Invasion of erythrocytes by *Babesia bovis*

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Invasie van erythrocyten door *Babesia bovis*
(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

GENERAL INTRODUCTION

"Reciteer in de naam van uw Heer, die geschapen heeft (Qur'an 96:1)"

INTRODUCTION

Members of the genus *Babesia* cause one of the most common parasitic infections worldwide in wild and domestic animals [98, 140]. Economically, bovine babesiosis, caused by *B. bovis*, *B. bigemina*, *B. major* and *B. divergens* forms a serious problem as it is widely distributed and threatens the health and safety of about 500 million cattle in tropical and subtropical regions of the world [98]. Although no *Babesia* species primarily infect humans, some of the species, like *B. divergens*, can be transmitted to humans [93]. *B. bovis* infection results in high mortality rates among susceptible cattle, and causes a virulent disease characterized by fever, anemia, anorexia and hypotensive shock syndrome. Parasitized erythrocytes are often sequestered in the capillary beds of the brain and lung, resulting in low peripheral parasitemia, but causing severe pathology like cerebral babesiosis and respiratory distress, which eventually can lead to death. The pathogenicity varies both between and within species but in many cases has a high rate of mortality in untreated animals.

THE APICOMPLEXA

Members of the phylum Apicomplexa cause parasitic diseases from which the most important disease, malaria, invokes major health problems in human populations in tropical and subtropical countries. Apicomplexan parasites are intracellular protozoan organisms that can invade a specific or even a wide range of different host cells, depending on the species. The characteristic common feature of all members of this phylum is the unique set of organelles localised at the anterior end of their invasive forms that have showed to play a crucial role in the invasion of the host cell [176]. These apical organelles are micronemes, rhoptries and dense granules. Besides these organelles they also contain polar rings and some species contain a conoid which lies within the polar rings. Apicomplexans are transmitted to new hosts by different vectors. Malaria parasites for instance, are transmitted by infected mosquitoes while *Babesia* species are transmitted by infected ticks.

THE GENUS *BABESIA*

Babesia organisms are protozoan parasites that are all transmitted by ticks to their specific vertebrate host. Completion of a life cycle and therefore the maintenance of *Babesia spp.* is completely dependent on both the tick and the vertebrate host [140]. The taxonomic classification of *Babesia spp.* places them in the phylum Apicomplexa and the order Piroplasmida. Piroplasms often have a pear-shaped appearance inside their host cell. The order of Piroplasmida is divided in Babesiidae and Theileriidae [98, 115]. Babesiosis and Theileriosis are both diagnosed largely on clinical signs and symptoms with confirmation by microscopic observation of the parasites with their specific host blood cells and often DNA-based tests, like reverse line blot [82]. True *Babesia* species exclusively invade erythrocytes, whereas *Theileria* species undergo a pre-erythrocytic cycle in lymphocytes. Examination of stained blood films shows *Babesia* organisms within red cells, where single and duplicated parasites are found. *Babesia* species are grouped into the small and large *Babesia* [98]. The invasive parasitic stage of *Babesia* species inside erythrocytes will grow and differentiate to trophozoites. The diameter of trophozoites divides *Babesia* species into small and large *Babesia*. Trophozoites of the small *Babesia* are 1.0 to 2.5 µm and include *B. bovis*, *B. gibsoni*, *B. ovis* and *B. divergens*. Trophozoites of large *Babesia* are 2.5 to 5.0 µm including *B. caballi* and *B. canis*. All apicomplexans have a complex life cycle with both asexual and sexual reproduction. Pathogenesis of *Babesia* species results from the asexual erythrocytic stage, where the parasite undergoes multiple rounds of invasion and replication in the host erythrocytes. The distribution of all the different *Babesia* species is governed by the geographical distribution of the tick vectors that transmit them.

LIFE CYCLE

The life cycle of *B. bovis* takes place in two hosts (Fig. 1). Asexual development takes place in the bovine host and sexual development in the tick vector [140].

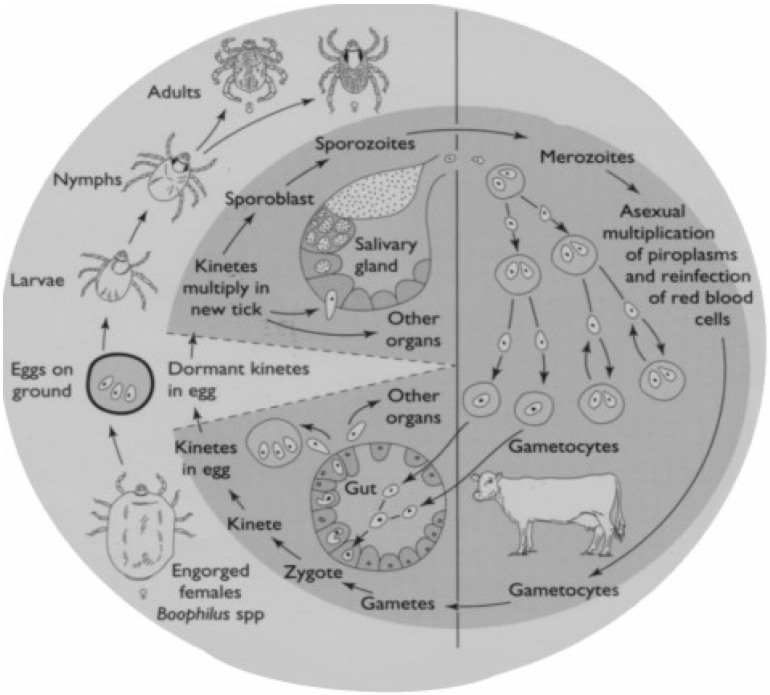

Fig. 1. Life cycle of *Babesia spp.* in the tick vector and vertebrate host [(140)]

Asexual stage

Babesia spp. infected ticks have accumulated mature sporozoites in their salivary glands. When an infected tick takes a blood meal on its host it will pass the content of its salivary gland, including the parasites, into the host. The sporozoite invades a red blood cell and will multiply itself by a single round of binary fission. In contrast to the malaria and *Theileria* life cycle, where sporozoites invade hepatocytes and white blood cells respectively, *B. bovis* sporozoites enter the red blood cell. Inside the red blood cell sporozoites differentiate to trophozoites. *Babesia* multiplies inside the red cell by a single round of binary fission after which the red cell ruptures and daughter parasites, called merozoites, are released into the bloodstream and directly invade a new red blood cell. This cycle is repeated over and over again and is called the merogony stage. During the merogony stage the parasite feeds on haemoglobin and takes up serum nutrients to be able to synthesize amino acids. Nutrients and hemoglobin are taken up by the parasite through pinocytosis at the area around the micropore. Once the parasite is inside the erythrocyte it will start replicating and at the same time will also modify the host cell making it a more suitable place for them. Structural alterations of infected host erythrocytes have been shown to take place. These changes are induced by the parasite and lead to formation of knob-structures [(2)]. Antigenic variation takes place of the proteins (VESA, variant erythrocyte surface antigen) localized on the

erythrocyte membranes [5]. Besides formation of merozoites, a few percent of the merozoites invading new erythrocytes differentiate into gametocytes. In apicomplexans like *Toxoplasma gondii* and *Plasmodium spp.* male (microgametocytes) and female (macrogametocytes) gametocytes have been found. In *Babesia spp.* no clear description has been given of gametocytes in the vertebrate host. In ticks that have ingested infected blood several types of parasite with long rays and spikes have been described that were originally called strahlenkörper. One type does not appear to develop further whereas others undergo further multiplication, even after they have emerged from the erythrocyte. These forms may represent micro- and macrogametes although firm evidence is lacking [140].

Sexual stage

Sexual development takes place in the gut of the tick, after a feeding tick has ingested gametocytes. Gametocytes will develop into gametes when taken up by the tick. Two morphologically different strahlenkörper will fertilize and form a zygote. The zygote is the only parasite stage that is diploid and directly after the first meiotic division the parasite is haploid again. The zygote differentiates into a kinete and can now pass the intestinal wall. Once outside the gut the kinete may enter the ovarium of an engorged female tick and infect the eggs. The eggs with the parasite inside will develop from larvae to nymph into adult. In these stages of the tick the kinetes will again infect the organs, including the salivary gland. Once a parasite has infected the salivary gland, a multinucleate but undifferentiated sporoblast is formed. If the tick will feed, sporozoites will mature and finally will bud off of the sporoblast (sporogony stage) [140].

SUSCEPTIBILTY TO INFECTION

Babesia spp. can infect various types of vertebrates, however *B. bovis* only infects cattle. In previous studies it was seen that *Bos taurus* species were more susceptible to *B. bovis* than *Bos indicus* species [19]. In enzootic areas the animals clinically affected are mostly susceptible cattle introduced for breeding purposes, for slaughter, or in transit. Severe clinical cases which occur in these cattle are caused by exposure to stresses such as parturition and starvation [98]. Animals that are aged, splenectomized or immunocompromised are more susceptible to Babesiosis than young and healthy animals. Protective immunity occurs after natural infection with most *Babesia spp.* If the infection recurs

repeatedly the immunity is permanent. If it is treated directly after infection and the protozoa are killed before antibodies are produced, no immunity occurs. If the infection is not treated, the protozoa survive in the host for a variable time, usually about six months, and then disappear below detectable levels. Animals under one year of age are infected predominantly with *B. bigemina* and those over two years of age by *B. bovis* [98].

MEDICATION AND VACCINATION

There is an efficient drug developed against *B. bovis* (Imidocarb diproprionate), however it is expensive and not always easily applicable in large herds roaming freely in areas of considerable size, especially in third world countries [139]. Moreover it has a 28-day withholding period for meat consumption and restrictions for lactating cattle. There is also a great risk of development of resistance against the drug when used extensively. Besides medication, life attenuated vaccines are used. Attenuated *B. bovis* strains used in vaccines are produced by multiple syringe passage of field isolates in splenectomized calves. Results presented sofar have suggested that a vaccine containing a single attenuated strain is not protective against heterologous challenge. A vaccine needs at least to contain two or three different isolates to be effective [23]. While attenuated live vaccines lead to significant decreases in mortality, they do not prevent infection or full protection against milder symptoms of the disease [54]. Live vaccines have been used routinely or experimentally in several countries [29, 54]. The observed decreasing efficiency of this method in some regions, the complicated production methods of live vaccines, the major risks involved by using blood-derived live vaccines like transmission of contaminating pathogens and the reported heterogeneity between and within natural isolates urges the search for efficient recombinant vaccines [24]. Development of a recombinant vaccine has focused on *Babesia* antigens that are recognized by antibodies or stimulate T-cells in naturally infected animals [24]. Intracellular parasites cause changes in the host cell by releasing and incorporating their own proteins into the host cell environment during the invasion and post-invasion steps, leading to conditions which favour survival of the parasite. Some of the proteins have already been shown to interact with the immune system and may be involved in eliciting a protective response. One of the candidate proteins that have been identified in *Babesia* species is rhoptry protein RAP-1 (rhoptry associated protein)[49, 168, 181, 203]. RAP-1, MSA-1 (merozoite surface protein) and MSA-2 have been found in *B.*

bovis and antibodies directed against these proteins have shown to inhibit invasion of merozoites into host cells [151, 152, 161, 211]. However, a recent study showed that a vaccination trial with RAP-1 of *B. bovis* did not result in protection [161].

STRUCTURE OF THE MEROZOITE

Babesia merozoites have a typical apicomplexan architecture (Fig. 2) and are morphologically similar in particular to *Plasmodium* merozoites which have been studied in much more detail. Mature merozoites are released free into the bloodstream by erythrocyte rupture after which they attach to and enter new erythrocytes within minutes. *B. bovis* merozoites are approximately 2 µm in length and 1 to 1.5 µm in width. Apicomplexan species have the characteristic of a normal eukaryotic cell (endoplasmatic reticulum, mitochondria, golgi apparatus, nucleus) and apicomplexa-specific organelles such as a conoid, polar rings, pellicles and the apical organelles [150]. In addition, apicomplexa possess an inner membrane complex (IMC) consisting of flattened membrane vesicles located just beneath the plasma membrane [150]. In the Apicomplexa, the trimembraneous structure (two membranes from the IMC and one from the plasma membrane) is called the pellicle. It runs from the anterior conoidal rings to the posterior end of the cell [150]. At the micropore this complex is interrupted. The micropore functions as a canal through which nutrients are taken up by pinocytosis. Beneath the IMC membranes microtubules are localized. In haploid forms of apicomplexan parasites there are two types of microtubules described: subpellicular microtubules and spindle microtubules. The subpellicular microtubules originate from the polar ring and end just below the nucleus, covering approximately two-third of the parasite length. The microtubules also help to form the shape of the parasite. Besides the cytoskeletal structures, the parasite also contains unique apical organelles. At the anterior end of the parasite rhoptries and micronemes are localized together with the conoid. The conoid is a small round-shaped structure composed of a spiral of unidentified filaments [150]. It is thought to play a mechanical role in invasion of host cells and is only found in some apicomplexans [150]. The conoid is approximately 250 nm in diameter and can be extended beyond the apical polar ring. Rhoptries are pear-shaped membrane-bound vesicles. Electron microscopy has shown that in their mature state rhoptries consist of two distinct parts: an electron-dense basal bulb and a

less dense rhoptry duct that ends just beneath the plasma membrane. The rhoptry is surrounded by a bilayer membrane.

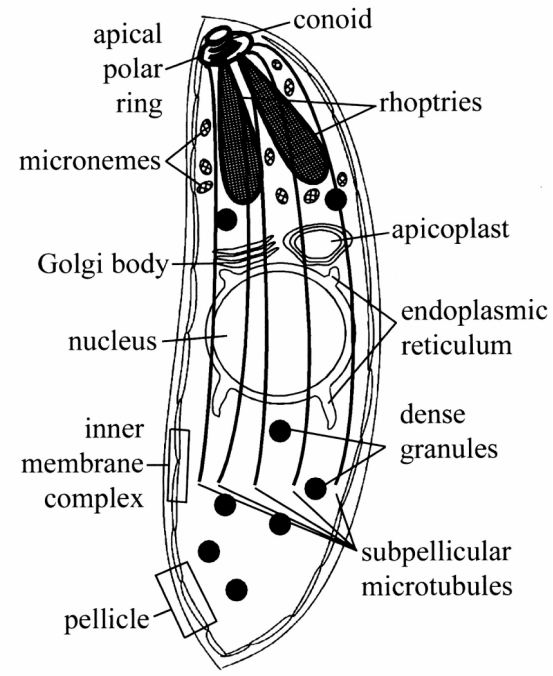

Fig. 2. A schematic picture of a general apicomplexan with most of the organelles [150].

In *P. falciparum* they are about 550 nm long and 250 nm in diameter [18]. Micronemes are much smaller than rhoptries and are about 120 nm long . Dense granules are often about 80 nm in diameter and have a densely granular content. They are localized between rhoptries and nucleus. Dense granules are known as spherical bodies in *B. bovis* of which usually only two are observed at a more posterior location. Rhoptries and micronemes release their protein content before completion of merozoite invasion, whereas dense granules release their proteins after invasion is completed. Microneme proteins are apparently used for host-cell recognition, binding, and possibly motility, rhoptry proteins for parasitophorous vacuole formation and dense granule proteins for remodelling the vacuole into a metabolically active compartment.

Merozoite membranes contain several proteins which are modified and largely cleaved off during erythrocyte invasion. The composition of the coat has not been clearly identified but in *P. falciparum* at least eight merozoite surface proteins (MSP-1 to MSP-8) have been described of which MSP-1 is the most abundant and best characterized [97, 109, 132, 147, 204]. MSP-1 undergoes extensive specific proteolytic cleavage after merozoite release, with the result that the molecule is cleaved into four fragments. It is inserted into the plasma membrane with a glycosylphosphatidylinositol (GPI) anchor [97].

HOST CELL INVASION

The first step in invasion is the apparent random interaction between the merozoite and the host cell (Fig. 3). Initial attachment is of low binding affinity and reversible, followed by reorientation on the erythrocyte surface bringing its apical prominence into contact with the host cell. The adhesion between parasite and the host cell is strong and irreversible. The apical organelles that are also localised at the anterior end of the parasite expel their contents. The invasion process is incompletely characterised in *Babesia*, but it is clear from studies performed in the closely related parasite *T. gondii* that microneme secretion takes place first and is probably involved in junction formation. The "moving junction" is a zone of attachment where host cell invagination will take place. It is characterized by a thickened host cell membrane with increased electron density. The parasite enters the host cell by pulling the moving junction down its body. Proteins involved during the junction movement are not known sofar in *Plasmodium spp.* Micronemes have been shown to contain specific ligands for host cell receptors. The first *P. falciparum* ligand identified in micronemes that binds to erythrocytes with high affinity was the erythrocyte-binding antigen 175 [30]. EBA-175 binds to glycophorin A on the surface of erythrocytes, and this interaction depends on sialic acid residues of the receptor [199]. Another transmembrane protein of this family, EBA-140, binds to glycophorin C and is also dependent on the presence of sialic acids [129]. Studies using a variety of mutant human erythrocytes and enzymatic treatments have shown that different isolates of *P. falciparum* are not totally dependent on sialic acid or glycophorin A for invasion [30, 61, 171]. Treatment of neuraminidase inhibits EBA-175 binding by removal of sialic acid [30]. Still some *P. falciparum* strains were able to invade a host cell after treatment with neuraminidase [61]. At least two pathways for *P. falciparum* merozoite entry into erythrocytes became evident from these investigations. Besides the sialic acid dependent pathways also a sialic-acid independent pathway is used by this apicomplexan species in order to invade their host cells.

Microneme secretion is followed by rhoptry release and at the same moment the parasite starts to invade the red blood cell. The erythrocyte membrane invaginates and the parasite begins to enter an enlarging parasitophorous vacuole. Once the parasite is inside the vacuole the erythrocyte and vacuole membrane will reseal [11, 207]. In contrast to rhoptry and microneme discharge which release their contents only at the apical prominence, dense granule release involves movement of the bodies to the

anterior lateral surface of the cell where they seem to fuse with the pellicle [9, 220]. In addition, most of dense granule release occurs after completion of invasion [220].

MICRONEME PROTEINS: TARGETING AND REGULATED SECRETION

Apart from a few characterized ligands binding to specific receptors on the erythrocytes in the case of some *Plasmodium* species, no such interactions have been identified for other apicomplexan species. Nevertheless, a range of micronemal proteins has been identified from species like *P. falciparum* and *T. gondii.* Many of these micronemal proteins contain domains that are conserved within species and between different genera, which are likely to serve similar functions in the invasion process. Approximately half of the known micronemal proteins are predicted to be type I membrane proteins with a single membrane spanning sequence situated near the C- terminus. Furthermore many microneme (MIC) proteins of *T. gondii* (MICs1-4 and 6-9) contain adhesive domains found in vertebrate proteins such as integrins, thrombospondin, kallikrein, and epidermal growth factor (EGF) [219]. For example, MIC2 possesses a single von Willebrand Factor type A domain (vWFA, also known as an I-domain of integrin) and five thrombospondin type I-like repeats [230]. vWFA-domains typically bind a subset of extracellular matrix proteins such as collagens, laminins, or heparin sulfate proteoglycans (HSPGs) [223]. MIC proteins often function in adhesive complexes. For example, TgMIC1 is complexed with TgMIC4 and TgMIC6. TgMIC1 and TgMIC4 are both essential for binding host cells and are responsible for the adhesive properties of the complex [32]. Although the host receptor is still unknown, TgMIC1 was recently demonstrated to be a lactose-specific lectin that presumably binds lactose-containing glycoprotein receptors on the surface of target cells [32]. TgMIC6 is the only protein in the complex that contains a transmembrane sequence and probably anchors the adhesive complex in the membrane. TgMIC6 is also required for correct targeting of the adhesive complex to the micronemes since genetic disruption of TgMIC6 caused mis-targeting of both TgMIC1 and TgMIC4 to the dense granules, which is the default secretory pathway in *T. gondii* and probably in other apicomplexan parasites too (reviewed in [32]). This result implicated that TgMIC6 probably contains microneme sorting signals. Disruption of the TgMIC2 cytoplasmic domain also mistargeted the M2AP protein that attaches to TgMIC2. Sequence data from

the cytoplasmic domain of TgMIC2 revealed two conserved motifs that were important for targeting of the proteins to micronemes [59].

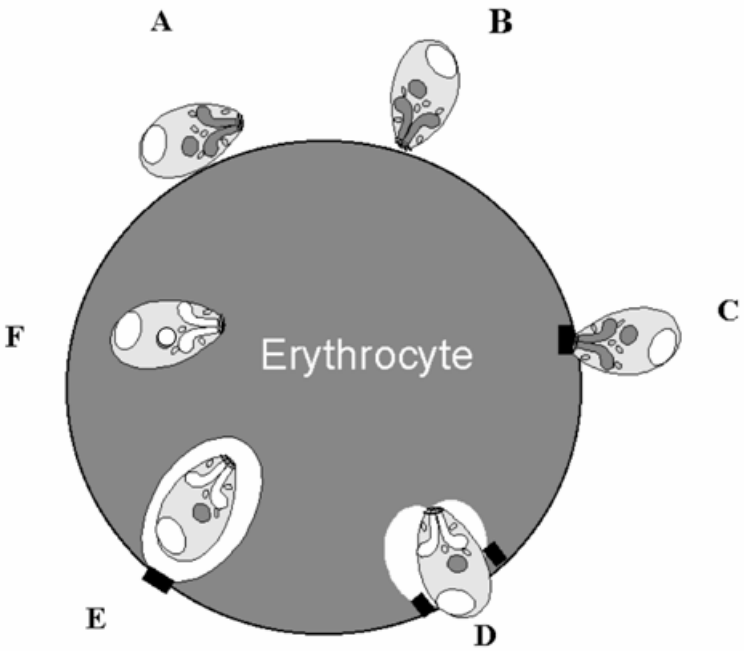

Fig. 3. Hypothetical model for the invasion by *B. bovis* as based upon the malaria model. (A) attachment; (B) apical reorientation; (C) emptying of micronemes and formation of moving junctions; (D) invagination of the membrane and emptying of the rhoptries; (E and F) sealing of erythrocyte membrane, formation of the parasitophorous vacuole and discharge of dense granules. The maintenance of a parasitophorous vacuole during the intra-erythrocytic life of *B. bovis* has not been well established.

The motifs contained tyrosine residues and a stretch of acidic residues that were also found in the cytoplasmic domain of TgMIC6. Furthermore, replacing the GPI anchor of surface antigen 1 (SAG1) of *T. gondii* by the transmembrane and cytoplasmic domain of TgMIC6 showed translocation of SAG1 to micronemes instead of dense granules. [20, 32].

The secretion of proteins from the three different apical organelles is a tightly regulated process. The precise mechanism of the secretion process is not known so far. Previous studies demonstrated that increases in intracellular calcium $[Ca^{2+}]i$ mediate microneme secretion in *T. gondii* [20, 128] and chelation of intracellular calcium blocks microneme secretion and invasion. Furthermore, reagents that raise *T. gondii* $[Ca^{2+}]i$ levels stimulate microneme discharge in the

absence of the host cells [31]. Also in *P. falciparum* invasion of merozoites was inhibited when Ca^{2+} was chelated [233]. Ca^{2+} is an essential regulator of microneme secretion since the membrane permeant Ca^{2+} -chelator, BAPTA-AM, completely blocked exocytosis of micronemes without affecting dense granules secretion [32]. BAPTA-AM also abrogated parasite attachment to host cells, supporting the presumption that micronemes are essential organelles in this process.

Microneme secretion is a regulated process and not just a release of their contents following binding of extracellular parasites to erythrocyte membrane receptors [89]. Apical membrane antigen 1 (AMA-1) and EBA-175 have both been found to be localized in micronemes, however the secretion of these proteins is differentially regulated. Expression of both proteins takes place at the same time during the schizont development. AMA1 is constitutively detectable on the merozoite surface [95, 158], whereas EBA-175 has never been shown to have a merozoite surface localization prior to invasion. Previous reports have shown that AMA-1 was secreted from the micronemes independently of EBA-175. The results indicated that there is a level of selectivity involved in microneme protein secretion, perhaps depending on specific signaling via the cytoplasmic-tail region. The cytoplasmic tail of PfAMA-1 was shown to contain the microneme targeting consensus motif YXX [63, 99], although it has not been proven whether this motif is important for correct subcellular localization. The cytoplasmic tail of EBA-175 also contains this motif but other studies have indicated that an EBA-175 protein, truncated at the 3' cysteine-rich domain and thereby lacking the transmembrane and cytoplasmic-tail regions was localized apically [59, 81], implying that motifs other than those found in the cytoplasmic tail may be involved in its subcellular localization. The function of N-terminal processing of PfAMA-1 is not known yet. It could be that N-terminal processing may be required to allow AMA-1 to move out of the micronemes and so allowing AMA-1 to bind to an escorter protein [179] or that this motif present within the N-terminal sequence may act as a microneme retention signal. In *P. falciparum* immature micronemes containing AMA-1 are transported from golgi cisternae to the apical region by travelling along f-MAST (*falciparum* merozoites-associated assemblage of subpellicular microtubules), whereas rhoptries and dense granules do not proceed by microtubules [10].

