Development of a novel subunit vaccine against East Coast fever based on the *Theileria parva* sporozoite surface protein p67

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Contents

Dedicated to my grand-mum, Madam Akakyeibire

Chapter 1

Introduction and scope of the thesis

East Coast fever

In the early parts of the 20th century East Coast fever (ECF) was identified as a tick-borne disease of cattle in South Africa and associated with cattle imported from East Africa in an attempt to repopulate the livestock that had been devastated by rinderpest [148]. ECF is a lymphoproliferative disease characterized by a number of diverse clinical signs and pathological lesions, all of which are associated with invasion of lymphoid and non-lymphoid tissues with parasitized lymphoblasts [38, 61]. The disease is caused by a protozoan parasite called *Theileria parva* and is endemic in the eastern, central and southern parts of Africa. The distribution of ECF is strictly associated with the distribution of the vector tick species (Fig. 1), mainly *Rhipicephalus appendiculatus.*
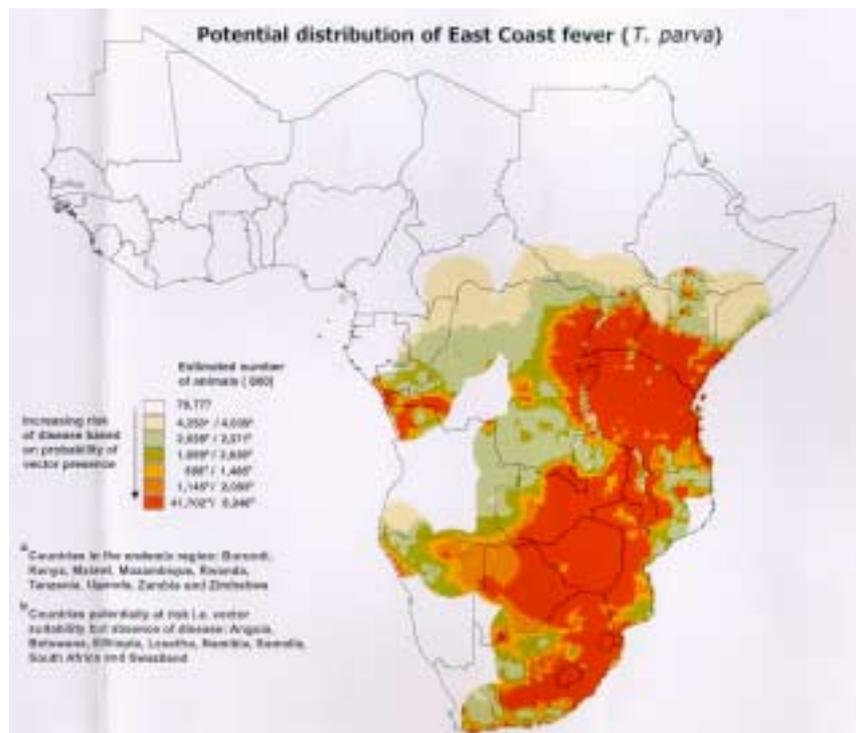
Fig. 1: **Distribution of ECF based on the probability of the presence of the vector, the brown ear tick.** [This picture originated from a poster designed and produced by Bruno Minjauw and others (ILRI) and presented jointly by DFID, AHP, FAO, ILRI and ICTTD. With permission from Sophie Carver, AHP, the University of Edinburg, Scotland, UK].

Epidemics of ECF in cattle, defined as occurrence of *Theileria parva* infections in previously or recently unaffected areas [148], are renowned for their devastating impact on the cattle population. These epidemics often strike with high morbidity and mortality, causing major loss and significant social and economic distress to the individual farmer. Extinction of entire herds is not uncommon especially when no measures are taken to mitigate the deadly impact of the outbreak [140]. As the livelihood of smallholder farms, often managed by women depends on one or two cattle, the financial burden due to loss of income and livestock products impacts on the quality of all aspects of family life. The disease is of considerable importance throughout the region resulting in significant losses in terms of cattle deaths and income, and represents a major constraint to the development of the livestock industry in eastern, central and southern Africa. For example *T. parva* costs farmers over US$ 170 million a year in direct losses [121, 130]. The spread of the infection is mainly through cattle movement, e.g. oxen working and/or feeding in neighboring or distant villages, making migration for distances of 15 km or more not exceptional [15, 16].

Theileria parva parasites have the ability, unique among protozoan parasites, to 'transform' the bovine lymphocytes they invade into cancer-like cells that proliferate uncontrollably, as leukaemia cells do, killing the animal within three weeks of infection. Pyrexia, generalized lymphodenopathy and terminal respiratory distress and/or diarrhea, due to pulmonary infiltration and edema, characterize ECF [114]. A consistent feature of the disease in its advanced stages is pronounced leukopoenia, which is reflected by extensive depletion of lymphocytes in the lymphoid organs. The severity of the disease in individual cattle is known to be influenced by a number of parameters, including infection dose, breed, and individual variation [114, 148]. The first clinical sign is persistent high fever that is used as a reliable indicator for early diagnosis. ECF exists in a number of epidemiological states in the field and exhibits a spectrum of syndromes ranging from those described above to sub-clinical infections, which are common, where heavy challenge occurs under conditions of endemic stability. The epidemiology is complicated by the presence of polymorphic traits between *T. parva* isolates and stocks, associated primarily with different clinical symptoms, and the presence of a wildlife reservoir in the African Cape buffalo (*Synerus caffer*) [116].

In the past, *T. parva* was thought to comprise three distinct sub-species: *T. parva parva* for parasites causing classical ECF; *T. parva lawrencei* for parasites causing Corridor disease and *T. parva bovis* for parasites causing January disease. It is now known that genetically *T. parva* is a single species and *T. parva* parasites are now classified according to their host species of origin, referred to as either cattle-derived or buffalo-derived [148]. The closely related *T. annulata,* a parasite of the Asiatic or water buffalo (*Bubalus bubalis*), is responsible for Mediterranean or tropical Theileriosis in cattle in areas extending from the Mediterranean and North Africa

through Asia to China [133]. Other *Theileria* species (e.g. *T. buffeli, T. orientalis* and *T. sergenti*) do not transform the host cells and cause diseases of lesser severity in various parts of the world in particular the Asian and African continents [192].

Life Cycle of *Theileria parva*

Theileria parva is a member of the phylum Apicomplexa, which includes many species of both medical (e.g. *Plasmodium* species; *Toxoplasma*) and veterinary (e.g. *Babesia*; *Eimeria*; *Theileria*) importance. The Apicomplexa are obligate intracellular parasites that, in general, have complex life cycles involving the invasion of a range of cells in one or more different hosts. For a detailed review of the classification and biology of *T. parva*, see references [38, 39, 145, 148, 179, 181]. The life cycle of *T. parva* (Fig. 2) resembles that of the malaria parasite, *P. falciparum*, in that these parasites undergo sequential development in nucleated cells and erythrocytes, the former involving cells of the lymphoid system. However, unlike malaria, the disease caused by *Theileria* is attributed mainly to the schizogenous stage of development in leukocytes, although anaemia also occurs in case of *T. annulata*.

Theileria parva is transmitted to cattle primarily by infected nymphal or adult ticks of the genus *Rhipicephalus appendiculatus* (the brown-ear tick) when the tick injects infective sporozoites into the new host while taking a blood meal. The sporozoites rapidly invade bovine lymphocytes by a receptor-mediated process and differentiate into schizonts [38, 61, 179 - 181]. This event is associated with transformation of the infected cell to a state of uncontrolled proliferation [61, 176]. Subsequent invasion of non-lymphoid tissues by parasitized cells and the associated immunopathological effects usually result in death of the animal within two to four weeks of infection [38, 114, 148]. During mitosis, the parasite body associates with the mitotic spindle and divides synchronously with the host nucleus so that the schizonts are distributed randomly to both daughter cells.

A proportion of the schizonts undergoes merogony to produce merozoites, which invade erythrocytes and develop into piroplasms, the form of *T. parva* infective to the tick. For the completion of the life cycle the piroplasms require to be ingested by larval and nymphal stages of the tick while feeding on an animal carrying piroplasms in the red blood cells. Infection in the tick is transmitted trans-stadially (i.e. from larva to nymph or nymph to adults) and unlike *Babesia* [67], there is no transovarian transmission.

LIFE CYCLE OF *THEILERIA PARVA*
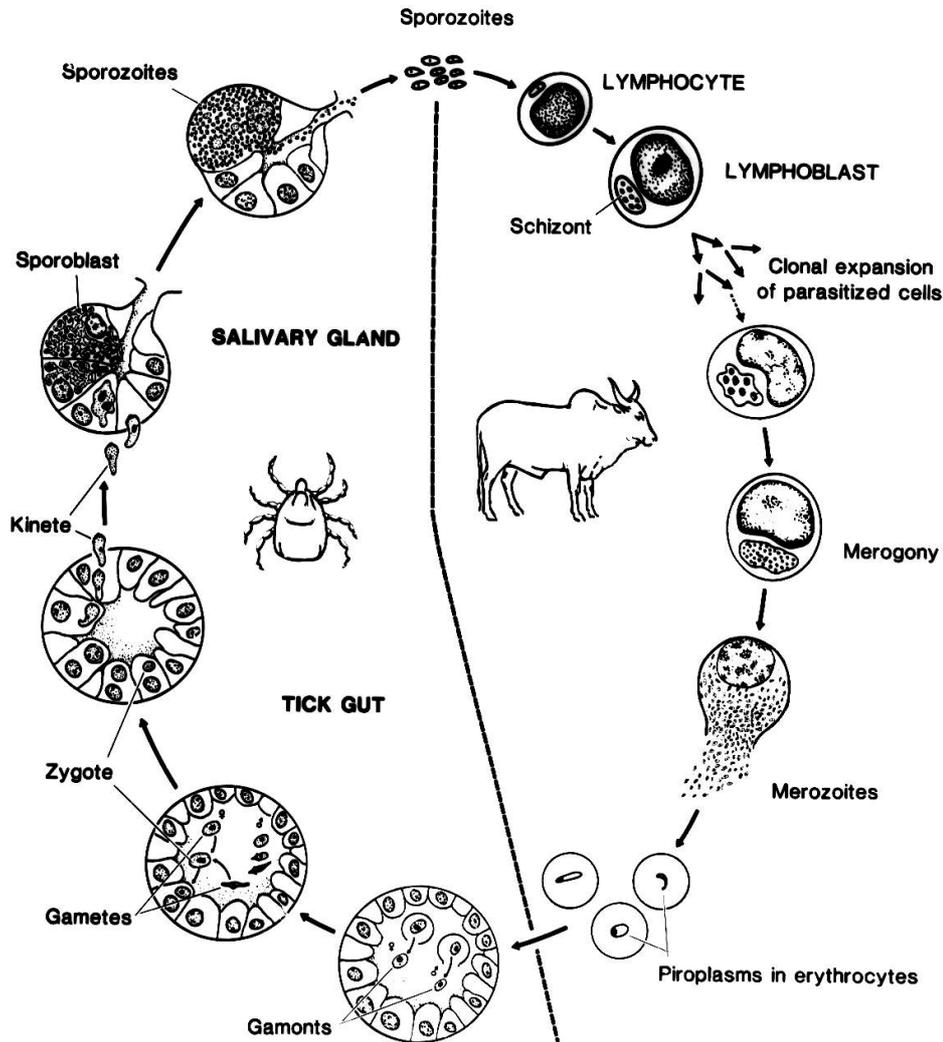
Fig. 2: Life Cycle of *Theileria parva* [148].

The life cycle in the tick continues through production of gametes that fuse to form a zygote. Zygotes penetrate the intestinal epithelium where they undergo reduction divisions to form mobile kinetes. The kinetes in turn migrate to the salivary glands where they undergo nuclear divisions without accompanying cytokinesis to form a complex syncytium, the sporoblast. Sporogony is completed when large numbers of the infective sporozoites are produced following four days of blood meal by the infected nymphal or adult tick and are ready to be released into the mammalian host.

Treatment and prevention of ECF

Currently ECF is controlled by a combination of techniques including chemotherapy, immunisation and vector control by use of acaricides on cattle as spray or cattle dips.

The first commercial drug against *Theileria* was halofuginone lactate (Terit®) followed by parvaquone (Clexon®). Subsequently, a more active analogue of parvaquone, buparvaquone (Butalex®), was synthesised. The three drugs are available but are extremely expensive. Costs of treatment of small indigenous breeds of low productivity may equal the value of the animal and only early treatment has a high probability of success.

Regular spraying or dipping of livestock in acaricide is quite effective in preventing ECF but the chemicals are expensive and due to environmental concerns and possible contamination of the food chain not desirable. Many of the farmers in the affected regions can hardly afford the cost of spraying and dipping even in communal dips and spray bays without any financial assistance. An addition to these problems is the ability of the ticks to develop resistance to the acaricides.

The only immunisation method used on a routine scale for the control of ECF is what is termed 'Infection and Treatment'. This is a live vaccination regimen where animals are given a needle injection of a lethal dose of *T. parva* and are simultaneously treated with a long-acting antibiotic, oxytetracycline. Immunity acquired can last for years, possibly even a lifetime, and resists homologous challenge, but not necessarily all strains encountered in the field. Immunized animals become carriers, thus increasing the infection rate in ticks and consequently the inoculation rate of other cattle. Although the techniques associated with the 'infection and treatment' method are far from being sophisticated and well within the reach of a reasonably equipped laboratory, the method demands skill, meticulousness and a great sense of responsibility and flexibility on behalf of the operators [202]. As with other live vaccines, problems related to the necessary cold chain (sporozoite stabilates have to be kept in liquid nitrogen until use in the field) are formidable obstacles in countries with poor infrastructure. In addition, there is a major concern over the introduction of exogenous vaccine strains to resident tick populations. Furthermore, the immunity engendered by the 'infection and treatment' method is strain-specific [202].

Thus from economic, social and environmental perspectives, the need for an alternative, such as a subunit vaccine against ECF, cannot be emphasised enough. The ultimate aim of the research described in this thesis therefore was to develop and optimise a subunit vaccine for ECF based on p67, the *Theileria parva* sporozoite surface protein. Besides, intensive efforts are focused on the identification of schizont-specific components for incorporation in a second-generation multi-component product [113, 114, 124,127]. The completion of the genome sequence of *T. parva* [145, 146] will further enhance the schizont antigen identification process. In addition, it offers new opportunities in combating infection and diseases by understanding the biology of these parasites and their hosts in greater detail.

Molecular basis of *T. parva* sporozoite-host cell interaction and approaches to vaccine development

A 20-25-nm thick surface coat covers sporozoites of *T. parva* and it is the interaction between this coat and the host cell surface molecules that are critical to parasite invasion [181]. For a detailed review on the biology and the morphological steps involved in the *T. parva* sporozoite invasion and/or interactions with the host cells, see references [176, 179 - 181]. The surface coat may have other functions in the biology of the sporozoites but the interest in its molecular composition has been directed primarily towards the development of a vaccine to block sporozoite entry and subsequent intracellular development [133, 135]. Sera from cattle repeatedly immunised with *T. parva* sporozoite lysates or from cattle that had been exposed to heavy parasite challenge in ECF endemic areas contain high titre antibodies capable of neutralizing *T. parva* sporozoite infectivity. These observations led to the hunt for sporozoite antigens that may be involved in the entry process [133]. Although these sera recognised five antigens on immuno-blots, a 67-kDa protein on the surface of sporozoites, designated p67, contained the major neutralising epitopes, as determined in studies with a panel of neutralising mAb [133]. The gene that encodes p67 has been cloned and characterized and it exists as a single copy gene interrupted by a 29-bp intron [141]. It encodes a 709 amino acid product that contains seven potential sites of N-linked glycosylation, although there is no evidence that the native protein is [141]. The p67 antigen is uniformly distributed on the sporozoite surface and is synthesised and assembled during the earlier stages of sporogony in the salivary glands [179]. P67-specific antiserum neutralises sporozoite infectivity from a range of different parasite stocks [143], indicating that p67 is conserved in cattle-derived parasites and underscoring the potential of p67 as a broad-spectrum vaccine. Monoclonal antibodies to both native and recombinant p67 also inhibit sporozoite invasion of target cells *in-vitro* [34, 141].

Anti-p67 specific antibodies block the initial binding event of the sporozoite to the host cell [179]. Furthermore, purified native and recombinant p67 can competitively block sporozoite entry, provided that the molecule is present in the incubation medium [177]. While the addition of exogenous p67 significantly inhibited sporozoite invasion, sporozoites that could bind entered host cells at levels comparable to control infections suggesting that exogenous p67 was affecting only the initial binding process [177]. These observation indicated that p67 may act as a ligand mediating the initial parasite-host cell interaction. Shaw *et al*., [177] also reported that the inhibition was readily and rapidly reversed by removing p67 from the medium implying that that at the molecular level, the interactions between individual p67 molecules and host cell surface molecules are relatively weak. In cell-to-cell adhesion processes, the rate of formation and the stability of the molecular interactions are dependent on the interplay of the association and dissociation rate constants. While at the molecular

level these individual interactions may be weak, *in vivo* they may be highly multimeric and involve large numbers of interactions between arrays of molecules on each cell surface. As *T. parva* sporozoites are uniformly covered by p67, it seems reasonable to conclude that parasite binding will involve the concerted participation of multiple interactions between, at least p67 and host of cell surface molecules. These studies thus provided direct and convincing evidence that p67 plays a critical role in sporozoite invasion.

The immunisation potential of p67 has been evaluated in cattle, using an *E. coli*-expressed recombinant form in which the p67 antigen was fused to the C-terminal 85 residues of the influenza A virus NS1 antigen [134]. Immunised animals generated high titres of p67-specific antibodies with strong *in-vitro* neutralising activity, and six of nine cattle (67%) were protected against severe disease following homologous challenge. Importantly, two of the immune animals developed a mild parasitosis, indicating that neutralisation was not complete. This has been a consistent feature in subsequent immunisation trials using recombinant p67 formulated in a number of adjuvant preparations. Five inoculations of 600 µg protein per inoculation were required to achieve this level of protection. Similar results were obtained with a baculovirus derived recombinant p67 vaccine [142]. Of considerable significance to the vaccine potential of p67 is the observation that the gene is highly conserved among cattle-derived isolates and that protection extends to heterologous parasite stocks [143].

The baculovirus-insect cell expression vector system

Since the discovery of the Baculovirus Expression Vector System (BEVS) in the early 1980s [157, 186, 187], the system has established itself as a powerful and versatile culture system for the production of numerous recombinant proteins in insect cells [96, 154]. Baculoviruses comprise a diverse group of arthropod viruses with high host specificity [120]. The best-studied member of this family, *Autographa californica* nucleopolyhedrovirus (AcMNPV), is a large enveloped virus with a double-stranded, circular DNA genome of about 130 kbp. The complete sequence of the viral genome has been determined [8]. This virus is non-pathogenic to vertebrates and can encapsulate large pieces of foreign DNA.

BEVS is based on constructing recombinant baculoviruses by replacing the coding sequence of the polyhedrin or p10 gene with the foreign DNA and then using these viruses as vectors for the infection of insect cells (Fig. 3). The polyhedrin gene has an extremely strong promoter, which is activated very late in the baculovirus infection cycle, and the gene encodes the occlusion-body matrix protein polyhedrin. This viral-encoded protein is the major component of the occlusion body, which embeds the wild-type baculovirus inside a protective para-crystalline coating.
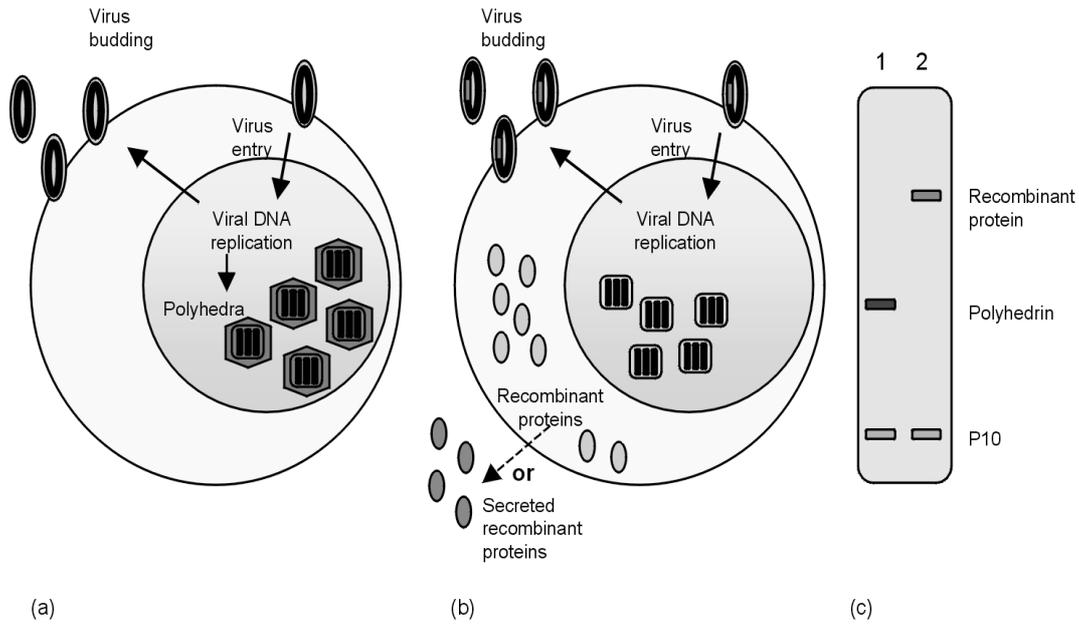
Fig. 3: Baculovirus expression system. Pannel (a) shows a wild type infection, resulting in the replication of the virus in the nucleus, the budding of progeny virus from the cell surface and the occlusion into polyhedra. In (b) infected cells are infected with a recombinant baculovirus overexpressing a heterologous gene under control of the polyhedrin promoter. In (c) a protein gel is shown for AcMNPV (1) and recombinant virus-infected cells (2) showing expression of p10, polyhedrin and a recombinant protein.

The polyhedrin gene is nonessential for viral replication and accumulates to high levels, comprising over 30% of the total protein content at the time of cell death [210]. During the early phase nucleocapsids bud at the plasma membrane, thereby acquiring an envelope. These budded or non-occluded viruses infect neighbouring cells and cause spread of infection in insect bodies or cell cultures. Lysis of infected cultured cells starts within 36 hours post-infection (p.i.) and is complete by 96 hours p.i.

Insect cell lines derived from *Spodoptera frugiperda* (Sf-9 and Sf-21) or from *Trichoplusia ni* (High Five™ , Tn368) are routinely used in research institutes and in the biotech industry for the production of baculovirus vectors and recombinant proteins. Insect cells provide post-translational processing similar to mammalian systems, including proteolytic cleavage, glycosylation, secretion, folding, phosphorylation, acylation and amidation. Expression of heterologous proteins using the BEVS has many advantages such as correct functionality of the foreign proteins, a high expression level for many proteins, the possibility of post-translational modification, easy vector construction, simultaneous expression of multiple genes and safety. The system is simple to use due to multiple commercially available expression kits [97].

Baculovirus transfer vectors

Traditionally, recombinant baculoviruses were constructed in two steps. First, the heterologous gene was inserted into a transfer vector that contained a promoter, usually from the polyhedrin or p10 gene, flanked by baculovirus DNA. This plasmid was then introduced into insect cells along with circular or linearized genomic viral DNA after which the generation of recombinants occurred. Several approaches have been developed to obtain recombinant baculoviruses easily and nowadays many modified viral DNA's [55, 90, 91, 109], and a huge variety of transfer plasmids are available [109, 117, 119, 164, 206]. Recent advances in the baculovirus expression vector technology include improvements to methods for dominant selection of recombinant viruses [118, 166], development of the system for production of multi-subunit protein complexes and vectors for easy and improved heterologous protein expression and production (e.g. bacmid and baculovirus display technologies). Recombinant vectors have also been engineered for transient and stable gene delivery/transfer into a broad spectrum of primary and established mammalian and other cells [95, 97, 100]. The application of modified baculovirus for *in vivo* gene delivery has also been demonstrated [188].

The baculovirus surface display method, which mimics the phage display system [13] allows heterologous proteins expressed in the BEVS to be projected on the surface of both infected insect cells and budded virions by fusing the foreign protein to the major baculovirus envelope glycoprotein, GP64 [51]. This method has been used successfully for the expression of viral surface antigens [50, 105, 128, 194]. This system has the capacity to produce near-native folding of recombinant protein [129]. Bacmid technology provides a rapid and efficient method to generate recombinant baculoviruses, and is based on site-specific transposition of a foreign gene into a baculovirus shuttle vector (bacmid) propagated in *E. coli* [108]. The generation of recombinant baculoviruses via the bacmid system eliminates the need for multiple rounds of plaque purification and reduces the time required to identify and purify a recombinant virus compared to the traditional methods involving homologous recombination.

Scope of the thesis

The aim of this thesis is to obtain the *Theileria parva* sporozoite coat protein (p67) in a near-native form and to develop a recombinant p67-based subunit vaccine against the deadly cattle disease ECF. Previous reports have showed the potential of p67 as a good and broad-spectrum antigen for inclusion in the development of a subunit vaccine against ECF [134, 142, 143]. P67 has been expressed in BEVS before but the expression level was very low and just a minor fraction of this product appeared to be correctly folded [142]. In addition, p67 was not transported to the cell surface. In all these reports, large amounts of the antigen ((300-600 µg)/inoculation) and more than

three inoculations were required to achieve a reasonable level of protection. Incorporating conformational epitopes may enhance the immunogenicity and efficacy of the p67-based subunit vaccine. Therefore, novel strategies have to be developed and incorporated into BEVS technology in order to express and produce heterologous proteins in more stable and correctly folded forms. In the first experimental chapter (**Chapter 2**) the aim is to overcome the low levels of p67 expression observed with full-length p67. Two sets of constructs are analyzed, where p67 regions are expressed as non-fused entities or as fusions to the C-terminus of green fluorescent protein (GFP). With these constructs various regions of p67 are expressed as cytoplasmic peptides. The idea behind this is that expressing full-length p67 via the secretory pathway may result in aggregation and bad processing of the recombinant protein.

In the next chapter the baculovirus surface display method is explored for the expression of an N- and C-terminal domains of p67 **(Chapter 3)**. The baculovirus surface display system was used to direct p67 through the export system to the outside of the cell, hopefully with conservation of it's native folding. To allow easy generation and screening of recombinant viruses the surface display system is combined with bacmid technology, enabling the generation of recombinant viral genomes in bacterial cells.

Transmembrane proteins are in general produced in relatively low amounts in BEVS. Therefore, another way of improving expression levels of p67 is sought in producing a secretory, soluble form of p67 (**Chapter 4**) using baculovirus vectors. In order to achieve this a hydrophobic region is deleted from the C-terminus that most likely serves as transmembrane domain, and the signal peptide is replaced by an insect signal peptide, derived from honeybee melittin. Furthermore, the purification of large amounts of p67 immunogens from the insect cell culture medium may be an easy-to-handle process for large-scale production of recombinant p67. In addition, baculovirus vectors with deletions in the *chitinase* and *v-cathepsin* genes are constructed in order to prevent breakdown of the recombinant protein by viral proteases.

The next step is to test the p67 antigens that have good levels of expression and a proper folding for their immunogenicity (**Chapter 5**). Therefore, the various GFP-p67 and GP64-p67 fusion proteins are tested in mice to select those antigens that give the highest levels of sero-conversion and generate neutralizing antibodies. The best immunogens are subsequently investigated for their ability to induce neutralizing antibodies in cattle. Finally, based on the sero-conversion experiments, two vaccine candidates are tested in an efficacy trial in cattle, which also includes two different types of adjuvants, in order to determine the level of protection conferred (**Chapter 6**). Finally the prospective of the new p67-based vaccines and additional questions that need to be addressed are discussed **(Chapter 7).**

Chapter 2

Fusion to green fluorescent protein improves expression levels of *Theileria parva* sporozoite surface antigen p67 in insect cells

SUMMARY

East Coast fever (ECF) is a fatal disease of cattle caused by the protozoan parasite *Theileria parva*. The development of a subunit vaccine, based on the sporozoite-specific surface antigen p67, has been hampered by difficulties in achieving high level expression of recombinant p67 in a near-authentic form. Therefore, two sets of recombinant baculovirus vectors were constructed. The first set, encoding various regions of p67, produced low levels of the corresponding p67 domains in High Five[TM] cells, despite the presence of large amounts of p67 RNA. The second set, consisting of p67 domains fused to the carboxy-terminus of green fluorescent protein (GFP) expressed significantly higher levels of p67 protein. The GFP:p67 fusion proteins were recognised by a sporozoite-neutralising monoclonal antibody (TpM12) raised against native p67 whereas non-fused full length p67 was not recognised. GFP-tagging, therefore, appeared to enhance the stability of p67 and to conserve its folding. The high-level expression of p67 domains in a more authentic form is an important step towards the development of an effective subunit vaccine against ECF.

Introduction

Theileria parva is a tick-transmitted protozoan parasite of cattle, which causes East Coast fever (ECF). This disease is of major economic importance throughout east, central and southern Africa. The brown ear tick, *Rhipicephalus appendiculatus*, transmits the sporozoite stage of the parasite. The sporozoites enter host lymphocytes where they develop into intracellular multinucleate schizonts resulting in a fatal lymphoproliferative and destructive disease [38, 148].

The sporozoite surface protein p67 synthesized during sporogony within the tick plays an essential role in the invasion process [35-37]. Monoclonal antibodies directed against p67 block [34] and soluble p67 competitively inhibits [177] invasion of bovine lymphocytes by the sporozoites. P67 contains 709 amino acid residues and has the characteristics of a transmembrane protein. It has a secretory signal sequence at the N-terminus, seven potential N-linked glycosylation sites and a hydrophobic C-terminal tail [141]. P67 is conserved among sporozoites isolated from different cattle-derived *T. parva* stocks [143] thus making it a good candidate for a broad-spectrum subunit vaccine against ECF.

To investigate its vaccine potential, p67 has been cloned and expressed in *Escherichia coli* [134] as a fusion to the non-structural (NS1) protein of influenza virus A. Cattle immunised with NS1-p67 generated high titres of p67 specific antibodies with a strong *in vitro* neutralising activity against *T. parva* sporozoites. On challenge with LD_{70} of stabilated sporozoites, 70% of the immunised cattle were protected against ECF. A relatively high dose of NS1-p67 was required to achieve this level of protection. In addition NS1-p67 was not recognised by TpM12, a neutralising monoclonal antibody raised against native p67 [132] These observations indicate that the *E. coli*-expressed p67 was not in a native conformation or it lacked characteristic post-translational modifications, such as glycosylation, which might be required for complete protective immune responses.

