modulation of tick blood ingestion and reproduction. Vaccine 24(19):4082-4095.

de la Fuente J, Almazan C, Blouin EF, Naranjo V, Kocan KM (2006b). Reduction of tick infections with *Anaplasma marginale* and *A. phagocytophilum* by targeting the tick protective antigen 4D8. Parasitol..Res. 100(1):85-91.

de la Fuente J, Almazan C, Naranjo V, Blouin EF, Kocan KM (2006c). Synergistic effect of silencing the expression of tick protective antigens 4D8 and Rs86 in *Rhipicephalus sanguineus* by RNA interference. Parasitol. Res. 99(2):108-113.

de Vos S, Zeinstra L, Taoufik O, Willadsen P, Jongejan F (2001). Evidence for the utility of the Bm86 antigen from *Boophilus microplus* in vaccination against other tick species. Exp. Appl. Acarol. 25(3):245-261.

Fragoso H, Rad PH, Ortiz M, Rodriguez M, Redondo M, Herrera L, de la Fuente J (1998). Protection against *Boophilus annulatus* infestations in cattle vaccinated with the *B. microplus* Bm86-containing vaccine Gavac. Off. Vaccine 16(20):1990-1992.

Galindo RC, Doncel-Perez E, Zivkovic Z, Naranjo V, Gortazar C, Mangold A J, Martin-Hernando MP, Kocan KM, de la Fuente J (2009). Tick 4D8 is an ortholog of the akirins described in insects and vertebrates. Dev. Comp. Immunol. 33(4):612-617.

George JE (2000). Present and future technologies for tick control. Ann. N.Y. Acad. Sci. 916:583-588.

Gitau GK, McDermott JJ, Katende JM, Perry BD (2000a). The epidemiology of *Theileria parva* infections on smallholder dairy farms in Kenya. Ann.N.Y. Acad. Sci. 916:265-270.

Gitau GK, McDermott JJ, Katende JM, O'Callaghan CJ, Brown RN, Perry BD (2000b). Differences in the epidemiology of theileriosis on smallholder dairy farms in contrasting agro-ecological and grazing strata of highland Kenya. Epidemiol. Infect. 124(2):325-335.

Jongejan F, Uilenberg G (2004). The global importance of ticks. Parasitology 129:S3-14.

Kocan KM, Manzano-Roman R, de la Fuente J (2007). Transovarial silencing of the 4D8 gene in three-host ixodid tick species after injection of replete females with 4D8 dsRNA. Parasitol. Res. 100(6):1411-1415.

Macqueen DJ, Johnston IA (2009). Evolution of the multifaceted eukaryotic akirin gene family. BMC. Evol. Biol. 9:34.

Maloo SH, Rowlands GJ, Thorpe W, Gettinby G, Perry BD (2001). A longitudinal study of disease incidence and case-fatality risks on small-holder dairy farms in coastal Kenya. Prev. Vet. Med. 52(1):17-29.

Manoutcharian K, Terrazas LI, Gevorkian G, Govezensky T (1998). Protection against murine cysticercosis using cDNA expression library immunization. Immunol. Lett. 62(3):131-136.

Melby PC, Ogden GB, Flores HA, Zhao W, Geldmacher C, Biediger NM, Ahuja SK, Uranga J, Melendez M (2000). Identification of vaccine candidates for experimental visceral leishmaniasis by immunization with sequential fractions of a cDNA expression library.

Infect. Immun. 68(10):5595-5602.
Nuttal PA, Trimnell AR, Kazimirova M, Labuda M (2006). Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. Parasite Immunol. 28(4):155-163.
Odongo D, Kamau L, Skilton R, Mwaura S, Nitsch C, Musoke A, Taracha E, Daubenberger C, Bishop R (2007). Vaccination of cattle with TickGARD induces cross-reactive antibodies binding to conserved linear peptides of Bm86 homologs in *Boophilus decoloratus*. Vaccine 25(7):1287-1296.
Opdebeeck JP (1994). Vaccines against blood-sucking arthropods. Vet. Parasitol. 54(1-3):205-222.
Rosenblum BB, Lee LG, Spurgeon SL, Khan SH, Menchen SM, Heiner CR, Chen SM (1997). New dye-labeled terminators for improved DNA sequencing patterns, Nucleic Acids Res. 25(22):4500-4504.
Samish M, Rehacek J (1999). Pathogens and predators of ticks and their potential in biological control. Annu. Rev. Entomol. 44:159-182.
Singh RA, Wu L, Barry MA (2002). Generation of genome-wide CD8 T cell responses in HLA-A*0201 transgenic mice by an HIV-1 ubiquitin expression library immunization vaccine. J. Immunol. 168(1):379-391.
Smooker PM, Setiady YY, Rainczuk A, Spithill TW (2000). Expression library immunization protects mice against a challenge with virulent rodent malaria. Vaccine 18(23):2533-2540.
Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22):4673-4680.
Willadsen P (1999). Immunological control of ectoparasites: past achievements and future research priorities. Genet. Anal. 15(3-5):131-137.
Willadsen P (2004). Anti-tick vaccines. Parasitology 129(Suppl)S367-S387.
Willadsen P, Bird P, Cobon GS, Hungerford J (1995). Commercialisation of a recombinant vaccine against *Boophilus microplus*. Parasitol. 110(Suppl):S43-S50.
Willadsen P, Riding GA, McKenna RV, Kemp DH, Tellam RL, Nielsen JN, Lahnstein J, Cobon GS, Gough JM (1989). Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*. J. Immunol. 143(4):1346-1351.
Xie WQ, Rothblum LI (1991). Rapid, small-scale RNA isolation from tissue culture cells. Biotechniques 11(3):324, 326-324, 327.
Zivkovic Z, Torina A, Mitra R, Alongi A, Scimeca S, Kocan KM, Galindo RC, Almazan C, Blouin EF, Villar M, Nijhof AM, Mani R, La Barbera G, Caracappa S, Jongejan F, de la Fuente J (2010). Subolesin expression in response to pathogen infection in ticks. BMC Immunol. 11:7.