THE MOTOR OF ACTIVE INVASION IN APICOMPLEXAN SPECIES

Merozoite entry into the erythrocyte is clearly an active process that requires motility and energy [150]. Mature erythrocytes do not show any phagocytic or receptor-mediated endocytic activities, indicating that the driving force is generated by the parasite. Cytochalasins, which prohibit invasion, inhibit actin polymerization suggesting the involvement of cytoskeletal structures and/or rearrangements in the transformation of force into movement when parasite invades its host cell. Treatment of merozoites with cytochalasins prior to invasion prevents invasion, but treatment of erythrocytes does not affect invasion, indicating that an actin/myosin motor of the parasite is important for invasion [145]. After cytochalasin treatment, merozoites are still able to attach to erythrocytes and form a tight junction, however movement of the merozoite into the erythrocyte is arrested. Fig. 4 shows two hypothetical models for which evidence is accumulating. Actin filaments were identified under physiological conditions when treated with jasplakinolide, which induces actin polymerization, mainly at the apical end of extracellular *Toxoplasma* tachyzoites [194]. Apicomplexan myosins are conserved in between species. *P. falciparum* has two actin genes. Pf-actin I is expressed throughout the life cycle, but Pf-actin II is expressed only in sexual stages [236]. Myosin is a motor protein that walks along actin filaments towards the end using energy derived from the actin-activated hydrolysis of myosin-bound ATP [173]. When an ATP molecule is hydrolyzed, a conformational change of the myosin head occurs such that force and movement are generated. Myosin motors have three domains. The amino terminus that contain a motor domain, the neck domain which binds to light chains and a tail domain that targets the MyoA to subcellular regions [144]. Five *T. gondii* and four *Plasmodium* myosins have been identified so far, from which MyoA and its homologues in *Plasmodium* species have been localized beneath the plasma membrane of the parasites [133, 174]. In *B. bovis* several myosin proteins have been described [122].

In the first model actin filaments remain stationary associated with the outer membrane of the IMC by a hypothetical protein [15]. The cytoplasmic domain of *P. falciparum* TRAP or *T. gondii* MIC2 directly or indirectly interacts with MyoA tail. The MyoA head domain interacts with actin filaments and moves along the actin filaments. As the myosin contracts down the spiralling actin filaments, it pulls along the attached protein toward the posterior end, where cleavage would occur to release the external portion of the protein. The second model is a more likely model considering the proteins that

have been identified recently (see below) [106]. In *P. falciparum* a myosin light chain homologue was identified and denoted Myosin A tail interacting protein (MTIP). MTIP anchors to the outer membrane of the IMC by a hypothetical protein that was recently identified as a docking protein in *T. gondii* and called *T. gondii* Myo-A docking protein (TgMADP) [91]. MTIP binds the tail domain of MyoA and the head domain of MyoA interacts with short actin filaments that are indirectly linked to the cytoplasmic domain of *P. falciparum* TRAP or *T. gondii* MIC2. Because MyoA is fixed to the IMC, the actin/TRAP complex is displaced from anterior to posterior resulting in a forward movement of the parasite.

Since the actin-myosin motor that drives motility is intracellular, at least one transmembrane bridge is required for apicomplexan species to link it to an extracellular ligand for cell locomotion. Current data suggest that most of the abundant proteins normally found on the surface of merozoites are linked to the membrane via a GPI moiety [113, 148]. Since this linkage only connects the proteins to a lipid inserted into the membrane and does not actually cross the bilayer, other types of protein probably perform this function. In *Plasmodium spp.* a protein was identified in the sporozoite stage, called the thrombospondin-related anonymous protein (TRAP) [182]. *Plasmodium* sporozoites and ookinetes, as well as the invasive stages of many other Apicomplexa, are also motile organisms that crawl along a substratum. This movement along a substratum, or 'gliding motility', is accompanied by the deposition of a trail of TRAP [197]. Gliding motility presumably involves attachment of the parasite ligands (i.e., TRAP) to the substratum. By virtue of their transmembrane domain, the TRAP proteins provide a connection from outside the cell to the intracellular parasite cytoskeleton. Presumably the C-terminal domain interacts with the myosin motor, which then associates with the actin filaments. The force generated by myosin motors will pull TRAP towards the posterior of the parasite and cause a forward movement of the parasite. Gene disruption studies confirm that TRAP plays essential role in both motility and cell invasion [212, 235]. The C-terminal regions of *Plasmodium* TRAP and *Toxoplasma* MIC2 are functionally homologous, suggesting that this type of protein is active in all apicomplexan species [111].

Recent evidence indicates that TRAP family proteins do not bind directly to the cytoskeleton, but rather recruit a complex that provides this vital link [106]. The C-terminal cytoplasmic domains of TRAP and MIC-2 bind specifically to aldolase, a glycolytic enzyme that is also a well-characterized actin-binding protein [106].

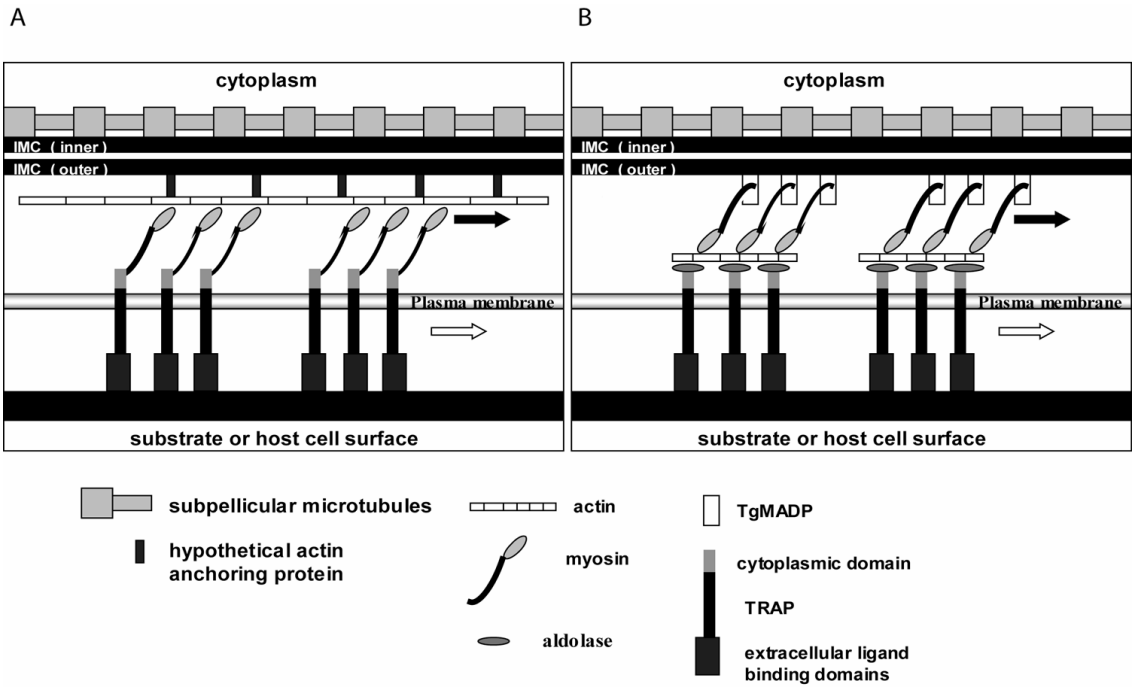

Fig. 4. Two different models proposed for the actin-myosin mechanism in apicomplexans. (A) In the first model filamentous actin remains stationary, bound to the outer membrane of the IMC by a hypothetical protein and the myosin/TRAP complex moves along those filaments. The tail domain of MyoA interacts with the cytoplasmic domain of TRAP/MIC-2. The MyoA head domain interacts with actin filaments and moves along posterior end. (B) In the alternative model MTIP/Myosin complex remains stationary bound to the outer membrane of the IMC by a hypothetical protein (recently found to be MADP in *T. gondii*). In this model actin binds with aldolase which links the complex to the cytoplasmic domain of TRAP. Interaction of actin with head domain of myosin A leads to movement of the parasite.

Aldolase binds directly to the C-terminal domain of TRAP proteins and in turn recruits actin filaments. The aldolase-TRAP interaction requires the conserved tryptophan residue near the C-terminus of the TRAP protein [(106)]. This study gives a molecular explanation for the previous observations that malaria parasites expressing a mutant form of TRAP lacking the tryptophan residue were unable to glide productively [(111)]. Aldolase serves to bridge the extracellular adhesive domains of MIC-2 or TRAP to the cytoskeleton, thus providing the link that was lacking to generate motility.

PROTEASES AND PROCESSING DURING MEROZOITE INVASION

In the previous paragraph microneme proteins have been described involved in invasion, however, besides these proteins, also parasite proteases were shown to be essential for merozoite invasion in *Plasmodium spp.* and *T.*

gondii. Protease inhibitors that are specific for serine and cysteine proteases have been shown to prevent erythrocyte invasion [17]. Protease activity present at the surface of the invading merozoite has shown to be involved in the processing of the MSP-proteins. The processing events of MSP-1 occur on the surface of merozoites at about the time that invasion is taking place [97].

Microneme proteins undergo proteolytic processing, which has mostly been studied in *T. gondii*. Proteolytic processing plays an important role in the maturation and activation of microneme proteins in apicomplexans. TgMIC-2, an adhesion proteins secreted during host cell invasion, belongs to the family of thrombospondion-related anonymous proteins (TRAP). Some microneme proteins are proteolytically processed during their transport and in addition are processed post-exocytosis. TgMIC-2 is processed by two types of proteases termed MPP1 (microneme protein protease 1) and MPP2 when released from the parasite surface [32]. The C-terminal cleavage of TgMIC-2 changes the adhesive properties of the protein drastically and is even thought to release the adhesive complexes from the parasite surface. The MPP1 cleavage site was localized near the C-terminus of TgMIC-2 ectodomain. Two different reports have shown that cleavage occurred within the transmembrane domain and mutations within the conserved TM domain prevented the release of the protein into the medium [164, 224]. However in a recent study mutations in the exodomain of MIC-2 directly upstream of the TM domain were also shown to be involved in cleavage and release of the protein from the cell surface. MIC-2 processing was dependent on a basic amino acid positioned 11 residues upstream of the predicted TM domain. Similar basic residues have been found also around 11-13 residues upstream of the TM domains of several microneme proteins, suggesting a similar process of cleavage [22]. These mutants of MIC-2 were still normally targeted to the micronemes, secreted upon stimulation and efficiently transported to the cell surface. When processing would be blocked, the exocytosed TgMIC-2 could create a long-lasting bridge between the host cell and parasite surface, and so interfering with sealing of the newly formed parasitophorous vacuole leading to blockage of parasite replication inside the vacuole. The microneme protein AMA-1 that is considered a vaccine candidate has also shown to be proteolytically shed in a soluble form during invasion from the parasite surface [101].

RHOPTRY AND DENSE GRANULE PROTEINS

The previous paragraphs have dealt in some detail with the involvement of micronemal proteins in the invasion pathway in chronological order. Such information is mainly lacking for rhoptry proteins. Rhoptries discharge their contents during the internalization of the parasite, releasing their proteins inside the parasitophorous vacuole and possibly into the erythrocyte membrane as well. Probably these proteins and possibly also lipid material play a role in the building of the parasitophorous vacuole. Quite a number of malarial proteins have been found to be transported to and stored in the rhoptry organelles [211, 242]. RAP-1 and RAP-2 are two proteins that have been localized in rhoptries and both proteins neither have transmembrane domains nor repeated amino acid motifs. After invasion the RAP-1/RAP-2 complex is apparently incorporated into the parasitophorous vacuole membrane (PVM) and /or the parasite plasma membrane of the newly formed ring-stage parasites [242].

Once the merozoite is inside the RBC, it is enclosed by the PVM and dense granules secrete their protein content. The dense-granule proteins associate with intravacuolar structures after their release. A few proteins have been identified in *Plasmodium* dense granules. One of these proteins is called RESA (ring-infected erythrocyte surface antigen), which, shortly after its release, becomes located under the membrane of the infected erythrocyte in association with the cytoskeleton (reviewed in [12]). The fusion of the dense granules and the release of their contents correlate with and perhaps trigger transformation into a growing intracellular ring-stage trophozoite that begins to feed on the hemoglobin of the RBC.

Despite the wealth of information which is accumulating rapidly, we are still far from a complete understanding of these very complex organisms and their relationship to the host cell. The close interaction between the parasite and host starts from the first moment the merozoites try to enter the host cell. Most of the proteins found in micronemes, rhoptries and dense granule proteins described are from the two major apicomplexan model systems *T. gondii* and *P. falciparum*. For *B. bovis* only one rhoptry protein RAP-1, three dense granules proteins (SBP-1 to SBP-3) and two merozoite surface proteins, MSA-1 and MSA-2, have been identified which were studied in detail and to date not a single micronemal proteins nor a receptor have been identified [74, 151, 152, 210, 211, 242]. A start to the identification of such proteins and the study of their role in invasion is the subject of this thesis.

OUTLINE OF THE STUDY

To know more about the invasion process of *B. bovis*, we first developed an in vitro invasion assay described in **Chapter 2** that is used as model to investigate the proteins that are involved during invasion. *B. bovis* merozoites were liberated from red blood cells by high voltage pulses, followed by fresh erythrocyte invasion in protein-free buffer. Besides developing the in vitro invasion assay, we also investigated the effect on invasion by inhibitors affecting cytosolic Ca^{2+} concentration or actin polymerisation. **Chapter 3** reports the host-specificity of *B. bovis* parasite and initial experiments have been done to know more about the surface receptors and ligands that the parasite needs to enter its host cell. Besides invasion in bovine red blood cells, we also provided red blood cells of multiple other species, including human red blood cells. Very few proteins involved in apicomplexan host cell invasion have been conserved across the genus border. Nevertheless, two such proteins have been identified from EST data that were generated in the laboratory parallel to this thesis. These proteins are considered as candidates to be incorporated into a recombinant vaccine for *P. falciparum*. AMA-1 is shown to be a protein that has been transcribed and expressed in the asexual merozoite stage. **Chapter 4** describes the *B. bovis* AMA-1 protein. We cloned and sequenced the complete AMA-1 gene, identified it in invasion supernatant material and total merozoite extracts and demonstrated a putative role in erythrocyte invasion. TRAP was also identified in *B. bovis* and further analysed in **Chapter 5**. The *B. bovis* proteins identified could only be characterized at molecular level and localized within the parasite. However we could not study the function of these proteins. The functional analyses of proteins require a transfection system of *B. bovis*. Several attempts and conditions that have been tried out to set up a transfection system for *B. bovis* are described in **Chapter 6**. Although many efforts have been taken, we were not able to develop a transfection system. *B. bovis* merozoites cultures that were transfected with human *dhfr* (dihydrofolate reductase) or *T. gondii dhfr*m2m3 plasmid were selected after weeks of drug pressure. However only spontaneous resistant *B. bovis* cultures for pyrimethamine and WR99210 were selected. These cultures were further analysed after observing that these cultures were selected over and over again. The *dhfr-ts* (dihydrofolate reductase-thymidylate reductase) of *B. bovis* wild type and resistant cultures were cloned, sequenced and compared. These analyses are described in **Chapter 7.** We detected a mutation in the *dhfr*

gene of *B. bovis* likely to be responsible for the resistance. In the final chapter, **Chapter 8**, the findings of the study described here are discussed.

CHAPTER 2

CHARACTERIZATION OF ERYTHROCYTE INVASION BY *BABESIA BOVIS* MEROZOITES EFFICIENTLY RELEASED FROM THEIR HOST CELL AFTER HIGH VOLTAGE PULSING

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ABSTRACT

Apicomplexa are a phylum of obligate intracellular parasites critically dependent on invasion of a host cell. An in vitro assay for erythrocyte invasion by *Babesia bovis* was established, employing free merozoites obtained after application of high voltage to parasitized erythrocytes. Invasion proceeds efficiently in phosphate buffered saline solution without requirement for any serum or medium components. Kinetics of invasion can be measured over a time span of 5 to 60 minutes after which invasion is completed at an average efficiency of 41%. The fast kinetics and high efficiency exceed those of most previously established apicomplexan invasion assays. Manipulation of intracellular calcium concentration inhibits invasion. Preincubation of merozoites at 37°C also reduces invasion, possibly by the premature secretion of protein. Proteins that are shed into the environment during invasion were directly detectable by protein staining after 2D gel electrophoresis. Limitations posed by the immunological detection of proteins released during in vitro invasion by other apicomplexan parasites can therefore be avoided by this method. A unique feature of the assay is the reversible uncoupling of invasion and intracellular development, the latter only taking place under serum-rich medium conditions. In addition, host cell attachment is uncoupled from invasion by cytochalasin B.

INTRODUCTION

Babesia bovis is an apicomplexan parasite of cattle causing major damage to the livestock industry in tropical and sub-tropical areas and is transmitted by ticks of the genus *Boophilus*. Unlike malaria sporozoites, which invade hepatocytes, the sporozoites of *B. bovis* directly invade erythrocytes upon which an asexual erythrocytic cycle of duplication, erythrocyte rupture and merozoite reinvasion is initiated. Like the plasmodial erythrocytic cycle this can cause severe pathology, which can ultimately lead to the death of the host. One clinical feature is cerebral babesiosis, characterized by clogging of the cerebral microvasculature with infected erythrocytes and associated inflammatory reactions. At least superficially this resembles human cerebral malaria caused by *Plasmodium falciparum* [163].

Host cell invasion by apicomplexan merozoites is a conserved and critical step that is amenable to intervention by the immune system. For *Plasmodium* species and *Toxoplasma gondii* the molecular mechanism of invasion has been investigated in some detail [31, 67, 196, 205]. Rhoptries and micronemes, characteristic apicomplexan secretory organelles containing molecules essential to host cell invasion, harbour a

complex mixture of proteins but for *B. bovis* only the rhoptry protein RAP-1 has been identified and studied in detail [152, 211, 242]. In addition, a function for the merozoite surface proteins MSA-1 and MSA-2 has been implied in *B. bovis* invasion [74, 151, 152]

In vitro invasion of host cells by other apicomplexan parasites is accompanied by the rapid release of parasitic proteins into the environment [31, 101]. A function in the invasion process is being assigned to an increasing number of these proteins. Studies on erythrocyte invasion by most *Plasmodium* species are hampered by the low yields of viable free merozoites and the long incubation times (up to 48 h) required for quantification. This is in marked contrast with the rapid, within minutes, invasion observed in vivo. Our aim was to establish an in vitro *B. bovis* invasion assay that is rapidly quantifiable and efficient in numbers of invading merozoites. A novel method of merozoite release, followed by erythrocyte invasion in a protein-free buffer, met with these criteria and allows direct detection of proteins secreted during invasion, in sufficient quantities for further characterisation and sequence identification. It should help to close the gap in molecular knowledge as compared to other related parasites and provide another apicomplexan model system for studying erythrocyte invasion.

MATERIALS AND METHODS

In vitro *culture of* B. bovis

B. bovis clonal line C61411, derived from an Israelian isolate, was cultured in vitro according the MASP method with slight modifications [121, 195]. Briefly, parasites were cultured in a settled layer of bovine erythrocytes at 5% packed cell volume (PCV) in M199 supplemented with 40% adult bovine serum and 26 mM sodium bicarbonate (M199S) under an atmosphere of 5% CO_2 in air at 37ºC. Culture volumes were 1200 µl in 24 well plates, 15 ml in 25 cm^2 flasks and 50 ml in 75 cm^2 flasks. Cultures were maintained at a parasitaemia between 1 and 5% by daily dilution. For invasion studies cultures were harvested in log phase at 8 to 12% parasitaemia.

Preparation of bovine serum and erythrocytes and B. bovis *immune serum*

Blood was retrieved from a selected Friesian-Holstein heifer, defibrinated by shaking for 10 min with glass beads and centrifuged for 30 min at 4ºC at 2000 g for pelleting erythrocytes. Serum was centrifuged again, frozen at –20ºC and stored until usage. Erythrocytes were washed 4 times in VyMs buffer, kept at 4ºC and used for maximally 14 days. Immune serum was obtained from a cow that was infected 3 times at three-month intervals with *B. bovis* clonal line C61411 [227].

In vitro *invasion assay*

B. bovis culture was centrifuged (2000 g, 10 min, 15ºC) after which the pellet was resuspended in an equal volume of cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2mM EGTA, 5 mM $MgCl_2$, 10 mM $KHPO_4/KH_2PO_4$ pH 8.0, 25 mM HEPES pH 8.0). Samples of 750 µl were subjected to five intermittent (10 seconds, 0ºC) high voltage pulses (2.5 kV, 200 Ohm, 25 uF) in a BioRad Gene Pulser with pulse controller using 4 mm BioRad cuvettes (165-2088). For removal of soluble extracellular components from the liberated merozoites and erythrocyte ghosts, 120 µl samples were washed 1 to 3 times by resuspension in 1 ml of buffer (as indicated in the text and legends) followed by centrifugation (2000 g, 2 min, 15ºC). After the last centrifugation the pellet was resuspended to a total volume of 120 µl which was subsequently added to 1080 µl of pre-conditioned bovine erythrocytes (in 24-well plates) and transferred to an atmosphere of 5% CO_2 in air at 37ºC to initiate invasion. Pre-conditioned erythrocytes consisted of 50 µl (PCV) bovine erythrocytes (washed in PBS for 3 times in addition to the 4 washes in VyMS buffer) suspended in 1030 µl of buffer (buffer indicated in the text) and incubated under an atmosphere of 5% CO_2 in air at 37ºC for 30 min. Invasion was measured at the indicated time points by counting the parasitized erythrocytes out of a total of 5000 erythrocytes in Giemsa stained smears. Invasion efficiency was calculated relatively to a control invasion performed in triplicates in M199S. For characterisation of the invasion process several chemicals were added to the pre-conditioned erythrocytes and/or pre-incubations of liberated merozoites were performed as indicated in the text and legends. These included BAPTA-AM (20 mM stock solution in DMSO, Sigma), Thapsigargin (1 mM stock in DMSO, Sigma), Cytochalasin B (400 µM stock in DMSO, Sigma) and Ionomycin (400 µM stock in DMSO, Sigma).

Viability determination of merozoites

Liberated merozoite suspension was added to an equal volume of 20 µg/ml 6-carboxyfluorescein diacetate (CFDA, Sigma) and incubated for 30 min at 20ºC at a dark place. Merozoites were resuspended in PBS after centrifugation (2000 g, 2 min) and examined under a UV.

Sample preparation and isoelectric focusing

The overlaying buffer of the invasion assay incubations was collected and centrifuged twice (10 min at 2000g and subsequently for 15 min 12000g) for removal of all cells. The final supernatant was concentrated and desalted over 3 kDa filters (YM-3, Millipore), precipitated in a final concentration of 10% TCA (dissolved in acetone) and dissolved in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 2% carrier ampholyte mixture pH 3-10NL (IPG buffer) and 20 mM DTT). IEF instrumentation, IPG gels and related reagents were from APharmacia Biotech, unless otherwise indicated. Protein (50 µg) with protease inhibitor (Complete, Roche) was loaded on 13 cm IPG strips (pH 3-10NL), rehydrated (10-14 h) and focused overnight (14-17 h) in an automated run (1 min 300 V, 90 min during which

voltage rises to 3500 V followed by continued focusing at 3500 V for a total of 35-40 KVh, IPGPhor^{TM}).

Second Dimensional Electrophoresis (2DE)

The strips were incubated in 10 ml of equilibration buffer (50 mM Tris, 6 M urea, 2% SDS, 30% glycerol, pH 8.8) containing 30 mM DTT for the first 15 min and replaced by equilibration buffer with 2.5% iodoacetamide for another 15 min. The second dimensional SDS gel was carried out in a Hoefer SE600 system. Silver staining was used to visualise proteins after 2DE. The images of the gels were acquired using LabScan v3.0 software on an Umax flatbed scanner and later analysed with Image Master 2D v3.01 software (APharmacia Biotech).

SDS-Polyacrylamide Electrophoresis and Western blotting

Sample proteins were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane, which was blocked with 5% skimmed milk powder in PBS, pH 7.2 for 1 h at 37ºC. All washes after each incubation step were done 3 times for 5 min with PBS/0.1% gelatine/0.05% Tween (PBS-GT). The blots were incubated overnight at 22-24ºC in immune sera diluted 1/500 or 1/1000 in PBS with 5% skimmed milk. After washing, the strips were incubated with anti-bovine total IgG coupled to alkaline phosphatase (diluted 1/30000 in PBS-GT) for 1 h at 22-24ºC. The colorimetric reaction was carried out for 10-15 min with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate diluted in 100 mM Tris/100 mM NaCl/5 mM $MgCl_2$, pH 9.5.

RESULTS

Kinetics of erythrocyte invasion by merozoites liberated by high voltage pulses.

Free merozoites were obtained by application of five subsequent 2500 V pulses to *B. bovis* infected erythrocytes (8 to 12% parasitaemia) that were resuspended at 50% PCV. Microscopic examination of Giemsa stained smears showed that 99.5% of the erythrocytes are lysed. Infected erythrocytes are hardly ever encountered but a substantial number of merozoites (<10%) reside in erythrocyte ghosts. At least 80% of the liberated merozoites were viable as defined by their ability to metabolise CFDA.