The baculovirus-insect cell expression system has been used in an attempt to express p67 in more authentic forms with the proper folding and modifications [142]. Attempts to generate a full-length p67 in insect cells were frustrated by the fact that recombinant p67 was produced at a low level, the product was only partially processed and not transported to the cell membrane. This insect cell-derived p67 was essentially produced in in a non-native form. Cattle immunized with the baculovirus-derived p67 showed a response similar to animals immunized with the NS1-p67 product. The lack of sufficient quantities of near-authentic recombinant p67 hampers

the evaluation of the full potential of p67 as a vaccine [142]. Waldo *et al.* [209] first suggested that green fluorescent protein (GFP) might enhance stability of GFP fusion proteins. Indeed in prokaryotic expression systems, GFP-tagging increased both stability and solubility of recombinant proteins [172]. Since the first expression of GFP with recombinant baculoviruses [170], GFP has been widely used as a fluorescence marker for gene expression, protein localization and trafficking, and protein-protein interactions by fusing its coding sequence to that of the protein of interest [216].

In this study we sought to evaluate whether GFP fusion could enhance the stability and hence the expression levels of p67 in insect cells as well as induce/preserve its native conformation, by expressing various p67 sub-domains either as separate open reading frames (ORFs) or as C-terminal fusions to GFP. We report here the high-level expression of p67 domains in a more authentic form in insect cells.

Materials and Methods

Cells and viruses

Trichoplasia ni High-Five^{TM} insect cells (Invitrogen) were maintained in Grace's supplemented insect medium (Invitrogen) with 10% foetal bovine serum (FBS) as monolayer cultures at 27°C. For routine cell maintenance, virus infection and propagation, standard procedures were followed [89, 193]. As control viruses the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), strain E2 [186], and the recombinant baculovirus, BEV-p67 [142] were used. BEV-p67 is a recombinant baculovirus carrying the complete p67 protein including its native signal and transmembrane sequences.

Construction of recombinant baculoviruses

Theileria parva cDNA sequences (Fig 1A) encoding the N-terminus (p67N$_{21-225}$), the central region (p67M$_{226-571}$), the C-terminus (p67C$_{572-651}$) as well as the full-length p67 protein (p67ΔSS$_{21-651}$) were PCR-amplified from a pMG1-p67 clone [134] using extended primers that introduced *Bss*HII/*Sal*I/*Sst*I or *Hin*dIII/*Cla*I restriction sites (see Table 1). In contrast to the complete p67 protein expressed by recombinant BEV-p67 [140], the p67ΔSS domain excluded the authentic p67 signal and transmembrane anchor sequences. The PCR products were cloned into pGEM-T (Promega) and sequences of the inserts were verified by automated sequencing.
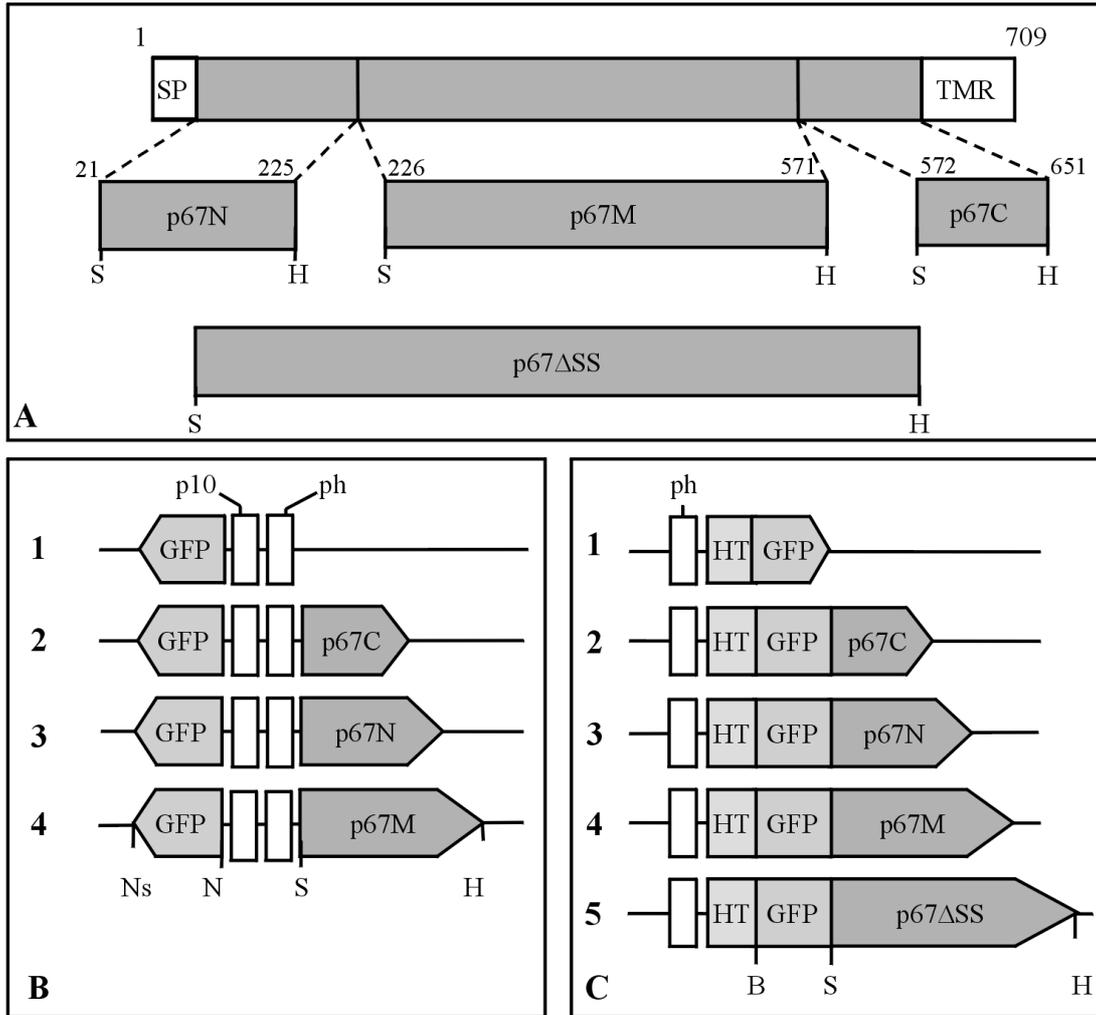
Fig. 1: Schematic representation of non-fused p67 (B) and GFP:p67 (C) fusion constructs.
(A) The *T. parva* p67 open reading frame: SP (amino acid 1-20) is the p67 signal peptide; p67N (amino acid 21-225) the amino terminus; p67M (amino acid 226-571) the middle region; p67C (amino acid 572-651) is the carboxy terminus and TMR represents the transmembrane region. (B) The various pFastBacDual constructs: GFP was inserted as a *Nco*I-*Nsi*I fragment downstream of the p10 promoter while the various p67 domains were inserted as *Hin*dIII-*Sst*I fragments under the transcriptional control of the polyhedrin (ph) promoter. (C) The various GFP:p67 fusion constructs: GFP was first cloned into the pFastBacHTb vector as *Bam*HI-*Sst*I fragment to generate the vector pFBhisGFP: the various p67 domains were then inserted into the pFBhisGFP vector as *Sst*I-*Hin*dIII fragments resulting in an end-to-end gene fusion of the p67 domains the C-terminus of GFP. The pFastBacHTb vector encodes a hexa-polyhistidine sequence allowing N-terminal His-tagging of the foreign protein to facilitate purification. S = *Sst*I, H = *Hin*dIII, Ns = *Nsi*I, N = *Nco*I, B = *Bam*HI.

The DNA sequence encoding GFP was amplified from a pBluescript-GFP clone (kindly provided by Gerard van der Krogt; Laboratory of Molecular Biology, Wageningen University) by using extended primers to introduce *Nco*I/*Nsi*I or *Bam*HI-*Cla*I/*Hin*dIII-*Sst*I sites (Table 1). The PCR products were cloned into pGEM-T (Promega). GFP was re-cloned into the plasmids pFastBacDual (pFBD) and

pFastBacHTb (pFBhis) (Invitrogen). GFP was cloned as *Nco*I-*Nsi*I fragment downstream of the p10 promoter in pFastBacDual generating the parental plasmid for non-fused expression, pFBDp10GFP (Fig 1B; 1). In the pFastBacHT vector, GFP was cloned as *Bam*HI-*Sst*I fragment generating the parental plasmid for the GFP fusions, pFBhisGFP (Fig 1C; 1).

To generate non-fused constructs, p67C, p67N and p67M were cloned as SstI-*Hin*dIII fragments downstream of the polyhedrin promoter in the pFBDp10GFP vector, generating the donor plasmids: pFBDp10GFP-php67C, pFBDp10GFP-php67N and pFBDp10GFP-php67M (Fig 1B; 2, 3, 4 respectively). Cloning of the p67 domains as *Sst*I-*Hin*dIII fragments into the pFBhisGFP vector resulted in in-frame end-to-end gene fusion of the p67 domains to the C-terminus of the GFP, generating the GFP:p67 fusion constructs pFBhisGFP:p67C, pFBhisGFP:p67N, pFBhisGFP:p67M and pFBhisGFP:p67ΔSS (Fig 1C; 2, 3, 4, 5). Recombinant baculoviruses encoding the non-fused p67 protein domains were generated via the Bac-to-Bac™ baculovirus expression system (Invitrogen), resulting in Ac-p67N, Ac-p67M, Ac-p67C and the control virus Ac-GFP. The recombinant baculoviruses Ac-hisGFP:p67N, Ac-hisGFP:p67M, Ac-hisGFP:p67C, Ac-hisGFP:p67ΔSS encoding the fusion proteins and the control, Ac-hisGFP was also generated [108].

Protein analysis

For the analysis of recombinant protein expression, 1×10^6 *T. ni* High Five™ cells were seeded into 35 mm^2 Petri dishes and infected with the various recombinant viruses at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell. At 72 h post infection (p.i.) cells were harvested and washed in 1-ml phosphate buffered saline (PBS) at 4°C. Finally the pellet was resuspended in 100-μl ice-cold PBS. Protein concentrations were determined using Bradford reagent (BIO-RAD). Samples containing 2.5-μg total protein were analysed in 15% (non-fused samples) or 10% (fusion proteins) SDS-PAGE gels as described by Laemmli, [98]. Proteins were visualized by Coomassie Brilliant blue (CBB) staining or subjected to Western analysis. Monoclonal antibodies, ARIII 22.7 and ARIII 21.4 [144] specific for p67N and C respectively, were used. The polyclonal antibodies, Rat 44 and bovine BJ 36 [144] raised against the complete p67 protein were used for p67M and p67ΔSS respectively. All antibodies were used at a dilution of 1:200. For the GFP:p67 fusion proteins, monoclonal antibodies against the polyhistidine tag (Sigma) or a polyclonal antibody against GFP (both at a dilution of 1 : 5000) were used. As the second antibody, rabbit anti-mouse immunoglobulins (for the monoclonals), anti-bovine immunoglobulins (for BJ 36), or swine anti-rabbit immunoglobulins (for the αGFP)

conjugated to horseradish peroxidase (HRP) (DAKO, A/S, Denmark) were used at a dilution of 1 : 5000. The HRP substrate, 4-chloro-1-naphthol (Bio-Rad) was used to detect the p67 recombinant proteins.

Table 1: **Oligonucleotides used for the amplification of p67 domains and GFP***

Name	Sequence from 5'to 3'	Characteristics
SUN-1F:	CGGATCCATCGATGCCATGGGCAAAGGAGA	Forward primer for GFP. Introduces *Bam*HI and *Cla*I sites.
SUN-1R:	CCAAGCTTGAGCTCTTCATCCATGCATGTG	Reverse primer for GFP. Introduces *Hin*dIII and *Sst*I sites.
GFP/F:	CATGCCATGGGCCATGGGCAAAGGAGA	Forward primer for GFP. Introduces *Nco*I site.
GFP/R:	TGCATGCATTTCATCCATGCCATGTG	Reverse primer for GFP. Introduces *Nsi*I site.
TP67/21-F:	GGCGCGCGTCGACGAGCTCATGCCTACGGAGGAACAACCAT	Forward primer for N-terminal domain of p67. Introduces *Bss*HII, *Sal*I and *Sst*I sites.
TP67/225-R:	CAAGCTTATCGATAAGATCTTGGCCCGATGTAGTT	Reverse primer for N-terminal domain of p67. Introduces *Hin*dIII and *Cla*1 sites.
TP67/226-F:	GGCGCGCGTCGACGAGCTCATGAATTCAAAACAACAGCAAACTG	Forward primer for the middle region of p67. Introduces *Bss*HII, *Sal*I and *Sst*I sites.
TP67/571-R:	CAAGCTTATCGATTGCTGCTCGTCCCGTACCTGAT	Reverse primer for the middle region of p67. Introduces *Hin*dIII, and *Cla*1 sites.
TP67/572-F:	GGCGCGCGTCGACGAGCTCTGGGAACGGGAGGGGGATCACTGAG	Forward primer for C-terminal domain of p67. Introduces *Bss*HII, *Sal*I and *Sst*I sites.
TP67/651-R:	CAAGCTTATCGATTCCAGCTGCTATTGTGGGCCCT	Reverse primer for C-terminal domain of p67. Introduces *Hin*dIII, and *Cla*I sites.

*Restriction sites are underlined

Purification and dot blot analysis

The polyhistidine tag was exploited to purify the fusion proteins using TALONspin IMAC columns (CLONTECH Laboratories). High Five^TM insect cells were infected with the recombinant viruses at a MOI of 10 PFU per cell and collected at 72 h p.i. Cells were concentrated and clarified cytoplasmic extracts were applied to the columns. After washing with buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0) containing 25 mM imidazole to remove unbound and weakly bound proteins, the polyhistidine-tagged GFP:p67 fusion proteins were eluted from the column by increasing the imidazole concentration to 200 mM.

To examine the antigenic authenticity of the insect cell-derived GFP:p67 fusion proteins dot blot analysis was performed. Five micrograms of the purified GFP:p67 fusion proteins and 10 μg of total cell lysate of infected *T. ni* High Five^TM insect cells were spotted on a nitro-cellulose membrane and allowed to dry. Proteins were denatured by adding 1/20 volume of β-mercaptoethanol and 1/4 volume of 4x sample buffer (40 mM Tris-HCl pH 8.0, 4 mM EDTA, 8% SDS) and incubation at 95 $^\circ$C for 10-min. Duplicate blots were incubated with TpM12 (1:50) or ARIII 22.7 (1:200) for 1 h at room temperature, washed and further incubated with goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (Amersham) at a dilution of 1:5000. Enhanced chemiluminescence (ECL) (Amersham) was used for detection. The insect cell-derived p67 expressed by Nene *et al*. [142], indicated as BEV-p67, was compared with these GFP:p67 fusion proteins. The same amount (5 μg) of purified *E. coli*-expressed p67N, and his: GFP proteins as well as mock and Ac-wt infected cell lysate containing 10 μg total protein were used as controls.

Transcript analysis

Trichoplusia ni High Five^TM insect cells (1.0 x 10^6) were infected with the recombinant viruses Ac-p67N, Ac-p67M, Ac-p67C, and Ac-GFP at a MOI of 10 pfu/cell and harvested at 24, 48 and 72 h p.i. Total RNA was isolated using the single-step acid guanidinium thiocynate-phenol-chloroform RNA extraction method described by Chomczynski and Sacchi [24]. Five μg RNA were resolved in 1.4 % agarose gel and Northern blot analysis was carried out as described in Pellé & Murphy [156]. The RNA was fixed to the filter by a UV light cross-linking. The filter was hybridized to a PCR-amplified p67 (full length) probe, labeled with α^{32}P-dCTP by random primed labeling with Klenow (Promega).

Results

Expression of p67 sub-domains in insect cells

To enhance the expression levels of p67 in insect cells, sub-regions of p67 rather than the complete p67 protein [142] were expressed. The domains of p67 expressed in this study were an N-terminal domain (p67N$_{21-225}$), the central region (p67M$_{226-571}$) and a C-terminal domain (p67C$_{572-651}$) as well as the full-length protein without its native signal sequence and transmembrane region (p67ΔSS) (Fig. 1A). The p67N, p67M and p67C domains were cloned and expressed as separate open reading frames (non-fused constructs) (Fig. 1B). In a second experiment, each p67 domain was fused to the C-terminus of GFP and expressed as a fusion protein (Fig. 1C).

Trichoplusia ni High Five^{TM} cells infected with recombinant viruses encoding these different p67 sub-domains s (non-fused and fused) were analysed in SDS-PAGE and stained with Coomassie Brilliant blue (CBB) or subjected to immunoblot analysis. In the Coomassie-stained gel of the non-fused p67 recombinants the p67 sub-domains p67N, p67M and p67C could not be detected (Fig. 2A, lanes 5-7). A protein with the expected electrophoretic mobility for GFP was observed for all recombinants (lanes 4-7). The medium of cells infected with Ac-GFP, Ac-p67N, Ac-p67M and Ac-p67C turned green as early as 24 h p.i. GFP expression was confirmed by the green fluorescence of the protein under UV-light and by Western blot analysis using αGFP polyclonal antibody (DAKO) (results not shown), indicating that the infection had been successful. These results indicate that the expression levels for the sub-domains were not significantly increased compared to the full-length p67 expressed in insect cells (lane 3).

In immuno-blot analysis A reaction with p67 antibodies was observed for BEV-p67 which was absent in the mock, Ac-wt and Ac-GFP samples (Fig. 2B, lanes 1-4). The monoclonal antibodies, ARIII 22.7, RAT 44 and ARIII 21.4, directed against p67N, p67M and p67C, respectively, showed that p67N, p67M and p67C proteins were expressed but at low levels and in unstable forms as indicated by the observation of degraded products (Fig. 2B, lanes 5-7). The major product of p67N was detected as a 45.0 kDa protein instead of the expected 22.0 kDa (Fig. 2B, lane 5). In Ac-p67M infected cells a 38-kDa protein was detected as expected for p67M protein (Fig. 2B lane 6). P67C was detected as two separate major proteins of 8.6 kDa, the expected mobility for p67C, and of 28.0 kDa, three times the predicted size of p67C (Fig. 2B, lane 7).
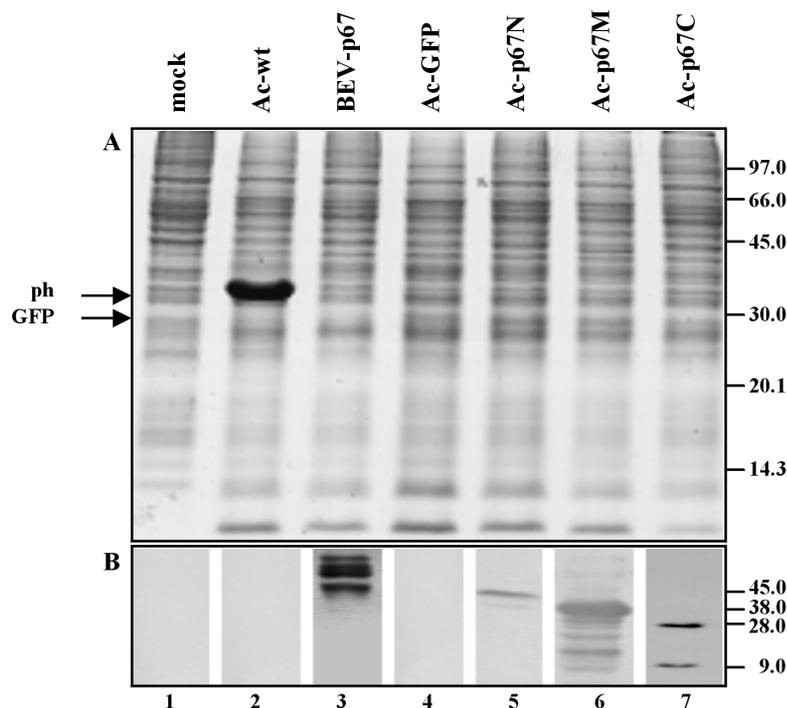
Fig. 2: SDS-PAGE and Western blot analysis of non-fused p67 proteins. *Trichoplusia ni* High Five[TM] insect cells were mock-infected (lane 1) or infected with Ac-wt virus (lane 2) or recombinant viruses BEV-p67 (lane 3), Ac-GFP (lane 4), Ac-p67N (lane 5), Ac-p67M (lane 6), Ac-p67C (lane 7) and harvested at 72 h p.i. Total protein (2.5 μg) was resolved in 15% SDS-PAGE and stained with Coomassie Brilliant blue (**A**) or subjected to immunoblot analysis using the p67N-specific monoclonal antibody ARIII 22.7, the p67M-specific polyclonal antibody Rat 44 or the p67C-specific monoclonal antibody ARIII 21.4 (**B**).

To investigate whether the low level of expression of p67 domains observed was due to low transcription, we examined p67 transcripts at different times post infection. Total RNA was purified from mock-infected and cells infected with Ac-wt, Ac-p67N, Ac-p67M and Ac-p67C viruses and subjected to Northern blot analysis using a p67-specific probe. The p67 probe did not hybridise to mRNA purified from mock and Ac-wt infected cells (Fig. 3 lanes 1 & 2). In the recombinant virus-infected cells, p67 transcripts were detected at 24 h p.i. and the amount increased at 48 h p.i. Transcripts were still observed at 72 h p.i. (Fig. 3, lanes 3-5). The large amount of transcripts was, however, not reflected in the level of p67 proteins observed (see Fig. 2A), suggesting that the low level of expression of p67 sub-domains might be due to instability of the end products. To overcome this problem, p67 sub-domains were expressed as carboxy-terminal fusion proteins to GFP (Fig. 1C). *T.ni* High Five[TM] cells were infected with the recombinant baculoviruses, Ac-hisGFP, Ac-hisGFP:p67N, Ac-hisGFP:p67M, Ac-hisGFP:p67C, Ac-hisGFP:p67ΔSS and total proteins from infected cell lysates were resolved in SDS-PAGE and stained with CBB or subjected to immunoblot analysis. In the control lanes no p67 specific proteins were observed (Fig.

4A, lanes 1-4). A 31-kDa protein corresponding to the predicted size of hisGFP was observed for Ac-hisGFP (Fig. 4A, lane 4). With the fusion constructs, significantly higher-levels of expression of p67 recombinant fusion proteins were observed in the CBB-stained gel (Fig 4A, lanes 5-8) as compared to the non-fused proteins. The hisGFP:p67N fusion protein (lane 5) migrated as a 75-kDa protein as opposed to a predicted size of 53-kDa. HisGFP:p67M was expressed in two forms, the expected 69-kDa protein and a 110-kDa protein (Fig. 4B, lane 6). HisGFP:p67C was expressed as a 39 kDa protein (Fig 4B, lane 7) and hisGFP:p67ΔSS as a 110 kDa protein (Fig. 4B, lane 8) as expected.
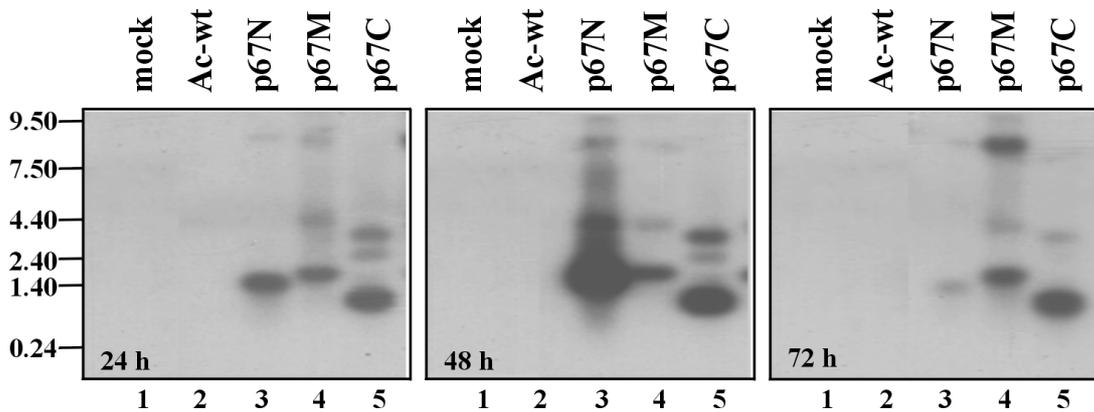
Fig. 3: Northern blot analysis of non-fused p67 transcripts. Total RNA (5 μg) was extracted from mock-infected *T. ni* High Five™ insect cells (lanes 1), or cells infected with Ac-wt virus (lanes 2) or the recombinant viruses Ac-p67N (lanes 3), Ac-p67M (lanes 4), Ac-p67C (lanes 5) at 24 h, 48 h, or 72 h p.i. Total RNA was resolved in a 1.4 % agarose gel and transferred to a Nylon filter. The filter was hybridized to a radioactively labeled p67 DNA probe. The sizes of an RNA marker are indicated in kilobases.

A monoclonal antibody directed against the N-terminal hexa-histidine tag (CLONTECH) was used to confirm expression of all the fusion proteins in an immuno-blot analysis. All the fusion proteins were detected at the same size as observed in the protein gel (Fig.4B lanes 4-8). Western blot analysis using either a polyclonal antibody against GFP or monoclonal antibodies directed against the different p67 domains gave similar results (not shown).

Conformation of p67 fusion proteins
The GFP-p67 fusion proteins were purified and the SDS-PAGE analysis of eluted fractions showed that the polyhistidine tagged proteins were efficiently bound to the column and that the fractions contained highly purified proteins (results not shown). An immuno-dot blot analysis was performed to investigate whether the GFP-tagging affected the folding of the p67 domains in the fusion protein. A monoclonal antibody,

TpM12, raised against native p67, and which does not recognise *E.coli* derived p67 [132, 134], was used as the first antibody in this analysis. Purified proteins as well as total cell lysate of cells infected with hisGFP:p67N and hisGFP:p67ΔSS were spotted on the blot.
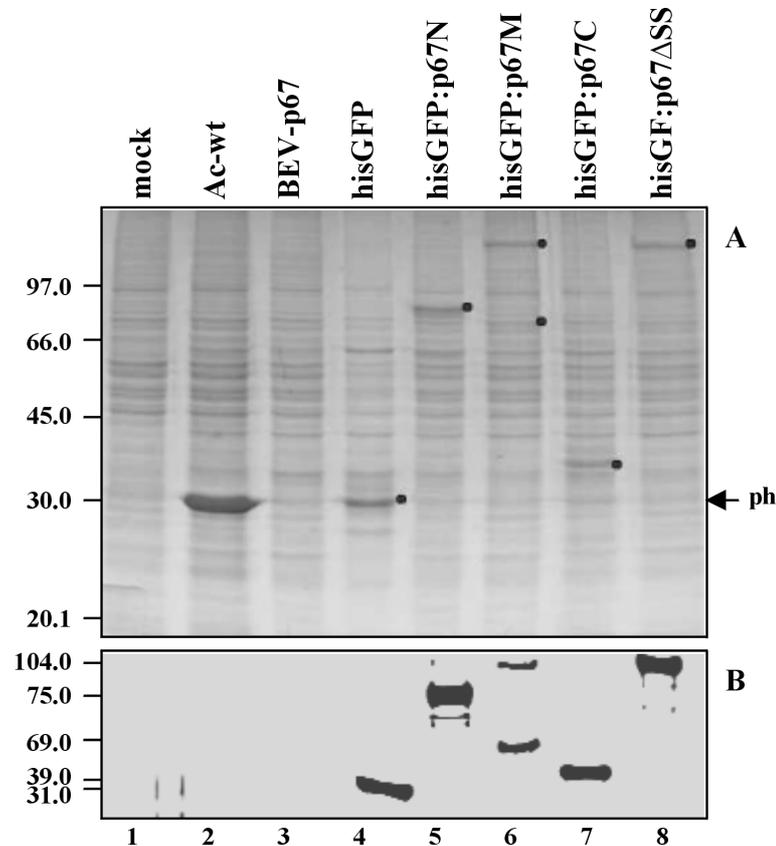
Fig. 4: SDS-PAGE and Western blot analysis of GFP:p67 fusion proteins. *T. ni* High Five™ insect cells (lane 1) were infected with the Ac-wt virus (lane 2), or recombinant viruses BEV-p67 (lane 3), Ac-hisGFP (lane 4), Ac-hisGFP:p67N (lane 5), Ac-hisGFP:p67M (lane 6), Ac-hisGFP:p67C (lane 7), Ac-hisGFP:p67ΔSS (lane 8) and harvested at 72 h p.i. Proteins (2.5 µg) were resolved in 10% SDS-PAGE and stained with Coomassie Brilliant blue (**A**) or subjected to immunoblot analysis using a monoclonal antibody against the hexa-histidine tag (**B**). The dots (in A) indicate the various GFP fusion proteins.

As controls, we used hisGFP, BEV-p67 and *E. coli*-derived p67N as well as a mock-infected cell lysate. Both denatured and non-denatured samples were analysed. As a control measure a duplicate blot was incubated with the monoclonal antibody, ARIII 22.7, that is raised against denatured p67 and which recognises a linear epitope in the N-domain [144]. TpM12 did not react with proteins (both denatured and non-denatured) in the mock, the BEV-p67, *E. coli*-derived p67N and the hisGFP samples (Fig. 5A, lanes 1-4). TpM12 reacted with non-denatured but not with the denatured samples of hisGFP:p67N and hisGFP:p67ΔSS (Fig. 5A, lanes 5 & 6), indicating that

the GFP-p67 fusion proteins were expressed in a near-native conformation. All the protein samples (both denatured and non-denatured) except for the negative controls reacted with ARIII 22.7 (Fig. 5B, lanes 1-6), confirming that p67 specific proteins had been spotted on all blots.
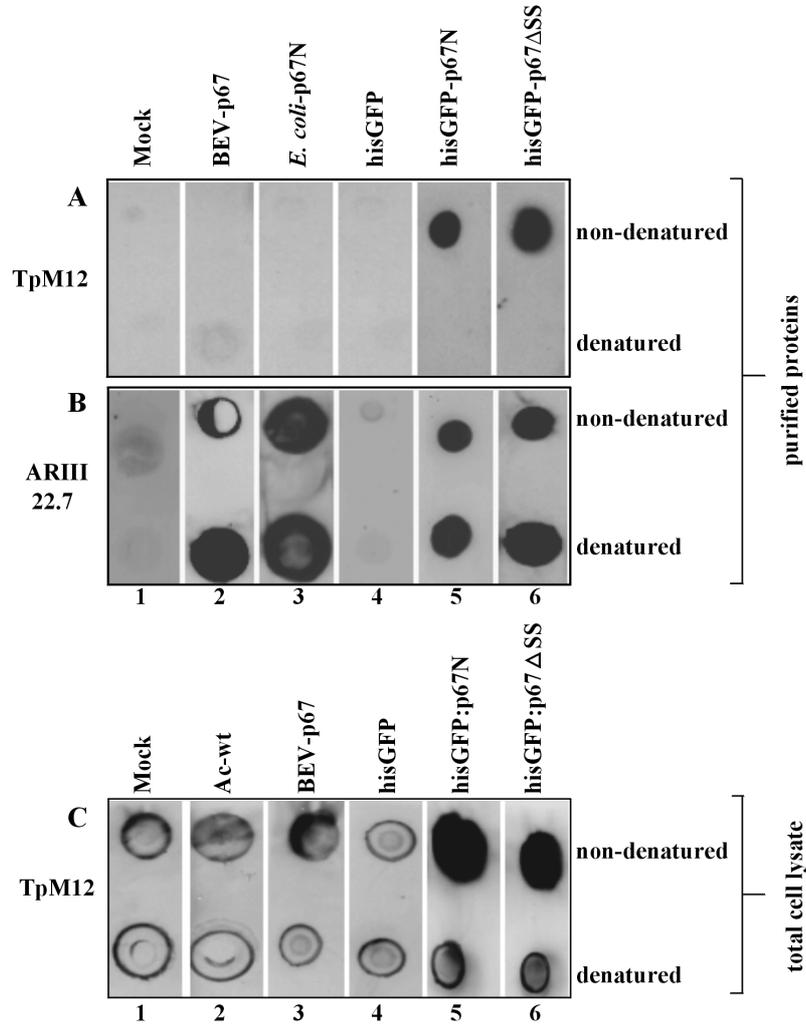
Fig. 5: Immuno-dot blot analysis of hisGFP:p67 fusion proteins. Both non-denatured and denatured purified proteins (A and B) or total cell lysate (C) of hisGFP:p67N and hisGFP:p67ΔSS were spotted on a nitrocellulose membrane and subjected to immunoblot analysis using monoclonal TpM12 or ARIII 22.7. TpM12 was raised against native p67 while ARIII 22.7 was raised against *E.coli*- derived p67.