The ability to invade host cells was examined by incubation of liberated merozoites with erythrocytes (5% PCV) under standard in vitro culture conditions. The ratio of free merozoites to erythrocytes was kept identical to the ratio before liberation (e.g. 8 to 12%). Fig.1a shows that invasion proceeds rapidly (0.91% parasitaemia at 15 min) reaching a plateau at 2.20% after 60 min. Invasion efficiency can be defined as the observed parasitaemia after reinvasion divided by the maximal number of

potentially invasion competent parasites (2x percentage of erythrocytes harbouring a duplicated parasite in the culture from which the liberated parasites were obtained). Six independent invasion experiments performed over a time span of several months yielded a mean invasion efficiency of 41.6 ± 15.6% (s.d.), reaching 0.8 to 3.8% absolute parasitaemia, after 60 min. Careful examination and focusing under the light microscope showed that many parasites were already in contact with erythrocytes (juxtaposed to, but often also looking as if being located on top of, an erythrocyte) after 15 min. The sum of parasites contacting and having invaded an erythrocyte after 15 min approximately equals (2.54 ± 0.14%) the number of parasites that have actually invaded after 60 min. Very few parasites contacting an erythrocyte are left after 60 min whereas the parasites that did not invade are laying free or clumped together. Fig.1b displays the effect of erythrocyte PCV on invasion. Relative parasitaemia becomes highest at a PCV of 1% but the absolute number of invaded merozoites is highest at a PCV of 8%, both after 10 min and 1 h.

B. bovis infections as well as in vitro cultures are asynchronous and methods for synchronisation are lacking. The span of a lifecycle has only been estimated from the average multiplication rate. In the current invasion assay it can be assumed that immature merozoites, liberated by high voltage, do not develop any further whereas only mature merozoites are invasion competent, thus providing a synchronised start. This does not result in synchronous growth (Fig.1c).

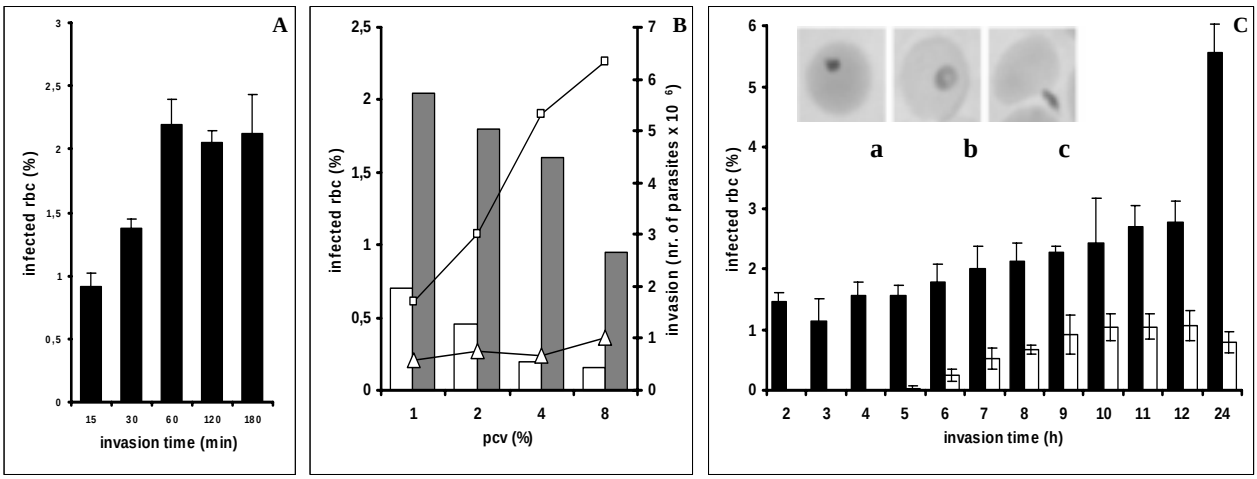

Fig. 1. A. Percentage of invasion plotted against time. **B**. Effect of packed cell volume on invasion. White bars represent percentage of invasion after 10 min, grey bars after 1 h. Lines indicate invasion in absolute numbers of parasites after 10 min (triangles) and 1 h (squares). **C**. Development of parasitaemia after invasion. Black bars indicate total percentage of invaded erythrocytes. White bars indicate the percentage of invaded erythrocytes harbouring a duplicated, double pear-shaped merozoite. Error bars indicate S.D. of triplicates. Invasion performed in M199S in all panels.

Upon the appearance of the first duplicated double-pear shaped parasites after 5 h, the onset of a gradual increase in parasitaemia is observed. This defines the minimal time span of a complete lifecycle at ~6 hours but a sudden burst of duplicated and subsequently reinvaded parasites is not observed, indicating a wide spread in lifecycle duration between individual parasites. In Fig.1c a 3.8-fold multiplication is reached after 24 h, which is within the range (3.5 to 5-fold in 24 h) that is observed in a continuously growing culture.

Effects of medium composition and merozoite pre-treatment on erythrocyte invasion

Dependence of *B. bovis* in vitro growth on a rich culture medium supplemented with 40% adult bovine serum (M199S) complicates dissection of the components involved in invasion, intra-cellular establishment and growth. Fig.2a shows that invasion can take place in absence of serum and medium. A 35% reduction in invasion efficiency was observed when performed in PBS instead of M199S (bar 1). Addition of 1 mM $CaCl_2$ restored relative invasion efficiency to 96% (bar 4). Washing of merozoites in PBS prior to invasion had no significant effect on efficiency (bars 2 and 5) whereas washing in presence of 1 mM $CaCl_2$ enhanced invasion by ~40% (bar 3 and 6). Repeated washing caused a gradual decline in efficiency but a three-fold wash and subsequent invasion in PBS/$CaCl_2$ still permitted comparable invasion levels as observed with non-washed merozoites in M199S (not shown). A pH from 7.5 to 9.0 was compatible with invasion with an optimum at pH 8.0 (Fig.2b). A sharp drop in invasion efficiency was seen at pH to 7.0. Invasion was abolished at 32^{o}C or 42^{o}C (not shown). Preincubation of merozoites for 5 min at 37^{o}C completely prevents invasion although no merozoite agglutination or changes in shape were observed by microscopy. 30 min preincubation of merozoites at 20^{o}C or 30^{o}C was much better tolerated as compared to 37^{o}C (Fig.2c). Light microscopy of Giemsa stained smears showed invaded parasites (either in PBS or in M199S) as condensed (pycnotic) intra-erythrocytic particles (Fig.1c, insert a). In M199S parasites rapidly (2 to 3 h) develop into trophozoite forms with a clear blue cytoplasm (Fig.1c, insert b), but after performing invasion in PBS any further development is blocked and parasites remain pycnotic. Normal development is rapidly restored when PBS is replaced by M199S after 2 or 4 h (Fig.2d). Addition of M199S after 24 h restored parasite growth only after a 24 h to 72 h lag phase (not shown).

Effects on invasion by inhibitors affecting cytosolic Ca^{2+} concentration or actin polymerisation.

Cytochalasins prevent actin polymerisation by capping the barbed end of actin and their inhibitory effect on host cell invasion of several apicomplexan parasites suggested the involvement of an actin/myosin motor system [25, 44, 60, 145]. *B. bovis* invasion is completely inhibited by 5 μM cytochalasin B (Fig.3a) and examination of Giemsa stained preparations identified many merozoites contacting an erythrocyte. Of these, many are spread out on the stained preparation as if the tip of the merozoite is contacting the erythrocyte (Fig.1c, insert c).

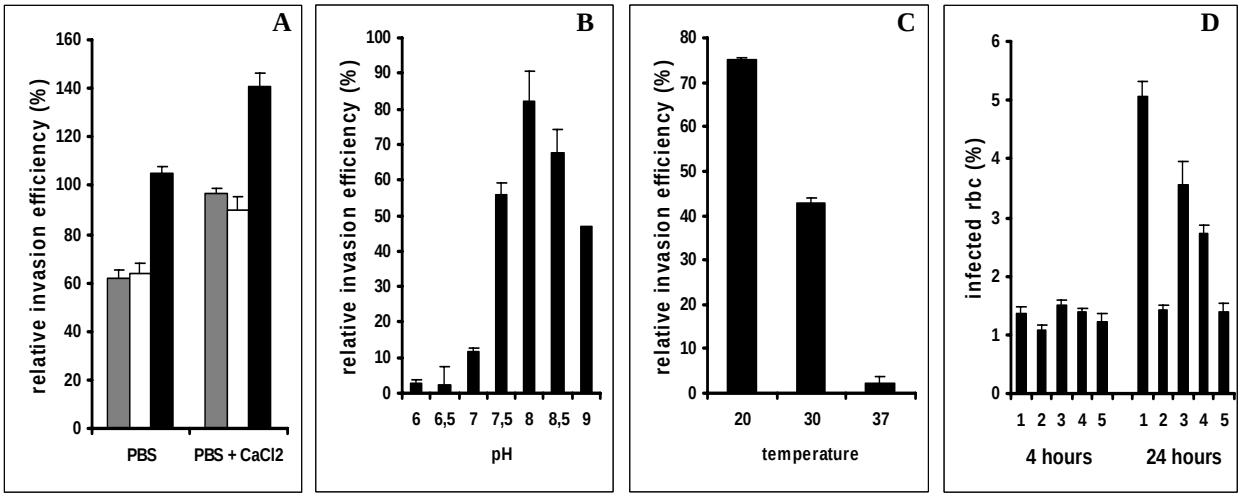

Fig. 2. A. Effect of medium composition on invasion. Merozoites are washed once in PBS (white bars) or in PBS + 1 mM $CaCl_2$ (black bars) or were not washed (grey bars) after lysis by high voltage. Immediately after washing merozoites were allowed to invade erythrocytes for 1 h in PBS pH 8.0 or in PBS pH 8.0 + 1 mM $CaCl_2$ as indicated. Invasion was plotted relative to invasion performed in M199S (control performed in triplicate), which was taken as 100%. **B.** Effect of pH on invasion. Invasion was performed for 30 min in PBS that was adjusted to the indicated pH and plotted relatively against invasion performed in M199S. **C.** Effect of preincubation of merozoites on invasion. Merozoites were incubated for 30 min in PBS pH8.0 prior to invasion at the indicated temperature, after which invasion was performed for 30 min in PBS pH8.0. Efficiency was plotted against invasion in PBS pH8.0 by merozoites that were not pre-incubated. **D.** Development of invaded parasites after addition of growth medium. Invasion was performed for 2 h in M199S (bar 1) or in PBS pH8.0 (bars 2 to 5). M199S or PBS pH8.0 was either not replaced (bars 1 and 2) or replaced by M199S after 2 h (bar 3), 4 h (bar 4) or 24 h (bar 5). Infected erythrocytes were counted after 4 h and 24 h as indicated. Replacement of PBS after 24 h (bar 5) showed at that time point abnormally developed parasites but within 72 h normal growth rate is restored (not shown). All assays were performed in triplicate and error bars indicate S.D.

1 mM extracellular Ca^{2+} had a small positive effect on invasion by *B. bovis* (Fig.2a). For examination of the effect of cytoplasmic free Ca^{2+} on invasion,

merozoites were preincubated with ionomycin (a Ca^{2+} ionophore giving rise to increased cytosolic Ca^{2+} by influx from the extracellular millieu or intracellular Ca^{2+} stores), BAPTA-AM (a specific chelator of cytosolic Ca^{2+}) or thapsigargin (an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPases that transport cytosolic Ca^{2+} back into the sarco/endoplasmic reticulum) [214]. Fig.3b shows that preincubation with 100 μM BAPTA-AM reduces invasion more than 10-fold whereas 1 μM thapsigargin reduces invasion 5-fold. Ionomycin had a moderate effect in presence or absence of extracellular Ca^{2+}, resulting in 40% inhibition after 30 min preincubation (Fig.3c). In all cases the viability of merozoites was not affected by the inhibitor treatment as indicated by their capacity to metabolise CFDA.

Invasion is accompanied by the rapid release of parasite specific antigens into the surroundings

B. bovis proteins shed at different time points during invasion were separated by SDS-PAGE, blotted and labelled by with *B. bovis* antiserum. Antigenic proteins of 60 and 64 kDa are released within 5 minutes upon transfer of the incubation mixture from 0 to 37°C (Fig.4). The same bands are also observed in a control experiment in which no erythrocytes were added to the liberated merozoites (Fig.4, compare lanes 3 and 8 to 4 and 9). During 60 minutes of invasion, shedding of antigenic proteins was continued, resulting in detection of additional antigenic bands with a gradually increasing intensity over time (lanes 9 to 12). In a control experiment where invasion is prohibited by the absence of erythrocytes, no gradual increase in the intensity and number of bands was observed (lanes 5 to 7).

Most of the protein in the samples is still derived from erythrocyte ghosts in the form of cytoskeletal proteins and associated haemoglobin that could not be removed by repeated washes at 0°C but partly became solubilized during the incubation at 37°C. This limits the amount of *B. bovis* derived proteins that can be loaded on a SDS-polyacrylamide gel and, in combination with the limited resolution obtained by 1D-SDS-PAGE, restricts identification of secreted/shedded proteins by direct protein staining. Employing the capacity and resolving power of 2D-protein gel electrophoresis, a large number of *B. bovis* specific major and minor protein spots could be detected after electrophoresis of in vitro invasion supernatants (Fig.5). Panel A shows the proteins present in 2 ml of supernatant derived from an experiment in which merozoites were allowed to reinvade for 1 h. Panel B shows a control experiment in which uninfected erythrocytes were lysed by high voltage and further washed and used for invasion under identical conditions as in Panel A. Thus, panel B shows all the erythrocyte proteins (mainly monomeric and multimeric haemoglobin B

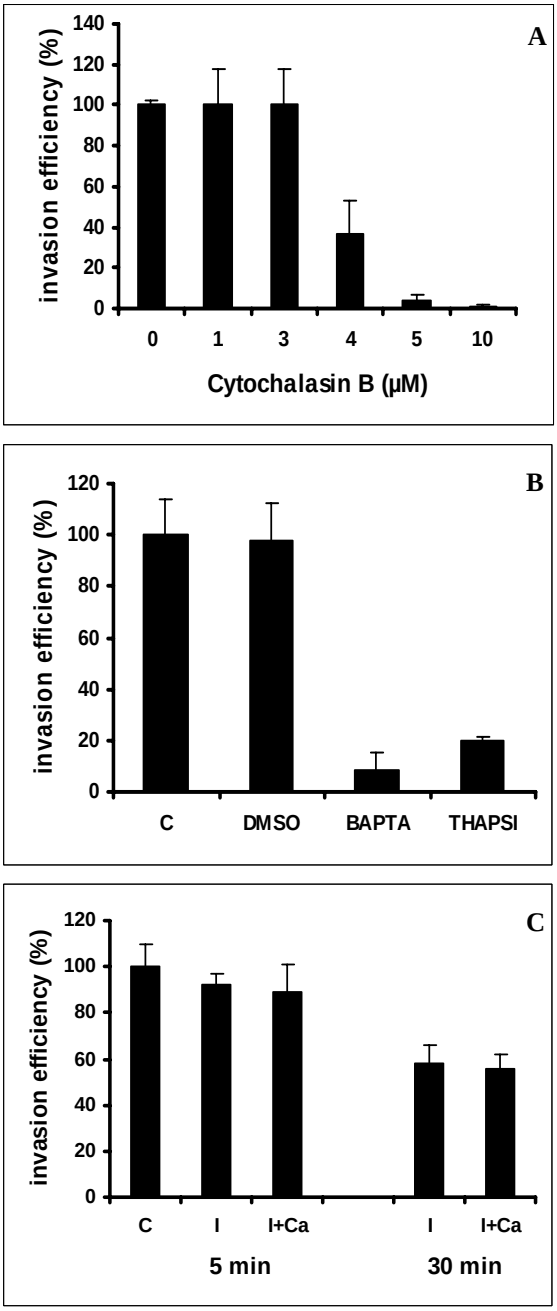

Fig. 3. A. Inhibition of invasion by cytochalasin B. Invasion was performed in M199S after 15 min preincubation of merozoites with cytochalasin B at the indicated concentration and determined after 1h. **B.** Effect of BAPTA-AM and thapsigargin on invasion. Invasion was performed for 1 h in M199S without preincubation (C) or after preincubation for 90 min at 20ºC in PBS + 0.5% DMSO, 90 min preincubation at 20ºC with 100 µM BAPTA-AM or 30 min preincubation at 20ºC with 1 µM thapsigargin. **C.** Effect of ionomycin on invasion. Invasion was performed for 1 h in M199S without preincubation (C) or after preincubation for 5 and 30 min respectively with ionomycin in PBS pH 8.0 or PBS pH 8.0 + 1 mM $CaCl_2$. Error bars indicate standard deviation (SD) of triplicates.

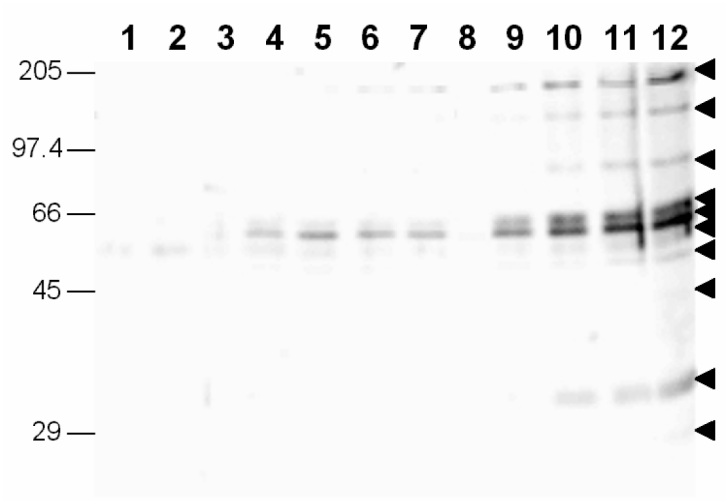

Fig. 4. Western blotting of proteins released during invasion of erythrocytes. Lanes 3 to 7, control incubations for determination of proteins released by free merozoites in the absence of erythrocytes. Lanes 8 to 12, invasion in PBS pH 8.0. Released proteins were harvested after 0, 5, 15, 30 and 60 min (lanes 3 and 8, 4 and 9, 5 and 10, 6 and 11, 7 and 12, respectively). Lane 2 is a control with non-infected erythrocytes used for invasion for 1 h. Lanes 2 to 12 were incubated with immune serum, Lane 1 is identical to lane 12 but incubated with preimmune serum.

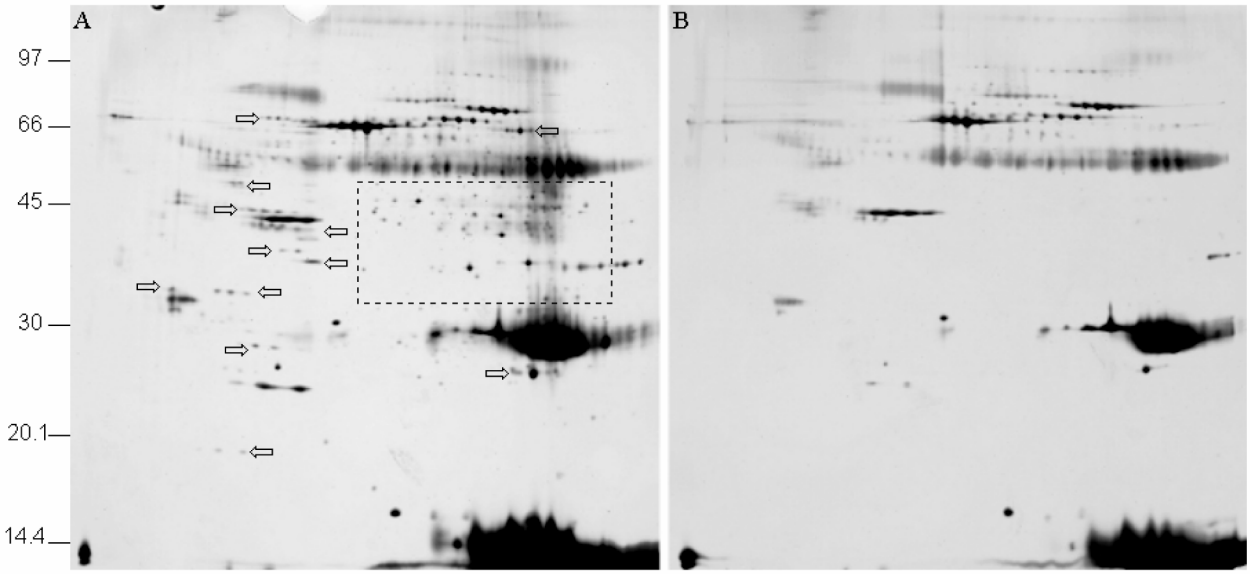

Fig. 5. 2D gel electrophoresis (12.5%) of proteins released during invasion. Panel A, supernatant of invasion of erythrocytes with liberated merozoites. Panel B, supernatant of "invasion" with high-voltage lysed extract of mock-infected erythrocytes. Invasion was performed for 1 h in PBS pH 8.0 in a volume of 15 ml. Merozoites were washed in PBS pH 8.0 for three times prior to invasion. Parasitaemia after invasion was determined at 0.84%. Arrows indicate prominent spots that cannot be detected in control of panel B. The boxed area contains many "invasion specific" spots.

and cytoskeletal proteins) that were not solubilized during the washing steps but only after incubation for 1 h at 37ºC (not any resemblance can be detected with a proteome map of cytosolic bovine erythrocyte proteins available at http://www.expasy.ch /ch2dothergifs/publi/rbc.gif, indicating that no significant lysis of erythrocytes takes place during invasion). Panel A shows in addition a large amount of spots that represent merozoite proteins released into the supernatant during 1 h of invasion. Merozoite derived proteins were particularly abundant in the boxed region of panel A. Arrows mark other prominent spots that are not present in the control. At least 110 spots are present in panel A that could not be detected in panel B (some of the fainter spots are close to the detection limit and may thus just have been missed in panel B).

DISCUSSION

Studies on *B. bovis* erythrocyte invasion may help in pinning down conserved and species specific aspects of the molecular mechanisms of apicomplexan host cell invasion [133, 156]. Here we have presented an invasion assay, based on the liberation of *B. bovis* merozoites by high voltage pulses, with the following hallmarks. (I) ~40% of the liberated "invasion-competent" merozoites invade an erythrocyte within 1 h. (II) Invasion proceeds efficiently in PBS thereby providing an excellent model system for studying the effects of exogenously added components without having to take into account the effects of the many undefined substances present in serum-rich media. (III) During invasion proteins are released that can be concentrated without using selective procedures like immunoprecipitation that otherwise have to be used to remove the excessive amounts of protein present in serum-rich media. (IV) The assay uncouples invasion and intracellular development, enabling systematic reconstitution of medium components required for intracellular growth.

These features allow more accurate and specific in vitro studies of *B. bovis* erythrocyte invasion than currently is the case for most *Plasmodium* species. Light microscopy indicates that within 5 minutes most parasites that will invade within the next hour have become attached to an erythrocyte whereas 41% of the maximum level of invasion is already reached after 15 min. A qualitative description of erythrocyte invasion by *P. knowlesi* has shown that invasion can be accomplished within minutes after initial contact but in contrast, invasion experiments by other *Plasmodium* species are typically performed in a time span ranging from 4 h to 48 h [71, 136, 160].

Studies on apicomplexans have shown that host cell receptors are rapidly secreted from micronemes upon initial cell-cell contact, followed by secretion of

additional proteins from rhoptries and release of plasma membrane-bound proteins [31, 36]. The results obtained for *B. bovis* fit into such a model. Immune recognised proteins of 60 and 64 kDa are secreted upon transfer to 37ºC followed by an accumulation of additional bands in the next hour of invasion. Immediate secretion of 60 and 64 kDa proteins is also seen upon transfer to 37ºC in absence of invasion (control with no erythrocytes added). This may be a premature secretion and could explain the reduced invasion that is seen after a brief preincubation of merozoites at 37°C. As preincubation at 20°C has no detrimental effect on invasion and does not lead to protein release, the rapid release of 60 and 64 kDa proteins could result from an enzymatically regulated event. This may have been triggered by interaction with host cell receptors that might also be present in the control in the form of erythrocyte ghost membranes.

Invasion in medium containing 40% bovine serum is fully productive as is shown by the subsequent growth rate. In the first 24 h this lies within the 3.5 to 5-fold multiplication range of a continuous growing culture. Invasion performed in PBS followed by a change to standard growth medium after 1 h is equally productive from which can be concluded that the merozoite carries all the requirements for optimal invasion. A large variation in intra-erythrocytic life cycle duration was determined with a minimal length of 6 h. After 11 h parasitaemia has increased from 1.55 % (x) to 2.7 % (y) with 1.7 % (z) of the erythrocytes harbouring a single trophozoite. Thus 35% ((z-(y-x))/x) of the parasites has not duplicated yet indicating that the time required to develop to a duplicated double pear-shaped meront is an important component in the asynchronous growth of *B. bovis*. This can be an inherent property of *B. bovis* development but could also be due to sub-optimal in vitro growth conditions.