Discussion

Low expression levels of *T. parva* p67 were observed by Nene *et al.* [142] in their attempt to produce large amounts of p67 in a more authentic form via the baculovirus-insect cell expression system. In the present study we expressed sub-domains (Fig. 1A) of *T. parva* sporozoite p67 instead of the complete p67 protein to obtain higher levels of expression. First, we expressed p67 sub-domains as non-fused (Fig. 1B)

proteins and in a second experiment, as fusions to the C-terminus of GFP (Fig. 1C). In both groups the native p67 signal peptide and transmembrane region were eliminated as this might limit expression levels [102]. Pepscan and computer analyses have predicted that antigenic sites of p67 were located in the N- and C-terminal regions [92, 144]. Bovine antibody responses to p67 are restricted to these two domains [92, 144]. Although the middle region of p67 does not appear to have linear B-cell epitopes, it contains several Th-cell epitopes [136]. The middle region was included in this study since it could be used to define T-cell epitopes when it becomes necessary to investigate the protective capacity of peptide-based vaccines in the future.

The non-fused proteins were expressed at the same low level (Fig. 2A) as the full-length p67 protein [142]. This may be due to inefficient translation of p67 transcripts or the instability of nascent p67 proteins since abundant transcripts were detected by Northern blot analysis (Fig. 3). A similar problem was seen in *E. coli*, where p67 expressed as fusion to the glutathione-S-transferase of *Schistosoma japonicum* [141] and the non-structural protein-1 (NS1) of influenza virus A [134] have failed to produce stable recombinant p67 protein.

A significantly higher level of expression of the p67 domains was obtained when fused to GFP, relative to the non-fused as well as full-length p67 [142]. Fusion to GFP either enhanced translation or, more likely, improved the stability of the p67 nascent protein products, thereby increasing the yield of p67 expression in insect cells. GFP has been shown to enhance the stability and increase the expression of fusion proteins in prokaryotic expression systems [172, 209]. Another indication that GFP could stabilise a fused protein was obtained by Akgul *et al.* [1] when they deleted a stability-regulating motif (the PEST sequence) from the Mc-1 protein [173, 175] but did not find any effect on the stability of a Mcl-GFP fusion protein.

The observed molecular mass of the hisGFP:p67N, hisGFP:p67M and hisGFP:p67ΔSS recombinant fusion proteins in SDS-PAGE was considerably higher than the calculated value. For example the apparent molecular mass of hisGFP:p67N was 75 kDa as opposed to the predicted size of 53 kDa. The exact reason for the anomalous behavior of these recombinant proteins in SDS-PAGE is not clear. It is known that p67 undergoes N-glycosylation in insect cell [142], but this could not explain the present observation since the signal peptide of p67 was eliminated in these constructs. The primary amino acid sequence of p67 may be responsible for the aberrant mobility since a similar difference between the observed and calculated molecular mass was observed for the non-fused p67 domains (Fig. 2B). Similar observations have been made with recombinant p67 production in both *E. coli* and

insect cells [141, 142]. A recombinant p67 in a near authentic form is highly desirable as it might function as a better immunogen than the previously tested NS1-p67 [134, 142]. An immuno dot-blot analysis using a monoclonal specific for native p67 indicated that GFP fusions of p67N and p67ΔSS had a near native folding, in contrast to the full length p67 expressed in insect cells in a non-fused form.

GFP is being used extensively as a visible marker in cell biology because it operates independently of cofactors and can be detected rapidly and easily. Due to its small size, GFP does not significantly increase the size of the chimeric protein neither does it interfere in general with the biological functions of even small proteins [23, 189, 190, 201, 209]. In this case, GFP fusion not only facilitated direct detection and monitoring of infected insect cells, and titration of recombinant viruses, it also increased expression levels of a recombinant protein, and appeared to conserve its natural folding properties. The high-level expression of near-authentic p67 domains is an important step towards the development of an effective subunit vaccine against ECF. Investigation of the immunogenic properties of these products and determining the level of protection against ECF in cattle will be the next step towards evaluating the vaccine potential of these GFP-p67 fusion proteins.

Chapter 3

Baculovirus surface display of *Theileria parva* p67 antigen preserves the conformation of sporozoite-neutralizing epitopes

Abstract

Theileria parva is an intracellular protozoan parasite that causes East Coast fever, a severe lymphoproliferative disease in cattle. Previous attempts to produce recombinant sporozoite surface antigen (p67) in bacterial or insect cells for vaccine purposes have not resulted in a correctly folded protein. Here, we report the expression of N- and C-terminal domains of p67 fused to the baculovirus envelope glycoprotein GP64 by cloning the appropriate p67 cDNA segments between the signal sequence and the major portion of GP64. To further advance the generation of such recombinants, existing surface display techniques were combined with bacmid technology. Chimeric proteins were present on the surface of budded viruses as judged by immunogold labelling and were exposed on the surface of insect cells, as concluded from immunofluorescence studies of infected, non-fixed insect cells. In non-denaturing dot blot experiments, a strong reaction was obtained between monoclonal TpM12 and baculovirus particles displaying the p67N-GP64 chimeric protein. This antibody, raised against native p67, also specifically recognised the surface of recombinant-infected cells. Apparently, a more native conformation was achieved than when p67 was expressed in *E. coli* or in conventional baculovirus expression systems. The baculovirus surface expression system, therefore, provides an improved way of expressing this *T. parva* sporozoite surface protein.

Introduction

The protozoan parasite *Theileria parva* is the causative agent of East Coast fever (ECF), a lymphoproliferative disease of cattle with a high mortality rate. The brown ear tick, *Rhipicephalus appendiculatus*, transmits the parasite during feeding. In cattle, the infecting sporozoites develop into schizonts, which induce transformation of lymphocytes [38]. Clinical signs such as pyrexia, lymphoadenopathy and panleukopenia are associated with the invasion of parasitized lymphoblasts into bovine tissues [75]. The current way of immunization of cattle involves vaccination with cryopreserved sporozoites and simultaneous treatment with long-acting antibiotic tetracyclines. The immunity achieved in this way, though often strain-specific, lasts for at least three years [124].

Immune responses against the infecting sporozoite and pathogenic schizont stages play a major role in mediating protection and immunity to ECF [114]. Cattle that recover from infection mount immunity to homologous strains. This immunity is thought to be dependent on a cellular immune response mediated by class I MHC-restricted CD8+ cytotoxic T-lymphocytes, and directed against schizont-infected lymphoblasts [114, 124]. On a single exposure, animals do not mount a detectable anti-sporozoite response, but on repeated challenge with infected ticks cattle generate high levels of antibodies that neutralize sporozoites *in vitro* [132]. These antibodies recognize p67, the major surface protein of sporozoites. A recombinant form of this antigen produced in bacteria has shown to provide protection in cattle [134]. Because p67 is invariant in parasites isolated from cattle and exhibits 76-96% sequence identity with stocks from buffalo, the antigen has a clear potential for development of a sub-unit vaccine against ECF. Pepscan analysis revealed that both murine and bovine epitopes cluster in the N- and C-terminal regions. Linear bovine B cell epitopes mapped within residues 25 to 296, and 577 to 591 while residues 105 to 221 contained three epitopes defined by neutralizing monoclonal antibodies. A further two epitopes were located in the C- terminal fragment between residues 617 and 631 [144].

Expression of p67 in *E. coli* as a fusion protein to influenza NS1, resulted in an insoluble protein [134]. In insect cells infected with a conventional recombinant baculovirus, p67 was expressed at low levels and mainly in a non-native conformation, and was not transported to the cell surface [142]. The protection achieved by these two recombinant proteins was only partial and relatively high doses of antigen were needed for induction of protective immunity. We believe that incorporating conformational epitopes would enhance the efficacy of the vaccine.

In this paper, we have expressed domains of p67 on the surface of budded baculovirus particles (BVs) in an attempt to achieve a more native folding of

immunodominant epitopes. The baculovirus surface display method [18, 51] is based on the expression of foreign proteins fused to the baculovirus GP64 protein. This major BV envelope protein is responsible for fusion of the viral envelope with endosomes of the insect host and for virus budding from infected insect cells [122, 152]. Baculovirus surface display has been used previously for the expression of viral surface antigens, such as HIV GP41 and GP120, rubella virus spike proteins and FMDV structural proteins [18, 50, 128, 194]. Indications for a near-native folding of recombinant proteins produced in this system have been given by the display of functional scFv fragments [129].

To allow easy generation and screening of recombinant viruses, we combined the existing surface display system with bacmid technology [108], enabling the generation of recombinant viral genomes in bacterial cells. This is the first report of the expression of a parasite antigen on the surface of baculovirus particles and may open new venues to develop vaccines against parasitic diseases.

Materials and Methods

Construction of transfer vectors
The coding sequence for enhanced GFP [25] was cloned as a *Nco*I-*Nsi*I fragment downstream of the p10 promoter in the pFastBacDual vector (Invitrogen Breda, The Netherlands). The *Kpn*I restriction site in the multiple cloning site (MCS) downstream of the p10 promoter was removed by digesting with *Nsi*I and *Kpn*I, and filling in with Klenow. After self-ligation, this resulted in the vector pFBD-GFP-Δ*Kpn*I. In this way, we were able to use a unique *Kpn*I cloning site between the gp64-signal peptide and major domain (see Figure 1). A p67 N-terminal domain corresponding to amino acids 21 to 225 was obtained by PCR from plasmid pMG1-p67 [134] with primers surf-3 and surf-4, generating *Kpn*I sites (Table I, Figure 1A). The C- terminal domain of p67, encoding amino acids 572 to 651, was amplified with primers surf-1 and surf-2 to introduce *Kpn*I sites on both ends (Table I, Figure 1A). The PCR products were verified by sequencing. The C-terminal domain was cloned into the *Kpn*I site of pBACSurf-1 (Novagen) to give plasmid pBACSurf-p67C. With this plasmid as template, the p67 C-domain flanked by the GP64 leader sequence (86 bp) and the GP64 major domain (1511 bp) was amplified using primers surf-5 and surf-6 (Table I) and the Expand Long Template PCR system (Roche). The PCR product was cloned between the *Eco*RI and *Hin*dIII restriction sites in the MCS of pFBD-GFP-Δ*Kpn*I, resulting in pFBD-GFP-GP64/p67C (Figure 1B). The sequence was verified. Removing the p67 C domain by KpnI digestion and replacing it with the N-domain generated plasmid pFBD-GFP-GP64/p67N. Self-ligation after *Kpn*I digestion resulted in an empty vector, pFBD-GFP-GP64, which was used as a control in the experiments described below.
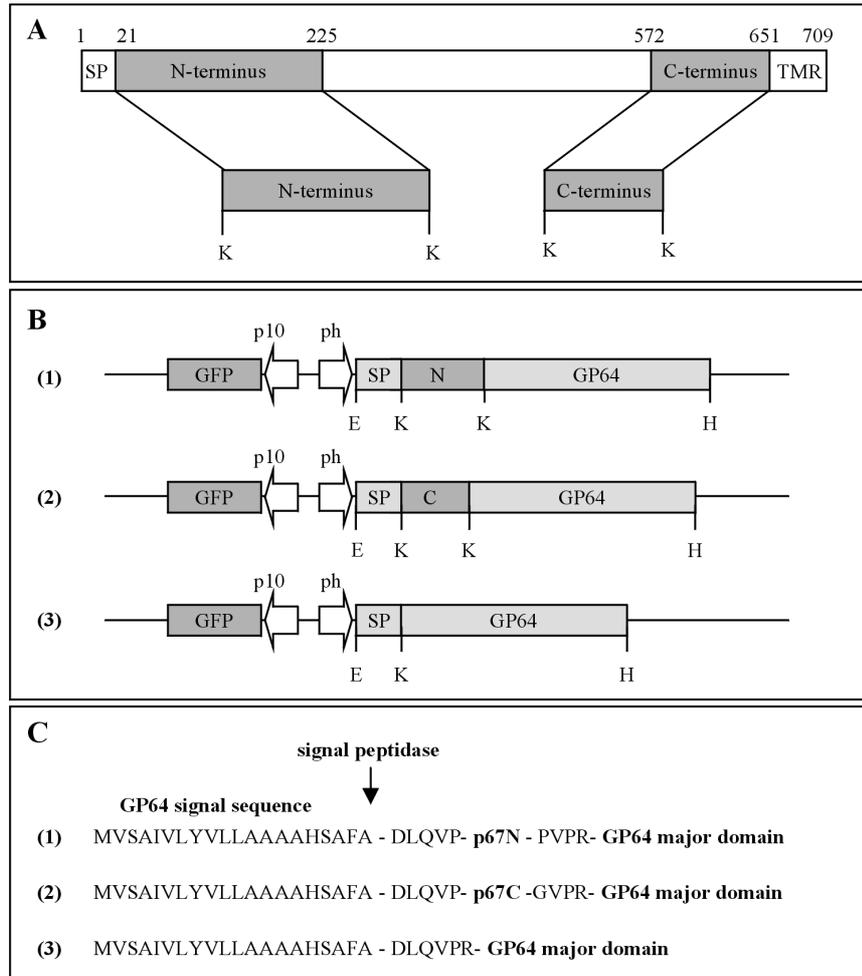
Fig. 1: Schematic representation of gp64/p67 chimeric constructs. (**A**) The *Theileria parva* p67 open reading frame in which the signal peptide (SP), and N and C-terminal domains, and a transmembrane region (TMR) are indicated. The N- and C-terminal domains were amplified as *Kpn*I fragments by PCR. The numbers refer to the corresponding position in the p67 amino acid sequence. (**B**) The various pFastBacDUAL constructs, in which N- (1) and C-terminal (2) domains of *T. parva* p67 were cloned between the GP64 signal peptide (SP) and the GP64 mature domain located downstream of the polyhedrin (ph) promoter. An 'empty' construct was made to serve as a control (3). The green fluorescent protein (GFP) sequence was cloned in the opposite orientation under control of the p10 promoter. Restriction sites used for cloning are indicated as follows: E: *Eco*RI, H: *Hin*dIII; K: *Kpn*I. (**C**) Predicted amino acid sequence of the primary translation products for the three constructs shown above. The first 20 amino acids represent the signal sequence and will be cleaved off by a cellular signal peptidase. Small spacer sequences are present between the different domains, as indicated.

Generation of bacmids and recombinant viruses

Escherichia coli DH10BAC cells (Invitrogen Life Technologies) were transformed with the plasmids pFBD-GFP-gp64/p67N, pFBD-GFP-gp64/p67C or pFBD-GFP-gp64 to generate recombinant AcMNPV bacmids. Putative recombinant bacmids were analyzed by PCR using the M13 reverse and surf-5 primers. Isolated bacmid DNA was

used to transfect *Spodoptera frugiperda* Sf21 cells [205], using Cellfectin (Invitrogen Life Technologies). This resulted in the recombinant viruses Ac-gp64/p67N, Ac-gp64/p67C and Ac-gp64. Recombinant viruses were grown to high titre stocks using standard procedure [89].

Table I. DNA sequence of oligonucleotides*

Primer name	Sequence 5' to 3'	Details
Surf-1	CC<u>GGTACC</u>AGGAACGGGAGGGGGATC	Forward primer for amplification of p67-C. Introduces *Kpn*I site.
Surf-2	CC<u>GGTACC</u>CCTTCTCCTCCAGCTGCTATTG	Reverse primer for amplification of p67-C. Introduces *Kpn*I site.
Surf-3	C<u>GGTACC</u>CATGCCTACGGAGGAACAACC	Forward primer for amplification of P67-N. Introduces *Kpn*I site.
Surf-4	C<u>GGTACC</u>GGAAGATCTTGGCCCGATG	Reverse primer for amplification of p67-N. Introduces *Kpn*I site.
Surf-5	G<u>GGAATTC</u>CAAGCAAGATGGTAAGC	Forward primer for amplification of gp64, anneals to gp64 signal sequence. Introduces *Eco*RI site.
Surf-6	CC<u>AAGCTT</u>AATAAATGTACTAATAACCC	Reverse primer for amplification of gp64, anneals to major domain. Introduces *Hin*dIII site.

*Restriction sites are underlined.

Immunofluorescence studies with non-fixed cells

Sf21 cells were grown in Grace's supplemented medium containing 10% FBS (Invitrogen Life Technologies). Sf21 cells were infected with Ac-GP64, Ac-gp64/p67N and Ac-GP64/p67C at a multiplicity of infection (m.o.i.) of 10 tissue culture infection dose 50 (TCID$_{50}$) units/cell. The cells were harvested and collected in 2 ml of Grace's supplemented medium with 10% FBS at 48 h post infection (p.i.). Infected non-fixed cells were incubated in this medium with monoclonal antibodies ARIII 22.7, recognising the N-domain of p67, or with ARIII 21.4, specific for the C-domain [144] at a dilution of 1 : 200 for 1 h at room temperature. Cells were washed three times with Grace's supplemented medium containing 10% FBS. Cells were further incubated with goat-anti-mouse IgG conjugated to Rhodamine Red X (Molecular Probes) for 1 h at a 1 : 200 dilution. Similar studies were also performed with monoclonal TpM12 at a 1 : 50 dilution. After extensive washing, the insect cells were viewed in a Zeiss LSM510 confocal laser-scanning Microscope (LSM510). GFP fluorescence was observed through excitation with blue laser light at 488 nm and emission through a 505-530 nm bandpass filter. Rhodamine was simultaneously visualised using green laser light at 545 nm for excitation and a 560 nm longpass filter for emmision.

Analysis of budded virions

Sf21 cells were infected with the various recombinant viruses at a m.o.i. of 10 TCID$_{50}$ units/cell and harvested 48 h p.i. Cell debris was removed by centrifugation at 3000 rpm for 5 min and filtration over a 0.45 µm non-pyrogenic filter. The filtrate was overlaid onto a 2.5 ml 25% sucrose cushion in 1 mM Tris, 0.01 mM EDTA, pH 8.0. (0.1 TE). Budded viruses (BVs) were pelleted by centrifugation at 25,000 rpm for 90 min at 4°C in a SW41 rotor. The pellet was suspended in 0.1 TE. Purified BVs were used for SDS-PAGE and dot blot analysis. Western blots were incubated with either monoclonal AcV5 recognising GP64 [63] diluted 1:1000, or monoclonals ARIII 22.7 or ARIII 21.4, diluted 1 : 200 and recognising p67 N- (amino acids 201-215) and C-specific (609-623) peptides, respectively [144]. Rabbit-anti-mouse immunoglobulins conjugated to horse-radish peroxidase (Dako A/S) were used as second antibody.

For immuno-dot blot analysis, a sample of the budded virus preparation of Ac-gp64/p67N or Ac-gp64 equivalent to 5 µg of total protein was spotted onto nitrocellulose membrane, either directly or after denaturation by boiling for 10 min in 10 mM Tris.HCl pH 8.0, 1 mM EDTA, 2% SDS and 5% β-mercaptoethanol. The filters obtained were incubated as described above using either monoclonal TpM12 or ARIII 22.7 [144]. These blots were developed using ECL (Pharmacia). As a control, Sf21 cells were infected with an AcMNPV recombinant expressing non-fused p67 and 5 µg total cell protein was blotted under both denaturing and native conditions (data not shown).

For immunogold labelling, Sf21 cells were infected with the various recombinants in Grace's medium without any supplements and the culture supernatant was replaced at 20 h p.i. The supernatant was collected at 36 p.i. and cleared from cell debris by centrifugation at 1000 rpm. The virus suspension was attached to nickel grids. After blocking in 1% (w/v) BSA in PBS for 20 min, grids were incubated for 1.5 h with the monoclonal antibodies ARIII 22.7 (N-specific), ARIII 21.4 (C-specific) or AcV1 (specific for GP64), all in a 1 : 200 dilution. The grids were then washed on 6 drops of 20 µl PBS-BSA for 5 min each and further incubated for 1 h with RAM coupled to 7 nm gold particles (Aurion). The grids were washed with PBS and negatively stained with 2% (w/v) uranyl acetate, pH 3.9 and examined in a Philips CM12 transmission electron microscope.

Results and Discussion

In the standard baculovirus expression system, the *Theileria parva* sporozoite surface protein p67 was expressed in low amounts in intracellular compartments and largely in a non-native form [142]. To overcome this hurdle, domains of p67 were inserted in frame into the open reading frame of the baculovirus GP64 protein between the coding sequences for the GP64 signal peptide and the GP64 mature protein (Figure 1).

The GP64 signal peptide is used to route the recombinant protein via the secretory pathway to the plasma membrane, while the GP64 portion directs the chimeric protein to the envelope of budded viruses. Since the antibody responses of bovine serum to p67 are restricted to N- and C-terminal domains [144], these two domains were considered as good starting points to test the baculovirus surface display technique for *T. parva* p67. The p67 N-domain comprises amino acid residues 21-225, thereby excluding the original p67 signal peptide (Figure 1A). The C-domain consists of residues 572-651 and does not contain the p67 transmembrane region, since the GP64 portion provides a functional analogue.

To avoid tedious rounds of plaque purification, recombinant viruses were generated using bacmid technology [108]. To this end, the GP64 sequences were amplified from the pBacSurf-1 vector (Novagen) and cloned downstream of the polyhedrin promoter between the *Eco*RI and *Hin*dIII sites of the pFastBacDUAL vector (Life Technologies). A *Kpn*I site, situated downstream of the signal sequence, was used to introduce the p67 N-and C-terminal domains (Figure 1B, 1C). The coding sequence for enhanced GFP was cloned downstream of the p10 promoter in order to follow transfection and infection processes, and to guarantee a simple read-out in virus titrations.

It has been reported that fusion of proteins to GP64 may adversely affect viral production or infectivity [18]. This appears to be dependent on the size of the protein, since larger proteins are more likely to interfere with GP64 trimer formation [151] and hence with incorporation into the virus particle. Apparently, since the titers obtained for the various viral stocks were within the normal range, the expression of GP64-p67 chimeric proteins did not significantly disturb virus budding and membrane fusion.

Immunofluorescence studies were performed in Grace's medium on non-fixed, living Sf21 cells. In this way antisera could only reach p67 domains which were exposed on the surface of the cells. With monoclonals specific for the N- and C-domains (ARIII 22.7 and ARIII 21.4, respectively), and with a second antibody conjugated to rhodamine Red X, a strong red peripheral fluorescence was observed (Figures 2A and C), which surrounded the cytoplasmic green GFP fluorescence (Figures 2B and D). In cells infected with the control Ac-GP64, only the green GFP fluorescence was observed with both monoclonal antibodies (data not shown). Cell surface expression has also been reported by Tami *et al*., [194], when expressing FMDV structural proteins fused to GP64. To analyse the conformation of p67N on the cell surface, monoclonal TpM12 was used in immunofluorescence studies with recombinant-infected non-fixed cells. A red fluorescence was observed at the cell surface for cells infected with Ac-GP64/p67N (Figures 2E and F), indicating that a conformational epitope in p67N was conserved.
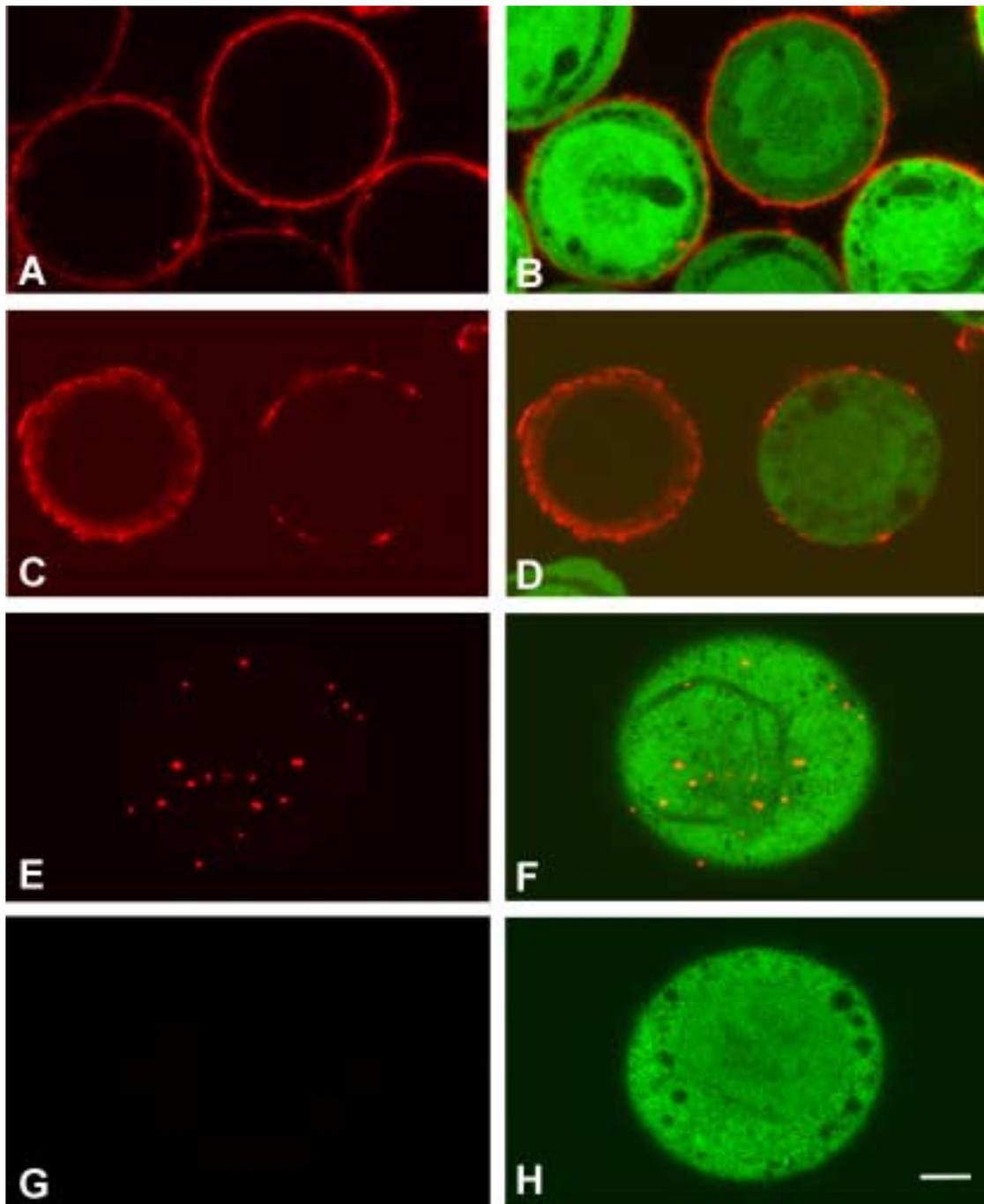
Fig. 2: Immunofluorescent surface labelling of non-fixed Sf21 cells infected with the various recombinant viruses. Forty eight hours post infection with Ac-GP64/p67N (A and B) or Ac-GP64/p67C (C and D), cells were incubated with the N-specific monoclonal ARIII 22.7 (A and B), or with ARIII 21.4, specific for the C-domain (C and D). Cells infected with Ac-GP64/p67N (E and F) and the control Ac-GP64 (G and H) were also labelled with monoclonal Tpm12. The left panel shows the red fluorescence due to labelling with rhodamine, whereas the right panel shows a combination of GFP and rhodamine fluorescence in the same cell. Figs. A-D show optical slices through the middle section of infected cells, while E-H show superficial slices. Bar represents 5 μm.

The amount of fluorescence observed was less with Tpm12 than with the other monoclonals. Therefore, we showed confocal images of the cell surface (Figures 2E-

2F) instead of optical slides through the middle of the cell. TpM12 did not react with cells infected with the Ac-GP64 control (Fig. 2G and H).
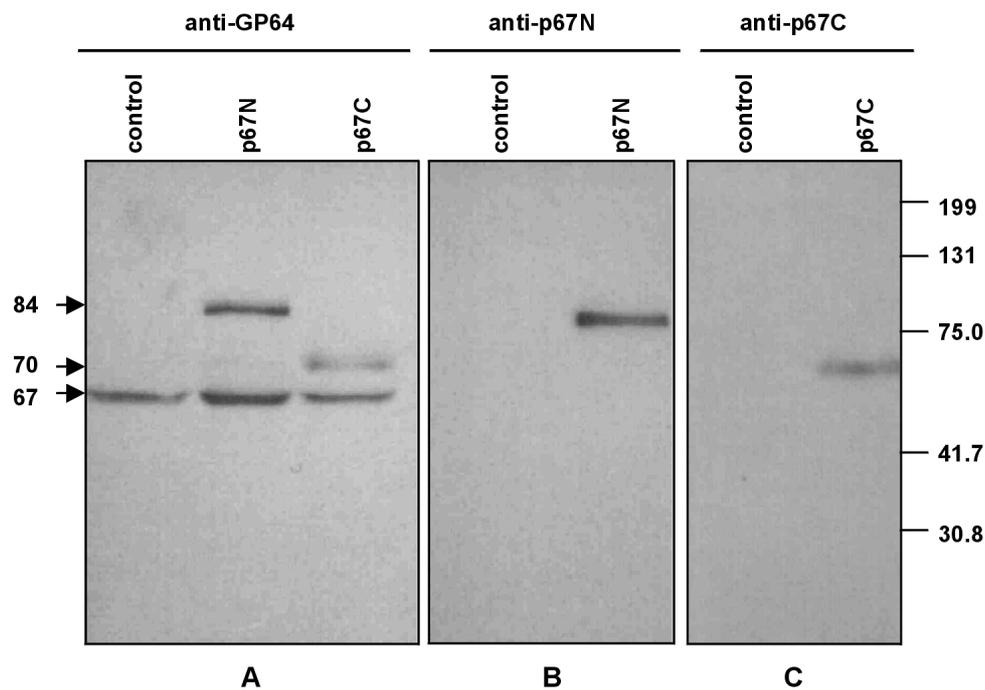
Fig. 3: Western blot analysis of AcMNPV recombinants. BVs were harvested from cells infected with either Ac-gp64, Ac-GP64/p67N or Ac-GP64/p67C and subjected to SDS-PAGE followed by Western blot analysis. In panel A, the filter was incubated with a monoclonal antibody recognising the AcV5 epitope of GP64. In panel B and C, monoclonals specific for the N- (ARIII 22.7), and the C-terminal (ARIII 21.4) domains were used, respectively.