5 µM cytochalasin B gives 95% inhibition whereas 3 µM has no effect. During a 48 hours period of growth, 0,4 µM of cytochalasin B inhibits *B. bovis* growth by 25%, possibly indicating a stronger effect on the function of contractile microfilaments during replication [122]. The effect on invasion was not measured by this method. Sensitivity is in between the values observed for *E. tenella* (82% inhibition by 10 µM) and *P. knowlesi* (~90% inhibition by 1 µM) [25, 145]. The frequent observation of merozoites contacting an erythrocyte by their tip is reminiscent of observations on *P. knowlesi* and *T. gondii* parasites that were preincubated with cytochalasin [60, 145]. Cytochalasins may prevent movement of the parasite into an invaginating vacuole by blocking an actin/myosin motor system [165] without blocking attachment to the host cell after apical reorientation. Studies on the role of Ca^{2+} in apicomplexan host cell invasion gave rise to a model in which protein secretion during invasion is a stimulus-coupled event with Ca^{2+} as a second messenger [34, 63, 136, 228, 233]. Chelation of intracellular Ca^{2+} by BAPTA-AM inhibits *B. bovis* invasion but is a harsh tool that will

also affect other biological processes. The strong inhibition by thapsigargin points at the involvement of the ER as this drug inhibits repletion of this calcium store. This effect of thapsigargin has been observed for other apicomplexans too, but recently it was shown that incubation with thapsigargin does not give rise to an increase in cytosolic Ca^{2+} making the effect of this drug on invasion still puzzling [(4)]. The observed stimulating effect of 1 mM extracellular Ca^{2+} could indicate the use of extracellular Ca^{2+} for transient increase of cytosolic Ca^{2+} but it may equally well positively affect essential protein interactions between host cell and merozoite during invasion.

During continuous in vitro cultivation of *B. bovis*, a mixture of proteins is shed that has been described as culture-derived exoantigens [(107, 149, 170, 191)]. Their origin and function are still largely unknown. The immunogenicity of these proteins provoked vaccination studies that have given promising results in other *Babesia* species as well. Most likely, the immunogenic proteins that are secreted during host cell invasion (Fig.4), are part of the proteins present in exoantigens. *B. bovis* exoantigens, harvested over periods of 48 h, will contain a lot of other parasitic proteins (e.g. from decaying parasites) and are usually studied with immunological tools as they are masked by an excessive amount of serum and host cell proteins. The *B. bovis* proteins released during in vitro invasion in PBS can be identified directly by 2-dimensional gel electrophoresis. The number of protein spots detected by far exceeds the number of antigenically detected proteins after 1D electrophoresis (10 in Fig.4) and also the number of antigenically detected exoantigens [(107, 149, 170)]. Identification and initial functional characterization of these additional proteins is now enabled by their visualisation by 2D electrophoresis. Identification of the proteins not detected by antibodies may also be relevant for vaccine development, the inherent immunogenicity of a parasite molecule does not correlate with its protective capacity [(238)].

In summary, the strength of the assay is its speed, efficiency and independency of medium composition. The effects of specific invasion inhibitors or conditions on the kinetics of secretion of individual proteins can be studied on a short time-scale (5 min to 1 h) by the use of 2D-electrophoresis in order to unravel the molecular mechanisms involved. The protein spots are amenable to identification by a proteomics approach considering the amounts that can be produced (the assay can without problems be performed in 50 ml quantities). Rare events can be monitored directly as the application of 50 ml of invasion supernatant to a 2D gel will allow the detection of 20 molecules of 60 kDa secreted by a single merozoite (assuming the invasion of 2% of the erythrocytes and a detection limit of 1 ng protein by silver staining).

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CHAPTER 3

BABESIA BOVIS MEROZOITES INVADE HUMAN, OVINE, EQUINE, PORCINE AND CAPRINE ERYTHROCYTES BY A SIALIC ACID–DEPENDENT MECHANISM FOLLOWED BY DEVELOPMENTAL ARREST AFTER A SINGLE ROUND OF CELL FISSION

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"En er is geen schepsel dat zich op aarde beweegt of zijn onderhoud berust bij Allah.
En Hij weet waar het zal verblijven en waaraan het zal worden toevertrouwd.
(Qur'an 11:6)"

ABSTRACT

Babesia bovis infections have only been observed in bovine species in contrast to *Babesia divergens* that also can infect humans, sheep and rodents. Using an in vitro assay that assesses invasion of erythrocytes by free merozoites after an one hour incubation period, it was shown that specificity is not determined by host-specific interactions associated with invasion. Human erythrocytes were invaded more efficiently than bovine erythrocytes whereas erythrocytes of sheep, pigs and horses were invaded only slightly less efficient. In contrast, goat erythrocytes were refractory to efficient invasion. Significant differences in invasion efficiency into erythrocytes from different individuals of the same species were observed. Erythrocytes from all species, except for goats, supported intracellular development of newly invaded merozoites and high numbers of duplicated parasites, located in a morphologically normal accole position, were present. Only in bovine erythrocytes subsequent rounds of invasion, leading to increased parasitaemia, took place. This suggests that host specificity is determined by factors operating late in the erythrocytic stage of the *B. bovis* lifecycle. Incubation of erythrocytes with neuraminidase prior to invasion led to a decrease in invasion efficiency of ~80%. This effect was observed for several species. The removal of either α(2-3)-linked or α(2-6)-linked sialic acid residues gave similar levels of reduction whereas simultaneous removal did not show an additive effect. Pre-incubation of merozoites with n-acetylneuraminyl-lactose decreased invasion efficiency by ~45% whereas addition just prior to invasion had no significant effect. The results demonstrate that invasion is dependent on the presence of sialic-acid containing membrane receptors on erythrocytes that interact with merozoite ligands that are probably already accessible during pre-incubation prior to invasion.

INTRODUCTION

Babesia bovis is a tick-borne apicomplexan parasite of cattle and together with *Babesia divergens* and *Babesia bigemina* it is responsible for major economic losses in the cattle industry over the world. Parasites invade host red blood cells where they multiply asexually, rupture the host cell and invade new ones. Invasion is one of the critical steps of the life cycle that is vulnerable to the immunological response of the host and therefore a potential target for vaccination. Erythrocyte invasion is well studied in *Plasmodium* species and was shown to be composed of an initial phase of random cell-cell contact, subsequent reorientation and specific receptor-ligand interaction, followed by active movement into a parasitophorous vacuole [(8)]. An

increasing number of receptor proteins are being identified [41, 46, 166, 176], some of which are being investigated as vaccine candidates [46, 108, 117, 208]. EBA-175 and EBA-140 are erythrocyte-binding proteins of *Plasmodium falciparum* that belong to the Duffy-binding-like-erythrocyte-binding protein family [62, 135, 166]. EBA-175 binds sialic acid residues on glycophorin A [57, 166] and antibodies raised against the recombinant sialic acid binding domain have been shown to block erythrocyte binding and invasion [125, 157, 169]. However, enzymatic removal of sialic-acid residues by neuraminidase leads to selection of *P. falciparum* laboratory lines that efficiently invade erythrocytes, apparently using sialic-acid-independent invasion pathways [13, 61, 68, 157, 178]. Other binding ligands like EBA-140, a paralogue of EBA-175 that binds glycophorin C, may functions in alternative invasion pathways [126, 135]. Knowledge about the invasion mechanism of *B. bovis* and proteins that are involved is limited. A rhoptry protein and two major merozoite membrane proteins have been implicated in invasion but no corresponding erythrocyte surface receptors are known [27, 74, 152, 242].

Whereas the rodent parasite *Babesia microti* or the bovine parasite *B. divergens* occasionally can infect humans, *B. bovis* is host-specific for bovines. In analogy to what has been demonstrated for *P. falciparum* host cell specificity may be dependent on highly selective binding of parasite ligands to erythrocyte surface proteins prior to invasion. Studies on possible erythrocyte ligands have recently been performed for *B. divergens* and *B. bigemina* by enzymatic modification of the erythrocyte surface [110, 245]. Pre-incubation of bovine erythrocytes with neuraminidase or trypsin significantly decreased in vitro multiplication of *B. bigemina*, whereas for *B. divergens* incubation with neuraminidase and α-chymotrypsin resulted in decreased growth.

Recently we established an in vitro invasion assay that uncoupled invasion from subsequent intracellular development [76]. In contrast to the above mentioned studies on *B. bigemina*, *B. divergens* and *P. falciparum*, which examined invasion indirectly after in vitro cultivation for up to 48 h, this assay directly determines invasion. In this way the requirement for surface receptors with specific properties was investigated by enzymatic modification of the erythrocyte surface and by competition for merozoite binding between erythrocytes and free glycans. In addition the ability of *B. bovis* merozoites to invade and develop in red blood cells of different species was investigated. The results demonstrated that *B. bovis* merozoites invade and develop in multiple species in contrast to the host specificity observed in nature.

MATERIALS AND METHODS

B. bovis in vitro *culture*

B. bovis Israel isolate (clonal line C61411) was cultured in vitro as previously described [121] with slight modifications [76, 121]. Briefly, *B. bovis* cultures were maintained in 24-well plates (1.2 ml total volume) or in 25 cm^2 bottles (15 ml total volume) containing medium (M199 with 40% bovine serum and 25 mM sodium bicarbonate) and bovine erythrocytes at 5% packed cell volume (PCV). Cultures were incubated at 37°C, 5% CO_2 in air and parasitaemia was kept between 1% and 12% by daily dilution

B. bovis in vitro *invasion assay*

Invasion was performed as described previously [76]. In short, *B. bovis* infected red blood cells (6 to 8% parasitaemia) were centrifuged (2000 g, 10 min., 15°C) and resuspended in an equal volume of VyMs buffer. 800 µl samples were submitted to five intermittent (10 seconds, 0°C) high voltage pulses (2.5 kV, 200Ω, 25 µF) in 4 mm BioRad cuvettes (165-2088) using a BioRad Gene Pulser with pulse controller. Invasion was performed in 24-well plates by addition of 0.20 ml of liberated merozoites to 1.0 ml of erythrocyte suspension (6% PCV in 25 mM sodium bicarbonate/PBS medium pH 8.0) and incubated under an atmosphere of 5% CO_2 in air. Giemsa-stained slides were prepared after 1 h invasion and a total of 5000 erythrocytes were counted to determine the level of invasion. In all invasion experiments that have been performed, a control was included of invasion in bovine erythrocytes from a standard donor animal. This donor was selected on the basis of a high in vitro growth rate and was used to maintain the *B. bovis* cultures for the last three years. Invasion efficiency was calculated relatively to this control invasion performed in triplicate. Intracellular development, following upon invasion performed as described above, was monitored for 48 h. After invasion for 1 h, PBS (containing 25 mM sodium bicarbonate) was replaced by RPMI medium containing 1 mg/ml albumax II, 25 mM HEPES, 25 mM bicarbonate, 1% glutamine and 50 µg/ml gentamycin. Medium was changed after 24 h. After 24 h and 48 h giemsa-stained slides were prepared and counted as described above.

Collection of sera and erythrocytes

EDTA red blood cells of human, bovine, caprine, ovine, porcine and equine origin were obtained and washed 3 times in VyMs. The buffy coat was removed after each centrifugation at 2000 g for 10 min and erythrocytes were stored in VyMs at 4°C for maximally 2 weeks. Sera were stored at -20 °C for later use.

Trypsin, α-chymotrypsin, neuraminidase and addition of N-acetylneuraminyl-lactose (Neu-Aclac) modification

Incubation of erythrocytes (50% PCV) with trypsin, α-chymotrypsin or neuraminidase was performed by addition of an equal volume of PBS (with 25 mM sodium bicarbonate) containing either L-1-Chloro-3-[4-tosylamido]-4-phenyl-2-butanone-treated trypsin (from bovine pancreas; Sigma T1426), α-chymotrypsin (Type I-S from bovine pancreas; Sigma 7762) or neuraminidase (from *Vibrio cholerae*; Sigma N6514) or neuraminidase (from *Arthrobacter ureafaciens*; Sigma N3642) concentrations indicated in the text followed by shaking (175 rpm) for 1 h at 37°C. Experiments were done in 24-well plates. Controls were performed under the same conditions in absence of enzymes.

Effect of bovine Neu-AcLac (Sigma A8681) on *B. bovis* cultures was investigated. The bovine NeuAc-lac consists of 85% of the NeuAc (2-3) Gal (1-4) Glu isoform and 15% of the NeuAc (2-6) Gal (1-4) Glu isoform. *B. bovis* infected red blood cells (6 to 8% parasitaemia) were liberated as described above. 200 µl samples were incubated with 1 mg/ml bovine NeuAc-Lac for 1 h at 20 °C and added to 1.0 ml erythrocyte suspension (6% PCV). Besides pre-incubation of NeuAc-Lac with *B. bovis* merozoites, NeuAc-Lac was also directly added to *B. bovis* merozoites at the same concentration together with bovine erythrocytes. Controls were performed under the same conditions in absence of compounds. After invasion, Giemsa-stained slides were prepared and counted as described above.

Statistical analysis

Invasion of *B. bovis* merozoites in erythrocytes of different species were tested by Kruskal-Wallis H-tests (these treat the values of percent inhibition of invasion in a non-parametric manner and compares the different groups to each other) and P-values were calculated manually (Fig. 1). Invasion in erythrocytes of three different persons collected on two different dates were compared with Scheffe's Post Hoc test (this test was used because values were normally distributed and more than 2 variables were compared) (Fig. 2A), invasion in three equines were tested with Kruskal-Wallis H-test (Fig. 2B) and invasion in three ovines with Sheffe's Post Hoc test (Fig. 2C). Invasion inhibition with different concentrations of trypsin (Fig. 5A), chymotrypsin (Fig. 5B) and 2-3 neuraminidase (Fig. 5C), 2-6 neuraminidase (Fig. 5D) in bovine erythrocytes was tested with Kruskal-Wallis H-test. Invasion inhibition in bovine erythrocytes with different forms of neuraminidase (2-3 or 2-6 isoforms) separately or combined together with or without α-chymotrypsin and trypsin were tested with Sheffe's Post Hoc test (Fig. 5E). Invasion in human or porcine erythrocytes with or without neuraminidase were tested with Mann-Whitney U test (these treat the values of percent inhibition of invasion in a non-parametric manner (Fig. 5F). Invasion inhibition in bovine erythrocytes modified with or without NeuAc-lac was tested with Sheffe's Post Hoc test (Fig. 6). P-values lower than 0.05 were considered statistical significant.

RESULTS

Invasion efficiency in non-bovine erythrocytes

Invasion of *B. bovis* merozoites into erythrocytes of different species was investigated in order to test the hypothesis that the strict specificity observed in vivo for bovine hosts is due to host-specific receptor-ligand interactions between merozoites and erythrocytes. Fig. 1 shows the efficiency by which high-voltage pulse liberated merozoites invade erythrocytes of human, bovine, caprine, equine, ovine and porcine origin relative to invasion in erythrocytes of a standard bovine donor (absolute invasion levels in between experiments performed over the year are variable, possibly because of variable, but unknown, factors determining the quality of liberated merozoites of a typical experiment [(76)]. The presented data are the mean of values obtained from at least three different individuals of each species, where each individual was tested twice in triplicate. Clearly, *B. bovis* merozoites are able to invade all tested species within 1 h, albeit at a wide range of efficiency. Surprisingly, human erythrocytes were invaded, on average, better than bovine erythrocytes. Caprine erythrocytes were poorly invaded whereas the relative efficiency of invasion in equine, ovine and porcine erythrocytes varied between 47% and 53%. Two sources of variation contributing to the standard deviations observed in Fig. 1 are displayed in more detail in Fig. 2. A comparison of individuals of the same species (n=3), relative to the standard bovine control, was made by performing triplicates with erythrocytes from two different blood collection dates. Clearly, the time point of collection has a minor effect on the standard deviation observed in Fig. 1.

In contrast, significant differences were found between individuals of the same species. Erythrocytes of three human individuals were invaded at an efficiency ranging between 98% and 170% (Fig. 2A). Significant variation in invasion efficiency occurred in between individual equines (Fig. 2B) and ovines (Fig. 2C). We conclude that *B. bovis* merozoites invade erythrocytes of all species tested, with significant differences in efficiency in between species as well as in between individuals of the same species.

Intracellular development in non-bovine erythrocytes

Having established the ability to invade non-bovine erythrocytes, the capacity for further intracellular development was investigated. In PBS no further development takes place but as demonstrated recently [(76)], a change to serum-rich medium conditions upon invasion performed in PBS, allows intracellular development resulting

in normal subsequent cycles of multiplication. Addition of medium containing the autologous serum, 1 h after invasion in erythrocytes of each of the different species, resulted in duplication and normal outgrowth only in bovine erythrocytes supplemented with bovine serum. The porcine and human autologous combinations showed no duplicated parasites after 24 h and a further decline in parasitaemia after 48 h (Fig. 3).

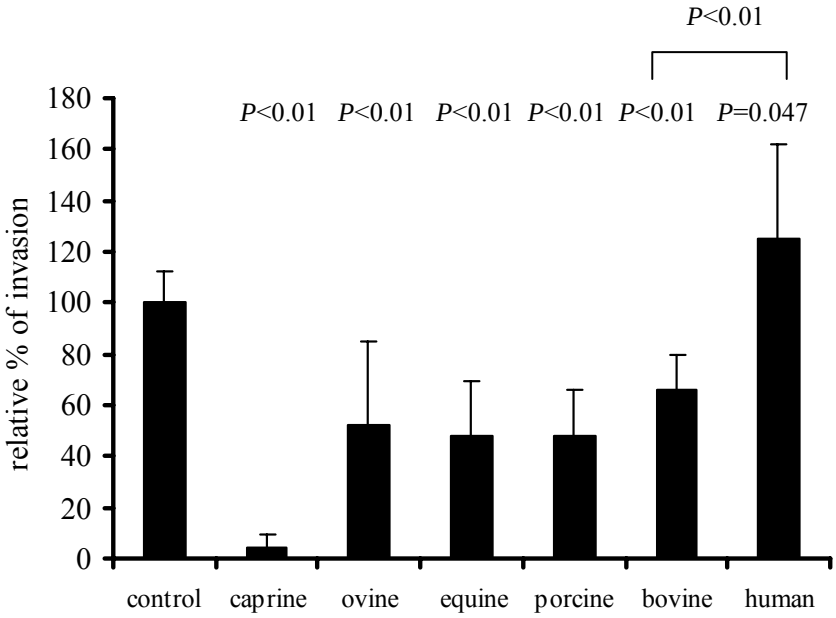

Fig. 1. Invasion of *Babesia bovis* merozoites in bovine, caprine, ovine, equine, porcine and human erythrocytes. Percentage invasion is shown relatively to invasion in standard bovine donor erythrocytes (control) that was considered as 100% invasion. Error bars indicate standard deviation.

To determine whether serum components are responsible for this lack of further development upon invasion in non-bovine erythrocytes, the experiment was repeated in artificial medium containing albumax instead of serum. As shown in Fig. 4, addition of this medium upon invasion gives rise to normal development and subsequent cycles inside bovine erythrocytes. Also, a large percentage of the parasites having invaded non-bovine erythrocytes after 1 h, have duplicated after 24 h and appear in the characteristic accole position normally observed in bovine erythrocytes. However, no increase in parasitaemia is observed after 48 h although most of the parasites still have a morphologically normal appearance at this time point. Human erythrocytes having been invaded by *B. bovis* merozoites were kept in in vitro culture for another 16 days under daily change of medium but Giemsa-stained smears showed a gradual decline of parasitaemia below detection level indicating that no rapid phenotypic adaptation to growth in human erythrocytes takes place.

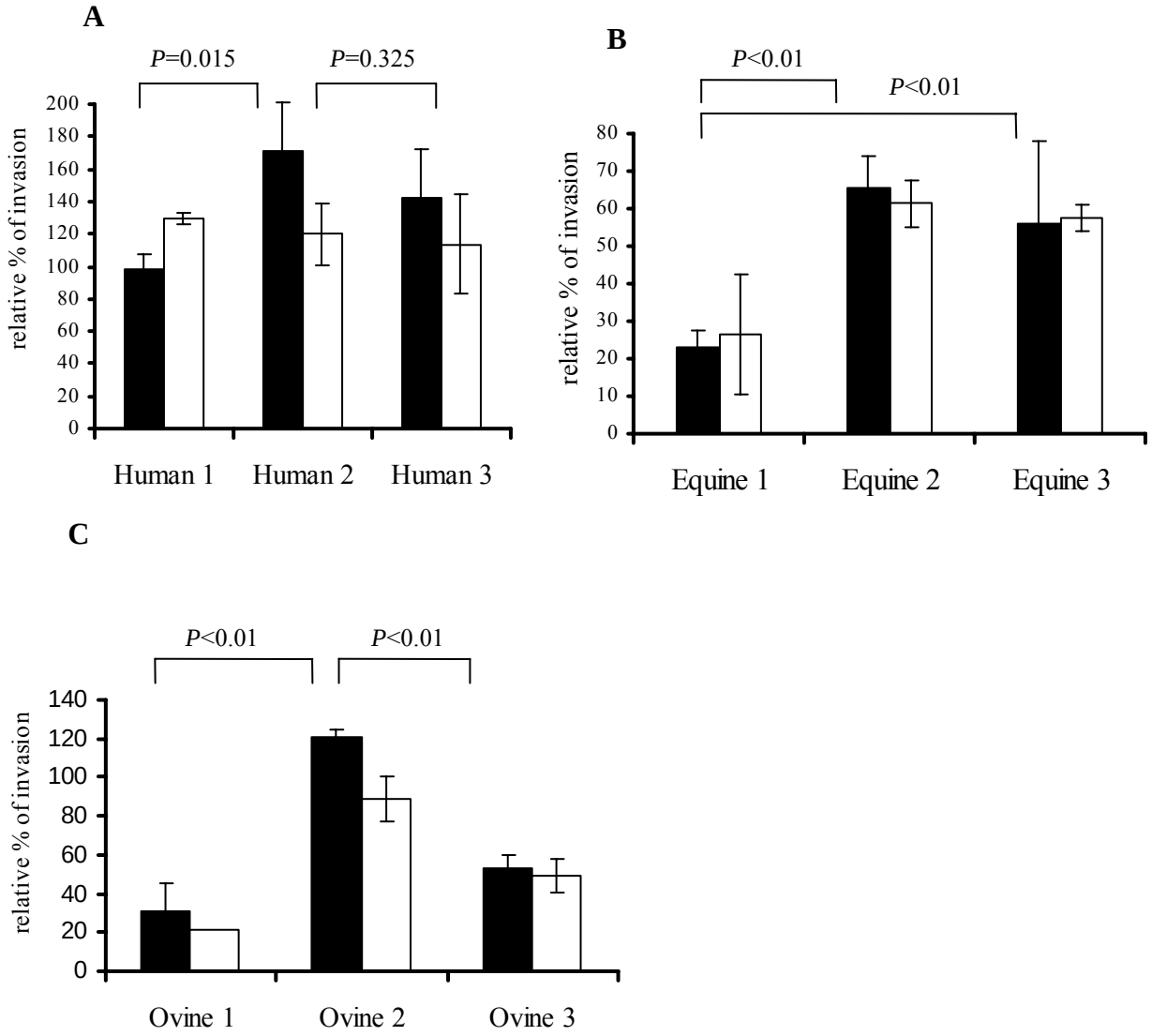

Fig. 2. Relative percentage of invasion in erythrocytes of individuals (triplicates) from two different time point (black bar versus white bar). Parasite invasion in erythrocytes of three individuals of human (A) equine (B) or ovines (C) are shown here. Origin is plotted relative to standard bovine donor erythrocytes. Error bars indicate standard deviation.

In conclusion, the albumax supplement contains factors enabling normal duplication within bovine and non-bovine erythrocytes whereas the sera of the non-bovine species may miss this factor or have inhibitory factors in addition that prevent duplication.

The effect of enzymatic modification of erythrocyte surface molecules prior to invasion

The effect of cleavage of the peptide backbone of erythrocyte surface proteins prior to invasion by proteases with different specificities like trypsin and chymotrypsin was examined (Fig. 5). Bovine erythrocytes were incubated for 1 h with a

concentration series of trypsin or α-chymotrypsin prior to invasion by *B. bovis* merozoites.