To determine whether the GP64-p67 chimeric proteins were incorporated into non-occluded virus particles, budded virions were harvested from infected insect cell cultures by centrifugation through a sucrose cushion (see Materials and Methods). The samples obtained were analysed by Western blotting (Figure 3). Labelling with a monoclonal antibody that is directed against the GP64 AcV5 epitope (Figure 3A) showed a protein of approximately 67 kDa in all recombinant viruses representing wild type GP64, expressed from the GP64 promoter. In the control Ac-GP64 this band is stronger due to over-expression of GP64 (Figure 3A, lane 1) driven by the polyhedrin promoter. In Ac-gp64/p67N budded viruses an additional protein of about 84 kDa was present, which was also observed with an N-specific monoclonal (Figures 3A and B, lanes 2). The recombinant virus expressing the C-domain showed an additional band of approximately 70 kDa, that was also detected with the C-specific monoclonal ARIII 21.4 (Figures 3A and C, lanes 3 and 2, respectively). The amounts of the recombinant p67-GP64 chimeric proteins were slightly less than that of authentic GP64 (Figure 3A, lanes 2 and 3).
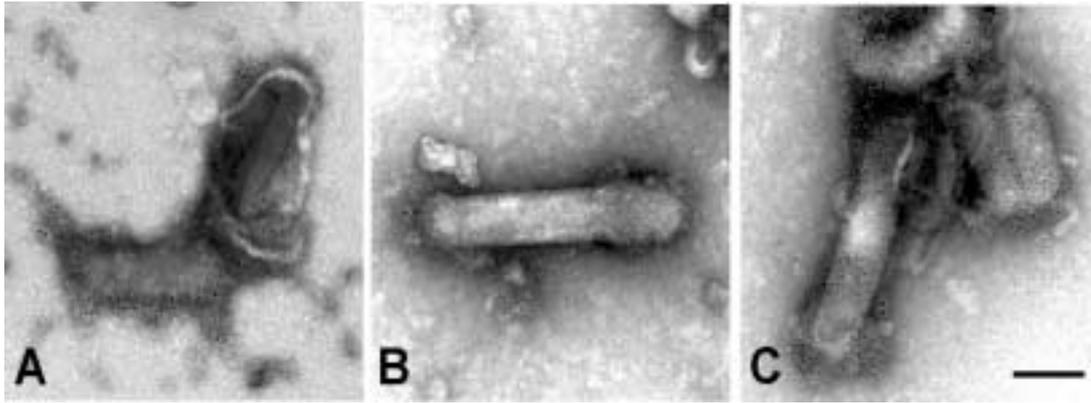
Fig. 4: Immuno-gold labelling of recombinant budded virions. Budded virions of recombinants Ac-gp64/p67N (A), Ac-GP64/p67C (B) were collected at 36 h p.i. and labelled with the N-specific monoclonal ARIII 22.7 (A) or the C-specific monoclonal ARIII 21.4 (B). As a positive control AcMNPV wild type was labelled with the GP64-directed monoclonal V1 (C). Rabbit-anti-mouse immunoglobulins coupled to 7 nm gold particles were used to visualise the bound antibodies. Bar represents 100 nm.

The presentation of chimeric proteins on the surface of budded virions was studied by immunogold labelling (Fig. 4). To this aim virions budded between 20 and 36 h p.i. were analysed for wild type AcMNPV, Ac-GP64, Ac-GP64/p67N and Ac-GP64/p67C. N- and C epitopes were detected on the surface of budded virions of the recombinants Ac-GP64/p67N and Ac-GP64/p67C, respectively (Figures 4A and B). The labelling was less intense with monoclonal ARIII 21.4, directed against C, but consistently present. Both monoclonals did not recognise the control virus Ac-GP64 (not shown). The GP64 epitope V1 was detected in all samples, as expected (Figure 4C for AcMNPV wild type). Recombinant virus preparations of earlier times post infection showed a high proportion of virions that did not present the p67-GP64 chimeric proteins, but only native GP64 (not shown). This can be explained by the fact that the first budding occurs much earlier than the onset of polyhedrin promoter-driven transcription. Since the polyhedrin promoter drove the synthesis of the recombinant proteins, the timing of expression is not optimal for incorporation in budded viruses.

The conformation of the p67 N-domain displayed on the surface of budded viruses was analysed by immunoblotting of budded virions directly, or after denaturation by boiling in SDS and β-mercaptoethanol-containing buffer. Both denatured and non-denatured virions reacted with monoclonal ARIII 22.7, but with a stronger signal in the denatured sample (Figure 5A and B). This might indicated that the folding of the protein in non-denatured samples partially masks the availability of a linear epitope. TpM12 reacted with the non-denatured virions, but not with denatured virions (Figure 5C and D), showing the preservation of a conformational epitope in the N-segment. Both monoclonals did not recognise budded virions of the control virus Ac-gp64 (Figure 5E and F), indicating that the reaction was specifically against the N-domain

of p67 and not against any other component of the budded virions. These results confirm the data obtained with the immune fluorescence studies on infected cells.
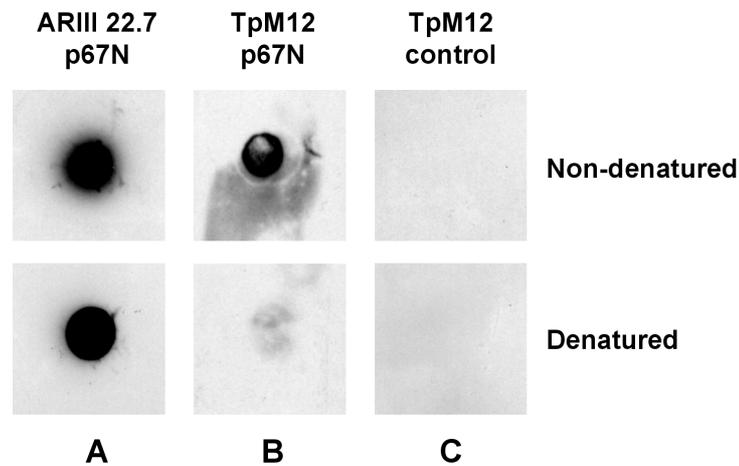
Fig. 5: Immuno-dotblot analysis of recombinant AcMNPV. Budded viruses were harvested 48 h post infection of Sf21 cells with Ac-GP64/p67N (panels A and B) or with the controls Ac-GP64 (panel C) and the equivalent of 5 µg of total protein was blotted onto a nitrocellulose membrane under non-denaturing or denaturing conditions. The blots were incubated either with monoclonal ARIII 22.7 (panel A), recognising a N-specific, linear epitope or with monoclonal TpM12, specific for native p67 (panel B and C).

When a lysate of insect cells infected with a non-fused baculovirus-p67 recombinant [142] was spotted under non-denaturing condition no reaction with TpM12 was observed (not shown). Previous experiments also showed that Tpm12 did not react with the majority of full- length e p67 expressed with conventional baculovirus vectors [142]. It was argued that the lack of recognition of the TpM12 epitope using these vectors was due to the fact that p67, despite the presence of a signal peptide, was not transported to the surface of insect cells but rather accumulated in intracellular compartments, a process that might affect its folding or processing. It is unlikely that this was due to lack of glycosylation, since glycosylation in p67 isolated from sporozoites has not been observed so far, although it contains seven potential sites of N-linked glycosylation [141]. Bacterially expressed p67 completely failed to react with this monoclonal [134, 142]. Transfected COS cells and bovine or murine cells infected with p67 recombinant vaccinia virus also failed to express p67 in its native form, and again p67 remained intracellular in these systems [142, and references therein]. Here, we showed labelling of p67 with TpM12 on the surface of recombinant infected cells, by immunofluoresent labelling of living cells (Fig. 2). Recombinant budded virions also reacted with TpM12 as shown in immunodot blot assays (Fig. 5). Also the stability of the protein seems to be increased compared to the expression of non-fused p67. Thus, the baculovirus surface display system appears to provide an improved way of expressing sporozoite surface protein p67, compared to bacterial or other conventional expression systems. Besides the more natural folding, the

particulate nature of the budded virus preparations is likely to enhance the immunogenicity of p67. In addition, isolation of budded virions is a much more easy procedure than purification of p67 from intracellular compartments.

The combination of surface display with bacmid technology as described in this paper has greatly accelerated the procedure of generating recombinant viruses, thereby facilitating the expression of antigenic domains for vaccine-related studies. The next step will be to test the immunogenic properties of the budded virions displaying the p67-GP64 chimeric proteins in cattle, and to determine whether it can protect against ECF and, if so, to evaluate the potential of p67-budded viruses under field conditions.

Acknowledgements

Chapter 4

Improved secretion of *Theileria parva* sporozoite surface protein p67 using a *chitinase* and *v-cathepsin* negative baculovirus vector

Abstract

East Coast fever (ECF) is caused by the protozoan parasite *Theileria parva* and is a serious constraint to the development of cattle industry in Africa. Towards the development of a sub-unit vaccine based on the p67 sporozoite surface antigen, baculovirus vectors were engineered aimed at increasing expression levels and producing a secreted form of p67. Therefore, the signal peptide of p67 was replaced by the honeybee mellitin (HBM) signal sequence and a putative membrane anchor domain (amino acids 659 to 709) was deleted. This resulted in secretion of p67 into the culture medium. Deletion of the *chitinase* and *v-cathepsin* genes by ET-recombination from the baculovirus vector showed a remarkable effect on the conservation of the integrity of the secreted p67. A large fraction of the secreted p67 recombinant protein was expressed in a glycosylated form as confirmed by infecting the cells in the presence of tunicamycin. The modified bacmid vector AcBacΔCC developed in these studies may be of great help for the expression of other secreted proteins in the baculovirus/insect cell system.

Introduction

East Coast fever (ECF) is an infectious disease of cattle caused by the protozoan parasite *Theileria parva.* The sporozoite stage of the parasite is transmitted to cattle by the brown ear tick *Rhipicephalus appendiculatus*. These sporozoites invade white blood cells and, after developing into schizonts, cause a massive proliferation of lymphocytes, leading to the death of the animals' [38]. Previous experiments have shown that the immunodominant sporozoite surface protein, p67, is a good candidate for the development of a broad-spectrum subunit vaccine against ECF [34, 132, 143]. *T. parva* p67 is entirely sequenced [141] and is conserved among various cattle-derived parasite stocks [143]. The exact function of p67 is not known but the molecule has been implicated in the attachment and subsequent entry of the sporozoites into bovine lymphocytes [178]. Hydrophobicity analysis of the molecule revealed hydrophobic N and C terminal tails [141] coinciding with a signal sequence and a putative transmembrane domain (TMR), respectively.

Vaccination with recombinant p67 produced in *E. coli* has been reported to give up to 50% protection in cattle against ECF but required high doses of recombinant protein [17, 134, 142]. The baculovirus expression system [89] was previously used to express full-length p67, but the expression levels obtained were very low. P67 was also not transported to the cell surface as in the parasite and it did not have a native form (BEV-p67) [142]. One possibility for the lack of transport of p67 in the baculovirus/insect cell expression system could be an inefficient recognition of the native p67 signal peptide by the protein translocation machinery in insect cells when compared to sporozoites. Fusion of p67 to the baculovirus fusion protein GP64 resulted in display of the GP64: p67 fusion protein on the surface of both infected cells and recombinant-budded viruses [83], suggesting that further improvements in transporting p67 to the cell surface can be made. Enhancement of cytoplasmic expression levels was achieved by fusing p67 domains to GFP [82]. In both cases p67 was expressed in near-authentic forms as it was recognized by a monoclonal antibody raised against native p67. This makes the recombinant p67 a better candidate as a vaccine.

In this paper, we aimed at improved expression of a secreted form of p67. We replaced the native signal sequence of p67 by the honey bee mellitin (HBM) signal sequence [194] and deleted the putative TMR, in order to direct the protein to the export pathway. Furthermore, the *chitinase* and *v-cathepsin* genes [8], encoding proteins which are involved in liquefaction of the insect at the end of the infection process and which are stored in large amounts in the ER, were deleted from the baculovirus genome to favor the secretion of p67 and to prevent degradation of the

protein. To facilitate the engineering of recombinant baculoviruses for this and other purposes, these deletions were made in the AcMNPV bacmid system.

Materials and Methods

Generation of p67 recombinant viruses

A p67 cDNA fragment encoding amino acid residues 18 to 651, thereby deleting the native signal sequence (aa 1-17) and the putative TMR (aa 651-709) was amplified by PCR using primers p67/18F and p67/651R (Table 1). A *Stu*I recognition site was added at the N-terminus of p67 (see Fig. 1A). The reverse primer contained the coding sequence for a polyhistidine tag flanked by *Sac*I and *Hind*III restriction sites. The obtained fragment was digested with *Stu*I and *Hind*III and cloned into pFastBacMelit, (kindly provided by Intervet International, Boxmeer, the Netherlands; Fig.1). This plasmid was constructed by inserting the honeybee melittin signal sequence (aa 1-21) [196] as a *Bam*HI–*Eco*RI fragment in pFastBac1 (Invitrogen). The resulting clone was designated pFB-HBM-p67[TMR⁻] (Fig. 1B). From this clone, plasmid pFB-HBM-p67[TMR⁺] was generated by replacing the *Stu*I-*Sac*I (aa 18-651) fragment with a PCR-amplified p67 fragment encoding amino acid residues 18 to 709, which includes the hydrophobic C-terminus (Fig. 1B). DH10B cells containing the wild type AcMNPV bacmid [108] were transformed with these two plasmids and recombinant bacmids were isolated. Standard PCR-based techniques were used to verify the nature of the bacmids, to transfect Sf21 insect cells [205] and to grow high titer virus stocks.

ET recombination

The neighboring *chitinase* and *cathepsin* genes (AcORF 126 and 127) [8] were deleted from the AcMNPV bacmid [108] by a single ET recombination event, a method originally described by Zhang *et al.* [219]. In our experiment the chloramphenicol resistance gene (Cm^R) was amplified by PCR from pBeloBac11 (New England Biolabs) with Cm^R-specific oligonucleotides flanked at the 5' regions by 50 nucleotides, which were homologous to the regions flanking the *chitinase/cathepsin* deletion site (CC-flanking F and R primers, Table 1). The primers were designed to delete the *chitinase* and *cathepsin* promoters together with large portions of the *chitinase* and *cathepsin* ORFs, leaving the flanking *lef-7* (acORF 125) and *gp64* (AcORF 128) genes intact (Fig. 1C). Recombination of the PCR product with the AcMNPV bacmid was performed in *E. coli* as described by Pijlman *et al.* [161] using the plasmid pBADαβγ [138] to supply the recombinase complex. Recombinant bacmid DNA was tested for insertion of the Cm^R resistance gene at the *chitinase/cathepsin* locus by PCR with the CC-forward and CC-reverse control primers (Table 1) followed by automated sequencing of the PCR product (BaseClear). The absence of the *chitinase* and *cathepsin* genes was verified with a CC-internal primer (Table 1) in combination with the CC-forward control primer. DH10B cells

containing the resulting AcBacΔCC bacmid were transformed with the Tn7 transposase helper plasmid pMON7124 [108]. A recombinant bacmid was generated using the pFB-HBMp67[TMR⁻] plasmid and used to produce recombinant viruses after transfection of Sf21 cells.
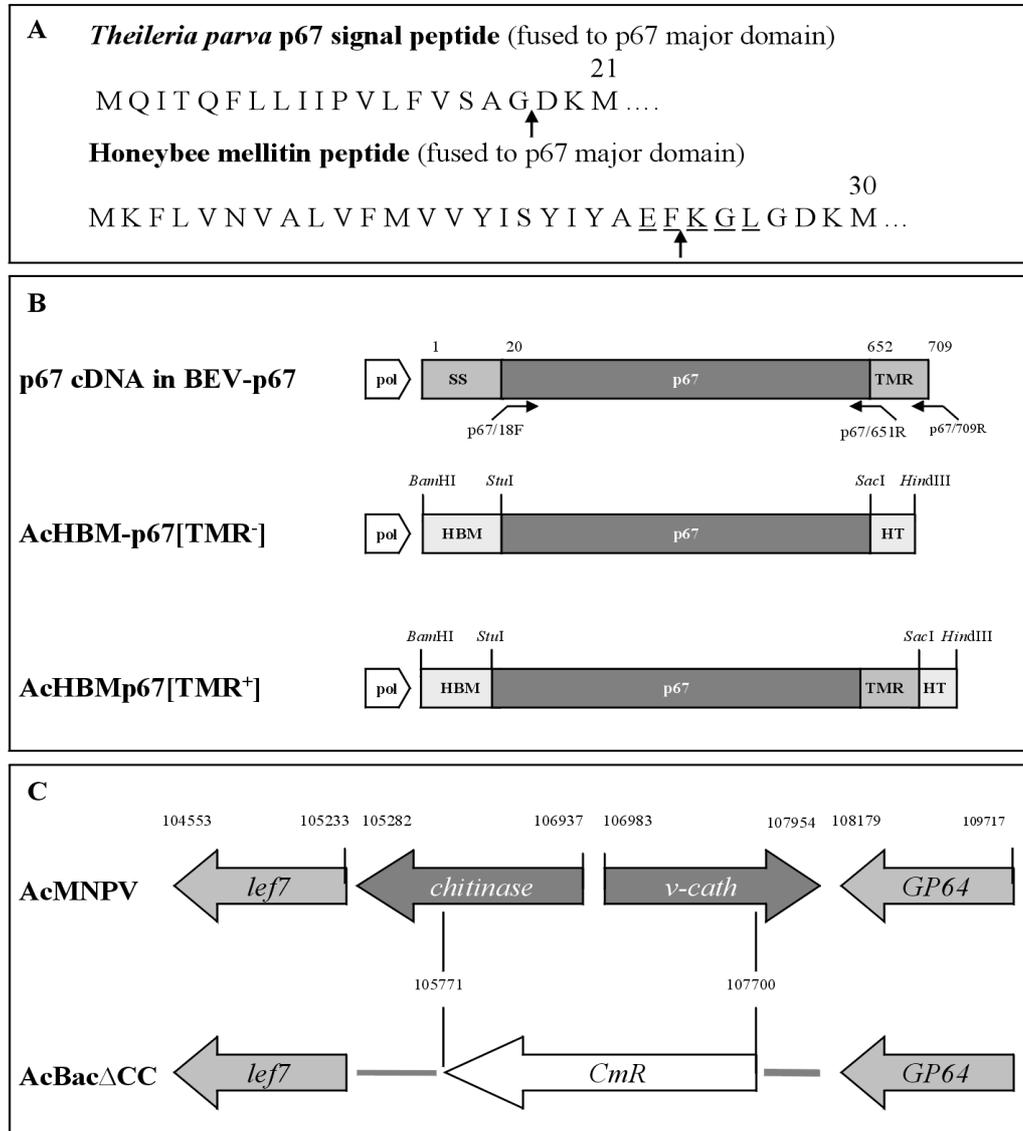
Fig. 1: P67 recombinant baculovirus vectors: (**A**) Amino acid sequence of the native signal peptide of *Theileria parva* p67 and the honeybee mellitin signal peptide fused to p67. Arrows indicate predicted sites for signal peptidase cleavage. In the case of HBM-p67, the *EcoR*I and *Stu*I restriction enzyme sites introduced the underlined amino acid residues. (**B**) Schematic representation of the various recombinant baculovirus vectors. All ORFs were placed into the AcMNPV genome downstream of the polyhedrin promoter (POL). SSp67: native p67 signal sequence; HBM: honeybee mellitin signal sequence; TMR: putative transmembrane region of p67; HT: polyhistidine tag. Arrows indicate the location and direction of PCR primers used to amplify p67 DNA fragments. (**C**) Genomic organization of AcMNPV wild type and the *chitinase* and *cathepsin* deletion mutant AcBACΔCC around the site of the deletion. The *chitinase* and *cathepsin* genes were partially replaced by the chloramphenical resistance gene. The numbers refer to the AcMNPV sequence numbering used by Ayres *et al.* [8].

Table 1: Oligonucleotides

Name	Sequence 5' to 3'	Purpose
p67/18 F	GA<u>AGGCCT</u>AGGGGACAAAATGCCTACGGAG	Amplification of p67 without native signal peptide. *Stu*I site
p67/651 R	GG<u>AAGCTT</u>ATGGTGATGGTGATGGTGGAGCTCTCCAGCTGCTATTGTGGGCC CTGTTGGAG	Amplification of p67 without TMR + histag. *Hin*dIII site.
p67/709 R	CC<u>GAGCTC</u>GTGGACGATGCTGATAATTATT	Amplification of p67 with TMR. *Sac*I site
CC-flanking F	CCGAGTTCCAACTTGGCGCGTAGACTGTTGTTTGGTAGCCCAAATCCGTGCCTGAGGTTTAAGGGCACCAATAACTG	ET recombination to delete chitinase and cathepsin
CC-flanking R	CCTGTTTATAGTTAACAATGTCGGCAGCGTCTATGGCCATAGGAATAGGGCCTCAGGTTCCTGTGCGACGGTTAC	ET recombination to delete chitinase and cathepsin
CC-control F	CCGTACATGGCGACGCCCACAA	Control PCR
CC-control R	CCGTCCTCTCCCCAATCCGTGC	Control PCR
CC-internal control	GGCGTCGTCTCAAAGTCAC	Control PCR

*Restriction sites are underlined

Protein expression

The expression of recombinant p67 protein was tested in *T. ni* High Five™ insect cells (Invitrogen) infected with the various recombinant viruses at a multiplicity of infection of 10 TCID50 units/cell using serum free Sf900II medium (Invitrogen). Both total cell lysates and medium samples harvested 48 h post infection (p.i.) and containing equal amounts of total protein were resolved in SDS-PAGE and blotted to a PDVF membrane (Milipore). For Western blot analysis, a bovine polyclonal p67-specific antiserum (BP300) was used diluted 1:1000. Rabbit anti-bovine immunoglobulins (Sanbio) conjugated to alkaline phosphatase were used as secondary antibodies, which were detected with NBT-BCIP (Roche).

Tunicamycin treatment

In vivo studies of the glycosylation status of recombinant p67 were carried out by addition of 10 µg/ml of tunicamycin (Sigma) 1 h p.i. to the culture media of *T. ni* High Five™ cells infected with AcHBMp67[TMR⁻] or AcΔCC-HBMp67[TMR⁻]. Duplicate cultures to which no tunicamycin was added were also set up. Cell lysates as well as supernatants were prepared 48 h p.i. and subjected to Western blot analysis

using a monoclonal antibody directed against the histidine tag (Clontech Laboratories).

Dot blot analysis

The authenticity of the insect cell-derived p67 proteins was examined by dot blot analysis as described by Kaba *et al.*, [82]. Volumes of cell lysate and supernatants containing 5 μg and 10 μg of total protein respectively, were spotted on a nitrocellulose membrane directly, and allowed to dry at room temperature. Duplicate blots were incubated with monoclonal antibodies TpM12 (1:50) or ARIII 22.7 (1:200) for 1 h at room temperature, washed and further incubated with goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (Amersham) at the concentration of 1 : 5000. Enhanced chemiluminescence (ECL) (Amersham) was used for detection. The insect cell-derived p67 expressed previously, BEV-p67 [142] was compared with the new recombinant proteins. As positive control we used 1.2 μg of purified hisGFP-p67N fusion protein [82]. Mock and AcMNPV wild type virus infected cell lysates containing 5 μg total protein were used as negative controls.
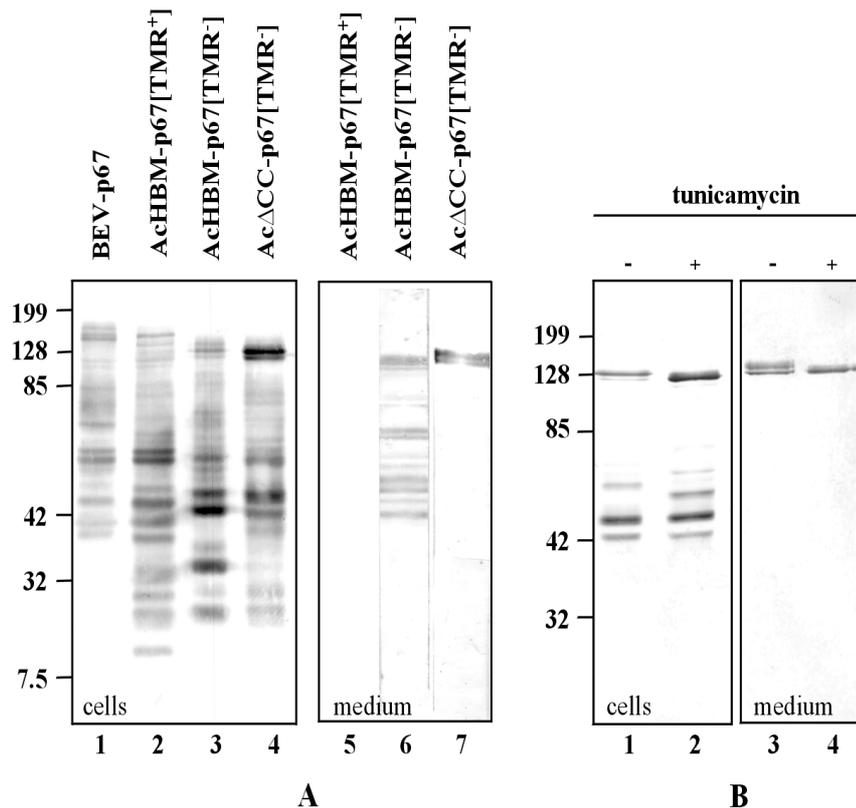
Fig. 2: Expression, secretion and N-linked glycosylation of recombinant p67 produced in T. ni High Five cells. P67 was detected by Western blot analysis using the bovine antiserum BP300. (**A**). Western blot analysis of lysates of cells (lanes 1-4) infected with the recombinant viruses BEV-p67, AcHBMp67[TMR$^+$], AcHBMp67[TMR$^-$] or AcΔCC-HBMp67[TMR$^-$], and in the culture medium

Results

Expression of a secreted form of p67 in insect cells

In order to direct p67 to the export pathway for expression on the surface of infected cells or in the culture medium the two recombinant baculoviruses Ac-HBMp67[TMR⁻] and HBMp67[TMR⁺] were constructed using the bacmid system. In both constructs the mature p67 domain, so omitting the native p67 signal peptide, was fused to the C-terminal end of the HBM signal. In one of the construct the hydrophobic C-tail, containing the putative TMR, was deleted. Both constructs contained a C-terminal his-tag to allow later purification (Fig. 1).

The expression of recombinant p67 in *T. ni* High Five cells infected with either construct was difficult to detect in standard Coomassie brilliant blue stained gels. Therefore, immunostaining was used to detect p67 on Western blots. In cells infected with either recombinant a multiplex of p67 specific polypeptides was found (Fig. 2A, lanes 2-3). These p67-specific polypeptides ranged in size between approximately 20 and 140 kDa, whereas the predicted size for the recombinant p67 protein would be 75.6 and 69.5 kDa for the plus and minus TMR constructs, respectively. The results were comparable with cells infected with recombinant BEV-p67 [142] Fig. 2A, lane 1) encoding full-length p67 with its own signal peptide and with TMR. The position of the bands in the gel was shifted about 10 kDa when the TMR was removed.

The higher mobility forms of p67 are most likely the result of proteolytic degradation. p67 specific peptides were also detected in the culture medium of cells infected with the recombinant virus AcHBMp67[TMR⁻], but were not secreted from cells infected with AcHBMp67[TMR⁺] (Fig. 2A, lanes 5-6). This result strongly suggests that the hydrophobic C-terminus or part there off indeed forms a transmembrane region and shows that the HBM signal is able to direct p67 towards the export pathway.

Prevention of protein degradation by a chitinase/cathepsin negative bacmid

To overcome the breakdown of secreted recombinant protein in order to improve the level of full length secreted protein we deleted the neighboring *chitinase* and v-*cathepsin* genes from the AcMNPV bacmid by ET recombination and produced a new recombinant virus AcΔCC-HBMp67[TMR⁻] (Fig. 1B and C). Western blot analysis (Fig. 2) showed that p67 secreted from cells infected with AcΔCC-HBMp67[TMR⁻] migrated as two separated forms with molecular masses of approximately 120 and 130 kDa, whereas in the presence of *chitinase* and *v-cathepsin*, multiple p67-specific polypeptides were found (Fig. 2A, lanes 6 and 7) differing in their migration in SDS-PAGE. This result showed that deletion of *cathepsin* and *chitinase* had a remarkable effect on the integrity of secreted p67. In cell lysates, p67 was also better conserved in the deletion mutant (Fig. 2A, lane 4).

Glycosylation of recombinant p67

The fact that recombinant p67 was detected as two forms of 120 and 130, respectively, when produced with AcΔCC-HBMp67[TMR⁻] kDa (Figure 2A, lane 7) prompted us to test whether the recombinant p67 protein was N-glycosylated in insect cells. Infections in the presence of tunicamycin, which blocks the first step in the formation of dolichol-linked precursors of N-linked oligosaccharides, resulted in the disappearance of the lower mobility form of 130 kDa, while the 120 kDa form remained (Fig. 2B) both in cells and supernatants. The results indicate that recombinant p67 is glycosylated, but that glycosylation can only account for a small shift in mobility.

Recognition by TpM12

To determine the authenticity of the recombinant HBM-p67 fusion proteins, a non-denaturing immuno dot blot analysis was carried out using a monoclonal antibody raised against native p67 (TpM12). A purified fusion protein containing the N-terminal domain of p67 fused to the C-terminus of GFP (hisGFP-p67N) [82] was used as positive control, the full-length recombinant p67 protein (BEV-p67) [142] as a negative control. The HBM-p67 fusion proteins expressed by the various recombinant viruses all reacted with monoclonal antibody ARIII 22.7, but not with TpM12 (Fig. 3), in contrast to hisGFP-p67N. The result was similar for cell lysates (Fig. 3A) and culture medium (Fig. 3B). This experiment showed that there is a difference between native p67 and the secreted recombinant form, which affects the TpM12 epitope. The nature of this difference is not clear yet.
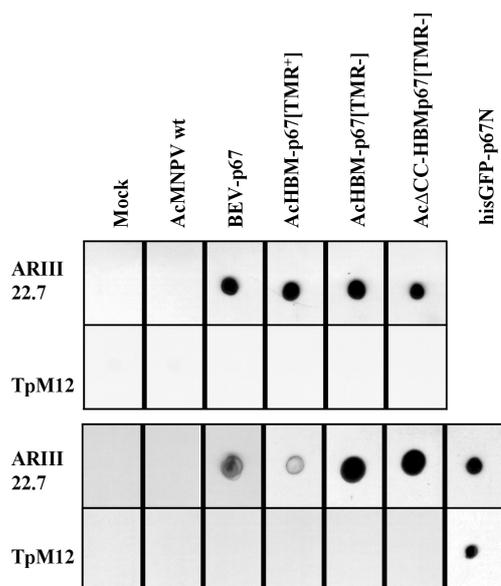
Fig. 3: Immuno-dotblot analysis of recombinant HBM-p67 fusion proteins. The blots were incubated either with monoclonal antibodies ARIII 22.7 or TpM12. The epitopes recognised by the antibodies are located in the N-terminal region of p67. ARIII 22.7 was raised against recombinant p67 whereas TpM12 was raised against native p67. Five micrograms of total protein from cell lysates (**A**) and 10 µg of total protein from the culture medium (**B**) were spotted onto nitrocellulose membranes under non-denaturing conditions. Purified hisGFP-p67N fusion protein [81] was used as a positive control for TpM12 detection.

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Discussion

This is the first report of secretion of p67 using baculovirus expression vectors. The most likely reason for the secretion of p67 into the culture medium as observed in this study is the deletion of the hydrophobic C-terminal region. Our results clearly suggests that the amino acid residues 652 to 709 (or part there off) function as membrane anchor domain. HBM might further enhance the expression levels and secretion of p67, but this cannot be determined from this study. The functionality of the HBM signal in insect cells has been shown before in *T. ni* High Five™ cells [14, 76, 102, 196].

A strategy described in literature to enhance the level of expression of secretory proteins is the use of baculovirus vectors with a deleted *chitinase* gene [165]. *Chitinase* is expressed in the late phase of virus replication and in conjunction with *v-cathepsin* (*v-CATH*) promotes liquefaction of the host in the latter stages of infection [57]. *Chitinase* translates into a secretory protein with an enormous burden on the protein translocation machinery and probably present keen competition with recombinant secretory proteins in entering the secretory pathway [165, 197]. *V-CATH* is synthesized as an inactive proenzyme, *pro-v-CATH*, that also accumulates in the ER of infected cells and is activated by proteolytic cleavage upon cell death [66]. *Chitinase* may function as a chaperone for the proper folding of pro-v-CATH in the ER [65]. Chaotropic agents like sodium dodecyl-sulfate (SDS) trigger processing of pro-*v-CATH* to active *v-CATH*, which may result in proteolysis of recombinant protein [64]. The *chitinase* and v-*cathepsin* genes are located in flanking positions in the AcMNPV genome [8] and were deleted simultaneously to favor the secretion of recombinant p67.

In this study the AcBacΔCC vector was used to express p67 fused to HBM signal sequence (AcΔCC-HBMp67[TMR⁻]). Our results showed no major increase in the level of expression and secretion of p67 protein from *T. ni* High Five™ cells infected with AcΔCC-HBMp67[TMR⁻]HT compared to those infected with the non-deletion virus AcHBMp67[TMR⁻]HT. A remarkable effect of the CC-deletion, however, was seen on the conservation of the integrity of both the intracellular and the secreted p67 protein (Fig. 2B). Addition of cysteine protease inhibitors (CPI, like E64) together with the SDS sample buffer during cell lysis can inhibit the pro-*v-CATH* processing [64]. In our studies we did not add any CPI to the cells or culture media during the sample preparation. Hence, we could demonstrate the positive effect of the deletion on the integrity of secreted p67. In our experiments, *v-CATH* most likely is responsible for the degradation of the recombinant protein as present in the extra-cellular medium at 48 h p.i. This observation contrast to what reported by Hom *et al.* [66] in which *v-CATH* appeared on the fourth day of infection. Intrinsic factors to the

experimental conditions such as different cell line, culture medium and incubation conditions used for the infections may have caused this discrepancy. The protective effect of the *v-cathepsin* deletion was less prominent in cell lysates. This result suggests that p67 may be broken down intracellularly during synthesis by other proteases, unless it is routed to the secretory pathway.

A prerequisite for the production of a highly immunogenic protein that matches the natural p67 produced by *T. parva* sporozoite is the correct folding of the newly synthesized polypeptide that results in the recognition by antibodies that specify topological epitopes on the molecule. The loss of reactivity of recombinant p67 expressed under the signaling of HBM signal peptide with the mAb TpM12 (Fig. 3) raised against native p67, is a strong indication that the antigenic properties may not be totally conserved. In contrast, p67 expressed as a cytoplasmic GFP fusion protein [82] retained the capacity to react with this antibody (Fig. 3B). The difference in recognition by TpM12 and the low mobility of the recombinant p67 in SDS-PAGE (Fig. 2) might indicate that recombinant p67 is post-translationally modified in the secretory pathway of insect cells. These modifications may make the recombinant protein different from the native p67 expressed on the surface of *T. parva* sporozoites.

Enzymatic deglycosylation of N-linked sugars with PNGase F failed to reduce the high mobility forms of purified p67 (data no shown). Our data seem to coincide with a report by Hsu *et al.*, [69] showing 1,3-fucosylation of recombinant proteins in *T. ni* High Five™ cells. Such fucosylation renders N-glycans resistant to hydrolysis with PNGase F [3]. Treatment with tunicamycin on the other hand showed that N-linked glycosylation was responsible for a shift in mobility corresponding to 10 kDa (Fig. 2B). This observation showed that glycosylation of p67 is only partly responsible for the low mobility of recombinant p67. Although the amino acid sequence contains seven potential N-linked glycosylation sites, there is no evidence that the native protein as expressed in the parasite is glycosylated (Nene, personal communication). The localization of these motifs on the amino acid sequence does not coincide with epitopes of the characterized mAbs [132, 144] that are currently used to evaluate the *in vitro* potential of recombinant p67 proteins.

Secreted recombinant p67, had a much lower mobility in SDS-PAGE as expected, which was only partly due to glycosylation. Furthermore, secreted p67 was not recognized by mAb TpM12, which was raised against native p67 (Fig. 3). The p67 coding sequence contains multiple codons for serine and threonine residues (18.2 %) [141] that might constitute potential phosphorylation sites. Predicted phosphorylation sites, situated along the molecule, might be phosphorylated by several kinases during the intracellular transport of the protein through the lumen of ER and Golgi compartments [21]. The importance of phosphorylation for epitope recognition has been described for several eukaryotic gene products [139, 158]. The mAb TpM12

used in this study recognizes an epitope between positions 169 to 183 (TKEEVPPADLSDQVP) [144]. This epitope contains the consensus motif TKEE for casein kinase II (CK2).   Steady state levels of CK2 phosphorylation have been reported for the ER/SR glycoprotein GPR94 when expressed in the baculovirus expression system, supporting the presence of functional protein kinases in the ER compartment.   The phosphorylation status of recombinant p67 is currently under study in relation with TpM12 recognition and the mobility in SDS-PAGE.

Chapter 5

Enhanced immunogenicity of novel baculovirus-derived *Theileria parva* p67 subunit antigens

Abstract

East Coast fever (ECF) in cattle is caused by the tick-borne protozoan parasite *Theileria parva*. The major sporozoite surface antigen of *Theileria parva* (p67) is an important candidate for inclusion in a subunit vaccine. Recently, we reported the expression and production of different parts of p67 as fusions to either GFP or to the baculovirus GP64 envelope glycoprotein in insect cells, which resulted in well-folded, stable proteins. The immunogenicity of these fusion proteins was examined in out-bred mice and cattle. In mice, the full length p67 molecule without its signal peptide and transmembrane region, but fused to GFP (HisGFP:p67ΔSS) was the best immunogen followed by the C-terminus of p67 fused to GP64 (GP64:p67C). These two immunogens also provoked a high level of sero-conversion in cattle when formulated in a proprietary water-in-oil or saponin-derived adjuvant with only 100 μg of protein and a single booster. The vaccine-elicited antibodies inhibited the infectivity of *T. parva* sporozoites in *in-vitro* neutralization assays. This study demonstrated that these new baculovirus–derived p67 vaccines were highly immunogenic, and that in combination with a suitable adjuvant, they have a clear potential to induce protective immunity in cattle.

Introduction

East Coast fever (ECF) is a lethal disease of cattle caused by the intracellular protozoan parasite *Theileria parva.* The disease poses a major threat to development of the livestock industry in eastern, central and southern Africa and the financial burden due to loss of income and livestock product impacts on the quality of all aspects of family life [127]. The need for a protective vaccine against ECF cannot therefore be emphasized enough. The brown ear tick (*Rhipicephalus appendiculatus*) transmits the infective sporozoite stage of the parasite to the mammalian host during blood feeding. These sporozoites rapidly invade bovine lymphocytes and, once inside, the sporozoites differentiate into schizonts. This differentiation event transforms the infected cell into a state of uncontrolled proliferation. Subsequent invasion of non-lymphoid tissues by parasitized lymphocytes and associated immunopathological effects usually result in death of the animal within 3 weeks after infection [38, 112].

Sera from cattle in areas endemic for ECF or from cattle hyper-immunized with sporozoite lysates contain sporozoite-neutralizing antibodies. Monoclonal antibodies (mAbs) to surface epitopes of this life-cycle stage can abrogate sporozoite infectivity, indicating that immune responses directed at the sporozoites stage may be protective *in vivo* [34, 132]. Cattle immune to ECF also develop cell-mediated responses directed at schizont-containing lymphocytes [114]. Previous data showed that six out of a group of nine cattle (67%) were immune to ECF when inoculated with a recombinant form of the *T. parva* sporozoite surface protein p67 produced in *E. coli.* Similar protection levels have been reported for cattle immunized with insect cell-derived p67 [142] or fragments of p67, including an 80 amino acid region from the C-terminal end of the protein [17]. These results showed that p67 is a promising candidate for the development of a subunit vaccine against ECF. However, it is worth noting that, in all these studies very high amounts of antigen (1.4 - 3.0 mg) were required and given in multiple inoculations (3-5) to provide this response [17, 134, 142]. For a vaccine, which should be used on a large scale, these practical drawbacks have to be addressed prior to the introduction of a p67 subunit vaccine.

In recent papers, we have described the expression of recombinant p67 in more authentic forms in the baculovirus-insect cell system by making fusions to green fluorescent protein (GFP) or to the baculovirus envelope glycoprotein GP64 [82, 83]. GP64 is a trans-membrane protein found on the surface of budded baculovirus virions (BVs) and is responsible for the fusion of the viral envelope with endosomes of the insect host, and involved in virus budding from infected insect cells [122, 152]. P67 fused to GP64, is displayed on the surface of infected cells and BVS [83]. Fusion of p67 fragments to the C-terminus of GFP resulted in a stable and high expression of p67 in the insect cell cytosol and allowed easy titration of the recombinant virus [82].

Our products, GFP fusion proteins or BVs, strongly bound to TpM12 [82, 83] a monoclonal antibody raised against native p67 [132] on immunoblots. TpM12 discriminates between correctly folded and misfolded p67 proteins and only binds to the former. Only a minute fraction of non-fused p67 expressed in insect cells weakly bound to TpM12 [142]. Other expression systems failed to produce TpM12-reactive p67 [17, 47, 60, 68, 112, 134]. Thus, fusion to GFP and GP64 represents a significant improvement in p67 expression.

In this study we examined the immunogenicity of five novel baculovirus-derived p67 fusion proteins by studying sero-conversion in out-bred mice induced by these antigens. Based on the results a selection of p67 antigens was tested in cattle in combination with two different adjuvants: a saponin-based and a water-in-oil emulsion. The antibodies obtained in mice and cattle were tested for their *in-vitro* anti-parasite activity in neutralization assays. The most promising results were obtained with the full length p67 (without the signal peptide and the transmembrane region) fused to GFP and with a C-terminal domain of p67 fused to baculovirus GP64.

Materials and Methods

Production and purification of p67 antigens

The various p67 recombinant proteins used as vaccines in this study are depicted in Fig. 1. The cloning, expression and analysis of these p67 fusion proteins have been previously described [82, 83]. The GFP-fusion proteins contained an N-terminal his-tag to facilitate purification. TALONspin^{TM} IMAC columns (CLONTECH Laboratories) were used to purify the GFP-p67 fusion proteins following the instructions of the manufacturer. For mid-scale production of recombinant p67 fusion proteins, *Trichoplusia ni* High Five^{TM} insect cells (InVitrogen) in the log phase of growth were seeded at a density of 2 x 10^7 cells per 175 cm^2 tissue culture flask, in Grace's supplemented insect medium (InVitrogen) containing 10% fetal bovine serum. Cells were grown as a monolayer culture and infected with the various recombinant viruses at a multiplicity of infection (MOI) of 10 plaque-forming units per cell. Cells were harvested 48 hours post infection by centrifugation at 240 x g in a Biofuge 22R (Heraeus) for 15 min at 4°C. Cell pellets were washed with ice-cold PBS and re-suspended in 5 ml of cold wash buffer (50 mM NaH$_2$PO$_4$; 300 mM NaCl, pH 7.0). The protease inhibitors Pefabloc (0.4 mg/ml; Boehringer Mannheim) and E-64 (N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine; 30 mg/ml; Roche) were added to the cell suspension. Cells were disrupted by ultra-sonication for 6 times 30 sec on ice. The sonicate was centrifuged for 20 min at 17300 x g in a SS-34 rotor (Sorvall) at 4°C and the supernatant applied to TALONspin columns and allowed to flow through by gravity. To remove any unbound and weakly bound proteins, the columns were treated first with 20 ml ice cold wash buffer followed by 1.2 ml elution

buffer-I (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 25 mM imidazole, pH 7.0). The proteins bound with high affinity were eluted from the columns with elution buffer-II (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 200 mM imidazole, pH 7.0). Glycerol and β-mercapto-ethanol were added to the eluted proteins to final concentrations of 10% and 0.5%, respectively, before storage at -80°C until further use. Before use, the proteins were dialyzed three times for 6 hours against 500 ml D100 buffer (10% glycerol, 20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 7-mM β-mercapto-ethanol and 100 mM KCl) at 4°C. The Bradford protein assay (BIORAD) was used to determine the concentration of purified proteins.
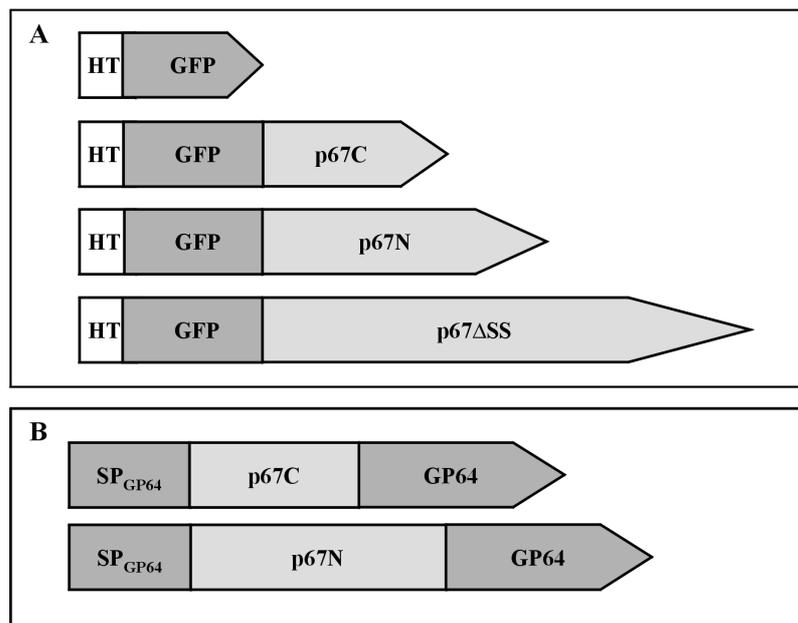
Fig. 1: Schematic representation of the various recombinant p67 constructs. (**A**) GFP fusion constructs; HT: (His)$_6$-Tag; GFP: green fluorescent protein; p67C: C-terminal region of p67 (amino acid residues 572-651); p67N: N-terminal region of p67 (aa 21-225) and p67ΔSS: full–length p67 without the sequences of its native signal and transmembrane domains (aa 21-651). For details on the construction of these constructs, see chapter 2. (**B**) GP64:p67 fusion proteins. SP: signal peptide of GP64; GP64: baculovirus major envelope protein of 64 kDa. Details are described in chapter 3.

For the purification of BVs that carry the GP64:p67 fusion proteins, *T. ni* High Five™ insect cells were grown in SF900-II serum free medium (InVitrogen) and infected with AcGP64:p67N and AcGP64:p67C, carrying N or C-terminal domains of p67 and harvested 48 h post-infection. BVs were purified over a sucrose cushion as previously described [83]. To obtain total cell extract for the sero-conversion in cattle the cell pellet was re-suspended in PBS and the cells were disrupted by ultrasonic treatment. The purified virus particles and total cell lysates were inactivated with Triton X-100 and gamma irradiated prior to vaccination. Triton X-100 was added to the virus sample to a final concentration of 0.2% and the samples were incubated for 28 hours at 28°C and then stored at 4°C.

Sero-conversion in mice

Twenty-four 3-4 week old, WL Swiss out-bred SPF female mice were immunized subcutaneously with 50 μg of the purified GFP-p67 fusion proteins or 150 μg of the purified, inactivated budded virus particles, each formulated with a proprietary water-in-oil emulsion designated "WOE". As a positive control, 100 μg recombinant p67C produced in *E. coli* [134] was used. 'Negative-control' mice received a formulation containing only the adjuvant. Five vaccine groups were categorized with three mice per group. All animals received a booster inoculation containing the amount of protein as described for the priming. Blood samples were collected at weeks 0, 6 and 10 after immunization for immunoblot analysis and for testing the ability to neutralize sporozoite infectivity *in vitro*.

Sero-conversion in cattle

For this experiment, eighteen 12-24 month old immuno-competent Holstein-Friesian oxen were immunized with 100 μg purified GFP:p67ΔSS protein (the full length p67 molecule without its signal peptide and transmembrane region spanning amino acids residues 21-651, fused to GFP) or, with 90 μg of total protein of purified BVs or total cell lysate prepared from insect cells infected with AcGP64:p67C after inactivation with Triton-X-100 and gamma irradiation. Five vaccine groups of three animals per group were tested. Each vaccine was formulated in a proprietary saponin-derivative designated 'SA' or in the water-in-oil emulsion (WOE) mentioned above. All animals received one boost inoculation containing the same amount of protein as that used in the prime inoculation, two weeks after the first immunization. Control cattle received a formulation containing only the adjuvant (SA + WOE). Serum samples were collected at day 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 and tested for antibody responses by Western blot analysis and ELISA (as described below).

Western blot analysis

Sera separated from the blood samples of mice and cattle were tested for the presence of p67-specific antibodies by determining their reactivity with total sporozoite lysate in a immunoblots. To this aim, the proteins of a total sporozoite lysate were resolved by size in 15% SDS-PAGE and transferred to a PVDF membrane following standard procedures [199]. Each membrane was then cut into 12 strips and each strip subjected to immunoblot analysis using the different serum samples. Each serum sample was tested in a series of two-fold dilutions for the mice sera and in 1 : 200 dilutions for the cattle sera. HRP-conjugated secondary antibodies were used to develop the immunoblots.

Direct ELISA

ELISA was used to measure the titers of serum antibodies elicited against the various p67 subunit vaccines as previously described [85]. P67$_{635}$, a recombinant form of p67 lacking the 74 C-terminal residues, was used as the antigen for coating the plates. As a

positive control, a standard bovine immune serum of known antibody titer was used while a pre-immune serum was used as the negative control. The antibody titer was designated as the highest dilution at which the mean absorbance, in duplicate wells, was three or more times the value of the negative control serum.

Sporozoite neutralization assays

Assays to measure the neutralization of sporozoite infectivity *in vitro* were performed as previously described [134]. Briefly, a suspension of sporozoites was incubated for 30 min with pre- or post-immunization sera at a final dilution of 1:50, and then included with bovine peripheral blood lymphocytes (PBMs). Cultures were examined on days 7, 10 and 14 and assessed for the percentage of infected lymphocytes. As a positive control for assay, the bovine serum G151 [134] was used, whilst the respective pre-immunization serum samples from mice or cattle were used as negative controls. PBMs were purified from the blood of five donor animals and infected with sporozoites *in vitro*. The most susceptible donor animal was selected and used in all subsequent experiments. Three independent assays were conducted for each sample and the mean percent inhibition was calculated.

Results

Antibody responses to p67 antigens in mice

Two sets of recombinant baculovirus vectors were constructed (Fig. 1) as described by Kaba *et al.* [82, 83]. The first set encoded various regions of p67 fused to the C-terminus of GFP and these antigens are designated 'GFP fusions'. In the second set, the p67 regions were fused to the baculovirus (AcMNPV) envelope protein, GP64 and these are designated 'GP64 fusions'. To determine which of the p67 vaccine constructs had the best potential to elicit high titers of sporozoite neutralizing antibodies, groups of three mice were immunized with purified proteins (GFP:p67N, GFP:p67C and GFP:p67ΔSS) or purified budded virus particles displaying GP64:p67N and GP64:p67C antigens. The antisera obtained were tested for their reactivity with sporozoite lysate in Western blots (data not shown) and scored as plus (+) for reactivity or as minus (-) for non-reactivity (Table 1). There were no observable differences among the individual mice used in each group with regards to the induction of antibodies as determined in Western blot analysis. Therefore the sera from each group were pooled for subsequent analysis.

With the exception of the GFP:p67C fusion protein, all vaccine antigens induced antibody responses to p67 in mice (Table 1). The bacterially produced recombinant p67 (p67C) product failed to generate high titer p67-specific antibodies in mice. GFP:p67ΔSS and GP64:p67C induced p67 antibody responses with the highest titers (1:5000). GP64:p67N and GFP:p67N vaccines elicited antibodies with lower titers

whereas GFP:p67C, GFP as well as adjuvant alone failed to induce any p67-specific antibody responses in mice as determined in Western blot analysis.

Table 1. Antibody titrations and sporozoite neutralization assays of mice sera

Antigen used for immunization	Dilution of serum used in Western blot analysis (Antibody titration)						Mean % inhibition (SNA)
	1:200	1:500	1:1000	1:2000	1:3000	1:5000	
GFP:p67ΔSS (P)	+	+	+	+	+	+	98.18 ± 0.11
Gp64:p67C (BVs)	+	+	+	+	+	+	90.00 ± 2.90
Gp64:p67N (BVs)	+	+	+	-			not done
GFP:p67N (P)	+	+	-				not done
GFP:p67C (P)	-	-					not done
P67C (B)	-	-					not done
GFP only (P)	-	-					12.38 ± 1.00
Adjuvant only	-	-					9.5 ± 1.25
Medium	N/A	N/A	N/A	N/A	N/A	N/A	7.59 ± 1.04

Values are means ± standard deviations (results of three independent assays);   P = purified protein; BVs = purified budded viruses; B = recombinant p67C produced in *E coli*;   N/A = not applicable;

In vitro neutralization activity of murine sera
To determine whether the antisera produced in mice could neutralize the infectivity of sporozoites *in vitro,* a serum neutralization assay (SNA) was conducted. Since antibodies raised against GFP:p67ΔSS and GP64:p67C showed the highest serum titers on Western blots (Table 1), these sera were selected for the SNA. Both sera strongly inhibited the invasion of bovine lymphocytes by *T. parva* sporozoites *in vitro*. GFP:p67ΔSS-elicited antiserum inhibited the sporozoite invasion of lymphocytes by 98%, whereas serum from mice immunized with the GP64:p67C resulted in 90% inhibition of sporozoite invasion (Table 1). An inhibition of 12% and 10% was observed for the GFP and adjuvant controls, respectively, and an inhibition of 8% for the medium control.

Antibody responses to p67 antigens in cattle
Based on the results of the seroconversion experiments in mice in combination with the promising SNA results, purified GFP:p67ΔSS and BVs containing GP64:p67C antigens were selected for immunogenicity studies in cattle. Given the difficulties involved in purifying large amounts of BVs for large scale immunization programs in cattle the immunogenicity of total cell extract (CE) prepared from infected insect cells

expressing GP64:p67C (GP64:p67C-CE) was also tested. GP64:p67C is abundantly expressed on the surface of insect cells [83]. Cattle were immunized with GFP:p67ΔSS and GP64:p67C formulated either in a proprietary water-in-oil (designated "WOE") or a saponin-based (designated "SA") adjuvant. Sera were collected weekly after the first immunization and analyzed by direct ELISA (Fig. 2) and Western blot analysis (Fig. 3). P67 antibody responses were determined by the reactivity of antibodies in each serum sample (1:200) with bacterial expressed $p67_{635}$ coated on the ELISA plate or total sporozoite lysate on the immunoblot.

The ELISA results (Fig. 2) showed that all the antigens tested induced high-levels of p67-specific antibody responses in immunized cattle. GFP:p67ΔSS induced significantly higher antibody titers than GP64:p67C (in either forms, BV or CE) with both adjuvants ($P < 0.05$). Antibody titers induced by GFP:p67ΔSS/WOE and GFP:p67ΔSS/SA formulated vaccines were not significantly different ($P < 0.05$) in cattle. Statistically, there was no difference between GP64:p67C-BV and GP64:p67C-CE, irrespective of the adjuvant used ($P < 0.05$). GP64:p67C-CE was the poorest immunogen. All vaccine-elicited antibodies reached an initial peak two weeks after the first immunization and began to level off afterwards. The boost inoculation with the same amount of protein (100 μg) resulted in a drastic increase in antibody titers attaining a new peak level around day 35. Cattle immunized with WOE-based vaccines maintained peak levels for more than 5 weeks in contrast to those immunized with SA-based vaccines, which decreased after the peak
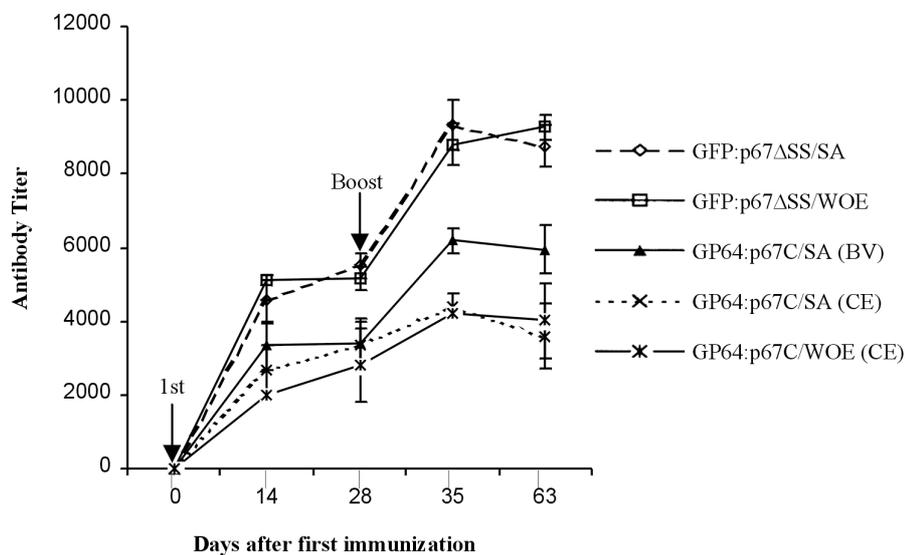
Fig. 2: Antibody responses to p67 antigens in cattle. Antiserum raised with the various immunogens GFP:p67ΔSS/WOE, GFP:p67ΔSS/SA, GP64:p67C/WOE (BV), GP64:p67C-BV/SA (CE), GP64:p67C/ WOE (CE), were titered against $p67_{635}$ (a recombinant form of p67 lacking the 74 C-terminal residues) by direct ELISA. Data are the average of three cattle with the error bars representing the standard error. Arrows represent days of first immunization (day 0) and booster (day 28). Pre-immune and adjuvant control sera did not react with $p67_{635}$ (data not shown). CE: cell extract, BV: budded virus, SA: saponin derivative, WOE: water in oil emulsion.
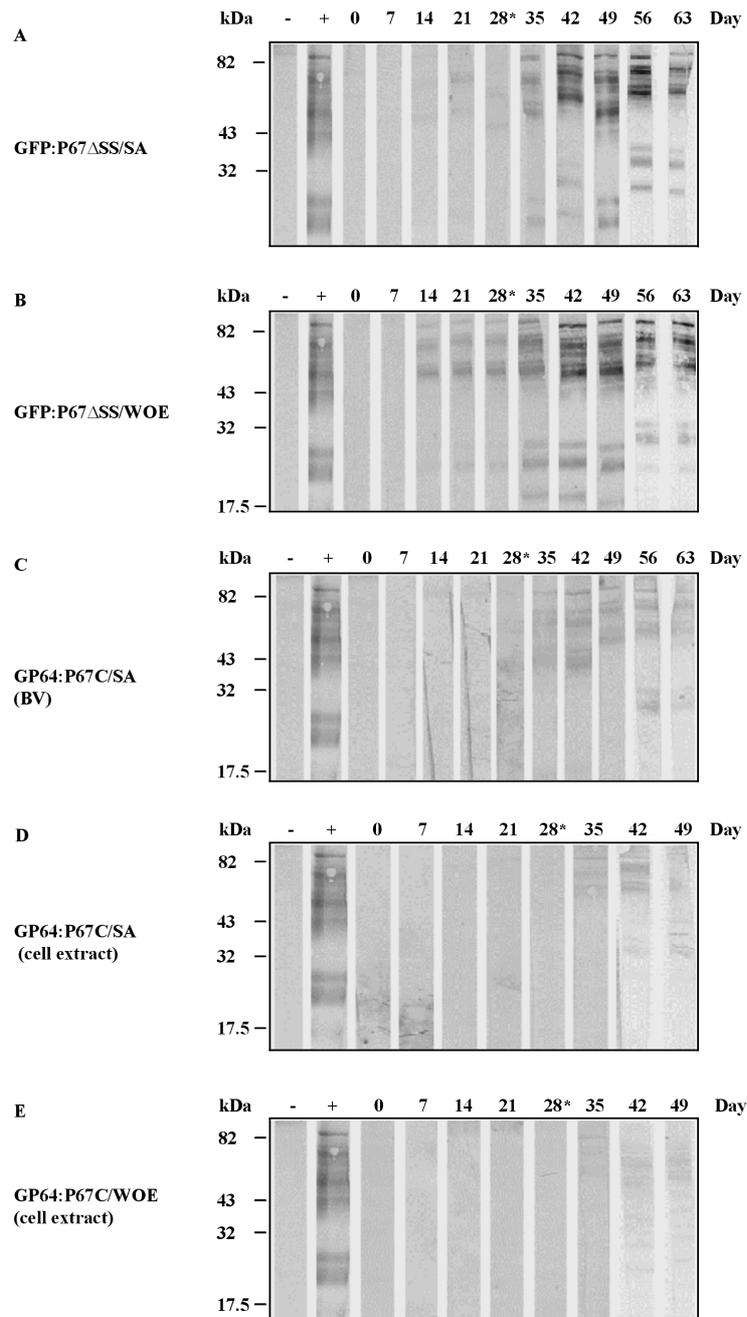
Fig. 3: Immunoblot analysis of antibody responses to p67 antigens in cattle. Total sporozoite lysate was resolved in 12% SDS-PAGE and subjected to immunoblot analysis using the various sera collected weekly from cattle immunized at day 0, and boosted at day 28 (*) with the vaccine antigens GFP:p67ΔSS and GP64:p67C. Sera were diluted 1 : 200. The negative control lane (-) was stained with serum from cattle immunized with a formulation containing only adjuvant whereas the positive control lane was stained with a polyclonal bovine antiserum, BP300, against p67. All other lanes were stained with sera collected at days 0, 7, 14, 21, 28, 35, 42, 49, 56, and 63 respectively. Purified GFP:p67ΔSS was formulated in SA (**A**) or WOE (**B**). AcGP64:p67C budded viruses (BV) were formulated in SA (**C**). Extracts of cells (CE) infected with AcGP64:p67C were formulated in SA (**D**) or WOE (**E**). Molecular size markers (kDa) are indicated.