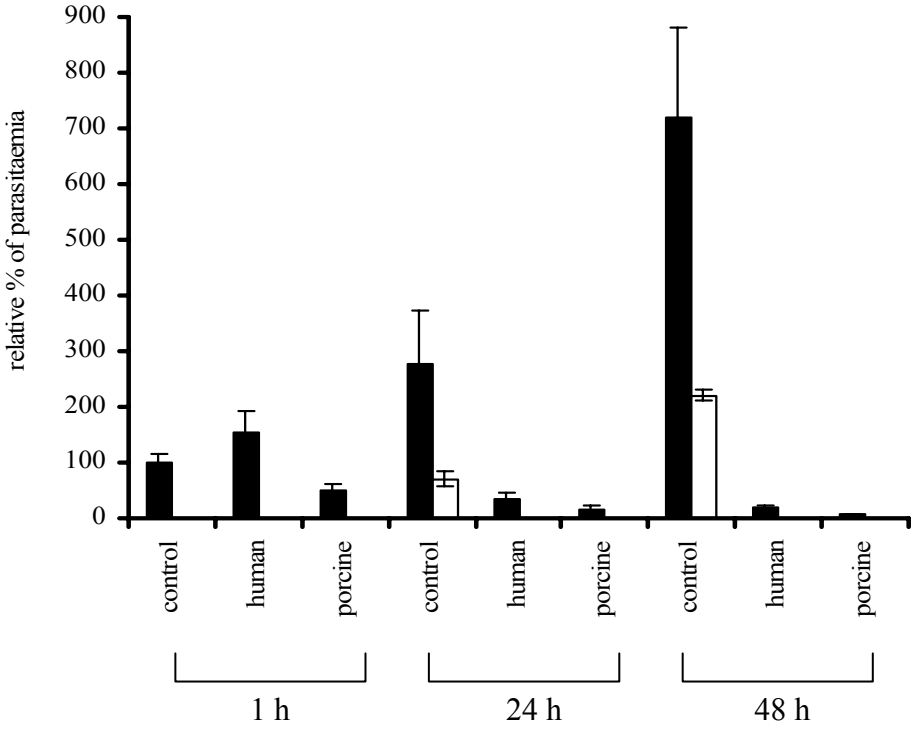

Fig. 3. Invasion and development of *Babesia bovis* merozoites in bovine, porcine and human erythrocytes after addition of autologous sera supplemented media. Black bars represent total parasitaemia as a relative percentage of the same control after 1 h, 24 h and 48 h. White bars indicate duplicated parasites. All values are indicated as parasitaemia relative to the parasitaemia obtained with standard bovine donor erythrocytes after 1 h. All experiments were done in triplicate and error bars indicate standard deviation.

Incubation with trypsin or α-chymotrypsin resulted in only a slight, but significant reduction (resp. 26% ± 2.2%, $p< 0.001$, 33% ± 5.7%, $p<0.001$) of invasion at the highest concentration tested (resp. 300000 U/ml trypsin, 3000 U/ml α-chymotrypsin).

Removal of sialic acid residues by preincubation of erythrocytes with neuraminidase resulted in a maximal reduction of invasion of 75 to 80 % at a concentration 30 U/ml. Remarkably, the use of neuraminidases with specificity for either NeuAc-α(2-3)Gal linkages (Fig. 5C) or NeuAc-α(2-6)Gal linkages (Fig. 5D) gave similar results whereas preincubation of erythrocytes with a combination of both neuraminidases or neuraminidase in combination with trypsin or chymotrypsin does

not reduce invasion any further (Fig. 5E). The dependence of invasion on surface exposed sialic acid groups as observed above was also shown after preincubation of erythrocytes of other species with neuraminidase (Fig. 5F).

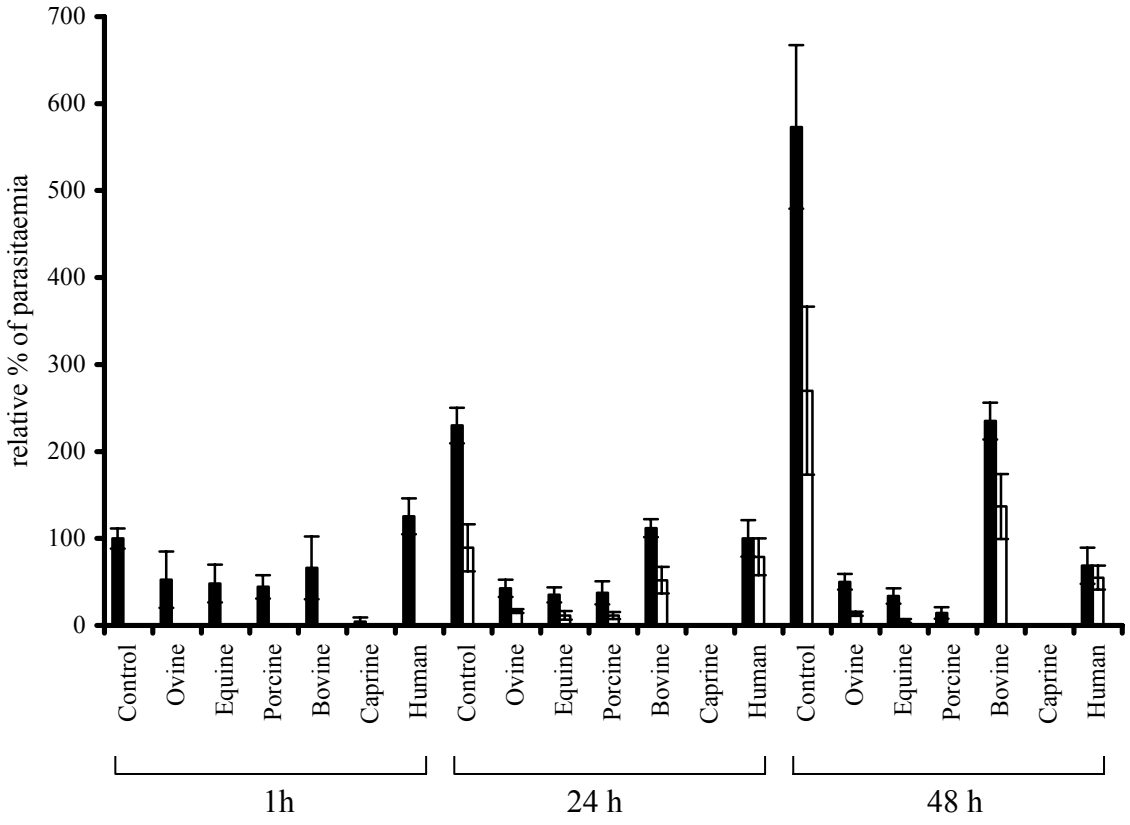

Fig. 4. Invasion and development of *B. bovis* merozoites in bovine, porcine, caprine, ovine, equine and human erythrocytes after addition of AlbumaxII supplemented medium. Black bars represent total parasitaemia as a relative percentage of the same control after 1 h, 24 h and 48 h. All values are indicated as parasitaemia relative to the parasitaemia obtained with standard bovine donor erythrocytes after 1 h. White bars indicate duplicated parasites. All experiments were done in triplicate and error bars indicate standard deviation.

To obtain further proof for the putative interaction of merozoites with sialic acid residues exposed on erythrocytes prior to or during invasion, sugars containing sialic acid residues were evaluated for their capacity to compete with invasion. Fig. 6 shows that direct addition of a mixture of NeuAc(2-3)Gal(1-4)Glu and NeuAc(2-6)Gal(1-4)Glu to the invasion assay gives a slight but statistically insignificant (P= 0.074) decrease in invasion. However, a 1 h preincubation of merozoites with the same mixture gives a significant 44% reduction in invasion.

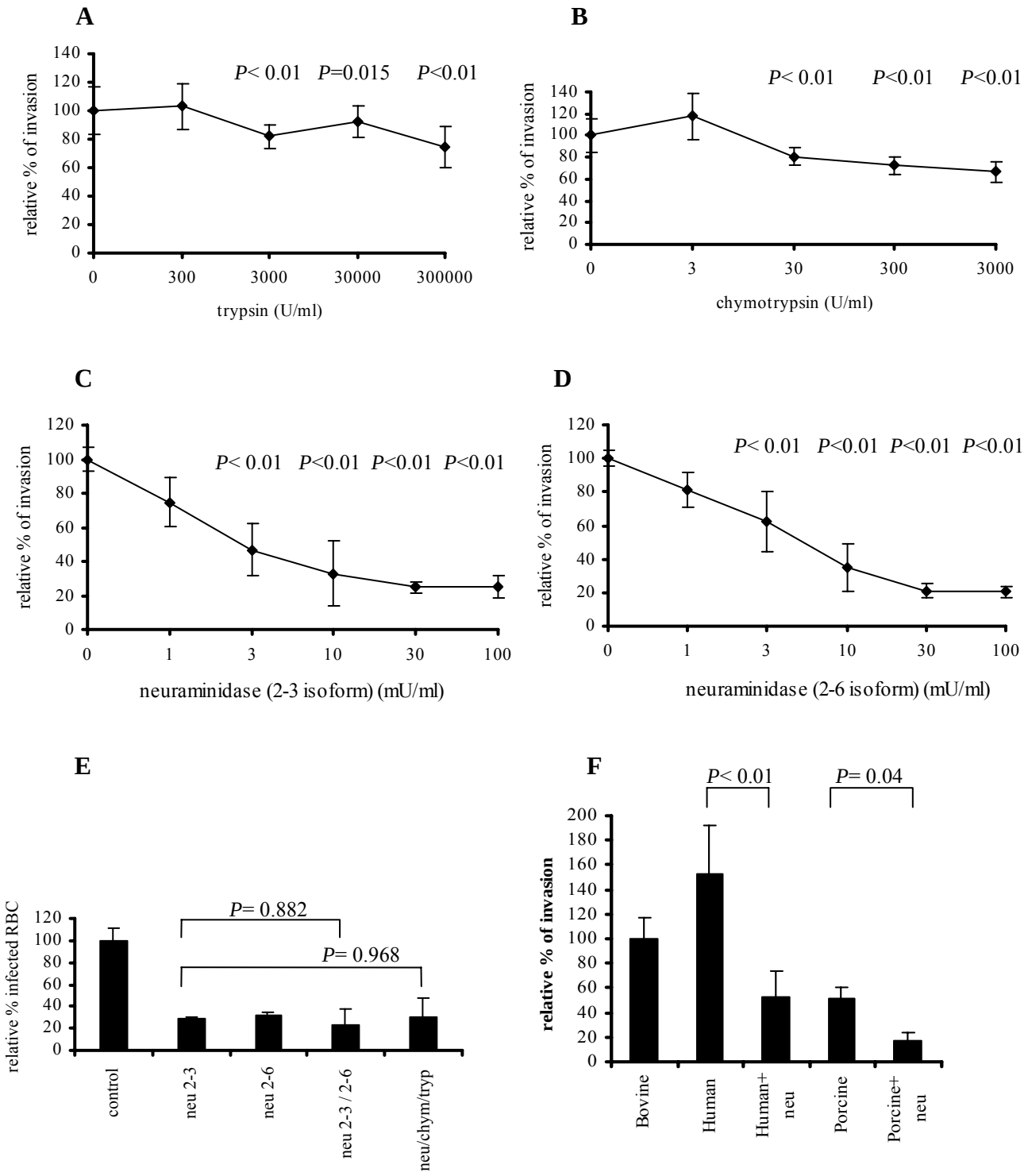

Fig. 5. Invasion in enzyme-modified erythrocytes. Invasion in standard bovine donor erythrocytes modified with different concentrations of (**A**) (0-300000 U/ml) trypsin, α-chymotrypsin (0- 3000 U/ml) (**B**), 2-3 neuraminidase (0-100 mU/ml) (**C**), 2-6 neuraminidase (0-100 mU/ml) (**D**), and combination of 2-3 and 2-6 neuraminidase (50 mU/ml) with or without trypsin (300000 U/ml) and chymotrypsin (3000 U/ml) as determined (**E**). (**F**) Invasion in human and porcine erythrocytes modified with neuraminidase (50 mU/ml) was also performed. All experiments were done in triplicate and plotted relative to the level of invasion in erythrocytes preincubated without addition of enzyme. Error bars indicate standard deviation.

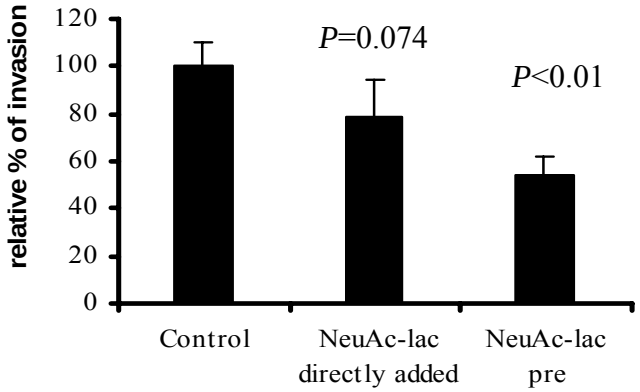

Fig. 6. Inhibition of invasion by N-acetylneuraminyl-lactose. Invasion in standard bovine erythrocytes with or without preincubating merozoites with NeuAc-lac (1mg/ml) for 1 h. All the experiments were done in triplicate and plotted relative to the level of invasion in erythrocytes preincubated without addition of NeuAc-lac. Error bars indicate standard deviation.

DISCUSSION

B. bovis belongs to a sub-set of apicomplexan parasites that are highly specific for host species and cell type. For *Plasmodium* species, specificity finds a molecular basis in selective receptor-ligand interactions involved in erythrocyte binding and invasion. For *Babesia* species, parasite ligands and erythrocyte receptors involved in erythrocyte invasion have not been identified. *B. divergens* is predominantly a parasite of cattle but is able to replicate in other hosts like humans [(102)] sheep [(37)] and rodents [(14)]. Likewise, *B. microti* causes disease both in rodents and humans. In contrast, *B. bovis* is strictly host specific for bovine species, with a reported preference for *Bos taurus* over *Bos indicus* [(19)]. Our current aim was to identify steps in the *B. bovis* life cycle that are incompatible with growth in other species. In vitro growth of *B. divergens* in erythrocytes of different origin was compared by hypoxanthine incorporation over periods of 48 h after sub-culturing [(244)] but the intrinsic inability of *B. bovis* for continuous growth in non-bovine erythrocytes required us to use specific assays in which consecutive steps in the life cycle were studied separately. *B. bovis* merozoites were shown to invade erythrocytes of a variety of species albeit at different levels of efficiency. On average, human erythrocytes were invaded more efficiently than bovine erythrocytes, whereas erythrocytes of goats were invaded at very low levels. Within species, considerable variation in the level of invasion was observed. Subsequent intra-

cellular development and duplication to form characteristic pear-shaped piroplasms in the accole position as normally occurring in bovine erythrocytes, was also observed in other species except for goat erythrocytes. Despite this apparently normal intra-cellular development within erythrocytes of multiple species, only in bovine erythrocytes a rapid rise in parasitaemia was observed in serum-free growth medium. As human erythrocytes are invaded efficiently, a developmental block in later stages could possibly explain this observation. For instance, egress from the host cell after intra-cellular maturation could be inefficient. For *Plasmodium* species the involvement of proteases has been demonstrated [84, 86] and possibly substrate-specificity contributes to species specificity. Alternatively, although morphologically normal duplicated piroplasms were observed, less than 50 % of invaded parasites may develop to this stage. In contrast to *Plasmodium* species, *B. bovis* exhibits only a two-fold multiplication per generation during asexual development and consequently, any step or combination of steps, less efficient than 50 % will lead to a decline in parasitaemia. The fact that no accumulation of duplicated parasites was observed on the long term gives some support to the latter option. All the other abnormal host erythrocytes are invaded at least 50 % less efficient than bovine erythrocytes implicating that in these erythrocytes the sub-optimal invasion would lead to a decline in parasitaemia even when subsequent intra-cellular development and egress are highly efficient. It also implicates that without the use of a direct method of estimating invasion after 1 h as employed here, invasion into abnormal host erythrocytes could not have been detected. The recently reported invasion of *B. divergens* into abnormal host erythrocytes [244] was studied by sub-culturing in these cells for extended periods, a method that clearly cannot work for *B. bovis*. Nevertheless a number of similarities can be noted when comparing to this work. For instance, subcultures of *B. divergens* performed slightly better in human erythrocytes than in bovine erythrocytes although, in contrast, for *B. bovis* this only holds for invasion and not for subsequent growth. Moreover, addition of autologous serum had a negative effect in the case of human and equine serum like shown here for *B. bovis*. On the other hand, ovine serum did support growth of *B. divergens*. A report of in vitro cultivation of *B. bovis* in bovine erythrocytes supported by equine sera showed a decreased parasite growth. The decline in growth observed in presence of equine sera could partly be relieved by the addition of hypoxanthine [159]. However, in vitro growth in abnormal host erythrocytes has never been described and in nature no *B. bovis* infections in non-bovine hosts have been described. The results presented here demonstrate that invasion and early intra-cellular development and duplication do not share this strict host specificity. The fact that *B. divergens* and *B. microti* infections are detected in humans, in contrast to *B. bovis*, might in part also be due to specificity of the tick vector. Whereas *Ixodes* species, transferring *B. divergens*

and *B. microti*, feed on humans as well as on other species, *Boophilus* species transmitting *B. bovis* are highly specific for cattle and seldom feed on humans.

In vitro growth of *B. bigemina* and *B. divergens* has been shown to be affected by removal of sialic acid residues by neuraminidase, most likely as a consequence of reduced invasion levels [110, 245]. Here we have directly established an inhibitory effect of removal of sialic acid residues on invasion of *B. bovis*. Specific removal of terminal sialic acid residues attached by an α(2-3) or an α(2-6) linkage to galactose, individually or combined, resulted in 75 to 80% inhibition of invasion, suggesting invasion by an erythrocyte recognition mechanism requiring the simultaneous presence of both linkage types. Neuraminidases of both specificities contain minor amounts (less than 0.5%) of the other specificity but the concentration curves of Fig. 5c and d indicate that such low level contaminations hardly influence cleavage specificity at the concentrations used. Whereas for *P. falciparum* the existence of parallel sialic-acid dependent and independent invasion mechanisms was suggested by an additive inhibitory effect of co-incubation with neuraminidase and proteases [68] such an effect was not observed for *B. bovis*. Maximum inhibition was already obtained by neuraminidase incubation alone, indicating that invasion is completely dependent on sialic acid and suggesting that inhibiton by trypsin and chymotrypsin might be due to an incomplete cleavage of erythrocyte membrane receptor protein(s) carrying sialic acid residues involved in invasion.

Erythrocyte membrane glycophorins carrying sialic acid residues have been shown to be specific receptors for *P. falciparum* merozoite binding ligands and contribute to the formation of a tight junction between merozoite and erythrocyte during the initial phase of invasion. The binding ligand EBA-175 becomes exposed on the merozoite membrane by secretion from micronemes only just before invasion actually takes place [200], putatively by a signal triggered by initial contact between erythrocyte and merozoite. Here, an inhibitory effect on invasion of *B. bovis* by a mixture of NeuAc(2-3)Gal(1-4)Glu and NeuAc(2-6)Gal(1-4)Glu was observed after preincubation with *B. bovis* merozoites but not after direct addition without preceding preincubation. This suggests that NeuAc(2-3)Gal(1-4)Glu and NeuAc(2-6)Gal(1-4)Glu binding to a putative binding ligand for sialic acid containing receptors on the erythrocyte membrane is relatively weak and requires time. Moreover, the putative binding ligand must be constitutively present on the merozoite membrane already during preincubation and not secreted only upon initial contact with fresh erythrocytes (in the latter case no difference in inhibition between invasion with and without preincubation should have been observed). Obviously, this does not exclude the presence of additional binding ligands secreted from internal organelles as in the case of *P.*

falciparum. In addition, a toxic effect of the added sugars during preincubation cannot be excluded due to the lack of a proper control for this possibility.

Invasion into erythrocytes of other species was also shown to be neuraminidase sensitive. This raises the question whether the amount of sialic acid residues present on membrane proteins is the main factor determining invasion efficiency. No detailed comparison of the total amount of sialic residues on membrane proteins of erythrocytes of different species has been reported. A recent report [3] demonstrated that from human erythrocytes ~80% more sialic acid could be released as from sheep and goat erythrocytes. This could explain the highly efficient invasion of human erythrocytes but does not explain why goat erythrocytes are hardly invaded at all. Possibly the type of linkage (e.g. α(2-3) or α(2-6)) also plays a role and might be different in between species. A known difference between goat erythrocytes and all other erythrocytes tested is their small size with an average diameter of 3.2 μm which is approximately half the size of bovine and human erythrocytes. Besides interspecies differences in invasion we also found significant and reproducible differences in between individuals of the same breed and species. Exploring differences in erythrocyte properties between such individuals offers the opportunity for a more detailed characterization of erythrocyte receptors employed for invasion by *B. bovis*.

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CHAPTER 4

ERYTHROCYTE INVASION BY *BABESIA BOVIS* MEROZOITES IS INHIBITED BY POLYCLONAL ANTISERA DIRECTED AGAINST PEPTIDES DERIVED FROM A HOMOLOGUE OF *PLASMODIUM FALCIPARUM* APICAL MEMBRANE ANTIGEN 1 (AMA-1)

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ABSTRACT

Apical membrane antigen 1 (AMA-1) is a micronemal protein secreted to the surface of merozoites of *Plasmodium* species and *Toxoplasma gondii* tachyzoites in order to fulfil an essential but non-characterized function in host cell invasion. Here we described the cloning and characterization of a *Babesia bovis* homologue denoted BbAMA-1. Overall similarity with *P. falciparum* AMA-1 was low (18%) but characteristic features like a transmembrane domain near the C-terminus, a predicted short cytoplasmic C-terminal sequence with conserved sequence properties and an extra-cellular domain containing 14 conserved cysteine residues putatively involved in disulfide bridge formation are typical for AMA-1. Rabbit polyclonal antisera were raised against three synthetic peptides derived from the N-terminal region and domain II and III of the putative extracellular domain and were shown to recognize specifically recombinant BbAMA-1 expressed in *Escherichia coli*. Immunofluorescence microscopy showed labelling of the apical half of merozoites with these antisera. Preincubation of free merozoites with all three antisera reduced invasion efficiency into erythrocytes by maximally 65%. Antisera raised against the N-terminal peptide detected a protein of 82 kDa on western blots and a protein of 69 kDa in the supernatant that was harvested after in vitro invasion, suggesting proteolytic processing and secretion to take place during or shortly after invasion. A combination of 2D-western blotting and metabolic labelling allowing the direct identification of spots reacting with the BbAMA-1 peptide antisera in combination with the very weak silver staining intensity of these spots indicates that AMA-1 is present at very low levels in *Babesia* merozoites.

INTRODUCTION

Babesia bovis is an obligatory intraerythrocytic bovine parasite that belongs to the phylum of apicomplexa. Although members of the apicomplexa infect different host and cell types, they share similar host cell invasion processes. In the case that extracellular merozoites enter erythrocytes, they form an initial reversible attachment, leading to reorientation of the merozoite to bring the anterior apical pole in contact with the plasma membrane of erythrocytes [67, 205]. A tight junction is formed through which the parasite will invade the red blood cell. The process is completed when the parasite is inside a parasitophorous vacuole of the red blood cell. From the first attachment till completion of the invasion process the parasite secretes proteins from apical organelles to the merozoite membrane and into the environment. Proteins

secreted from micronemes, rhoptries and dense granules are thought to play a central role in invasion and the establishment of infection of apicomplexan parasites [31, 205]. This supposed critical function and their exposure to the immune system, when localized on the surface of the merozoite, has marked them as potential vaccine candidates [7]. One of the candidates is the apical membrane antigen-1 (AMA-1) [63, 89, 101, 117, 158, 221], which is expressed in the late schizont stage of the asexual life cycle of the *Plasmodium falciparum* parasite [158]. AMA-1 is a type I integral membrane protein with three characteristic structures: (i) a N-terminal, cysteine-rich, ectodomain, (ii) a single transmembrane domain and (iii) a C-terminal cytoplasmic tail. The ectodomain is organized into domains I, II and III by the formation of disulfide bridges between conserved cysteine residues. Full-length *P. falciparum* AMA-1 (83 kDa) is a micronemal protein [100] that is transported to the merozoite surface membrane as a 66 kDa protein upon proteolytic cleavage in the N-terminal ectodomain [100]. During invasion of merozoites, *P. falciparum* AMA-1 is further processed to 44 kDa and 48 kDa soluble fragments [100, 101]. Although the biological function of AMA-1 is unknown, the subcellular localization, stage-specific expression and secretion during host cell invasion suggest that it is involved in merozoite invasion. A strong correlation was found between protection and *P. falciparum* AMA-1 antibodies that were generated against different peptide sequences [186]. Furthermore, passive transfer of rabbit AMA-1 antibodies protected mice against *P. chabaudi* infection [6] and antibodies against *P. reichenowi* [116] and *P. vivax* AMA-1 [115] were shown to inhibit red blood cell invasion. Recently, eight peptides of the *P. falciparum* AMA-1 protein were mapped that have specific erythrocyte binding activity [77, 225]. An AMA-1 homologue is present in all *Plasmodium* species studied and *T. gondii*, which supports the suggestion that this protein is involved in an essential function [39, 63, 70, 116, 131]

Here we report the complete sequence of the *B. bovis* AMA-1 cDNA. We have studied the protein on 1D and 2D western blots, by immunofluorescence microscopy and analysed inhibition of in vitro invasion by antisera directed against specific regions.