In Western blot analysis, vaccine-elicited antibodies in sera bound to p67, revealing multiple bands (Fig. 3) consistent with that observed in previous reports [17, 132, 134, 141, 142, 144]. P67-specific antibody responses were observed at day 21 after first immunization in cattle immunized with GFP:p67ΔSS/SA (Fig. 3A). In cattle immunized with GFP:p67ΔSS/WOE (Fig. 3B), p67-specific antibody responses were observed as early as day 14 after first immunization. Hence, WOE induced p67-specific responses seven days earlier than SA for GFP:p67ΔSS. In cattle immunized with GP64:p67C/SA (BVs and cell extract; Figs. 3C and D) a similar response was first observed one week after the boost on day 35. First responses for immunizations with GP64:p67C/WOE (cell extract) were also seen one week after the boost inoculation (day 35, Fig. 3E). When animals were inoculated with cell extracts with GP64:p67C, there was no clear difference between WOE and SA with regards to the timing of induction of p67-specific responses (Fig. 3D and E). Due to a limited amount of purified budded virus we did not immunize animals with GP64:p67C-BV formulated in WOE. As expected, cattle immunized with GFP or adjuvant controls did not induce p67-specific antibody responses in mice or cattle (data not shown). Overall, GFP:p67ΔSS was the best antigen followed by GP64:p67C-BV, and GP64:p67C-CE (Figs. 2 and 3). GFP:p67ΔSS/WOE immunization led to a rapid antibody induction (14 days post-immunization) and a long sustained peak titer (>5 weeks; see Fig. 2).

Table 2: Neutralization of sporozoite infectivity by p67 vaccine-elicited antibodies in cattle

Antigen/Adjuvant	Mean % inhibition (SNA)
GFP:p67ΔSS/SA	95.00 ± 2.50
GFP:p67ΔSS/WOE	97.00 ± 0.25
GP64:p67C/SA (CE)	69.90 ± 2.63
GP64:p67C/WOE (CE)	69.75 ± 5.25
GP64:p67C/SA (BV)	76.00 ± 1.25
Adjuvant alone	14.67 ± 2.50
Pre-immune serum	15.00 ± 1.50
Medium	8.75 ± 0.90

Values are means ± standard deviations (results of three independent assays)

CE = Cell extract; BV= Budded virus

Sporozoite neutralization activity of bovine sera

Table 2 shows the SNA results obtained with sera from cattle immunizations. Sera from animals immunized with GFP:p67ΔSS/WOE caused an average inhibition of 97%, whereas GFP:p67ΔSS/SA-elicited antibodies resulted in 95% inhibition followed by GP64:p67C-BV/SA (76%), GP64:p67C-CE/SA (70%) and GP64:p67C-CE/WOE (70%) in decreasing order. The positive control serum G151 gave around 100% inhibition. Both pre-immune and sera from animals immunized with adjuvant

alone resulted in only 15% inhibition whereas in medium alone it was 9% (Table 2). These new p67-based vaccine antigens, especially those for GFP:p67ΔSS induced antibodies with higher inhibition activity comparable to published data [134, 144] as well as that observed with the positive control.

Discussion

Previous research has implicated that the *T. parva* sporozoite surface coat protein p67 is crucial for the attachment and subsequent entry of *T. parva* sporozoite into host lymphocytes (for review see references 179 – 181]. The possibility of using p67 as a subunit vaccine against ECF has been shown before [17, 134, 142], but the amount of antigen needed (>1 mg) and the number of boosts required was too high to make it a suitable vaccination regime. In recent papers we described the expression and production via the baculovirus expression system of more authentic recombinant p67 proteins, which reacted with a monoclonal antibody raised against native p67 [82, 83], and we speculated on their potential as improved vaccines.

To select the most immunogenic recombinant p67 proteins, we first immunized mice with five baculovirus-derived p67 protein constructs, in which domains of p67 were fused to either GFP or to the baculovirus envelope protein GP64 (Fig 1). The baculovirus-derived p67 fusion proteins were immunogenic in mice with the exception of GFP:p67C (Table 1). GFP:p67ΔSS and GP64:p67C, induced antibodies with the highest titers. In contrast to GFP-p67C, p67C fused to the baculovirus major envelope protein GP64 and projected on the surface of the baculovirus particle was highly immunogenic and induced p67-specific antibody responses in mice with titers comparable to that induced by GFP:p67ΔSS (Table 1). Therefore, the baculovirus surface display system might be a better alternative with regard to the expression of relatively small immunogens than GFP-fusions. High levels of antibody were elicited to the GFP portion of the GFP-p67C fusion protein in mice (data not shown). Since p67C comprises only 80 amino acid residues and a small portion of the fusion protein, it seems that most antibodies are directed to the GFP and not p67C.

The fact that high antibody titers were provoked in mice, especially by GFP:p67ΔSS and GP64:p67C, does not *per se* make these antigens good vaccine candidates if these elicited antibodies cannot neutralize sporozoite infectivity. In the past, high antibody titers did not always translate into high neutralization sporozoite infectivity and/or protection [60, 68, 134, 142, 143]. Table 1 shows that sera elicited by GFP:p67ΔSS and GP64:p67C had very strong sporozoite neutralizing activity in an *in vitro* assay. The percent inhibition obtained was remarkable since only small amount (2 x 50 μg) of antigen were used in a single prime and boost immunization. The failure of the bacterially produced p67C to elicit antibodies of comparable titers in mice despite the fact that at least twice (2 x 100 μg) as much of this protein was used, could be due to the difference in conformation of these proteins effecting the quality of antibodies

elicited. Thus, the present results show that, at least in mice, these p67 fusion constructs not only provoke high levels of serum conversion but also induce antibodies with the potential to prevent sporozoite invasion of lymphocytes.

Based on the mice data, the immunogenicity of GFP:p67ΔSS and GP64:p67C was tested in cattle, using two different adjuvants, WOE and SA. The levels of anti-p67 antibodies raised were comparable to the levels observed in previous studies [17, 134, 141, 142], but the amount of antigen used per inoculation was much lower (100 µg versus 300-600 µg) and only a single boost was given, compared to 2 or 4 boosts given before. One major reason for this superiority is likely to be due to proper folding in these new recombinant proteins resulting in the conservation of native epitopes [82, 83] and possibly to a longer half-life *in vivo*. Another reason could be an improvement in adjuvant and formulation. The positive correlation between neutralizing efficacy and the presence of conformational epitopes has been demonstrated [43, 86, 99, 163]. The antibody levels obtained with GFP:p67ΔSS were significant higher than with GP64:p67C ($P < 0.05$). This may be related to the absolute amount of p67 present in the various samples used for immunization. In addition, even though the middle region (present in GFP:p67ΔSS) has been reported to have no B-cell activity it might contain some Th-cell epitopes that enhance B cell responses [141, 144].

The strong *in vitro* anti-parasite activity observed for mice and cattle sera converted with GFP:p67ΔSS and GP64:p67C (Tables 1-2) suggests that these novel antigens elicit high quality antibodies in high quantities. These are essential properties for a vaccine and may be critical in protection, given that *T. parva* sporozoites are available for direct antibody-based intervention only for a short period of time. *T. parva* sporozoites, once deposited in the host rapidly invade host lymphocytes and internalize within 10 minutes. It is also important to note that the severity of ECF is dose dependent [114] and the number of sporozoites exceeding the infectivity threshold and the capacity of the animal to acquire immunity will influence this. Thus large amount of antibodies of high quality and neutralizing capacity is desirable.

GFP:p67ΔSS formulated in WOE induced antibody responses within 14 days after first immunization compared to 21 days for GFP:p67ΔSS formulated with SA (Figs. 3A and B). This accelerated development of antibody responses could be advantageous in situations where ECF is endemic and rapid vaccination of unprotected animals is required. In our studies WOE was a better adjuvant than SA in rapid induction and sustaining of high titer antibodies (Fig. 2). The exact mode of action of both WOE and SA are not known. But water-in-oil or oil-in-water emulsifiers are known to enhance the induction of humoral responses better than saponin-based adjuvants [2, 26, 88, 149].

In conclusion, we have developed highly immunogenic baculovirus-derived recombinant p67 antigens that induce high levels of antibodies in cattle and can serve as a vaccine. Even more important, the vaccine-elicited antibodies produced significant anti-parasite activity as evidenced by high levels of inhibition of *T. parva* sporozoite infectivity *in vitro*. Overall, the GFP:p67ΔSS fusion protein was the best immunogen, followed by GP64:p67C (BV). Our results indicate that WOE was a better adjuvant than SA with respect to rapid induction and sustaining of anti-p67 antibodies at peak level. The potential of these formulations to provoke protective immunity against ECF will be analyzed in subsequent efficacy trials in cattle. If positive, the improved expression of p67 in combination with an improved application might lead to the first recombinant vaccine for the control of East Coast fever in Sub-Saharan Africa.

Acknowledgements

Chapter 6

Improved protection against East Coast fever with novel baculovirus-derived p67 subunit vaccines

Summary

The *Theileria parva* sporozoite surface antigen p67 is the basis for the development of an anti-sporozoite subunit vaccine for the control of East Coast fever (ECF) in cattle. The vaccine potential of two novel baculovirus-derived recombinant p67 constructs with a near-native conformation was evaluated in an efficacy trial. Boran calves were immunized with an affinity-purified his-GFP-p67 fusion protein (GFP:p67ΔSS) or with total insect cell extracts containing GP64-p67C, a protein fusion between a C-terminal domain of p67 and the baculovirus envelope protein GP64. The former vaccine was formulated either in a water-in-oil (WOE) or a saponin-based (SA) adjuvant and the later in SA. Both GFP:p67ΔSS and GP64:p67C induced antibodies with high ELISA titers, that neutralized sporozoite infectivity with high efficiency in *in-vitro* assays. Upon challenge with cryopreserved *T. parva* sporozoites, eleven of thirteen (11/13) animals immunized with GFP:p67ΔSS formulated in WOE were protected, while immunization with the GFP:p67ΔSS/SA formulated in SA conferred protection to ten of thirteen (10/13) calves. Nine of thirteen (9/13) animals immunized with GP64-p67C were protected. In comparison, 10/15 controls were unprotected and were euthanased or treated. Level of protection correlated positively with the *in-vitro* neutralization assays of *T. parva* sporozoites. A significant level of protection resulting in an average 67% reduction in incidence of severe ECF for the three immunized groups over the controls was achieved with only two inoculations using 25 - 50 µg of recombinant p67 per inoculation. These ECF subunit vaccines consisting of insect cell-derived recombinant p67 in a near-native conformation, when formulated in a suitable adjuvant, can be applied in a simple vaccination regime.

Introduction

Theileria parva is a tick-borne protozoan parasite that infects T and B-lymphocytes of cattle. Once inside the lymphocyte, the parasites rapidly develop into schizonts, transforming the target cells into clonally proliferating lymphoblasts. The host cells and schizonts divide synchronously and the parasitized lymphocytes invade tissues throughout the body, resulting in a severe and often lethal disease known as East Coast fever (ECF). A proportion of the schizonts differentiates into merozoites, which invade erythrocytes and give rise to piroplasms, the tick-infective stage [38]. The disease is of major economic importance because of the considerable losses caused in eastern, central and southern Africa [121]. Direct losses comprise mortality and loss of production (milk, draught power and growth). Indirect losses are costs incurred for disease control, such as acaricides for tick control and theileriocides for chemotherapy. Currently, vaccination against the disease involves the simultaneous use of a lethal dose of cryopreserved sporozoites and a long-acting oxytetracycline. A major limitation of this vaccination strategy is that it requires a cold chain, a rare facility in endemic areas, and that the immunity engendered is often parasite strain specific [202]. The quest for a subunit vaccine for the control of ECF therefore continues [114, 127].

Primary exposure of cattle to *T. parva* does not result in significant antibody responses to the sporozoites. However, sera from cattle repeatedly challenged or hyper-immunized with the parasite or sporozoite lysate contain high titers of sporozoite-specific antibodies capable of neutralizing infectivity of sporozoites *in-vitro* [113, 114]. Although these sera react with an array of *T. parva* antigens in immunoblots, the sporozoite surface coat protein p67 has been shown to bear the neutralizing epitopes [34, 36, 132, 134, 141]. Previously, the potential of p67 as a broad-spectrum vaccine against ECF has been demonstrated [134, 141-144]. *Theleria parva* exists as different cattle-derived and buffalo-derived strains [143, 148].

The success in developing a p67-based subunit vaccine has been hampered by problems associated with expressing sufficient amounts of recombinant p67 in a near-authentic form, protein stability and solubility in a variety of bacterial and eukaryotic protein expression systems, including the baculovirus/insect cell system [47, 60, 68, 134, 141, 142]. An 80 amino acid peptide corresponding to a C-terminal region of p67 exhibited improved stability and the degree of protection was comparable to full length p67 protein produced in *E. coli*. However, this still required doses as high as 450 µg per inoculation [17] in a multiple vaccination scheme with two boosters, two months apart.

Recently, we described the successful expression of p67 in near-native forms via the baculovirus-insect cell expression system [82, 83, 89]. In those experiments, p67 was

fused to green fluorescent protein (GFP) and expressed as a cytoplasmic protein in insect cells. GFP fusion enhanced the stability and hence the expression levels of p67. Furthermore, the TpM12 epitope, a marker for the native form of p67, was preserved [81]. Similar results were obtained when domains of p67 were fused to the baculovirus envelope protein GP64, resulting in the display of p67 on the surface of infected insect cells and budded baculovirus particle [82]. Amino acids 21 to 651 of p67 fused to GFP (GFP:p67ΔSS) and amino acids 572 to 651 fused to GP64 (GP64:p67C) induced high titers of antibodies in mice and cattle and the sera obtained neutralized sporozoite infectivity very efficiently in *in-vitro* assays [84].

In this paper the efficacy of these two recombinant proteins as p67-based subunit vaccines in cattle is evaluated in detail in combination with a saponin-based (SA) or a water-in-oil (WOE) adjuvant. These novel baculovirus-derived p67 antigens need only a single booster and much lower amounts of antigen than required before [17].
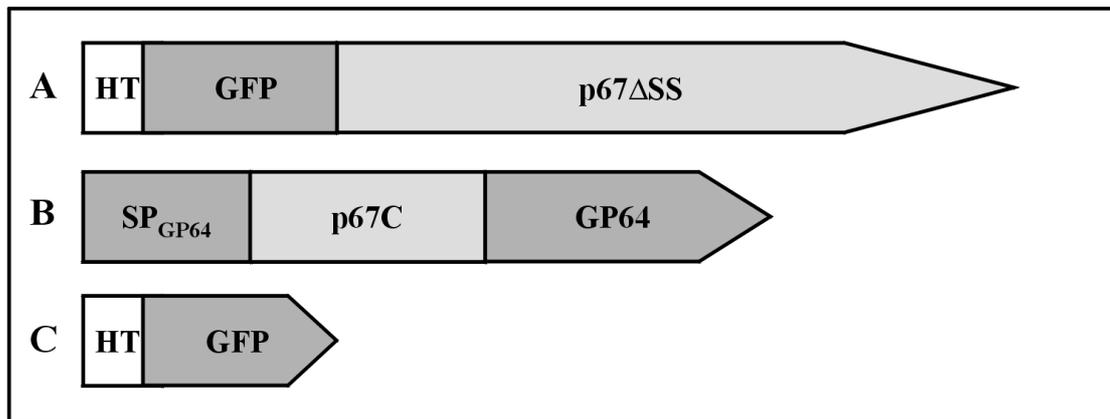
Fig. 1: Schematic representation of the best recombinant p67 construct used in efficacy trial in cattle. (**A**). The full-length $p67_{(21-651)}$ protein without its signal and transmembrane domains, fused to the C-terminus of GFP. HT: N-terminal polyhistidine tag; GFP: green fluorescent protein. (**B**). The C-terminus of $p67_{(572-651)}$ sandwiched between the signal peptide (SP) and the main domain of the baculovirus major envelope protein, GP64. Numbers in brackets (subscript) represents the range of p67 amino acid residues included in the construct. (**C**). GFP with N-terminal his-tag used as negative control. (For detail description of these constructs, see ref: [82, for **A & C** and 83 for **B**).

Materials and Methods

Expression, purification and preparation of baculovirus-derived p67
The construction and expression of recombinant baculoviruses encoding the two p67 antigens (AcGFP:p67ΔSS and AcGP64:p67C) as well as the non-fused GFP control construct (AcGFP), have been described previously [82, 83]. GFP and GFP: p67ΔSS have his-tags incorporated at their N-terminus to facilitate purification. For the preparation of the GFP and GFP:p67ΔSS vaccines, 2 x 10^7 T. ni High Five™ cells were seeded into 75 cm^2 tissue culture flasks in a monolayer and infected with the

recombinant baculoviruses AcGFP:p67ΔSS or AcGFP at a multiplicity of infection of 10 plaque-forming units per cell. At 48 h post-infection (p.i) cells were harvested and washed once in cold phosphate-buffered saline (PBS) and re-suspended in 5 ml wash buffer (50 mM NaH_2PO_4; 300 mM NaCl, pH 7.0). To purify the GFP and GFP:p67ΔSS proteins, the cells were disrupted by ultra-sonic treatment and purified over his-tag affinity purification columns as described previously [83].

To prepare the GP64:p67C vaccine, cells were infected with the recombinant baculovirus AcGP64:p67C, as described above. After 48 h, the cells were harvested, washed and resuspended in PBS and then disrupted by ultra-sonication. To inactivate remaining baculoviruses according to current GMO regulation, the total cell extract was treated with Triton X-100 as previously described [83], and gamma-irradiated. The protein concentration of the purified proteins (GFP and GFP: p67ΔSS), as well as the total protein content of the cell extract, were determined using Bradford reagent (Bio-Rad). The amount of recombinant p67C protein contained in the total cell extract was estimated by resolving known amounts of affinity-purified GFP:p67C protein alongside the GP64:p67C in SDS-PAGE, and then comparing the intensity of the bands detected in immunoblot analysis using a monoclonal specific for an epitope in the p67C terminal portion (ARIII 21.4) [144]. The vaccines were formulated in two different proprietary adjuvants, a saponin-derived adjuvant (SA) and a water-in-oil emulsion (WOE) (Intervet International B.V., Boxmeer, The Netherlands).

Experimental animals and parasites
Fifty-four male Boran cattle (*Bos indicus*), aged 4-6 months, from the International Livestock Research Institute's (ILRI) Kapiti Ranch, an ECF-free region near Nairobi, were used in this study. The animals were kept under strict acaricide control of ticks at the ILRI Campus at Nairobi, Kenya, and given hay, supplementary calf pellets and water *ad libitum.* All animals were tagged and confirmed to be free of antibodies to *Theileria parva* by ELISA as described by Katende *et al.,* [85] prior to the immunization experiments. The Muguga stock of *T. parva,* originally obtained from the East African Veterinary Research Organization, Muguga, Kenya, was used in this study. A bulk stabilate (# 4133) of cryopreserved sporozoites was prepared following the procedure previously described by Morzaria *et al.* [126] from adult *Rhipicephalus appendiculatus* ticks infected with the *T. parva (Muguga)* stock.

Immunization, challenge and monitoring of cattle
Animals were randomly assigned to three vaccine groups of 13 animals each and two control groups of 8 and 7 animals, respectively. Group 1 and 2 animals were inoculated with 50 μg of purified GFP:p67ΔSS protein formulated in SA or WOE, respectively. Group 3 animals were primed with total cell extract equivalent to 50 μg total protein from infected insect cells expressing GP64:p67C, formulated in SA. The total cell extract contained about 23 μg of GP64:p67C per dose. Control Groups 4 and

5 were inoculated with 50 μg of GFP in SA or WOE, respectively. Each animal in all the five groups received a single booster, containing the same type and amount of antigen as described for the prime, 28 days after the first immunization. Thus, each animal in groups 1, 2, 4 and 5 was immunized with a total (prime + boost) of 100 μg of GFP:p67ΔSS (Group 1 and 2) or GFP (Group 4 and 5), whereas each animal in Group 3 received a total of about 46 μg of GP64:p67C fusion protein. Immunizations were administered sub-cutaneously. Prior to challenge, 31 days after the booster, all the animals were given new tags and regrouped randomly. The code for this re-tagging/reshuffling was kept a secret till the experiment was terminated, in order that the investigation remained blind (Randomized double-blind placebo-controlled trial). All were given a syringe challenge of 1 ml of *T. parva* (Muguga) sporozoites corresponding to a lethal dose 70 (LD_{70}), administered sub-cutaneously over the parotid lymph node (local drainage lymph node). The challenge was given at that time (31 days after the booster) to reduce any possible effect of the adjuvant alone.

All experimental animals were monitored daily for changes in rectal temperatures and for other clinical manifestations of ECF as previously described [171]. On day 5 after challenge, and daily thereafter, needle biopsies were made from the parotid lymph node, and examined for the presence of schizonts. Biopsy smears were also prepared using fluid from the pre-scapular lymph node and blood from the jugular vein, from the day after the parotid lymph node was found to be positive for schizonts. Blood smears were stained with Giemsa and examined for piroplasm parasitaemia by counting the number of infected red blood cells (RBC) per 1000 cells. Total white blood cell counts (WBC) were determined from blood taken from the jugular vein before the challenge and 3 times per week, thereafter. Serum was collected from each animal every week for ELISA and immunoblot analysis. Animals were euthanased if they became severely affected by ECF, the predominant signs of which included duration and level of pyrexia and parasitosis, severe changes in haematology and general body condition affecting appetite and respiration rate [171]. Animals less severely affected were allowed to recover.

Analysis of anti-p67 antibody responses in cattle
Anti-p67 specific antibody titers in serum were determined by direct ELISA using $p67_{(635)}$ [134] as an antigen, as described previously by Kaba *et al.*, [84]. Briefly, ELISA plates were coated with 150 ul (2.5 μg/ml) of antigen, blocked with 0.1% Tween20 in PBS, and tested with appropriate dilutions of sera, collected from both immunized and control cattle on day 0 and weekly thereafter. Antibody titers (geometric mean) for each animal, were determined based on the highest dilution of the samples that generated an optical density (OD) value greater than the cutoff value (geometric mean of pre-immune sera + 3 SD). The mean antibody titer for each group was then calculated using the titer determined from the sera collected a day before the sporozoite challenge.

Sporozoite neutralization assays (SNA) were performed using pre-immune sera and sera taken a day before the sporozoite challenge as previously described [134]. Briefly, a suspension of sporozoites was incubated for 30 min with pre- or post-immunization sera at a final dilution of 1:50, and then included with bovine peripheral blood lymphocytes (PBMs). Cultures were examined on days 7, 10 and 14 and assessed for the percentage of infected lymphocytes. Percent inhibition was determined as (average of three separate counts) the number uninfected lymphocytes of 400 counted, times 100. As a positive control for this analysis, the bovine serum G151 [134] was used, whilst the pre-immunization serum samples served as negative controls. Three independent assays were conducted for each animal and the mean percent inhibition was calculated.

Detection of T. parva DNA by p104 gene PCR assays

Needle biopsies from the parotid lymph node at the site of sporozoite inoculation were collected from non-reacting animals into 1 ml of Dulbecco's phosphate-buffered saline 8-10 days after the sporozoite challenge. In addition, blood samples were collected into EDTA vacutainers (Becton Dickson, Oxford, UK). DNA was purified from lymphocytes using a tissue DNA purification kit (Promega) following the manufacturer's protocol. Purified DNA (50 ng) was amplified in a 50 μl reaction volume following the procedure described by Skilton *et al.* [183]. For a positive control, 10 ng of piroplasm DNA was used. The dried blood spot p104 gene PCR technique described by Skilton *et al.*, [183] was used to analyze the blood samples. Cleaned filters and/or filters spotted with blood from uninfected cattle were used as negative controls. Blood from ECF-infected animals BT 169 [183] and TM 413 was used as positive controls and spotted on two filters, respectively. TM 413 was an animal from the current experiment showing schizont parasitaemia.

PCR amplification from both the lymphocyte DNA and blood samples was performed using the p104 primer-pair IL755 (5'-TAAGATGCCGACTATTAATGACACC-3') and IL3231 (5'-ATTTAAGGAACCTGACGTGACTGC-3') following the procedure described by Skilton *et al.* [183]. PCR products were resolved in a 2 % agarose gel and transferred onto Hybond-Nylon membrane (Amersham Pharmacia Biotech). The filter was hybridized to a nested probe generated by PCR from *T. parva* Muguga piroplasm DNA with p104 gene primers IL 4234 (5'-GGCCAAGGTCTCCTTCAGA ATACG-3') and IL3232 (5'-TGGGTGTGTTTCCTCGTCATCTGC-3') and labeled with α^{32}[P]-dATP using the Mega-primer random priming kit (Amersham Pharmacia Biotech).

Statistical analysis

A number of variables, derived from parasitological, haematological, and rectal temperatures were used for the statistical analysis. Details of how each variable was

derived, the statistical analysis that followed, and how the statistically derived ECF reaction indices in a range 0 - 10 were calculated as previously described [171]. ECF indices were then analyzed by a one-way analysis of variance for the five groups of cattle. Animals were further divided into five disease categories based on the ECF index scores [171] calculated from the pathological and clinical data collected for each animal. The range of ECF index scores for four of these classification groups was chosen as follows: 0.01 - 3.49, mild reactors (MR); 3.5 - 5.99, mild-moderate reactors (MR/MODR); 6.0 -7.99, moderate-severe reactors (MODR/SR), and 8.0 - 10.0, severe reactors (SR). An animal showing a MODR/SR or SR reaction was defined as having severe ECF, a condition likely to result in the need for treatment for ECF when the disease is contracted in the field. This is equivalent to an ECF index score between 6 and 10 as indicated above. An immunized animal with a score less than 6 was considered to be 'protected'. Animals showing no parasitological reaction (score 0) were classified as NR (non-reactors). The proportions of severe ECF (MODR/SR or SR) in each of the immunized groups were compared with the average proportions in the controls by a chi-square (χ^2) test.

Results

Expression and purification of p67 recombinant proteins
Based on previous sero-conversion experiments in mice and cattle [84], two novel baculovirus-derived p67 vaccines were chosen for efficacy tests in cattle. Both recombinant proteins, GFP:p67ΔSS (Fig 1 A) and GP64:p67C (Fig 1 B) were capable of inducing highly effective neutralizing antibodies in both mice and cattle. GFP:p67ΔSS contains amino acid residues 21 to 651 of p67 fused to the C-terminus of GFP. GP64:p67C encodes the C-terminus of p67 (amino acid residues 572 to 651) fused to the baculovirus envelope protein, GP64. Both GFP:p67ΔSS and non-fused GFP (negative control; Fig 1 C) were purified on his-tag affinity columns and the purity was checked by SDS-PAGE and Western blot analysis (data not shown). The average yield of both GFP and GFP:p67ΔSS was approximately 300 μg protein per 1.6×10^7 *T. ni* insect cells. The GP64-p67C vaccine was prepared from total insect cell extract from GP64-p67C-infected cells. The total cell extract was estimated to contain 45% GP64:p67C. The vaccines were formulated in either SA or WOE.

Immunization and serological analysis
Sera collected on a weekly basis were analyzed by immunoblotting, ELISA and sporozoite neutralization assays. For immunoblotting, recombinant p67 or total sporozoite lysate were resolved on SDS-PAGE and subjected to immunoblot analysis using pooled sera from each group. The sera from the two control groups were combined for this analysis. Sera from animals immunized with p67 recombinant proteins (Groups 1, 2 and 3) bound to both recombinant and sporozoite p67, whereas

sera from Groups 4 and 5, as expected, failed to bind to p67 in immunoblot analysis (data not shown).
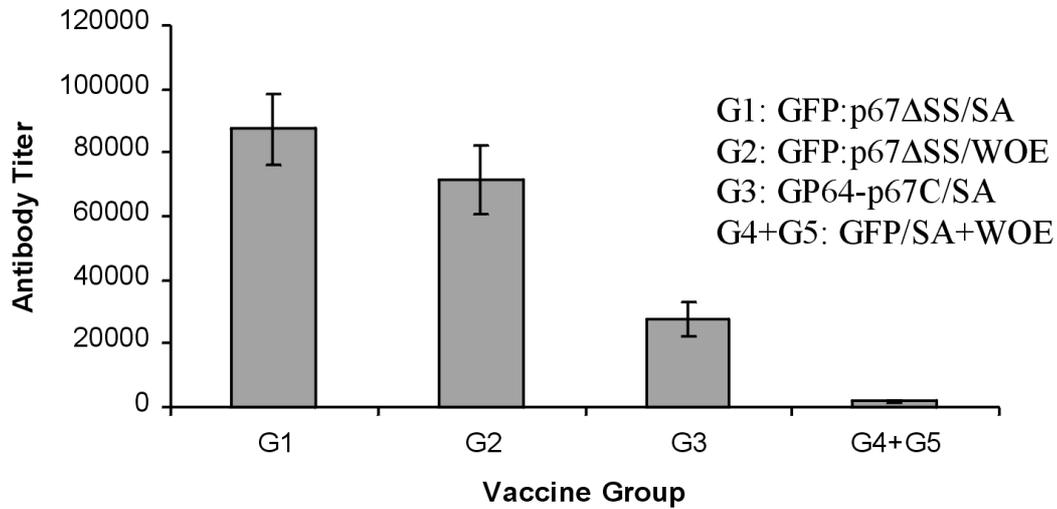
Fig 2: Mean antibody responses to vaccine formulations in p67-immunized and control cattle. Antibody titers for all animals were determine using the serum collected 60 days after first immunization (that is a day before animals were challenged with *T. parva* stabilated sporozoites). The graph was generated using the mean antibody titers. Antibody titers for the two control groups were combined. G1: group 1, G2: group 2, G3: group 3 and G4+G5: group 4 plus group 5.