MATERIALS AND METHODS

B. bovis in vitro *culture*

B. bovis Israel isolate (clonal line C61411) was cultured in vitro in bovine erythrocytes as previously described [76]. Briefly, *B. bovis* cultures were maintained in 24-well plates (1.2 ml total volume) or in 25 cm^2 bottles (15 ml total volume) containing medium (M199 with 40% bovine serum and 25 mM sodium bicarbonate and bovine erythrocytes at 5% packed cell

volume (PCV). Cultures were incubated at 37°C, 5% CO_2 in air and parasitaemia was kept between 1% and 12% by daily dilution.

For metabolic labelling, *B. bovis* culture (8 to 10 % parasitaemia) was centrifuged (2000 *g*, 10 min, 15°C) washed once with PBS and resuspended in RPMI 1640 medium without methionine and cysteine containing 20 mM TAPSO (Sigma) and 1% Glutamine (Sigma). ^{35}S labelled methionine and cysteine (167 µCi/ml) were added followed by incubation for 18 h at 37ºC in 5% CO_2 in air.

B. bovis in vitro *invasion*

Invasion was performed as described previously [76] with slight modifications. *B. bovis* infected red blood cells (6 to 8% parasitaemia) were centrifuged (2000 *g*, 10 min, 15°C) and resuspended in an equal volume of VyMs buffer (4°C). Samples (800 µl) were submitted to five intermittent (10 seconds at 0°C in between pulses) high voltage pulses (2.5 kV, 200Ω, 25 µF) in 4 mm cuvettes (BioRad) using a BioRad Gene Pulser with pulse controller. The lysed samples (800 µl) were washed with 8 ml of PBS containing 25 mM sodiumbicarbonate ((PBSbc) pH 8.0, 20°C) followed by centrifugation (1800 *g*) for 10 min at 15°C. A second similar wash was performed except that centrifugation speed was lower (1300 *g*). The final merozoite pellet was resuspended in 800 µl PBSbc. Invasion was initiated by addition of 1 volume of resuspended merozoites to 9 volumes of suspended bovine erythrocytes (5.5% PCV in PBSbc pH 8.0, preincubated for 60 min at 37°C in 5% CO_2 in air) and was performed in 24-well plates (final volume 1.2 ml), in 25-cm^2 flasks (15 ml) or in 80-cm^2 flasks (50 ml) at 37°C, 5% CO_2 in air. Giemsa-stained slides were prepared after 1 h and parasitized erythrocytes out of a total of 5000 erythrocytes were counted.

In vitro *inhibition of invasion by rabbit antisera*

B. bovis merozoites (200 µl), liberated by high voltage pulsing and resuspended in PBSbc as described above, were incubated with 40 µl of rabbit antisera for 1 h at 20°C. After 1 h, 960 µl of suspended bovine erythrocytes (6.25% PCV in PBSbc, preincubated for 60 min at 37°C in 5% CO_2 in air) were added, followed by 1 h of incubation after which Giemsa-stained slides were prepared and counted to determine the level of invasion. The used rabbit antisera were raised against synthetic peptides derived from the BbAMA-1 sequence and a control serum raised against an unrelated peptide (YAGRLFSKRTAATAYKLQ), named peptide C. Peptides were linked to Maleimide activated Keyhole Limpet Haemocyanin (KLH; Pierce) prior to immunization. Pre-immune sera were also included in the test.

Preparation of total merozoite protein extracts and proteins solubilized upon invasion

Samples of merozoites (800 µl), prepared as described above for in vitro invasion, were partially separated from erythrocyte ghosts by filtration over 1.2 µM polypropylene prefilters (Millipore). Filtered merozoites were pooled and washed twice in 20 volumes of PBSbc followed by centrifugation at 2000 g for 20 min at 4°C. After the second wash the

pellet was resuspended in an equal volume of PBSbc and divided in aliquots of 200 µl that were centrifuged (10000 *g*, 5 min at 4°C) and stored as 100 µl cell pellets (2×10^9 merozoites) at -20°C after removal of supernatant. Frozen merozoite pellets were thawed just before use and lysed, reduced and alkylated by using a total protein extraction kit (Proteoprep, Sigma) according to the manufacturer's instructions and finally obtained in 1.7 ml of buffer compatible with direct application on SDS-polyacrylamide gels or isoelectrofocusing (IEF) strips. Insoluble material was removed by centrifugation at 16000 g for 3 min at 4°C. As the extracts contained considerable amounts of erythrocyte proteins, control extracts were prepared in the same way but starting with a culture of non-infected erythrocytes.

Proteins solubilized upon invasion were obtained by gently removing the overlaying buffer after 1 h of in vitro invasion as described above. The samples were centrifuged (2000 *g*, 10 min, 4 °C) after which the supernatant was centrifuged again at high speed for removal of membrane fragments (20 min, 12000 *g*, 4°C). The final supernatant was dialysed (Pierce; Snakeskin®pleated dialysis tubing) overnight against 10 mM Tris NaCl, pH 7.5.

Construction and screening of a B. bovis *cDNA library*

A cDNA library was constructed from 5 µg *B. bovis* mRNA using the λZAP-cDNA® Synthesis Kit (Stratagene) according to the manufacturer's instructions. cDNA fragments of 0.5 to 4 kb were collected by gel filtration on a sepharose CL-4B column and ligated into the *Eco*RI / *Xho*I site of λ uniZAP-XR Express vector. Giga pack III Gold was used for packaging into phage particles followed by transformation of *Escherichia coli* XL-1 Blue MRF'cells. A total of 3×10^5 plaques were obtained of which an amplified library was made.

The cDNA library was screened with probe F1. Oligonucleotides p1 (5'–ccacggctctggaatctatgtc-3') at position 329 and p2 (5'-caaaaggatacctatatttggtac-3') at position 703 (derived from an EST available at www.sanger.ac.uk and numbered here according to the numbering of Genbank Acc. No AY486101) were used to amplify probe F1 by PCR in a 50 µl volume containing 0.2 mM dNTP, 20 pmol μl^{-1} of each primer, 100 ng *B. bovis* genomic DNA and 0.5 U Taq DNA polymerase in standard buffer (Promega). Amplification was performed for 30 cycles (92°C for 30 sec, 58°C for 30 sec, at 72°C for 30 sec) preceded by initial denaturation for 3 min at 95°C and a final elongation at 72°C for 10 min. The fragment was purified from agarose gel and labelled with 50 µCi [α-^{32}P]dATP (3000 Ci $mmol^{-1}$), using a Random Primer labelling kit (Roche). 1.2×10^6 plaques were screened by standard procedures [187] for obtaining the AMA-1 sequence. After 2 cycles of plaque purification two clones were *in vivo* excised for isolation of the phagemids inserts as described in the manufacturer's instructions (Stratagene) and sequenced on both strands, using automated cycle sequencing with the dye terminator method (ABI PRISM dye terminator kit, Pharmacia). A full-length AMA-1 cDNA including the non-coding 5'-end was obtained with GeneRacer™ kit with a specific primer 5'-gatgaaatgggatcgaggaagtcg-3' (Invitrogen) according to the manufacturer instructions and the obtained clone was sequenced on both strands.

Expression of recombinant BbAMA-1 in E. coli

A cDNA clone of BbAMA-1 was amplified by PCR using primers that introduced a *Bam*HI site prior to base 1 (numbered from the first base of the initiation codon) and a *Hin*dIII site after base 1504. Primers p3 (5'-cccggatccatgcagttacataacaaa-3') and p4 (5'-gggaagcttctgagcaaaggaaatagg-3') with *Bam*HI and *Hin*dIII sites were used to clone the AMA-1 PCR products in vector pET-32a (Novagen) allowing their expression as a fusion product with a N-terminal thioredoxin domain and an internal 6-histidine tag. After PCR (1min 94°C, 1 min 55°C, 1 min. 72°C; 30 cycles) on cDNA clones, the fragment was gel purified, ligated in pET-32a vector and used for transformation of *E. coli* NovaBlue strain. Plasmids containing the appropriate insert were used to transform expression host strains, BL21 (DE3). Fusion proteins with thioredoxin were obtained after induction with 1 mM of isopropyl-β-D thiogalactosidase (IPTG) for 4 hr at 37°C as shown by analysis of total cell samples at 0 and 4 hr after induction (Fig. 2). Bacterial pellets were heated at 95°C in SDS-polyacrylamide (SDS-PAGE) sample buffer containing 2% (v/v) β-mercaptoethanol, run on 10% SDS-PAGE minigels, and Coomassie Blue-stained to confirm expression.

Peptide selection and immunisation

Synthetic peptides for immunisation were derived from the full length BbAMA-1 sequence by selection of amphiphatic alpha-helices having a high probality for surface localization and several charged residues using the Protean software package (Lasergene) for protein sequence analysis. Peptides were selected from the N-terminal region (aa 46-60; cysteine-AFHKEPNNRRLTKRS: peptide 1), domain II (aa 395-409; cysteine-RGVGMNWATYDKDSG: peptide 2) and domain III (aa 453-467; cysteine-YVEPRAKNTNKYLDV: peptide 3). The peptides were synthesized and coupled to KLH as carrier protein according to manufacturer's recommendations (Pierce). The peptide-carrier conjugate was used to generate rabbit polyclonal antisera.

Three groups of NZW-rabbits, each group containing two rabbits, were immunized five times subcutaneously at 3-week interval for eight months. Before the first immunisation blood serum was collected from each rabbit, which was used as negative control. Each rabbit was injected with 250 µg peptide that was coupled to 250 µg of KLH and equal volume of adjuvant Stimune® (ID-DLO, The Netherlands), in a total volume of 1000 µl. Sera were tested periodically for reactivity by ELISA. Plasmaforeses were performed one week after the immunization starting at month 4.

ELISA

The production of rabbit antibodies against the peptides was monitored by ELISA. Ninety-six-well microtiter plates were coated with 150 ng of peptide 1, peptide 2 or peptide 3 per well in 0.1 M Tris-HCl pH 8.0, incubated 30 min at 37°C and blocked for 1 h with PBS/0.25% BSA at 37°C. Consecutive dilutions (1:50 to 1:50000) of individual rabbit sera were incubated for 1 h at 37 °C. The plates were washed and swine anti rabbit-IgG Horseradish Peroxidase (HRP)-conjugated (DAKO) (1:2000) secondary antibody was

incubated for 1 h. The plates were washed and developed for 45 min with ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)]- peroxidase substrate (Roche biochemicals). The OD_{405} was recorded, and comparative ELISA titres were calculated.

SDS-polyacrylamide electrophoresis and western blotting

Total merozoites extracts or proteins solubilized upon invasion were separated on a 10% SDS-PAGE and transferred to a PVDF membrane (Immobilon™-P, Millipore). The blot was blocked with 5% skimmed milk diluted in phosphate-buffered saline containing 0.05% Tween (PBST) for 1h at 37°C. The rabbit's antisera were diluted (1:500) in PBST containing 2% skimmed milk and incubated overnight at 4°C. The blot was washed with PBST and then incubated with anti- rabbit-immunoglobulins HRP (1:10000) (DAKO) for 1 h at 37°C. After being washed with PBST, the blot was developed either with TMB MB substrate kit (Lucron Bioproducts b.v.) or with enhanced chemiluminescence ECL plus (Amersham).

Two dimensional electrophoresis

Total merozoite extract (300 µg) and invasion supernatant (100 µg) were dissolved in rehydration solution (7M urea, 2 M thiourea, 4% CHAPS, 2% carrier ampholyte mixture pH 4-7 IPG buffer) and 20 mM DTT) and loaded on 13 cm IPG strips (pH 4-7). The procedures for isoelectric focusing (IEF) and subsequent two-dimensional SDS-PAGE electrophoresis (2D SDS- PAGE) were performed as described previously [(241)]. Silver staining was used to visualise proteins after 2D SDS-PAGE. Images of the gels and blots were acquired using LabScan v3.0 software on an Umax flatbed scanner and were digitally analysed using ImageMaster® 2D v4.01 software (Amersham Biosciences).

Immunofluorescence assay

The recognition of *B. bovis* merozoites by anti-AMA-1 antibodies was tested by indirect immunofluorescence assay (IFA). Thin blood smears were fixed with methanol. Primary incubation with polyclonal rabbit anti-AMA-1 peptide (1:5 to 1:160) for 35 min was followed by three wash steps of 5 min with PBS. Slides were incubated with goat anti-rabbit IgG coupled to fluorescein isothiocyanate (1:80, Nordic) for 30 min. The slides were washed, and vectashield solution (Vector laboratories) was applied and parasites were visualized using a UV fluorescence microscope with FITC filters (455/ 515 nm). IFA titres were determined as the last serum dilution with a positive recognition of the parasite compared to the negative preimmune serum diluted 1:5.

RESULTS

Identification and cloning of a full length cDNA encoding B. bovis *AMA-1*

A *B. bovis* EST, displaying 33.1% identity to domain I of *P. falciparum* AMA1 over a stretch of 145 amino acids, was identified by BLAST analysis. Probing a *B. bovis* cDNA library with a 350 bp PCR product derived from this EST resulted in the cloning and sequencing of a 2036 bp cDNA containing an open reading frame of 1818 bp and a 3'non coding region of 189 bp terminating in a polyA-tail. To determine the 5' capped end of the full-length mRNA, total mRNA was dephosphorylated after which the 5' caps, which are left intact, were removed by tobacco acid pyrophosphatase (TAP) followed by ligation of a specific RNA oligonucleotide. Subsequently, nested PCR on first strand cDNA allowed the cloning and sequencing of a 755 bp fragment derived from the 5' end of the *B. bovis* revealing an 5' untranslated region of 246 bp preceding the first methionine codon of the 1818 bp ORF.

Comparison of B. bovis *AMA-1 with* T. gondii *and* Plasmodium *AMA-1*

A conceptual translation of the 1818 bp ORF predicted a 67.2 kDa protein with a pI of 6.35 which has been aligned with the full-length sequences of *P. falciparum, P. vivax* and *T. gondii* AMA-1 (Fig. 1). Reminiscent of the known AMA-1 proteins, the hydrophobic N-terminal 39 amino acids of *B. bovis* AMA-1 were predicted to form a signal peptide (SignalP2.0) whereas the hydrophobic stretch from Ile-523 to Trp-541 is likely to form a transmembrane region with a predicted topology of a type Ia membrane protein (TMHMM2.0) leaving a 64 amino acid cytoplasmic C-terminus. The signal peptide cleavage sites and terminal residues of the transmembrane segments as predicted by SignalP2.0 and TMHMM2.0 for all four AMA-1 proteins have been precisely aligned in Fig. 1. The predicted signal peptide of *B. bovis* AMA-1 is nearly twice as long as those of the other species. However, it cannot be excluded that translation initiation does not start at the first ATG codon. The transmembrane segment of *B. bovis* AMA-1 is 2 to 4 amino acids shorter as the other transmembrane segments whereas the boundaries of the four segments are remarkably conserved. The cytoplasmic domains of the displayed AMA-1 molecules are of similar length (53 to 64 aa) but only the 30 C-terminal residues show considerable similarity (e.g. 43.3% identity between *B. bovis* and *P. falciparum*). The extracellular domain of *P. falciparum* AMA-1 has been shown to be organized in three structural domains (I to III) that are stabilized by 8 intra-domain disulfide bridges (as illustrated in Fig. 1) between 16 cysteine residues (12 of which are conserved in *T. gondii*). Fourteen of these cysteine residues are easily aligned with cysteine residues in the *B. bovis* sequence and only the third and sixth cysteine residues of domain III, forming

disulfide bridge IIIc, are absent. Whereas domain I (Pro96 to Pro303) and II (Met-304 to Glu-438) constitute the best conserved regions of AMA-1 (respectively 40.3% and 29.3% identity between *P. falciparum* and *B. bovis*, excluding gap positions) the remaining sequences, including domain III, show very little conservation. *P. falciparum* AMA-1 is special when compared with AMA-1 of other *Plasmodium* species and *T. gondii* in having an N-terminal prodomain that is cleaved off during merozoite maturation. *B. bovis* AMA-1 is the first that also has an extended N-terminal sequence although it is shorter than its *P. falciparum* homologue. Disregarding gap regions, the full-length AMA-1 sequences of *B. bovis* and *P. falciparum* are only slightly more similar to each other (28% identity) as to *T. gondii* (25% identity in both cases) but the alignment of *T. gondii* AMA-1 to *P. falciparum* and *B. bovis* requires the introduction of 24 gap regions whereas *P. falciparum* and *B. bovis* AMA-1 align with 14 gaps.

Recognition of recombinant BbAMA-1 by antisera against short, BbAMA-1 derived, peptides

To enable further studies on the BbAMA-1 protein, rabbits were immunized with KLH-linked synthetic peptides (see Fig. 1) that were derived from the N-terminal domain (peptide 1, Ala-46 to Ser-60), domain II (peptide 2, Arg-395 to Gly-409) or domain III (peptide 3, Tyr-453 to Val-467). All three antisera specifically recognized a recombinant fusion product of thioredoxin and the extra-cellular domain of BbAMA-1 (Met-1 to Ser-501) that was expressed in *E. coli* BL21 cells (Fig. 2). Polyacrylamide gel electrophoresis of total cell lysates obtained before (lane 1) and after (lane 2) induction with IPTG identified the recombinant fusion product as an 80 kDa product (calculated size 65 kDa) that was recognized by all three immune sera (lanes 5, 8, 11) and not by pre-immune sera (lanes 4, 7, 10) on immuno-blots. Immune recognition was specific for the BbAMA-1 part of the fusion product as a recombinant fusion product of *B. bovis* rab5 (lane 3) (298 to 1801, Genbank Acc No AY324137) expressed in PET32a was not recognized (lanes 6, 9, 12). Also, immune recognition was peptide-specific and not due to antibodies induced by the KLH carrier protein used for immunization as antiserum raised against a KLH-linked synthetic peptide unrelated to AMA-1 did not recognize the BbAMA-1 recombinant fusion product (lane 13).

Immunofluorescence microscopy

To localize BbAMA1, immunofluorescence studies using rabbit antisera against the three KLH-linked peptides were performed on *B. bovis* in vitro cultures attached to

glass slides by methanol fixation (Fig. 3). Incubation with pre-immune sera (panels A, C, E) did not result in any specific staining of parasites above a background signal of faint fluorescence derived from infected as well as non-infected erythrocytes. In contrast, immune sera resulted in specific staining of parasites in any microscope field examined (panels B, D, F). Fluorescent parasites were detectable with antisera against all three peptides at a dilution of 1:5. The strongest fluorescence was obtained with the anti-domain III peptide serum, which still gave a signal at a 1:20 dilution at which the other two sera only gave rise to a background signal. Although intra-erythrocytic *B. bovis* parasites and free merozoites are small (~1 by 2 μm) a maximal magnification allowed a clear visualization of the staining pattern. Especially the duplicated, double pear-shaped, parasites showed more intense staining at the apical side of the parasite (see typical example in panel G).

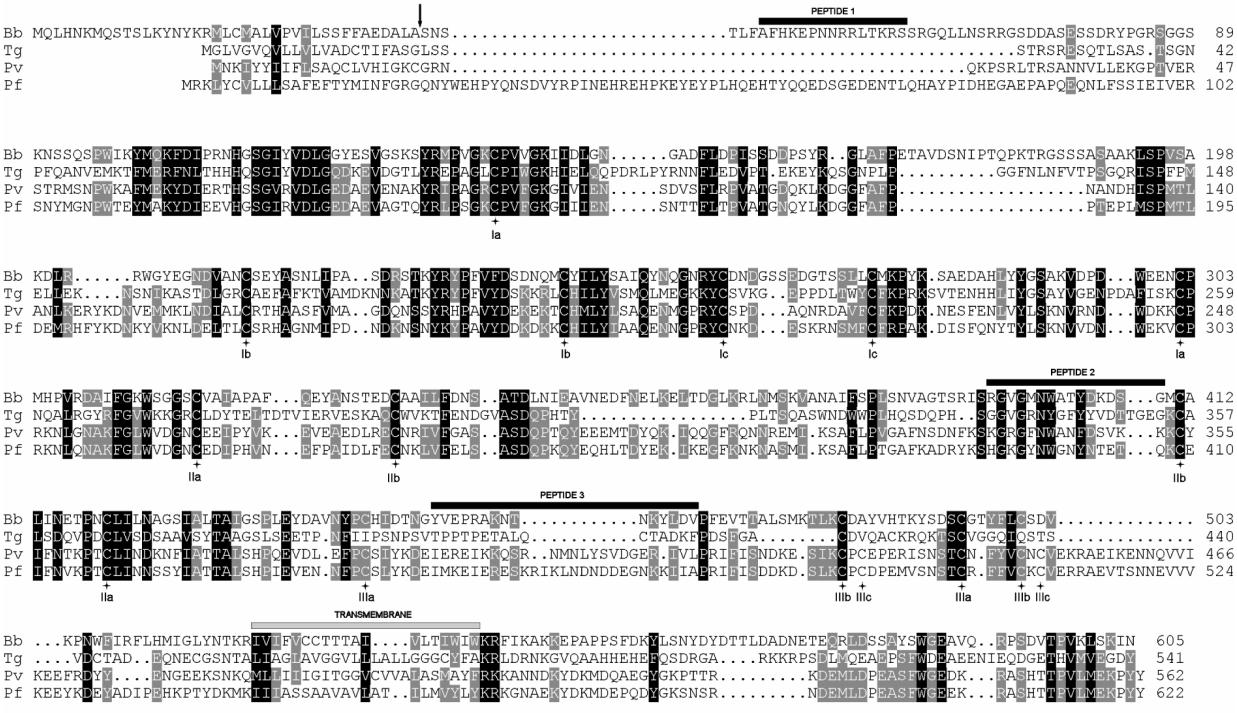

Fig. 1. Multiple sequence alignment AMA-1 of *B. bovis* (BbAMA-1) with *T. gondii* (TgAMA-1), *P. vivax* (PvAMA-1) and *P. falciparum* (PfAMA-1). Similar and identical residues are shaded. Black shading indicates similarity in all four species, gray shading in three species. Synthetic peptides 1, 2 and 3 (black bars) are indicated. The signal peptide cleavage site is depicted with an arrow and the transmembrane region is indicated by a grey bar. Cysteine residues forming disulfide bonds in PfAMA-1 are indicated according to their domain (I, II or III) and the bond (a, b, c).

Inhibition of in vitro *invasion by peptide-specific antisera*

AMA-1 of *P. falciparum* and *T. gondii* is secreted from apically located micronemes and thought to be involved in host cell invasion [77, 90] . A *B. bovis* in vitro invasion assay, allowing to study the invasion of erythrocytes by free merozoites in a protein free buffer within a timespan of 1 h, was used to assess the effect of antisera directed against the three peptides derived from different domains of BbAMA-1. Free merozoites were pre-incubated for 1 h at 20°C with the three anti-BbAMA1 sera and the control serum directed against a non-related peptide after which invasion was started by the addition of erythrocytes. All three antisera against the BbAMA-1 specific peptides gave rise to significant inhibition of invasion whereas pre-immune sera and a control antiserum had no significant effect on invasion efficiency. The strongest effect (65% ± 13% inhibition) was observed by the antiserum directed against the n-terminally located peptide whereas antisera directed against domain II or III peptides showed less inhibition.

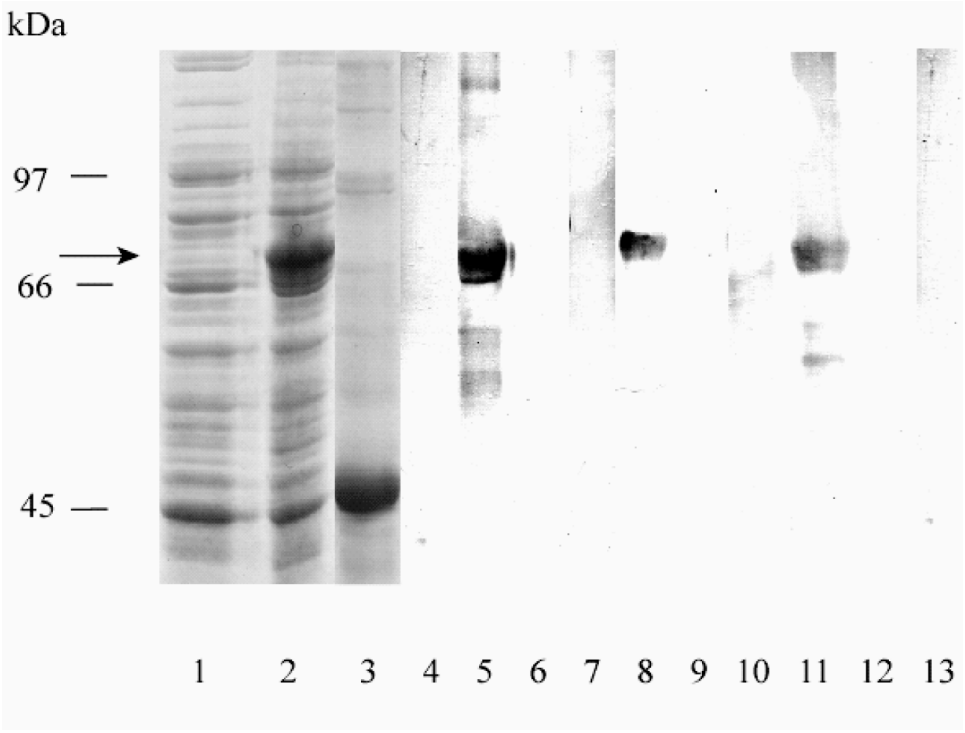

Fig. 2. Western blots of recombinant BbAMA-1 probed with polyclonal rabbit antisera against synthetic peptides. Coomassie Blue-stained SDS-polyacrylamide gel containing *E. coli* lysates of uninduced cells (lane 1) and induced (lane 2, 3) cells expressing *B. bovis* AMA-1 (lane 2) and *B. bovis* rab5 (lane 3). Immunoblots of recombinant BbAMA-1 (lanes 4,5,7,8,10,11,13) were incubated with pre-immune serum (lane 4, 7, 10), immune serum against peptide 1 (lanes 5, 6), peptide 2 (lanes 8, 9) and peptide 3 (lanes 11, 12) or with *B. bovis* rab5 antisera (lane 13). Lane 6, 9 and 12 shows immunoblots of *B. bovis* recombinant protein rab5 with the different AMA-1 antisera. Molecular weights are indicated.