Sera collected from all thirty-nine animals immunized with p67 recombinant proteins a day before *T. parva* stabilated-sporozoite challenged (day 60 after first immunization) showed direct ELISA titers ranging between 1 : 8000 and 1 : 128000 (Fig.2; Table 1). The mean antibody titers were compared amongst p67-immunized groups and that of the combined mean for the control groups by a two-tailed t-test. The mean titers of GFP:p67ΔSS/SA or GFP:p67ΔSS/WOE immunized groups were significantly different from that of GP64:p67C (P < 0.001). There was no significant difference between the mean titers of GFP:p67ΔSS/SA and GFP:p67ΔSS/WOE immunized groups (Table 1).

Sera from p67-immunized cattle had strong anti-parasite activity as evidenced by the neutralization of sporozoite infectivity *in in-vitro* assays (Table 1). Although not different the other immunized groups, GFP:p67ΔSS/WOE-immunized animals induced antibodies with the highest average of sporozoite neutralizing activity (89%) followed by GFP:p67ΔSS/SA and GP64-p67C/SA in that order (Table 1). Pre-immune sera from all experimental animals and that from cattle immunized with non-fused GFP (GFP alone) failed to neutralize sporozoite infectivity *in-vitro* (Table 1). These observations are consistent with previous sero-conversion experiments [83]. In total 11 animals (5/15 controls and 6/39 immunized animals) were ethanased between days 20 and 22 post-challenge, so that the length of the period over which measurements might have been taken for these animals was truncated. Monitoring

was continued for the rest of animals to day 27. Two animals from the control group were treated after day 27.

Table 1: Mean antibody responses for vaccine formulations in immunized cattle and neutralization of sporozoite infectivity

| Group | Vaccine formulation | Total number of animals | ELISA | | SEM (x 10³) | SNA |
| | | | Titer (x 10³) | | | Mean % inhibition* |
			Mean	Range		
1	GFP:p67ΔSS/SA	13	87.4	16-128	± 11.2	81.7 (± 14.1)
2	GFP:p67ΔSS/WOE	13	71.4	32-128	± 10.9	88.9 (± 12.1)
3	GP64-p67C/SA	13	27.7	8-64	± 5.7	76.4 (± 15.6)
4	GFP/SA+WOE	15	1.7	1-4	± 2.6	22.4 (± 3.7)
	Pre-immune	54	N/A	N/A	N/A	17.5 (± 1.0)

* Value in brackets represents ± SEM.   SNA: Sporozoite Neutralization Assay;

SEM: standard error mean

Eleven of 13 animals immunized with GFP:p67ΔSS/WOE (85%) were protected whereas 10 of 13 animals immunized with GFP:p67ΔSS/SA (77%) were protected (Table 2). GP64-p67C/SA induced protection in nine of thirteen (69%). Eight animals showed no parasitological reaction (NR). Five of these were immunized with GFP:p67ΔSS/WOE and three with GFP:p67ΔSS/SA (Table 2). Between two and four of the 13 animals in the three immunized groups (average 23%) had severe ECF compared with six and four animals, respectively, from the two control-groups (average 67%). There was no significant difference between the proportions of animals with severe ECF in the two control groups. Hence, these two groups were combined for further statistical comparison with the immunized groups. On the average, 67% of the controls had severe ECF. This corresponds exactly to the infectivity expected with the LD_{70} sporozoite stabilate challenge given to the cattle, suggesting that inoculation with the adjuvants alone had no influence on level of immunity. The proportion of animals with severe ECF in each of the immunized groups was compared with the average proportion in the controls by a χ^2 test (Table 3). P-levels ranged from 0.06 to < 0.01. There were no significant differences among the three immunized groups when comparing either the proportions with severe ECF or the mean ECF indices shown in Table 2.

ECF indices were also analyzed by one-way analysis of variance. In this analysis each of the GFP-p67ΔSS-immunized groups (Groups 1 and 2) was significantly different from the average of the controls when compared by a two-tailed t-test (P < 0.01) but not the GP64:p67C group, which had no non-reactors. Nevertheless, there were no significant differences among the immunized groups. These data follow from the fact

that the level of protection achieved by GP64:p67C, as indicated by the mean ECF index, was intermediate between the two GFP:p67ΔSS groups and the control groups (Table 2).

Table 2: Distribution of ECF reactions, proportion of animals with severe ECF and mean ECF indices for all p67-immunized and control groups

Vaccine formulation	Number of animals						Severe[b] ECF	Range of ECF indices	Mean ECF Index
	NR	MR	MR/ MODR	MODR /SR	SR*	Total			
GFP:p67ΔSS/SA	3	3	4	2	1	**13**	3/13	0.17-8.37	3. 72
GFP:p67ΔSS/WOE	5	2	4	0	2	**13**	2/13	0.21-8.80	3.44
GP64-p67C/SA	0	6	3	1	3	**13**	4/13	2.07-8.92	4.89
Total	**8**	**11**	**11**	**3**	**6**	**39**	**9/39**	-----------	**4.02**
GFP/SA	0	2	0	5	1	**8**	6/8	2.95-7.28	6.01
GFP/WOE	0	0	3	0	4	**7**	4/7	3.43-9.14	6.83
Total	**0**	**2**	**3**	**5**	**5**	**15**	**10/15**	----------	**6.39**
SED[a]	----	-----	--------	---------	-----	-------	---------	----------	**0.96**

[a] Standard error difference between each of the immunized group means (13 observations) and the average of the two control means (15 observations)

[b] An individual animal with an ECF index score ≥6 (MODR/SR or SR) is defined to have severe ECF.

*All animals falling in the SR category were euthanased.

Table 3: Comparison of animals immunized with p67 recombinant proteins with the combined control groups in a chi-square test

GFP:p67ΔSS in SA	$\chi^2 = 5.32$	(P < 0.05)
GFP:p67ΔSS in WOE	$\chi^2 = 7.48$	(P < 0.01)
Gp64-p67C in SA	$\chi^2 = 3.59$	(P = 0.06)

An apparent correlation between antibody titers in the serum-ELISA data, and level of protection as described by the ECF index could not be found among individual cattle in these studies, although mean antibody titers for the three immunized groups followed the same trend as mean ECF indices (Table 1, 2). However, a positive correlation between the level of inhibition of sporozoite infectivity observed in *in-vitro* assays and the level of protection achieved in individual cattle was apparent. Immunization with full-length p67 antigen provided significant protection regardless of the adjuvant used. Given an average LD of 0.7 in the controls, the average reduction in incidence of severe ECF resulting from immunization of GFP:p67ΔSS (19%) compared with controls (67%) was [(67-19)/67]% = 72% and the odds ratio 0.12. Immunization with GFP:p67ΔSS thus provided significant protection with both types of adjuvant used.
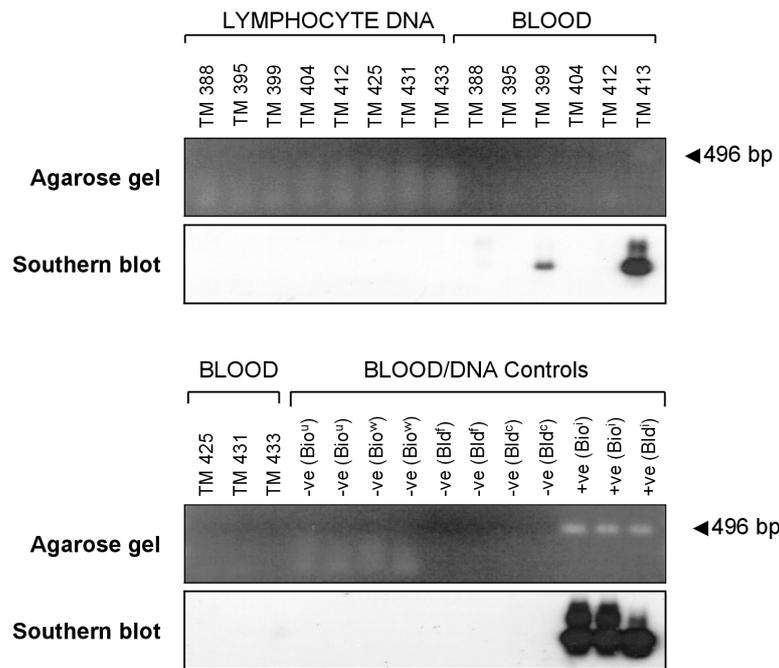
Fig 3: Detection of *T. parva* DNA in lymph node biopsies and blood taken from non reacting p67- immunized cattle by p104 PCR assay. Upper panels: An ethidium bromide-stained agarose gel of amplified PCR DNA using p104-specific primers [181]. As template samples of lymphocyte DNA or blood were used derived from from animals TM388, TM395, TM399, TM404, TM412, TM413, TM425, TM431 and TM433, as well as *T. parva* piroplasm DNA, blood from the positive control animal BT169. As negative controls for blood samples, clean filters and filters spotted with uninfected bovine blood, and for biopsies, clean water and DNA from uninfected bovine lymphocytes was used. Lower panels: Southern blots of the agarose gel probed with a PCR product generated by PCR from *T. parva* piroplasm DNA with p104-specific primers. Bio: Biopsy (DNA); Bld: blood; c: clean blood; f: clean filter; w: water; i: piroplasm DNA from an infected animal; u: piroplasm DNA from an uninfected animal.

PCR detection of T. parva DNA in NR-cattle

To determine if the non-reacting animals were solidly immune and completely cleared the experimentally infected parasites before they could develop, the *Theileria parva* p104 gene-based PCR assay for detecting parasite DNA in blood or in lymph node biopsies was employed. *Theleria parva* DNA was detected by PCR in the blood of only one NR-animal (TM 399; Fig. 3) but not in DNA extracted from lymphocytes obtained from the same animal, indicating that sporozoites had not extensively invaded lymphocytes. As expected, *T. parva* DNA was also detected in a mild-reactor (animal number TM 413) that was included as a positive control for this analysis from the current study (Fig. 3, upper panels). All positive control samples produced a strong specific PCR signal while none of the negative control samples produced a PCR signal. The probe obtained by PCR with p104-specific oligonucleotides

hybridized to all the positive controls as well as TM 399 but not to the rest of the NR-animals and the negative controls in a Southern hybridization (Fig. 3, lower panels).

Discussion

In previous papers, we described the development of two novel baculovirus-derived p67-based fusion proteins [82, 83] capable of provoking efficient p67 specific neutralizing antibody responses in mice and cattle [84]. To evaluate the protective efficacy of these recombinant proteins, GFP-p67ΔSS and GP64-p67C, Boran cattle were immunized and subsequently challenged with a lethal dose (LD$_{70}$) of *T. parva* sporozoite stabilate, and monitored for the manifestation of clinical ECF. The influence of two proprietary adjuvants, SA (saponin-derivative) and WOE (water-in-oil emulsion) on the efficacy of the proteins as immunogens was examined.

The exact mode of action of the water-in-oil adjuvant is unclear but the results obtained here suggest that in cattle, it enhanced the induction of GFP:p67ΔSS-specific antibodies of a similar if not better, neutralizing quality than SA. In addition, p67-specific responses induced by GFP:p67ΔSS/WOE were seen earlier and were maintained longer at a high level than GFP:p67ΔSS/SA antibody responses (data not shown). Water-in-oil emulsions have been shown to provide short-term depots and induce good antibody response [26]. It is possible that WOE influenced the generation of an immune response at several levels including the mobilization of appropriate antigen presenting cells to the injected site, enhancing efficient antigen processing and presentation, and influencing the cytokine and co-stimulatory signals necessary for an optimal immune response. WOE might also have exerted its influence by simply boosting relevant antibody responses to neutralizing epitopes on p67 [2].

Pepscan^{TM} data reported by Nene *et al*., [144] showed that the major linear bovine B-cell epitopes were mapped in residues 25-39, 73-175, 281-296, 577-591 and 617-671 of p67. Three of the sporozoite neutralizing epitopes are located between 105-221 and two between 617-631 and antibodies to any one of these epitopes of p67 have been shown to inhibit sporozoite entry into host cells [144]. It is therefore possible that the combined effect of these adjuvants and the conformation of the recombinant p67 protein greatly enhanced the bovine immune response to the neutralizing epitopes which are present in the GFP:p67ΔSS vaccine, thereby optimizing its potential compared to the full-length p67 used in previous studies [17, 134, 142].

Immunized cattle generated high p67-specific antibody titers, both to his-tag affinity purified GFP:p67ΔSS or to a crude insect cell extract containing GP64:p67C protein (Fig. 1; Table 1). GFP:p67ΔSS formulated in SA generated similar antibody titers to GFP:p67ΔSS formulated in WOE (Table 1). GP64:p67C/SA induced a lower mean antibody titer. Nevertheless, there was wide variation in p67 specific antibody

responses among p67 immunized cattle (1: 8000 to 1:128000) (Table 1) and no apparent correlation was observed between the levels of antibodies in individual animals and their susceptibility to challenge. Qualitative differences in the antibody responses among individual animals, such as the ability to recognize neutralizing epitopes, most likely accounted for the variable protection. The wide variation in antibody responses is most likely due to differences resulting from the genetic background of the animals. Genetic variations or the ambient health stage of the animal may further influence the levels of neutralizing antibodies induced in immunized animals and the capacity of an animal to acquire immunity.

Upon challenge with *T. parva* sporozoites, an average 77% of immunized cattle fell into the 'protected' category, whereas 67% (in good accordance with LD_{70} expectation) of the control group succumbed to severe ECF and were either euthanased or treated (Table 2). Eleven of thirteen animals (85%) immunized with GFP:p67ΔSS/WOE and 10/13 (77%) of GFP:p67ΔSS/SA-immunised animals were protected (Table 2). GP64:p67C/SA induced protection in nine of thirteen animals (69%) (Table 2). Previous studies [17, 134] have reported that p67C produced in *E. coli* induced comparable antibody titers and protective immunity as the full-length p67 molecule. However, in this study we observed an apparent superiority of purified GFP:p67ΔSS over GP64:p67C. This superiority might be due, at least in part, to differences in purity and the amount of antigen used [207], or to difference in the constructs themselves (GFP versus GP64 fusions) possibly effecting the folding of the protein. It is also possible that when given with WOE, the antibody titers of GP64:p67C might increase to levels comparable to GFP:p67ΔSS. On the other hand, it is not unlikely that when properly folded and given with an optimal adjuvant, immunological responses might be improved when epitopes distributed over the full-length p67 are present [167].

Levels of protection obtained in previous studies using the full-length p67 protein or fragments of it [134, 142], as well as a p67 peptide vaccine incorporating an 80 amino acid region from the C-terminal end of p67 [17] have averaged about 50%. Our experimental protocol reduced the number of inoculations to two from the three inoculations employed in the previous studies. The amount of antigen required per inoculation was also reduced drastically from 300-600 μg used previously, to 25-50 μg. The reduction in the amount of antigen as well as in the frequency of immunization needed to provide significant protective immunity to ECF is a significant advancement over previous p67 vaccine efficacy trials [17, 134, 142]. Further more, our data suggest a correlation between anti-p67 neutralizing antibody titer and induction of protective immunity to ECF. This has not been observed in previous studies with recombinant p67 [17, 134, 142]. The most likely explanation for this difference may be found in the near-native authenticity/conformation of the p67 protein used in this study in combination with the adjuvants.

The mechanism of the protective immunity induced by the GFP:p67ΔSS or GP64:p67C is not clear but antibody responses appeared to have played a role in reducing the infectivity of sporozoites as evidenced by the lack of schizont parasitosis in the eight non-reacting cattle. Perhaps, by simply binding to the sporozoites and blocking attachment to host receptors (lymphocytes), thereby interfering with establishment in the predilection site on the lymphocytes and hence with their entry [34, 141, 176 - 181, 215]. The fact that only one of the non-reactors showed the presence of parasite DNA on PCR amplification, indicated that the parasites failed to establish/develop in these animals. GFP:p67ΔSS or GP64:p67C specific antibodies most likely contributed to sporozoite neutralization and clearance *in vivo* by enhancement of phagocytosis of sporozoites by opsonization [134]. It is known that bovine IgG1 and IgG2 induce phagocytosis by homologous neutrophils and macrophages [134]. It has been reported that antibody-dependent cell-mediated cytotoxicity (ADCC) plays a role in the clearance of *Trypanosoma theileri* infection in cattle [200] and it is possible that GFP:p67ΔSS or GP64:p67C specific antibodies operate through ADCC as well to clear the sporozoites.

An important feature of a p67-based immunization strategy for ECF is that a limited breakthrough of infection may be desirable. The longevity of the antibody responses generated against these novel recombinant p67 proteins remains to be defined and it is not known whether p67-specific responses will be boosted by field challenge. Its worth noting at this point, that p67-specific antibodies in a sero-conservation in Holstein Friesian by these novel recombinant p67 proteins persisted for more than ninety-six days after first immunization [84]. In the present study animals were challenged thirty-one days after the booster, whereas in all the previous experiments animals have always been challenged 14 days after the final booster [17, 134, 142].

Cellular responses to the schizont stage of the parasite are believed to persist for up to three years or more [202]. Thus a controlled development of schizonts in immunized cattle (as seen with MR or MR/MODR ECF reactions) might be considered more desirable and sustainable than complete protection. Whether this is achievable using these novel baculovirus-derived p67 subunit vaccines under field conditions will await the results of further immunization and challenge experiments in the laboratory and field. In addition, protection against heterologous parasites stocks and species remains to be evaluated. There is ample evidence that ticks in the field can deliver heterologous challenge [125].

In conclusion, we have developed novel recombinant p67-based vaccines, GFP:p67ΔSS and GP64:p67C, against ECF. GFP:p67ΔSS performed slightly the better (though not significantly so) resulting in a reduction of 72% in severe ECF for LD$_{70}$ in challenge dose, compared to 50% achieved in earlier experiments [17, 134,

142]. When combining all three immunized groups the reduction in severe ECF was 67%. More importantly GFP:p67ΔSS induced this significant protective immunity to immunized cattle with much lower (12 - 15 fold lower) amounts of antigen required per dose and with just one booster. Our results also showed that the choice of adjuvant did not significantly influence the performance of the GFP:p67ΔSS vaccine.

Acknowledgements

Chapter 7

General Discussion

Introduction

Historically, vaccines have been one of the most cost-effective and easily administered means of controlling infectious diseases in humans and animals. In veterinary practice, they have prevented economic losses, increased life span of livestock [4, 10] and have considerably improved animal welfare. Vaccine development had its roots in the work of Edward Jenner (1749-1823), who discovered how to protect people from smallpox by inoculation with cowpox [42], and Louis Pasteur (1822-1895), who developed the first rabies vaccine. These pioneering efforts subsequently led to vaccines against diseases that had once claimed millions of lives worldwide [4]. Currently, most human and veterinary vaccines are based on killed or attenuated pathogens [10, 198].

Live vaccines can potentially replicate in the host, but are typically attenuated in their pathogenicity in order not to cause disease. Live vaccines may elicit both humoral and cellular immunity, and only a single or few doses may give lifelong protection [54] One major drawback of attenuated live vaccines is the risk of reversion into their original pathogenic forms or recombinant virulent strains, especially in immuno-compromised individuals or young animals or infants. Moreover, it is possible that some live vaccine strains can be transmitted from the vaccine to a non-vaccinated individual or animal [10, 54].

Ideally, killed vaccines should not be capable of replicating in the host and can therefore not multiply, or revert to pathogenicity, or transmit the disease [54]. The immunogenicity of a killed vaccine usually has to be enhanced using its presentation by an adjuvant system, and multiple doses are normally necessary for obtaining long-term protective immunity. The production of killed vaccines requires large-scale culturing *in vitro* of the disease causing microorganism, which can be associated with both safety risks and may not always be cost-effective [10, 54]. In the preparation of killed vaccines, one major problem is to achieve effective killing without complete destruction of the protective antigens [10, 54]. Thus, killed vaccines can still contain few copies of the live pathogen. In terms of safety killed vaccines are preferred. These problems together with consumer demands for chemical-free food and concerns regarding the environment and animal welfare led to a growing need for improvements of existing animal vaccines in terms of increased efficacy and improved safety. Novel technological possibilities, combined with increased

knowledge in related areas, such as immunology and molecular biology, nowadays allow for new vaccine development strategies. Recombinant DNA techniques are now dominating the strive for ideal vaccines, which should be safe and cheap, heat-stable and easy to administer, preferably as a single-dose, and capable of inducing a broad immune response with life-long memory [104]. Potential challenges in the development of subunit vaccines are that they are often poorly immunogenic and have short *in vivo* half-life [10, 54, 104]. Subunit vaccines are designed to include only the antigens required for protective immunization and to be safer than whole-inactivated or live-attenuated vaccines. However, the purity of the subunit antigens and the absence of the self-adjuvanting, immunomodulatory components associated with attenuated or killed vaccines often result in weaker immunogenicity.

Table 1.   Veterinary parasite vaccines presently commercially available*

Parasite	Trade make	Manufacturer
Avian coccidiosis	Paracox	Schering PloughAnimal Health
Avian coccidiosis	Coccivax	Schering PloughAnimal Health
Avian coccidiosis	Livacox	Biopharm (Czech Republic)
Avian coccidiosis	Immunocox	Vetech
Toxoplasmosis in sheep	Toxovax	Intervet
Giardiosis in dogs	GiardiaVax	Fort Dodge
Anaplasmosis in cattle	Anaplaz	Fort Dodge
Lungworm	Huskvac	Intervet
Lungworm	Dictol	Schering PloughAnimal Health
Boophilus microplus	TickGard	Biotech Australia
Boophilus microplus	Gavac	Heber Biotec S. A., Havana, Cuba

* Taken from Dalton and Mulcahy, (2001)

Thus far, only one subunit vaccine has been licensed for human use, the surface antigen of the hepatitis B virus produced in yeast [203], in contrast to the many such vaccines developed against veterinary viruses and bacteria [12, 27, 29, 72]. Presently, there also exist a number of anti-parasite (endo-/ecto-parasites) subunit vaccines in veterinary practice, e.g. the recombinant Bm86-vaccine directed against the cattle tick vector*, Boophilus microplus* [22, 46, 195, 213] and the recombinant 45w and EG95 oncosphere proteins against *Taenia ovis* and *Echinococcus granulosis*, respectively [103]. An overview of anti-parasite vaccines currently on the market is given in Table I. However, similar vaccines against protozoan parasites such as *Plasmodium*, *Theileria*, *Babesia*, *Cryptosporidia* are still elusive mainly due the complexity of their pathogenesis and life cycles. Extensive research is going on in this area but most vaccines are still in the earlier stages of development [7, 28, 150, 162, 214]. Similarly, until recently many veterinary pathogens and immune responses to these pathogens

have been poorly characterized. As a result, it has been difficult to develop effective vaccines [10].

The research described in this thesis aimed at the development of a subunit vaccine against East Coast fever, a fatal cattle disease caused by the protozoan parasite *Theileria parva*. As described in the experimental chapters, this research led to the development of a recombinant vaccine against ECF based on the sporozoite surface protein p67. Several aspects, which have not yet been experimentally addressed in this thesis, will be discussed. These aspects include the longevity of the protection induced by p67-based recombinant sub-units, the advantages and problems related to the baculovirus-insect cell expression system and the contribution of this research to the further exploitation of the baculovirus expression vector system vis-à-vis other reports. The chapter will also include safety aspects and will conclude with the future perspectives of the p67-based vaccine development both from a technical point of view as well as the impact that it may have on animal husbandry and poverty alleviation of smallholder and/or poor farmers in sub-Saharan Africa.

Prospect of novel p67-based ECF subunit vaccines

The studies conducted in this thesis led to the development of p67-based recombinant vaccine candidates of which GFP:p67ΔSS is the most promising giving 85% protection against ECF when cattle were challenged with *T. parva* sporozoites by needle injection in an efficacy trial (Chapter 6). Important remaining questions regard the likely performances of this vaccine under field conditions. In particular, the longevity of the induced protective immunity will be crucial to its sustainability. Ideally vaccines should be capable of stimulating a potent and long-lasting immunity after a single vaccination. In this respect it is important to understand for how long period the antibodies generated against recombinant p67 remain in the serum and are able to stand guard against *T. parva* sporozoites during re-infection.

This is certainly true for cattle, which reach a relative long lifetime. Nearly all studies that have been conducted before on recombinant p67-based vaccines have been directed at examining the early immune events and were frequently terminated after 28 days [17, 47, 60, 68, 134, 142, 143]. Results from all these earlier studies indicate that under laboratory conditions anti-p67 specific antibody titers and protection appear to decline relatively quickly, within 2-3 weeks after immunization [113].

In many acute viral infections, individuals induce a protective humoral response that is characterized by pre-existing antibodies, which can persist for a long time - in the range of a few months to years [185]. Despite the extensive documentation of prolonged antibody responses following vaccination or acute infection, the mechanisms behind long-term antibody production are not fully understood. The simplest explanation is that antibody levels are boosted by repeated exposure to the

pathogen. Although such sustained antibody production has not been studied for ECF with regard to animals immunized with the new baculovirus-derived recombinant p67-based vaccines developed in this thesis, in our studies anti-p67 antibodies remained at peak titers for at least two months (>96 days after first immunization) both in the Holstein-Friesian cattle used in sero-conversion experiments (Chapter 5) and in the Boran cattle used in the efficacy trial (Chapter 6). In general, the half-life of serum immunoglobulins is less than 2-3 weeks [184]. Therefore the antibody levels could only be maintained by continuous antibody production. Foot-and-mouth disease virus (FMDV) subunit vaccines with various types of formulations in oil have previously been shown to promote good protection in ruminants and pigs with immunity lasting for more than six months [12, 27].

A p67-based immunization strategy against ECF is supposed to be based on neutralizing antibodies limiting the severity of the infection by reducing the numbers of sporozoites capable to invade lymphocytes. This will provide a controlled development of schizonts in immunized cattle, as seen in animals with mild or moderate ECF reactions. Although a sustained antibody production to p67 has not been reported yet for ECF, animals immunized by infection and treatment or recovering from natural infections, have showed that cellular responses to the schizont stage of the parasite persist for over 3½ years after immunization [113]. Thus, if the immunized animals in the field are exposed to ticks carrying the parasites, it can be assumed that these animals will experience additional exposure to sporozoites before the vaccine based immunity wanes completely. In other words, if the field challenge caused by the ticks boost ECF immunity, these animals might be expected to resist further infection, removing or at least minimizing the need for re- or multiple-component vaccinations.

This effect has been demonstrated under laboratory conditions, where animals that underwent mild-moderate clinical reaction generated potent parasite specific CTL responses and were found to be solidly immune to ECF following re-challenge with a lethal dose (LD_{100}) of *T. parva* sporozoites [134, 135]. In the absence of repetitive natural infections that can function as boosts, repeated booster injections with recombinant p67 may be required to maintain an adequate level of neutralizing antibodies; the frequency of which will depend on the longevity of the vaccine elicited antibodies and the life-span of farm animals. In the case that animals are not boosted by field challenge or by re-injection, exposure may eventually result in the establishment of infection, the severity of which will depend on the remaining level of immunity. In the case of sufficient field boosters endemic stability may establish, at least in the short term, and only new calves and replacement stock would require vaccination. In contrast, under conditions of infrequent challenge, at the extreme of endemic instability, it is more likely that those animals that withstand primary

exposure in the field will be susceptible to subsequent infections and such a situation will necessitate a more frequent vaccination regimen [113].

Another explanation for long-term antibody production may be intermittent re-exposure to the antigen, as caused for example by a low-grade persisting pathogen/virus infection, or by persisting antigen itself, in the form of antigen-antibody complexes that are sequestered and retained on the surface of follicular dendritic cells (FDC) [94, 185]. Antigen trapped on the surface of FDC can lead to continuous antibody production by stimulating memory B cells to become antigen-secreting cells (ASC).

In a p67-based subunit vaccination optimal adjuvants should trap p67 antigen for instance in the form of immune-stimulating complexes exposing the antigen intermittently over a longer period [11, 30, 31, 48, 147, 167, 168]. Two different kinds of proprietary adjuvants (Intervet International B.V., The Netherlands) were used in this study; a saponin-based and a water-in-oil emulsion adjuvant. The exact modes of action of these adjuvants are not known but water-in-oil emulsions in general provide good short-term depots and induce good antibody responses while saponin-based adjuvants are known to induce strong Th1 and Th2 and excellent CTL responses [26]. Useful reviews of classification and the mode of action of adjuvants can be found in Cox and Coulter, [26], Vogel, [207] and O'Hagan *et al.*, [149]. The immunological basis for the enhanced ECF protection achieved in this study is not completely clear. It is tempting to speculate that the improved conformation of p67 we obtained contributed to the improved efficacy of the vaccine. Finally, long-term antibody production we observed (Chapter 6) may also be maintained by long-lived plasma cells [185]. It is not known whether plasma cells relevant for protection against ECF have a long life span, but if so, the requirement for long-term antigen retention may be less critical in sustaining immunological memory.

An important topic for further study is whether or not animals immunized with p67 can withstand a tick-delivered challenge. Since sporozoite infection rates in ticks are extremely variable [113, 115, 169], it is essential that p67-induced protection is sufficiently robust to accommodate the most likely challenge under different field conditions. In addition, the success of a p67 subunit vaccine will depend not only on its protective capacity but also on how it performs under different geographic locations and epidemiological states of the disease. Its efficacy may also be affected by changes in management practices arising from a reduced threat of disease, that alter the intensity of parasite challenge. For example, in contrast to intensive management systems, the establishment of endemic stability will be desirable in extensive management systems, where tick challenge is maintained by carrier animals and migrating wildlife. The frequency of exposure to infected ticks will diminish as intensive management systems (e.g. zero grazing) spread within endemic areas. Under

these conditions, maintenance of immunity will depend entirely on vaccination, the frequency of which will be determined by the longevity of vaccine-elicited antibodies and immunity engendered by the subunit vaccines. Resolution of these issues will require careful evaluation of the vaccines under different circumstances, applying molecular diagnostic tools and epidemiological methods [113, 115, 202].