Detection of BbAMA1 secreted into the surroundings during erythrocyte invasion

Despite the easy recognition of recombinant BbAMA1 on Western blots by antisera against all three BbAMA1 derived peptides (Fig.2), only minor amounts could be detected directly in *B. bovis* extracts by sensitive chemoluminescence staining methods. The best antiserum for this purpose appeared to be the one raised against peptide 1 (N-terminal peptide) that detected a *B. bovis* specific band of 82 kDa in total merozoite extracts (Fig. 5, panel A, lane 1) and a smaller band of 69 kDa in the protein pool secreted after invasion of liberated merozoites into fresh erythrocytes for a period of 1 h (lane 2). Antisera against the other two peptides occasionally recognized bands of comparable size, but with very weak signal. In addition variation in between repeated experiments also included the occasional detection of other minor bands (results not shown) by sera against peptides 2 and 3, making the results obtained on western blotting by the latter two antisera as yet inconclusive. A reaction of anti-peptide 1 serum against proteins of 55 kDa to 60 kDa was observed in lanes 1 and 3 containing the merozoite extract and invasion supernatant as well as in control lanes containing only erythrocyte proteins (Fig.5, lanes 2 and 4). Additional controls showed that several other unrelated peptides, when linked to KLH, induced rabbit antisera reacting with a group of erythrocyte cytosolic proteins at this position (result not shown). Merozoite extracts still contain erythrocyte cytosolic proteins derived from a fraction of erythrocytes not lysed during isolation of merozoites whereas proteins secreted during invasion have been shown to be contaminated with erythrocyte cytosolic proteins [(76)] as a small number of erythrocytes become lysed during invasion.

To obtain additional proof for the erythrocytic nature of these bands and the *B. bovis*-specific nature of the 69 kDa band secreted upon invasion, two-dimensional (2D) gel electrophoresis was employed. Western blots of 2D gels were probed with anti-peptide 1 (Fig. 5, panel B) and anti-peptide 3 (panel D) sera and matched to a silver-stained image of another gel (panel F).

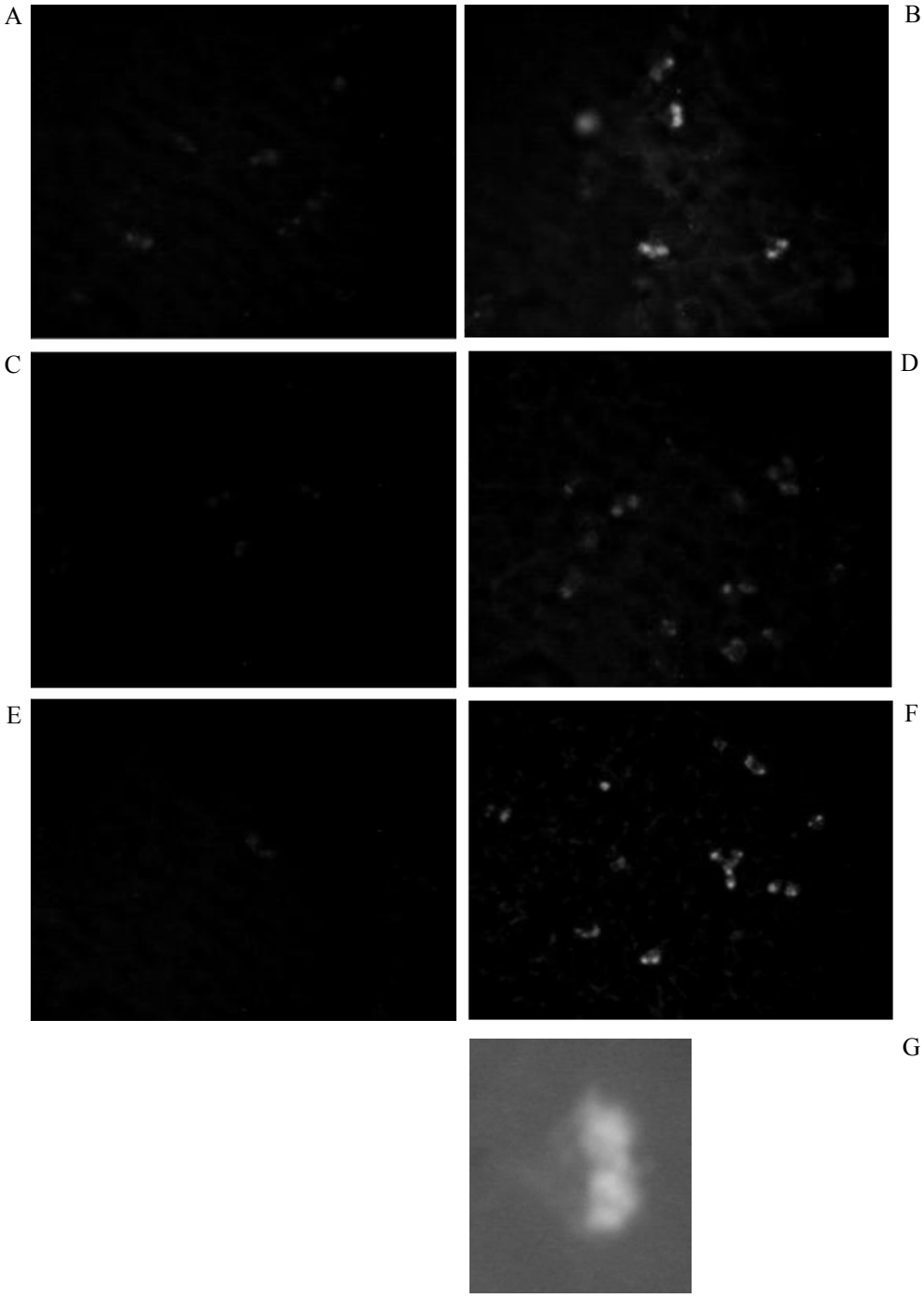

Fig. 3. Immunofluorescence reactivity of antiserum against *B. bovis* AMA-1 incubated with methanol-fixed *B. bovis* infected bovine erythrocytes. Slides were incubated with immune serum against peptide 1 (B), peptide 2 (D) and peptide 3 (F) and the corresponding preimmune sera (A, C, E). An enlargement of duplicated *B. bovis* merozoites showing immunofluorescence is shown (G). For coloured picture see page 112

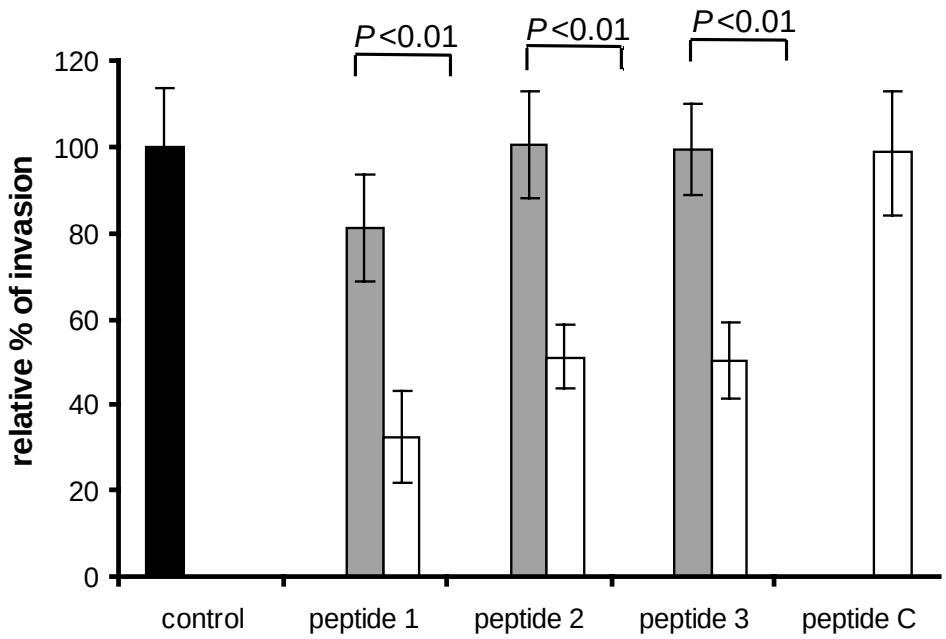

Fig. 4. Inhibition of erythrocyte invasion by *B. bovis* merozoites with antisera raised against four different synthetic peptides. The black bar indicates invasion of *B. bovis* merozoites that were directly added to medium after liberation and is considered as the 100% value against which all incubation are plotted. White bars represents invasion of erythrocytes by *B. bovis* merozoites after pre-incubation with immune sera against anti-peptide 1, anti-peptide 2, anti-peptide 3 and anti-peptide C, whereas grey bars shows invasion of *B. bovis* merozoites after pre-incubation with pre-immune serum. Each bar represents the average value of six individual experiments and error bars indicate standard deviation. Data were tested by the non-parametic test of Kruskal-Wallis. Pairwise comparisons of the groups were calculated by post-hoc analysis as advised by Kruskall-Wallis and p-values are indicated.

Subsequent metabolic labelling of parasites with ^{35}S prior to invasion allowed the localization of *B. bovis* specific spots (Panel G). Erythrocytic proteins of 55 kDa to 60 kDa (non-labelled) were recognized by both antisera and were also present in controls carrying only erythrocyte cytosolic proteins (panels C and E). In addition, spots specifically reacting with anti-peptide 1 serum (panel B, arrow) and anti-peptide 3 serum (panel D, arrow) were detected on the 2D western blots, matching the most abundant spot of a row of four metabolically labelled spots of 69 kDa that in turn matched a row of weak spots on the silver-stained gel. We conclude on basis of this set of data that AMA-1 is a low abundance protein of which a 69 kDa form is secreted upon invasion of erythrocytes by *B. bovis* merozoites.

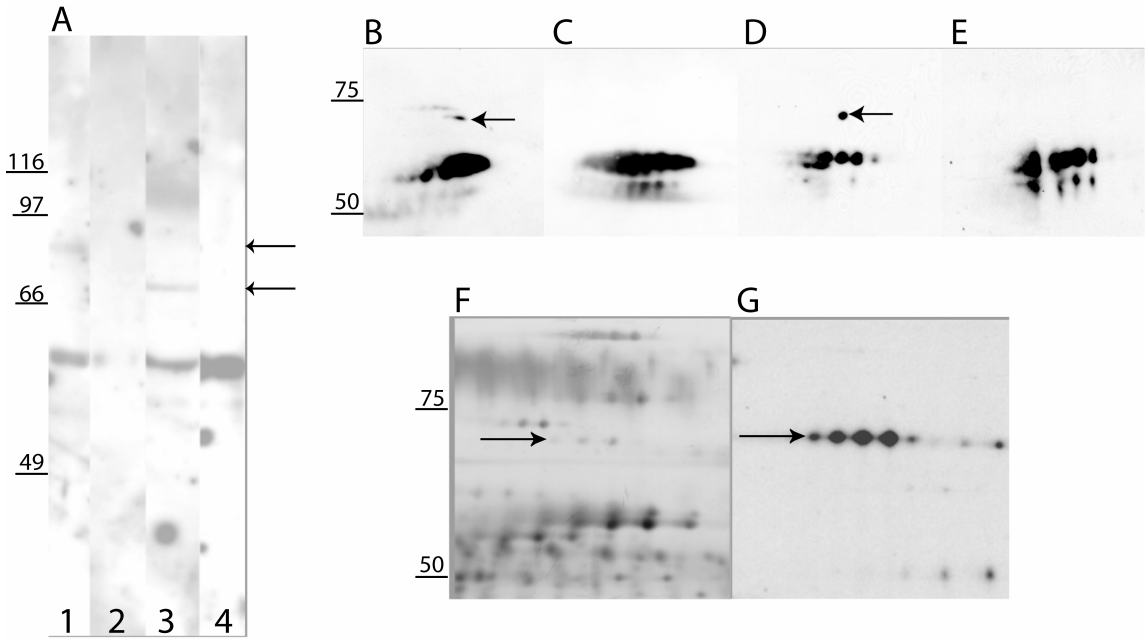

Fig. 5. Western blot analysis of total merozoites and invasion supernatant, incubated with sera raised against AMA-1 peptides after SDS PAGE 1D (panel A) and 2D SDS PAGE (panel B-G). Panel A; total merozoites (lane 1) and invasion supernatant (lane 3) were incubated with anti-peptide 1 antiserum. The arrows indicate bands of respectively 82 kDa (lane 1) and 69 kDa (lane 3). Lanes 2 en 4 represents an erythrocyte control incubated with anti-peptide 1 antiserum. Panel B and D represents western blot of 2D gels loaded with invasion material and incubated with respectively anti-peptide 1 and anti-peptide 3 antisera. Panel C and E represent erythrocyte controls incubated with the same two antisera. A silver-stained image of a 2D gel loaded with invasion supernatant using metabolically labelled *B. bovis* was run in parallel (panel F) and was subsequently exposed to film (panel G). The arrows indicate *B. bovis* AMA-1 specific spots in total merozoite and invasion supernatant.

DISCUSSION

Apicomplexan organisms are defined by a common set of apically located secretory organelles required for host cell invasion by a mechanism displaying many conserved features. Despite the conserved nature of the invasion process, only very few proteins involved show significant similarity when comparing *T. gondii* with *Plasmodium* species. AMA-1 represents one such protein conserved between *T. gondii* and *P. falciparum* and here we report the cloning of the *B. bovis* homologue that we named BbAMA-1. Overall, *B. bovis* AMA-1 is only slightly more similar to *P. falciparum* AMA-1 as to *T. gondii* AMA-1 (resp. 28%, 25%). Most notably, these proteins share 10 conserved cysteine residues that have been shown to form disulfide bridges and were suggested to stabilize the proposed domains I and II of the PfAMA-1 ectodomain [96, 155]. However, a few conserved residues like the four cysteine residues

forming two of the three disulfide bridges present in domain III of PfAMA-1 may represent a functional feature conserved between *B. bovis* and *Plasmodium* species that is lost in *T. gondii*. Also the number of insertions/deletions is much smaller in a pairwise comparison of *B. bovis* and *P. falciparum* as in comparison to *T. gondii*. Sofar, molecular phylogenetic analyses have not unambiguously resolved the evolutionary relationship between the genera of *Toxoplasma*, *Babesia* and *Plasmodium* and it is tempting to hypothesize that some features of the AMA-1 ectodomain may be slightly more conserved between *Babesia* and *Plasmodium* in relation to their identical host cell target, the erythrocyte.

Like other micronemal proteins, AMA-1 has a short cytoplasmic domain varying between 64 aa (*B. bovis*) and 53 aa (*P. falciparum*) of which the 30 C-terminal residues are remarkably similar indicating a conserved function. The cytoplasmic tail of micronemal proteins has been suggested to function as an organellar targeting signal [(59, 99)] but a more recent study has shown that removal of this domain still leads to correct localization of *P. falciparum* micronemal proteins like EBA-175 and TRAP [(81)] . The cytoplasmic tail might be involved in binding to other intracellular proteins that may govern the timing of secretion, which is not synchronous for different micronemal proteins [(89)]. Alternatively it may be involved in transmitting a signal upon contact of the ecto-domain with a host cell ligand or be involved in linkage to cytoskeletal structures like recently shown for members of the TRAP-family of micronemal proteins [(106)].

PfAMA-1 becomes proteolytically processed at several positions giving rise to a complicated pattern of bands as recognized by polyclonal and monoclonal antisera [(10)]. A type I transmembrane protein with an aberrant mobility of 64 kDa (predicted Mw is 83 kDa) is created after cleavage of the signalpeptide. Upon translocation to the apical region a propeptide of 72 aa is removed leaving a protein migrating as 66 kDa on polyacrylamide gels [(100)] . TgAMA-1 does not possess such a propeptide whereas BbAMA-1 was shown here to contain an N-terminal region intermediate in size between TgAMA-1 and PfAMA-1. Peptide 1 directed antisera recognized a 82 kDa band in *B. bovis* total merozoite extracts suggesting an aberrant mobility (predicted Mw from signal peptide cleavage site to C-terminus is 62.7 kDa) as observed for PfAMA-1. Whether a propeptide is cleaved remains to be solved as Western blots probed with anti-peptide 2 and 3 sera yielded inconclusive results, potentially due to the small amounts of BbAMA-1 present.

Several forms of soluble PfAMA-1 are continuously released from the merozoite surface into the extracellular milieu. A 48 kDa form results from cleavage at a position 29 residues N-terminal to the transmembrane region [(100)] whereas a 52 kDa form probably results from cleavage just besides or within the transmembrane region

[100]. Further processing gives rise to a 44 kDa protein [100]. Here we have shown the release of a 69 kDa form of BbAMA-1, recognized by anti-peptide 1 and 3 antisera, upon invasion into erythrocytes suggesting the occurrence of cleavage N-terminal to the transmembrane region for BbAMA-1 as well. Analysis on 2D gels allowed the mapping of this band to a row of spots of low abundance and provided definite proof of the *Babesial* origin of these spots by making use of metabolic labelling. Whereas on 1D gels only anti-peptide 1 serum consistently recognized this band, also peptide 3 directed antisera recognized these spots on 2D gels, probably due to the higher amount of protein loaded onto a 2D gel and arguing against the cleavage of a propeptide.

Immunofluorescence showed that BbAMA-1 was localized at the merozoites surface, specifically distributed around the apical region, like the N-terminal processed form of PfAMA-1. PfAMA-1 has been shown to be localized near the periphery of micronemes in developing schizonts, most likely as a transmembrane protein [10] . Upon release of mature merozoites secretion from micronemes results in spreading of PfAMA-1 over the external side of the plasma membrane. Immunofluoresence studies indicated that PfAMA-1 remains mainly confined to the apical half of the merozoite. Upon erythrocyte invasion traces of PfAMA-1 were shown to be carried into the host cell but immunofluorescence soon fades away [69]. Immunofluoresence studies with BbAMA-1 anti-peptide sera resulted in staining of the apical part of *B. bovis* parasites present in asynchronous in vitro cultures. Staining showed a punctuate pattern and can represent plasmamembrane located BbAMA-1 as well as location in apical organelles. This staining pattern as well as the fact that the more intense staining was mainly detected on apparently mature and duplicated intra-erythrocytic forms suggest a similar localization and temporal expression as observed for PfAMA-1.

The inability to make knock-out mutants suggests a critical function for AMA-1 in *T. gondii* [90] and *P. falciparum* [221]. Several lines of evidence suggest a role of AMA-1 in host cell invasion. Monoclonal and polyclonal antisera inhibit host cell invasion [6, 90, 96, 116] as do peptides binding specifically to AMA-1 [114, 123]. Also, substitution of the PfAMA-1 gene with the *P. chabaudi* AMA-1 gene results in *P. falciparum* parasites that better invade mouse erythrocytes [221]. Antisera directed against BbAMA-1 derived peptides specifically reduced the in vitro invasion efficiency of *B. bovis* indicating that AMA-1 is indeed located on the surface of merozoites and accessible to antibodies. Invasion inhibition may result of blocking some specific function of AMA-1 or may involve inhibition of proteolytic processing as recently shown for PfAMA-1 [69] . Alternatively, antibodies may just crosslink merozoites although the low dilutions required to obtain an immunofluorescent signal argue against this option. Peptides derived from loop 1 of Domain III of PfAMA-1 have been shown to induce antibodies that inhibit *P. falciparum* growth [153]. The

immunodominant epitopes in these peptides are located in the region that is aligned with peptide 3 of BbAMA-1 in Fig. 1.

The epitopes recognized by other invasion blocking antisera or peptides have not been mapped yet. Antisera directed against peptide 1 (from the N-terminus) and peptide 2 (from domain 2) also gave rise to invasion inhibition of *B. bovis* indicating that inhibitory antibodies can be directed against epitopes over the full length of the ectodomain. The peptides were selected on basis of their prediction as forming amphiphatic α-helices with a high surface probability and a considerable amount of charged residues. Studies on population genetics of the AMA-1 gene in endemic areas have provided evidence for selective pressure operating on domain I and III indicating them as a target for protective immunity [45, 175]. Remarkably, AMA-1 sequences are most conserved between genera in domain I (see Fig. 1), implicating strong functional constraints, whereas the population studies mentioned above have indicated this region of PfAMA-1 as the most variable.

PfAMA-1 is considered as one of the prime candidates for incorporation into a recombinant vaccine [69] for reasons briefly discussed above. Soluble parasitic antigens (SPA) of *B. bovis* secreted into the environment during in vitro culture have been shown to confer protective properties against *B. bovis* infection in cattle [170] although no individual antigens contributing to this effect have been isolated. The results presented here have identified BbAMA-1 as such a secreted protein and the inhibition of invasion by rabbit antibodies directed against it, indicates it might be one of the protective components of SPA. Further proof obviously requires immunization experiments of cattle using native or recombinant BbAMA-1. Genetic studies on BbAMA-1 diversity in the field will help in determining its potential as a vaccine component.

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CHAPTER 5

A *BABESIA BOVIS* MEROZOITE PROTEIN WITH A DOMAIN ARCHITECTURE HIGHLY SIMILAR TO THE THROMBOSPONDIN RELATED ANONYMOUS PROTEIN (TRAP) PRESENT IN *PLASMODIUM* SPOROZOITES

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submitted for publication

"And God Created every living creature from water. Some of them walk on their bellies, some walk on two legs, and some walk on four. God creates whatever He wills. God is Omnipotent (Qur'an 24:45)"

ABSTRACT

Recognition and invasion of host cells is a key step in the life-cycle of all apicomplexan parasites. The thrombospondin-related anonymous protein (TRAP) of *Plasmodium* sporozoites is directly involved in both processes and shares conserved adhesive domains with micronemal transmembrane proteins of other apicomplexans. Here, we report the cloning and characterization of a *Babesia bovis* TRAP homologue (BbTRAP). It was predicted to be a type I transmembrane protein containing a van Willebrand Factor A domain (vWFA), a Thrombospondin type 1 domain (TSP1), a simple repeat region, a conserved transmembrane region and a conserved cytoplasmic C-terminus, thus closely resembling the domain arrangement of *Plasmodium berghei* TRAP (PbTRAP). In contrast to the sporozoite protein PbTRAP, BbTRAP was shown to be present during the asexual erythrocytic cycle, being located mainly at the apical side of merozoites. Polyclonal rabbit antisera directed against synthetic peptides derived from the TSP-1 domain or the C-terminal end of the ectodomain were shown to inhibit erythrocyte invasion in vitro. Both antisera recognized a 75 kDa protein in merozoite extracts as well as in a protein fraction that was secreted into the extracellular milieu during in vitro invasion of erythrocytes.

INTRODUCTION

Babesia bovis is an apicomplexan parasite of cattle causing major suffering and economical loss worldwide. Its biology resembles that of *P. falciparum* in having an asexual erythrocytic cycle of reproduction that is responsible for pathogenesis with clinical features like anaemia, renal failure and the development of severe cerebral babesiosis characterized by sequestration of parasitized erythrocytes in the microvasculature of the brain [(98)]. The asexual life cycle of *B. bovis* is simple, in replicating by a single binary fission and, remarkably, is initiated by direct invasion of eyrthrocytes by sporozoites. Thus, *B. bovis* lacks a pre-erythrocytic lifestage in its vertebrate host in contrast to *Plasmodium* species and the even more closely related *Theileria* species of which sporozoites invade hepatocytes or white blood cells respectively. Apicomplexan parasites invade their host cells by employing molecules located at the cell surface and in apical secretory organelles. These organelles are localized at the anterior end of the invasive stages and are named micronemes, rhoptries and dense granules [(18, 67, 176)]. Micronemal proteins are crucial in mediating attachment and invasion and some of them become rapidly secreted directly upon initial contact with the host cell surface [(142)]. *Plasmodium* species use different

micronemal proteins comparing sporozoite and merozoite invasion of host cells. Apart from invading host cells, sporozoites display a characteristic movement called gliding motility [112, 142, 212], as demonstrated in vitro by analysis of motion on artificial surfaces. The processes of invasion and gliding motility of sporozoites may depend on the same set of molecules. In *Plasmodium* sporozoites thrombospondin-related anonymous protein (TRAP) [134, 212] is a candidate ligand for interaction with host cell or substrate receptors. TRAP is a type I transmembrane protein that carries two adhesive domains in its extracellular region, an A-domain of von Willebrand (vWFA) factor and a motif similar to the type 1 repeat of thrombospondin (TSP1). Recombinant TRAP of *P. falciparum* (PfTRAP) binds to sulphated glycosaminoglycans (GAGs) and human hepatocytes [154, 182] using the TSP1 domain for adhesion and heparin is a binding ligand for the vWFA domain [137]. Disruption of the *P. berghei* TRAP gene by transgenetics resulted in loss of gliding motility and invasion of mosquito salivary glands and to reduced infection of host hepatocytes [212]. Expression of mutated forms of PfTRAP in transgenic *P. berghei* sporozoites led to similar conclusions and also implicated the importance of the vWFA domain in this process [235]. However, specific mutagenesis of the vWFA and TSP-1 domains of *P. berghei* only impaired cell invasion without affecting gliding motility [134].