Given the information regarding the immunnological mechanisms deployed by *T. parva*-immune cattle, current research strategies for the development of improved vaccines against the parasite are based on a multi-component formulation targeted at both the sporozoites and schizont stages [114]. Since p67 vaccines produced in earlier studies may not meet expectations as a stand-alone product. Research on a recombinant vaccine for ECF also focuses on stimulating cell-mediated immunity using a schizont-based approach. Some efforts have also been directed to developing an anti-tick vaccine based on the so-called 'concealed' antigens present in the gut of ticks [153, 212]. Typical examples and proof of principle, are the TickGard (Biotech, Australia) and Gavac (Heber Biotec S.A., Cuba) vaccines presently on the market based on the 89 kDa glycoprotein from *Boophilus microplus* [22, 46, 195, 213]. However, from the studies described in this thesis, it is tempting to speculate that, assuming on the performance of the novel recombinant vaccine in the field mimics the one in the laboratory (Chapter 6), this p67-based vaccine could provide effective protection against *T. parva* as a standalone vaccine for ECF.

Exploiting the benefits of the baculovirus expression system to improve the Theileria parva subunit vaccine

The possibility of using a recombinant protein subunit as a vaccine depends on the ability to produce large quantities of this antigen in a form, which is immunogenic and comparable to the native antigen. The baculovirus expression system (BEVS) offers an excellent tool for the high-level production of heterologous immunogens. The system can very well serve as production system for human and animal vaccines [32, 89, 206]. It is also very versatile and can be easily tailored towards specific technological needs. However, the expression of complex secretory or membrane-bound glycoproteins, such as *T. parva* p67, are generally expressed at low-level compared to cytoplasmic or nuclear proteins in the baculovirus-insect cell system. The exact reasons for this are unclear, but a widely discussed possibility is that heterologous signal peptides might be inefficiently recognized by the protein translocation machinery in host cells [76] or by preferential synthesis and clogging of the ER by host secretory proteins such as the viral chitinase [57]. This could be one of reasons for the low expression levels of p67 observed by Nene et al., [142]. Other possible reasons could be rates of transcription and translation as well as post-translational modification [80].

In the presented studies the bacmid technology, which allows the generation and testing of various recombinant baculoviruses within a workable time frame, was combined with application of GFP-fusion of the foreign gene and the baculovirus surface display. In the latter strategy, the N- and C-terminal domains of p67 were fused to the baculovirus envelope protein GP64. Fusion of different regions of the p67gene to GFP led to significantly higher expression levels, probably by increasing the stability of the expressed protein (Chapters 2 and 3). GFP-fusion also allowed for easy monitoring of expression levels, targeting and titration of recombinant viruses. Furthermore, fusion to GFP and GP64 was beneficial a better folding of the protein, as evidenced by the conservation of immunological properties of the recombinant p67 proteins (Chapter 2, 3, 5). Recombinant p67 proteins were also tagged with polyhistidine to facilitate purification of the expressed proteins.

Further improvements of the BEVS to allow a better secretion of recombinant proteins from insect cells may be the use of insect secretion signals such as the honeybee melittin signal sequence [196] which was exploited in Chapter 4. The native signal peptide of full-length p67 without its transmembrane region was replaced with the honeybee melittin signal sequence, resulting in the secretion of recombinant p67 into culture medium. Deletion of the viral chitinase and *v*-cathepsin genes for the viral genome further improved the integrity of the recombinant p67 (Chapter 5). Low levels of expression can sometimes be increased with optimization of time of expression and multiplicity of infection (MOI) and other relative adjustments [70, 220]. Improper folding of proteins and the occurrence as intracellular aggregates may result from high expression, late in the baculovirus infection process and may be solved by harvesting cells at earlier times after infection. Alternatively, early baculovirus promoters instead of very late promoters might be used to drive foreign gene expression [77, 123].

Health risk and environmental concerns

Due to consumer demands for chemical-free food and concerns regarding the environment and human and animal welfare coupled with the present requirements from the regulatory agencies e.g. U.S. Food and Drug Administration (FDA) and the World Health Organization (WHO) for the exact specifications of vaccine preparations and the mechanisms to obtain immunity, it will probably be increasingly difficult to get new classical (live-attenuated or killed) vaccines accepted, in particular, for human use. Subunit vaccines take the advantage of the possibility of using only parts of the infectious microorganism to raise a protective immune response, and since subunit vaccines cannot replicate in the host, there is no risk of pathogenicity provided that the subunit is not a toxin. The composition of a subunit vaccine can be defined more specifically, which is a significant step in terms of safety considerations and minimization of side effects. Recombinant-DNA technology allows controlled production and purification of recombinant protein subunits (to be used as vaccines) in heterologous hosts including insect cells via baculovirus vectors

[9, 10, 32, 40, 45, 54, 104, 106]. As already mentioned above, baculoviruses do not affect human and other vertebrates as reviewed by Kost and Condreay [97] including fish [101]. So far, over 3000 different proteins have been expressed using BEVS including several presenting interest as diagnostic tools (e.g. Puumala virus, Herpes simplex virus, Parvovirus, Hantaan virus, Ebola), therapeutic treatment and human and veterinary vaccines against for instance Hog Cholera virus, Canine Parvovirus, dengue, flu, malaria, and production of anti-Rhesus immunoglobulines [6, 29, 33, 40, 52, 71, 72, 74, 81, 107, 211].

BEVS based-products are allowed [155] and recombinant subunit proteins produced in via BEVS and purified over affinity columns have been tested in humans [59]. However, it is essential to obtain more information on insect cells before crude preparations derived from the baculovirus-insect cell expression system may become acceptable in human vaccines, if ever [56]. Adding to cautious move is the findings of Wu *et al.*, [217], that baculovirus replication in the insect cell nucleus is prone to the generation of defective viral genomes by deletion. Similar findings have been reported by Pijlman *et al.* [160, 161]. The replication behavior of these defective viruses may be unpredictable. These findings are especially important when using recombinant baculoviruses as insecticides or gene therapy vectors [218]. An alternative approach could be to identify the capsid proteins involved in intracellular and nuclear transport and to use this knowledge to develop synthetic gene transfer system that will not only target a transgene to the nucleus but also transport it into the nucleus. However, for the production of veterinary vaccines, the BEVS may be an ideal system as the registration requirements are less stringent than for humans. Various successful veterinary vaccines have been produced and a few are marketed internationally, for example the vaccine against the Hog Cholera virus in swine and the Canine Parvovirus in dogs [29, 72].

Social impact of a successful p67-based vaccine against ECF

The immediate beneficiaries of this research will be laboratories, companies, and other agencies engaged in animal health research program developing vaccines against intracellular pathogens. In addition to developing an effective vaccine against East Coast fever, other economically important parasitic infections of animals for which technologies developed in this study could be applied include anaplasmosis, babesiosis, and cowdriosis as well as related diseases in humans like malaria. The development of these novel vaccines for East Coast fever will contribute to improved health of cattle and as a consequence increased productivity of both local cattle, and enable use of more highly productive exotic and crossbred animals. Increased livestock production contributes to poverty alleviation through sale of livestock products such as meat and milk, which are the only sources of income, many landless poor African smallholders have available to them [121]. In Kenya smallholder farmers produce between 75% and 80% of the total marketed milk [49, 131].

Removing disease constraints to livestock production makes an important contribution to food security because livestock products contain micronutrients such as vitamins B12, which are necessary for health, and in particular for children's growth and development of their cognitive capabilities. Livestock are the only assurance against crop failure and thus make another critical contribution to food security. In addition, livestock provide draft power without which crop production would be severely compromised. Because women account for 80% of smallholder livestock production in sub-Saharan Africa, the impact of improved animal health afforded by the development of the East Coast fever vaccine, will be of particular benefit to them [121]. Furthermore this research has a positive environmental impact resulting from reduced reliance on the use of chemicals (acaricides) for disease (tick) control.

Future perspectives of p67-based research

Further research involving the longevity of the protective immunity induced by p67-based vaccination and performance of the vaccine in the field has been discussed above. In relation to the characterization of the antibodies generated, it has been reported for earlier experiments, that protection in animals immunized with recombinant p67 formulated in adjuvant did not correlate with titer, avidity, isotype, or peptide specificity of the antibody response. It has therefore been suggested that cell-mediated mechanisms play an important role, possibly through the elaboration of cytokines [136]. In the study described in this thesis, a positive correlation was found between the results of sporozoite neutralization assays and the protection. In other words the titers of neutralizing antibodies elicited by these new recombinant p67 antigens correlated positively with the level of protection they conferred to immunized animals. It will be interesting therefore to characterize the antibodies generated by the new baculovirus recombinant p67 antigens. For example to map-out all the epitopes on the new recombinant p67, linear and/or conformational, and determine which of these are neutralizing. Determine the avidity of these antibodies and identify (classify) the various isotypes of immunoglobulins. Knowledge about characteristics and kind of antibodies generated by these antigens will shed more light on mechanism of immunity underlying the protection.

Most if not all studies about p67, have concentrated on vaccine development aspects. Knowledge about the exact function and biochemical properties of p67 will enlightened us about the biology of *T. parva* and may be also of other protozoan parasites of medical importance. Detection of recombinant p67 proteins produced in both prokaryotic and eukaryotic expression systems has always presented a characteristic multiplex banding pattern in SDS-PAGE [17, 82, 83, 134, 142] often difficult to interpret. The p67 domain fused to GFP gives rise to two bands, one of which could be a dimer (Chapter 2) [82]. Secreted p67 migrates in SDS-PAGE as a 130 kDa band as opposed to the expected 85kDa band (Chapter 4). It would be interesting to analyze the post-translational modifications (e.g. glycosylation,

phosporylation) of p67 produced in the baculovirus system in more detail, both in the cytoplasmic and secreted forms in comparison to native p67 present in sporozoites and also study the possible dimerization of this protein. These types of studies should be performed by biochemical analysis and modifications should be analyzed in relation to epitope recognition.

The nature of the binding of sporozoites to lymphocytes is still unclear, but we are now nearer to identifying the host cell molecule(s) recognized by the invading parasite [179 - 181]. The GFP-p67 fusion peptides developed in this study may help in elucidating a possible receptor. The identification of other sporozoite surface molecules will not only be important for vaccine development, but will also help us to understand the invasion process. Preliminary data using FACS analysis shows that GFP-p67 fusion peptides bind to bovine lymphocytes in a measurable way. Proof of binding phenomenon will not only be interesting in terms of localizing the receptor binding site and the invasion domain on p67, but could also led to the identification of the receptor(s) on bovine lymphocytes. The binding of GFP-p67 fusion to lymphocytes could be done using direct fluorescence with non-bovine lymphocytes serving as negative controls. To this aim the existing GFP-fusion domains viz.; GFP:p67N, GFP:p67M and GFP:p67C, could be used to test the binding and ability to block invasion. Using site-directed mutagenesis, new mutants could then be generated to study and to identify motifs or amino acid residue(s) that may be necessary for the binding process. The use of monoclonal antibodies with known epitopes to block the binding could also help the identification of the binding sites on the lymphocytes. Pull-down experiments such immunoprecipitation with GFP-p67 purified fusion proteins or possibly purified BVs of the GP64:p67N or C fusion could also help in the identification of the receptor on the bovine lymphocytes. Similar experiments could be carried out with *T. annulata* SPAG-1 protein [19, 20] to search for conserved domains and possible binding sites.

A model for vaccines against animal and human parasites

These experiments if successful will not only contribute more to recent advances made in the understanding of the biology of *T. parva* but also other *Theileria* species, in particular, *Theileria annulata* and *Babesia* as well as other related parasites of medical importance, *Plasmodium* for example (19, 137]. Although a very effective cell-culture derived vaccine exists for *T. annulata* [162], the search for a subunit vaccine continues [20] and findings in this research may be applicable to *T. annulata* research. The prime candidate for inclusion in a subunit vaccine against *T. annulata*, is the major sporozoite surface antigen (SPAG-1) [19, 20]. The nucleotide sequences of SPAG-1 and p67 have about 54% homology [141] and could even be linked to generate a common vaccine against the two pathogens.

An equally important application might the possible use of this animal parasite model for human health. Tremendous effort and significant investments are currently being made to develop vaccines against major human parasites such as *Plasmodium* and *Schistosomia* [53]. Time and again, researchers are confronted with a basic lack of understanding of the ecological, epidemiological and immunological mechanisms that determine host-parasite relationships. Methodological and ethical constraints often limit the study of complex interactions in the human host using an integrated, holistic approach. Several of the malaria vaccine candidates which progressed to the into phase I/II clinical trials are based on the circumsoporozoite and merozoites [5, 191] surface proteins. Successful in this research and better understanding of the biology of *T. parva* could be beneficial to the quest for a malaria vaccine. In addition, an in-depth knowledge about the biology of *T. parva* might also be of special interest to cancer researchers, because of its ability, unique among protozoan parasites to 'transform' the cattle cells it invade into cancer-like cells that proliferate wildly, as leukemia cells do, killing the animal within three weeks of infection.

Finally, it should be noted that *T. parva* is only one of the problems cattle owners (in the affected regions in Africa, or worldwide) have to deal with, although in many situations its is a factor of major economic importance. Theileriosis cannot be seen in complete isolation from other tick-borne associated diseases like babesiosis for example, and immunization against ECF in the end should be part of an integrated control program for the whole package. Control strategies depend on several factors, including the value of the cattle, their susceptibility to theileriosis and to other tick-borne and tick-associated diseases, the level of infestation by the tick species in the area, and the percentage of pathogen carrying ticks. The type of theileriosis (ECF, January disease, and Corridor disease) and the presence or absence of buffaloes, the natural reservoir of these diseases also determines control strategies [202]. For instance in South Africa wild buffaloes may mix up with domestic cattle. And this is certainly the case with the Massai cattle in MaSai Mara National Park in Kenya, the Massai's are allowed to graze their cattle within the park. Hence, direct contact even occurs allowing very easy transmission of new infectious diseases. In addition, the control strategy in an endemic area is different from the approach to prevent expansion of theileriosis and/or its vector into a normally theileriosis free area.

Concluding Remarks

The new recombinant p67 molecule GFP-p67ΔdSS produced in this study is very similar, if not identical, to the authentic sporozoite p67, is highly immunogenic and is able to induce a high level of protective immunity to cattle against ECF. The work described in this thesis represents a significant advancement over earlier experiments using either NS1-p67 [134], or baculovirus-p67 [142] as well as peptides encoding 80 (p67C) and 250 (p67N) amino acid fragments of p67 [17]. For the first time a simple

protocol in p67-vaccination has been described requiring only a single booster and small amounts of antigen.

The advantages/benefits of the baculovirus-insect cell expression system have been optimally exploited to drastically improve the expression levels and the fidelity of recombinant p67 produced via the system. In addition further development of new transfer vectors tailored for easy and optimal expression, secretion and characterization of heterologous proteins with high fidelity.

Progress towards the development of a p67-based subunit vaccine against *T. parva* has never been closer and more promising, but a few questions need to be answered before the kick-off. For example, efficacy trials under field conditions as well as studies towards the longevity of the protection engendered. This work has also shown that conformational epitopes, optimal adjuvants and formulations are essential to the performance and success of a p67-based subunit vaccine, at least under laboratory conditions. GFP-p67ΔSS has the potential to constitute a standard-alone p67-based subunit vaccine against ECF.

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Summary

Theileria parva is an intracellular protozoan parasite and the causative agent of a lethal cattle disease, called East Coast fever (ECF). This disease poses a major constraint on improvement of cattle production in Eastern, Central and Southern Africa, especially for smallholder farmers. The protozoa are transmitted to cattle in the form of sporozoites by the brown-ear tick, *Riphicephalus appendiculatus*. The sporozoites invade lymphocytes, where they develop into schizonts. In addition, they induce a large-scale uncontrolled proliferation of the lymphocytes, leading to severe clinical symptoms, like weight loss, pyrexia, anaemia, terminal respiratory distress and finally death ensues within two to three weeks, if the animal is not treated. The disease can be cured with antibiotics, and this is the basis for the current method of vaccination, called "Infection and Treatment", where animals are injected with *T. parva* sporozoites and are, simultaneously, treated with antibiotics over a longer period of time. This vaccination method, however, is far from convenient, since the production of large amounts of sporozoites is very time consuming and a cold environment is needed to keep the sporozoites alive. In addition, the use of a live vaccine has pertinent risks, especially when the instructions for antibiotic treatment are not strictly followed and the immunity engendered is strain-specific.

The research described in this thesis was aimed at the possibilities of developing a subunit vaccine against East Coast fever, based on the production of *T. parva* sporozoite surface major protein p67. This protein is present on the outside of sporozoites and plays a crucial role in the entry of sporozoites into lymphocytes and is the major antigen producing neutralising antibodies. The first objective was to produce large amounts of p67 in a near-authentic conformation. Production of recombinant p67 in bacterial expression systems had failed to produce correctly processed protein and large amounts were needed to achieve a reasonable (70 %) level of protection. The baculovirus-insect cell expression system forms a valuable alternative for the expression of large amounts of near-authentic and immunologically active proteins. Previous attempts, however, to produce p67 in insect cells resulted in low levels of recombinant protein, which had a conformation different from the native p67 protein. Again large quantities were needed to protect cattle against ECF.

In the research described in thesis several types of novel baculovirus vectors were constructed to produce different regions of p67 in insect cells. In the first set of vectors, various domains of p67 were expressed as separate entities, but this resulted in low levels of expression. For the second set, domains of p67 were fused to the carboxy-terminus of the "green fluorescent protein" (GFP), a visible marker, leading to a considerable increase in yield of recombinant p67. In addition, GFP:p67 fusion polypeptides were recognised by a monoclonal antibody (TpM12), which was raised against native p67 and capable of neutralising sporozoites. On the contrary, only a small portion of full length, non-fused p67 expressed in insect cells was recognised by

this antibody. Fusion to GFP, thus, appeared to increase the stability of p67 and to result in a more native configuration of the recombinant protein. In a third set of baculovirus vectors, N and C terminal domains of p67 were fused to the baculovirus envelope protein GP64. This resulted in the display of recombinant p67 on the outside of insect cells as well as on the surface of budded baculovirus particles. The TpM12 epitope was also conserved when p67 was fused to GP64.

P67 could also be expressed as a secreted soluble protein. The rationale behind this experiment was to ultimately facilitate the purification of the recombinant protein. This was achieved by removal of a putative transmembrane domain and fusion of p67 to a specific signal peptide derived from honeybee melittin. Deletion of the viral genes, *chitinase* and *v-cathepsine* from the baculovirus genome enhanced the integrity and increased the stability of this secreted p67 protein. Unfortunately, the secreted form was no longer recognised by TpM12, and hence, had a conformation different from p67 in sporozoites. Therefore, the secreted p67 was not tested in further immunological studies.

In order to select the best recombinant p67 products for extensive vaccine trials, the various fusion proteins combining domains of p67 with GFP or GP64 were tested in mice for their immunogenicity and, especially, the ability to induce neutralising antibodies. In mice, the p67 molecule, lacking both its signal peptide and transmembrane region, and fused to GFP (GFP:p67ΔSS) gave the best humoral immune response, followed by the p67 C-terminal domain coupled to GP64 (GP64:p67C). These two immunogens were tested in cattle, in combination with a water-in-oil or a saponin-based adjuvant. Also in cattle, a high level of sero-conversion was obtained using a total of 100 μg recombinant p67 for immunisation divided over two needle injections. Moreover, the antisera raised in mice and cattle neutralised the infectivity of *T. parva* sporozoites in an *in-vitro* assay. Subsequently, in Kenya Boran cattle were vaccinated with GFP:p67ΔSS or with GP64-p67C. After a primary immunisation followed by a single booster, *T. parva* stabilated sporozoites were injected to test whether the vaccines protected the animals from ECF. Eighty five percent of the animals was protected from the lethal disease (ECF) using a much lower dose of recombinant protein than was used in the earlier studies.

The research described in this thesis exploited the versatility of the baculovirus-insect cell expression system and showed that an ECF subunit vaccine based on recombinant p67, in a better conformation and formulated in an optimal adjuvant, can be used effectively in a vaccination program. Both of the proteins tested are good candidates for the development of a commercial ECF subunit vaccine and may contribute substantially to improvement in cattle productivity and poverty alleviation in sub-Saharan Africa.

Samenvatting

Theileria parva is een ééncellige, intracellulaire parasiet en de veroorzaker van een lethale aandoening in koeien. Deze ziekte wordt in het Engels aangeduid als East Coast fever (ECF) en vormt een belangrijke beperkende factor voor de veeteelt in Centraal en Zuidelijk Afrika, vooral onder arme boeren. De protozoa worden overgedragen in de vorm van z.g. sporozoïten door de teek *Riphicephalus appendiculatus*. Deze sporozoïten dringen witte bloed cellen (lymfocyten) binnen, waar ze zich ontwikkelen tot schizonten. Tevens zetten ze de lymfocyten aan om zich ongebreideld te delen, hetgeen leidt tot ernstige klinische symptomen, zoals vermagering, koorts, bloedarmoede, en uiteindelijk sterfte. De ziekte kan bestreden worden met antibiotica, en daarop is het huidige vaccinatiebeleid gestoeld, door tegelijk met een dosis *T. parva* een langdurige antibioticumkuur te geven. Deze methode van vaccineren is echter niet practisch omdat het leveren van grote hoeveelheden sporozoïten erg tijdrovend is en ze bovendien in een koude omgeving moeten worden bewaard om in leven te blijven. Bovendien neemt het gebruik van een levend vaccin risico's met zich mee, zeker wanneer het antibioticum-regime niet strikt nageleefd wordt.

Het in dit proefschift beschreven onderzoek was gericht op de mogelijkheden van de ontwikkeling van een zogenaamd "subunit" vaccin, gebaseerd op het transmembraan eiwit p67 van *T. parva* en de expressie in insektencellen. Dit eiwit bevindt zich aan de buitenkant van de sporozoïten en speelt een cruciale rol bij het binnendringen van de lymfocyten en het oproepen van neutraliserende antilichamen. De eerste hindernis die genomen moest worden was het in grote hoeveelheden tot expressie brengen van p67 in een meer authentieke vorm. Productie van recombinant p67 in bacteriele cellen had namelijk niet geleid tot een correct gevouwen eiwit en bovendien was er erg veel van nodig om een redelijke mate van bescherming te krijgen. Het baculovirus insectencelsysteem vormt een alternatief expressiesyseem, dat in het bijzonder geschikt is voor de expressie van grote hoeveelheden eiwit in de juiste immunologische conformatie. Eerdere pogingen om het volledige p67 eiwit in insectencellen te produceren leverde echter geen hoge expressieniveaus en de conformatie van het recombinante p67 eiwit was anders dan van het natieve eiwit. Ook hiervan waren grote hoeveelheden nodig om koeien te beschermen tegen ECF.

In dit promotieonderzoek werden verschillende nieuwe typen baculovirusvectoren geconstrueerd om delen van het p67 eiwit in insectencellen te maken. In de eerste set werden verschillende domeinen van p67 tot expressie gebracht, maar dit resulteerde in lage expressieniveaus. Voor de tweede set werden delen van p67 gefuseerd aan het carboxy-einde van het "green flourescent protein" (GFP), een zichtbare marker, en dit gaf een aanzienlijk hogere opbrengst aan p67 eiwit. De GFP:p67 fusie-eiwitten werden bovendien herkend door een monoclonaal antilichaam (TpM12), dat opgewekt is tegen natief P67 en in staat is sporozoïten te neutraliseren. De fusie met

GFP leek dus niet alleen de stabiliteit van het eiwit te vergroten maar ook te leiden tot een natuurlijkere conformatie. In een derde set baculovirusvectoren werd een N- of een C-terminaal domein van p67 gefuseerd aan het baculovirus envelopeiwit GP64 om een nog betere conformatie te bewerkstelligen via intracellulaire processing. Dit resulteerde in expressie van p67 op het oppervlak van zowel geïnfecteerde cellen als van virusdeeltjes. Ook bij fusie aan GP64 bleef de TpM12 epitoop intact.

In de laatste serie recombinanten werd p67 tot expressie gebracht in een vorm, die secretie van dit eiwit in het celkweekmedium mogelijk maakte. Het idee hierachter was dat op deze manier het eiwit eenvoudiger te zuiveren zou zijn. Verwijderen van het transmembraandomein van p67 en fusie aan een specifiek signaalpeptide ontleend aan honingbij melittine leverde inderdaad een secretoire vorm van p67. Deletie van twee virale genen, *chitinase* en *v-cathepsine,* uit het baculovirusgenoom voorkwam de afbraak en verhoogde de stabiliteit van het uitgescheiden p67 eiwit. Helaas bleek de secretoire vorm niet herkend te worden door TpM12, en dus een afwijkende conformatie te hebben vergeleken met het sporozoït-eiwit, en daarom werd dit product niet verder getest.

Om uit de verschillende fusie-producten van p67 met GFP en GP64 de beste kandidaten te kiezen voor uitgebreide vaccinatiestudies werden alle fusie-constructen eerst in muizen getest op immunogeniciteit, en in het bijzonder op hun vermogen neutraliserende antilichamen op te wekken. In muizen bleek het p67 molecuul zonder signaalpeptide en ontdaan van het transmembraandomein als fusie met GFP (GFP:p67ΔSS) de beste humorale immuunrespons te geven, gevolgd door de C-terminus van p67 gefuseerd aan GP64 (GP64:p67C). Vervolgens werden deze laatste twee immunogenen ook in koeien getest, in combinatie met een water-in-olie of een saponine adjuvant. Ook in koeien werd een hoge mate van sero-conversie gevonden bij immunisatie met 100 µg recombinant P67 eiwit verdeeld over twee toedieningen in de vorm van injecties. De antilichamen opgewekt in muizen en koeien bleken bovendien de infectiositeit van *T. parva* sporozoïten in een *in-vitro* test te neutraliseren.

Vervolgens werd in Kenia een vaccinatie-experiment uitgevoerd, waarbij Boran koeien gevaccineerd werden met GFP:p67ΔSS of GP64-p67C. Na een primaire immunisatie gevolgd door een booster, werden sporozoïten geïnjecteerd om te zien of de immunisatie de dieren zou beschermen tegen ECF. Vijfentachtig procent van de dieren werd beschermd en de hoeveelheid p67 antigen die hiervoor nodig was veel geringen dan bij in het verleden geteste recombinant P67 preparaten (geproduceerd in *E. coli of* in insectencellen).

Dit onderzoek heeft gebruik gemaakt van de uitgebreide mogelijkheden van het baculovirusexpressiesysteem en aangetoond dat een ECF subunit vaccin gebaseerd op

recombinant p67 met een natuurlijke conformatie en bij formulering in een geschikt adjuvant, doeltreffend gebruikt kan worden in een vaccinatieprogramma. Beide geteste eiwitten zijn goede kandidaten voor de ontwikkeling van een commercieel ECF subunit vaccin en kunnen een belangrijke bijdrage leveren aan de armoede bestrijding in Afrika.

CurriculumVitae:

Stephen A. Kaba was born one of five brothers and two sisters to Mr. and Mrs. Kaba in Navrongo, Ghana, on Monday, November 10th, in the year of our Lord, one thousand nine hundred and sixty-five. He graduated from the Tamale Secondary School (TASMASCO) with the West African Examination Council Certificates of (both Ordinary and Advanced levels), in 1985 and 1987 respectively. He worked as a classroom teacher under the National Service Scheme for three years (1987 - 1990) before enrolling for his first-degree program in Agricultural Sciences in the Kwame Nkrumah University of Science and Technology Kumasi, Ghana. He obtained his Bachelor of Science degree [B.Sc. (Hon.) Agric.] in March 1995. He specialized in Animal Science and Husbandry.

He then joined the Ghana Education Service as a General Science and Mathematics tutor at St Jerome Senior Secondary School, Abofour in the Offinso District, Ghana. From August 1996 to January 1998 he studied for his Master of Science degree in Biotechnology (MSc. Biotechnology) in the Wageningen University under the sponsorship of the Netherlands Fellowship program (NFP). Mr. Kaba undertook his MSc. thesis research work in the Laboratory of Virology in the Molecular Virology/Biotechnology of insect viruses (Baculovirus) group where he first worked on the *Theileria parva* sporozoite surface protein, p67. He also worked on plant molecular biology including Agrobacterium based -transformation of Arabidopsis in the Laboratory of Molecular Biology, Wageningen University.

In April 1999 he was awarded a 4-year research grant by the to undertake a PhD research by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO) in field molecular Parasitology and vaccine development. This project was in the framework of collaboration between the Laboratory of Virology, Wageningen University, the Netherlands and the International Livestock Research Institute (ILRI), Nairobi, Kenya.

List of publications

Kaba, S.A., J.C. Hemmes, V. Nene, A.J. Musoke, , J.M. Vlak and M.M. van Oers (2003). Baculovirus surface display of *Theileria parva* p67 antigen preserves the conformation of sporozoite-neutralizing epitopes. Protein Engineering16, 73-78.

Kaba, S.A., A.M. Salcedo, P.O. Wafula, A.J. Musoke, J.M. Vlak and M.M. van Oers (2003). Improved secretion of *Theileria parva* sporozoite surface protein p67 using a *chitinase* and *cathepsin* negative baculovirus vector. In preparation.

Kaba, S.A., Schaap, D., Roode, E.C., Nene, V., Musoke, A.J., Vlak, J.M., and Van Oers, M.M. (2003). Enhanced immunogenicity of novel baculovirus-derived *Theileria parva* p67 subunit antigens. Veterinary Parasitology, submitted.

Kaba, S.A., A.J. Musoke, D.C. Schaap, A.N. Vermeulen, J. Rowlands, V. Nene, J.M. Vlak, and M.M. van Oers (2003). Improved protection against East Coast fever with novel baculovirus-derived p67 subunit vaccines. In preparation.

Kaba, S.A., V. Nene, A.J. Musoke, J.M. Vlak, and M.M. van Oers (2002). Fusion to green fluorescent protein improves expression levels of *Theileria parva* sporozoite surface antigen p67 in insect cells. Parasitology 125, 497-505**.**

Kaba, S.A., A.J. Musoke, V. Nene, J.M. Vlak and M.M. van Oers (2002). Enhanced expression of the *Theileria parva* sporozoite surface antigen p67 in the baculovirus-insect cell system as a fusion to GFP. 2002. Abstract book of the XIIth International Congress of Virology, Paris, France, p. 315.

Kaba, S.A., V. Nene, M.M. van Oers and J.M. Vlak (2001). Fusion to green fluorescent protein improves expression of the *Theileria parva* sporozoite surface antigen p67 in insect cells. Abstract Book of the 34th Annual Meeting of the Society for Invertebrate Pathology, Noordwijkerhout, The Netherlands, p. 41

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