Several micronemal proteins of other apicomplexan genera contain vWFA and/or TSP-1 domains in variable numbers of tandemly repeated copies. These include *Toxoplasma gondii* MIC-2 [75, 230], *Eimeria tenella* Etp100 [218] and *Cryptosporidium parvum* TRAPC1 [206]. Like *Plasmodium* sporozoites, *T. gondii* tachyzoites and sporozoites of *Eimeria* species display gliding motility [25, 72, 197]. In addition, *T. gondii* has been shown to adhere to GAGs like *Plasmodium* sporozoites [33]. A number of studies have linked this class of micronemal proteins to a critical function in host cell invasion and motility [22, 103, 134, 205, 213] and inspired a search for proteins containing similar domains in *B. bovis*. Only three *B. bovis* proteins have sofar been associated with erythrocyte invasion of which two constitute the MSA-1 and MSA-2 families of merozoite surface proteins [74, 151, 152, 210] whereas the other is the rhoptry protein RAP-1 (152, 211, 242). Here, we report on a *B. bovis* protein, with an architecture remarkably similar to TRAP proteins of *Plasmodium*, that is present at the apical end of *B. bovis* merozoites and is a target for invasion inhibitory antibodies.

MATERIALS AND METHODS

B. bovis in vitro *culture*

In vitro culture of *B. bovis* (Israël strain, clonal line C 61411) in bovine erythrocytes were maintained in 24-well plates (1.2 ml total volume) or in 25 cm^2 bottles (15 ml volume) as previously described [76]. Cultures were allowed to grow in M199 medium supplemented with 40% normal bovine serum and 25 mM sodium bicarbonate together with bovine erythrocytes at 5% packed cell volume (PCV) at 37°C, 5% CO_2 in air. The parasitaemia of the in vitro culture was kept between 1 and 12% by daily dilution.

B. bovis in vitro *invasion*

B. bovis invasion was performed under the conditions that have been described previously [76] with some modifications. *B. bovis* in vitro culture (6 to 8% parasitaemia) was centrifuged (2000 *g*, 10 min, 15°C) and the pellet was resuspended in an equal volume of VyMs buffer (4°C). Samples (800 µl) were submitted to five intermittent (10 seconds at 0°C in between pulses) high voltage pulses (2.5 kV, 200Ω, 25 µF) in 4 mm cuvettes (BioRad) using a BioRad Gene Pulser with pulse controller and washed with 8 ml of PBS containing 25 mM sodiumbicarbonate (PBSbc, pH 8.0, 20°C) followed by centrifugation (1800 *g*) for 10 min at 15°C. The merozoite pellet was resuspended in 8 ml PBSbc and centrifuged again (1300 *g*, 10 min, 15°C). The final merozoite pellet was resuspended in 800 µl PBSbc. Invasion was started by addition of 1 volume of resuspended merozoites to 9 volumes of suspended bovine erythrocytes (5.5% PCV in PBSbc pH 8.0, 20°C, preincubated for 60 min at 37°C in 5% CO_2 in air) and was performed in 24-well plates (final volume 1.2 ml), in 25-cm^2 flasks (15 ml) or in 80-cm^2 flasks (50 ml) at 37°C, 5% CO_2 in air. Giemsa-stained slides were prepared after 1 h and parasitized erythrocytes out of a total of 5000 erythrocytes were counted.

Inhibition of in vitro *invasion by rabbit antisera*

B. bovis merozoites (200 µl), prepared as described above, were incubated with 40 µl of rabbit antisera at 20°C. After 1 h, 960 µl of suspended bovine erythrocytes (6.25% PCV in PBSbc, preincubated for 60 min at 37°C in 5% CO_2 in air) were added and incubated for 1h (37°C in 5% CO_2 in air) in 24-well plates (Greiner) after which Giemsa-stained slides were prepared and counted (5000 erythrocytes) to determine the level of invasion. Induction of rabbit antisera against synthetic peptides derived from the BbTRAP sequence and a control serum raised against an unrelated peptide (YAGRLFSKRTAATAYKLQ), named peptide C has been described below.

Preparation of total merozoite protein extracts and proteins solubilized upon invasion

Samples of merozoites (800 µl), prepared as described above for in vitro invasion, were partially separated from erythrocyte ghosts by filtration over 1.2 µM polypropylene prefilters (Millipore). Filtered merozoites were pooled and washed twice in 20 volumes of PBSbc followed by centrifugation at 2000 g for 20 min at 4°C. After the second wash the pellet was resuspended in an equal volume of PBSbc and divided in aliquots of 200 µl that were centrifuged (10000 *g*, 5 min at 4°C) and stored as 100 µl cell pellets (2×10^9 merozoites) at -20°C after removal of supernatant. Frozen merozoite pellets were thawed just before use and lysed, reduced and alkylated by using a total protein extraction kit (Proteoprep, Sigma) according to the instructions and obtained in a final volume of 1.7 ml. Insoluble material was removed by centrifugation at 16000 g (3 min, 4°C). As the extracts contained considerable amounts of erythrocyte proteins, control extracts were prepared by the same procedure, starting with a culture of non-infected erythrocytes. Soluble proteins secreted or shed upon invasion were obtained by gently removing the overlaying buffer after 1 h of in vitro invasion followed by centrifugation (2000 *g*, 10 min, 4 °C) after which the supernatant was centrifuged again at high speed for removal of membrane fragments (20 min, 12000 *g*, 4°C). The final supernatant was dialysed (Pierce; Snakeskin®pleated dialysis tubing) overnight against 10 mM TrisNaCl, pH 7.5.

Screening a B. bovis *cDNA library for TRAP homologues*

PCR primers were derived from a *B. bovis* EST clone (available from www.sanger.ac.uk/Projects/B_bovis/) matching the TRAP-family protein Em100 (Acc. No. A48569). Forward primer A1 (5'–tgtggtagatgaatctgctagtatatc-3') at position 143 and reverse primer A2 (5'- ctatgccacggcattcagcaacattta -3') at position 592 were incubated in 50 µl standard buffer (Promega) containing 0.2 mM dNTP, 20 pmol μl^{-1} of each primer, 100 ng *B. bovis* genomic DNA and 0.5 U Taq DNA polymerase. Amplification was performed for 30 cycles (92°C for 30 sec, 58°C for 30 sec, at 72°C for 30 sec) preceded by initial denaturation for 3 min at 95°C and a final elongation at 72°C for 10 min. A 450 bp fragment was purified from agarose gel and labelled with 50 µCi [α-^{32}P]dATP (3000 Ci $mmol^{-1}$), using a Random Primer labelling kit (Roche). The fragment was used for screening 1.2×10^6 plaques of a *B. bovis* cDNA library [79] by standard procedures [187]. After 2 cycles of plaque purification five clones were excised *in vivo* for isolation of the phagemids inserts as described in the manufacturer's instructions (Stratagene). Clones c1 and c2 containing the largest inserts were sequenced on both strands, using automated cycle sequencing with the dye terminator method (ABI PRISM dye terminator kit, Pharmacia). A full-length TRAP cDNA including the non-coding 5'-end was obtained with GeneRacer™ kit (Invitrogen) according the manufacturer's instructions. A single clone g1 was sequenced on both strands.

Expression of recombinant BbTRAP in E. coli

Genomic DNA of *B. bovis* was used to amplify part of the *BbTRAP* gene, from base 145 (Accession number AY486102, numbering starting at the ATG initiation codon) to base 1964, by PCR using forward primer A3 (5'-cccgaattcgtggtagatgaatctgct-3') containing an *Eco*RI site and a reverse primer A4 (5'-cccgtcgactgcctcgccccaaatgttgt-3') with a *Sal*I site. After PCR (1 min 94°C, 1 min 55°C, 1 min. 72°C; 30 cycles) the BbTRAP PCR product was purified as described above and ligated into the *Eco*RI-*Sal*I site of pET-32a vector (Novagen) allowing expression as a fusion product with a N-terminal thioredoxin domain and an internal 6-histidine tag. Ligation was used for transformation of *E. coli* NovaBlue strain. As control *B. bovis* rab5 (residues 298 to 1801, Genebank Acc no AY324137) was expressed in pET-32a. Plasmids containing the appropriate insert were used to transform *E. coli* BL21 (DE3). Fusion proteins with thioredoxin were obtained with maximal yield after induction with 1 mM of isopropyl-β-D thiogalactosidase (IPTG) for 4 hr at 37°C. Bacterial pellets were heated at 95°C in SDS-polyacrylamide (SDS-PAGE) sample buffer containing 2% (v/v) β-mercaptoethanol, run on 10% SDS-PAGE minigels and stained with Coomassie Blue.

Peptide selection and immunisation

Peptide sequences were selected from the BbTRAP amino acid sequence by searching for amphiphatic alpha-helices having a high probability for surface localization and containing several charged residues with the Protean software package (Lasergene) for protein sequence analysis. Two peptides were selected: the first from the TSP-1 domain (aa 255-269; cysteine-: PGKRTRALLDLRMIE: peptide 1) and the second from the C-terminal end of the ectodomain (aa 547-561; cysteine-VYDDHPEESENTGIN: peptide 2). The peptides were synthesized and coupled to maleimide activated keyhole limpet haemocyanin (KLH) as carrier protein according to the manufacturer's recommendations (Pierce). The peptide-carrier conjugate was used to generate rabbit polyclonal antisera. Two groups of NZW-rabbits, each containing two rabbits, were immunized subcutaneously at 3-week interval for eight months. Before the first immunisation blood serum was collected from each rabbit, which was used as negative control. Each rabbit was injected with 250 µg peptide coupled to 250 µg of KLH and equal volume of adjuvant Stimune® (ID-DLO, The Netherlands), in a total volume of 1000 µl.

Plasmaforeses were performed one week after immunization, starting at month 4. The production of rabbit antibodies against the peptides was estimated periodically by ELISA. Ninety-six-well microtiter plates were coated with 150 ng of peptide 1 or peptide 2 per well in 0.1 M Tris-HCl pH 8.0, incubated 1 h at 37°C and blocked for 1 h with PBS/0.25% BSA at 37°C. Consecutive dilutions (1:50 to 1:50000) of individual rabbit sera were incubated for 1 h at 37 °C. The plates were washed and swine anti rabbit-IgG Horseradish Peroxidase (HRP)-conjugated (1:2000, DAKO) secondary antibody was incubated for 1 h. The plates were washed and developed for 45 min with ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)]- peroxidase substrate (Roche biochemicals). The OD_{405} was recorded, and comparative ELISA titres were calculated.

SDS-polyacrylamide electrophoresis and western blotting

Total merozoite extracts or proteins solubilized upon invasion were separated by 10% SDS-PAGE and transferred to PVDF membrane (Immobilon™-P, Millipore). The blot was blocked with 5% skimmed milk diluted in phosphate-buffered saline containing 0.05% Tween (PBST) for 1 h at 37°C. Rabbits antisera were diluted (1:500) in PBST containing 2% skimmed milk and incubated overnight at 4°C. The blot was washed with PBST and incubated with anti-rabbit-immunoglobulins conjugated to HRP (1:10000, DAKO) for 1 h at 37°C. After washing with PBST, the blot was developed either colorimetrically with TMB MB substrate kit (Lucron Bioproducts b.v.) or with enhanced chemiluminescence ECL plus (Amersham).

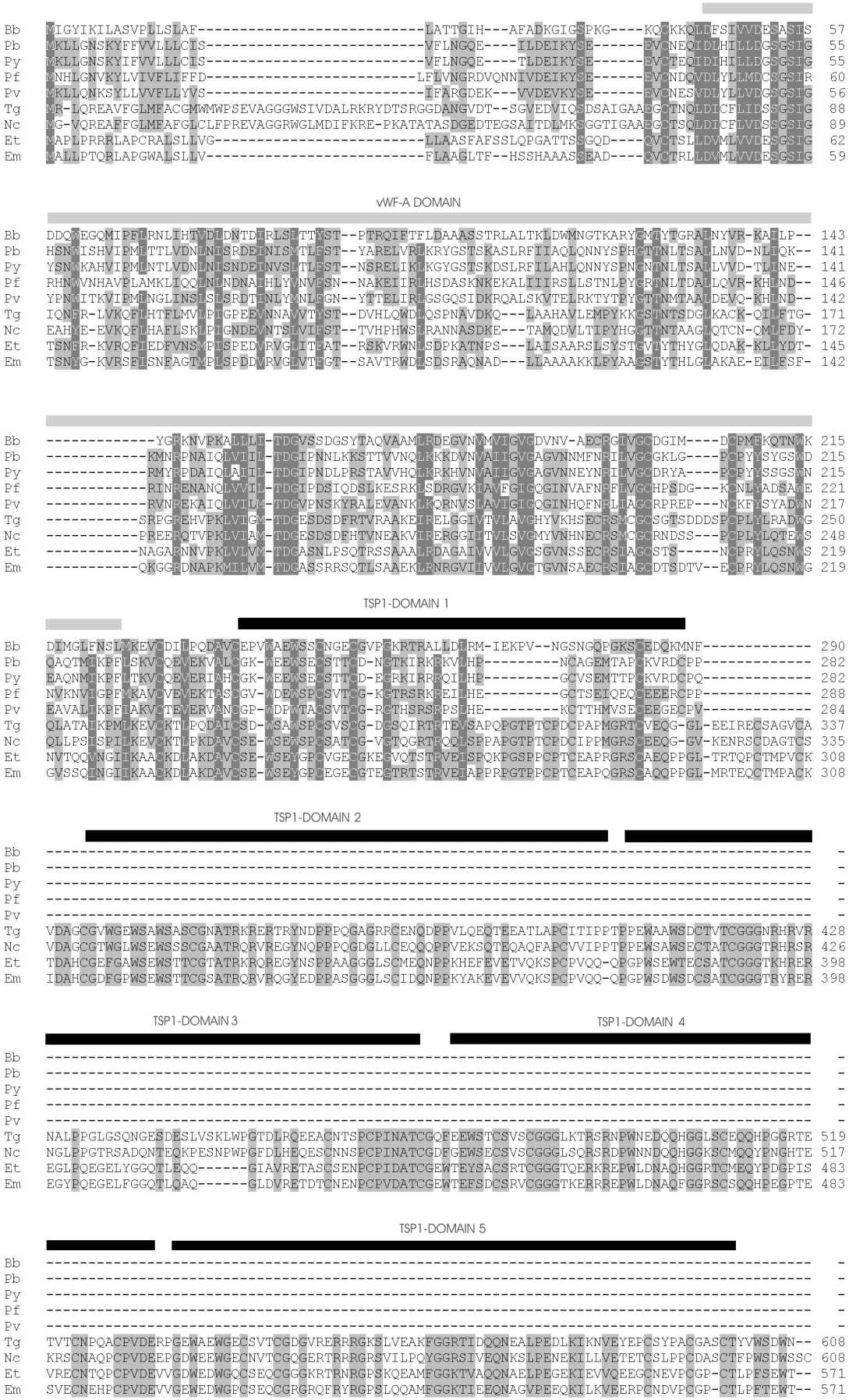
Immunofluorescence assay

Indirect immunofluorescence assays were performed with *B. bovis* merozoites infected red blood cells fixed as thin blood smears with methanol. Slides were incubated for 35 min with polyclonal rabbit anti-TRAP antisera (1:5) or preimmune serum (1:5), washed three times for 5 min with PBS and then incubated for 35 min with goat anti-rabbit IgG antibodies coupled to fluorescein isothiocyanate (1:80, Nordic). The slides were washed, and vectashield solution (Vector laboratories) was applied and parasites were visualized using a UV fluorescence microscope with FITC filters (455/ 515 nm).

RESULTS

Cloning and sequence analysis of a full length cDNA encoding B. bovis *TRAP*

Blastx analysis of a *B. bovis* EST dataset (available from www.sanger.ac.uk) identified a single EST of 600 bp matching with the highest Blast score to the Em100 antigen of *Eimeria maxima* (Acc. No. AAA29076). Similarity extended over a region of 188 amino acids (35% identity) corresponding to the vWFA domain (also referred to as the I-domain from integrins) present in the N-terminal part of Emp100 and other antigens of the apicomplexan TRAP-family named after *P. falciparum* PfTRAP. The encoded protein was denoted BbTRAP and cDNA sequencing allowed to assemble a full-length 2105 bp mRNA sequence from a 1990 bp cDNA clone isolated from a *B. bovis* cDNA library and a 725 bp cDNA clone obtained by specific priming of a RT-PCR reaction on the 5' capped end of the BbTRAP mRNA (described in Materials and Methods). The BbTRAP mRNA was composed of a 100 bp 5' UTR, a 37 bp 3' UTR and a 1968 bp open reading frame encoding a protein of predicted MW of 71.3 kDa. Prediction of a signal peptide using SignalP2.0 (most probable cleavage site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TSP1-DOMAIN 1
TSP1-DOMAIN 2
TSP1-DOMAIN 3
TSP1-DOMAIN 4
TSP1-DOMAIN 5

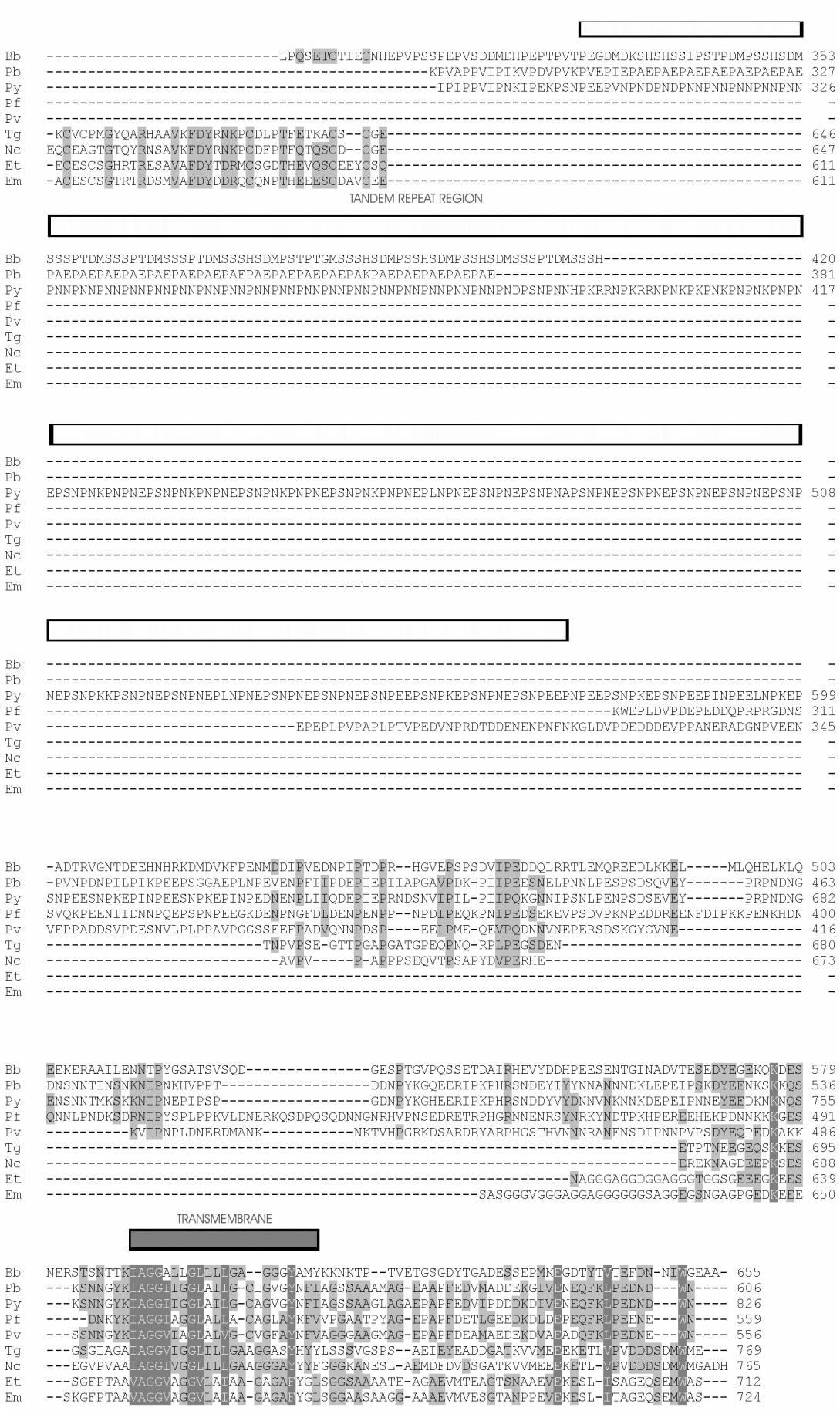
TANDEM REPEAT REGION
TRANSMEMBRANE

Fig. 1. Amino acid sequence aligment of TRAP-family members *B. bovis* (BbTRAP) was aligned with *P. berghei* TRAP (Pb, Acc. No. AAB63302), *P. yoelli* TRAP (Py, Acc. No. Q01443), *P. falciparum* TRAP (Pf, Acc. No. CAA31440), *P. vivax* TRAP (Pv, Acc. No. AAC97485), *T. gondii* MIC2 (Tg, Acc. No. AAB63303, *N. caninum* MIC2 (Nc, Acc.No. AAF01565), *E. tenella* Etp100 (Et, Acc. No. AAD03350) and *E. maxima* Em100 (Em, Acc. No. AAA29076). Positions sharing eight or nine identical or similar residues are shaded dark grey whereas four to seven similarities are shaded light grey (similarity groups: VLIM; YF; RK; EQ; DN; ST). The vWFA domain (grey bar, similar to pfam domain 00092) and TSP-1 domains (black bar, similar to pfam domain 00090) are indicated as well as the repeat region (open bar) and the predicted transmembrane region (grey bar).

between A26 and F27) was in agreement with similar predictions for other proteins of the TRAP family and gives an expected MW of the mature protein of 68.6 kDa. Figure 1 presents the predicted amino acid sequence of BbTRAP, as a representative of the Piroplasmida, in alignment with TRAP family members derived from the Eimeriida (*T. gondii, Neospora caninum, E. maxima, E. tenella*) and the Haemosporida (*P. falciparum, P. berghei, P. yoelli, P. vivax*).

Like all *Plasmodium* TRAP proteins, BbTRAP has one TSP1 domain whereas the Eimeriida TRAP proteins have five tandem TSP1 domains. BbTRAP possesses the remarkably conserved hydrophobic region, predicted (using TMHMM2.0) to be a transmembrane region with a type I membrane protein topology, that was noted before [(183)] and a conserved cytoplasmic domain of 45 amino acids. The region directly N-terminal to the transmembrane domain is poorly conserved in between the different species except for the closely related *P. berghei* and *P. yoelli.* These two species also have a region of imperfect tri-peptide repeats, directly C-terminal to the TSP1 motif, of which the first residue is always a proline. BbTRAP shares this overall protein architecture with PbTRAP and PyTRAP in having 11 imperfect hepta-peptide repeats at this position of which the majority consensus is given by the sequence MSSSH[S/T]D. At positions 2 and 5 of this repeat a proline residue is frequently present.

Recombinant BbTRAP is recognized by rabbit antisera raised against synthetic BbTRAP peptides

A recombinant fusion product (rBbTRAP) of thioredoxin and BbTRAP (residues Val-49 to Ala-654) or Bbrab5 was expressed in *E. coli* BL21 cells (Fig. 2). Polyacrylamide gel electrophoresis of total cell lysates obtained before (lane 1) and after (lane 2) induction with IPTG identified a recombinant fusion product of 80 kDa (predicted size 77.8 kDa). Total cell lysate containing rBbTRAP was transferred to PVDF membrane that was probed with sera from rabbits that were immunized with KLH-linked synthetic peptides derived from the TSP1 domain (Pro-255 to Ser-269) or

a region just preceding the transmembrane domain (Val-547 to Asn-561). Both immune sera (lanes 5 and 8) recognized rBbTRAP whereas bands around 58 kDa are most likely caused by recognition of *E. coli* proteins as pre-immune serum (lane 4) and BbTRAP immune sera against both peptides give the same signal at this position with both rBbTRAP (lanes 5 and 8) and recombinant *B. bovis* rab5 (lanes 6 and 9). Sera raised against *B. bovis* rab5-derived peptide linked to KLH did not recognize rBbTRAP (lane 10).

Immunofluorescence microscopy

BbTRAP was localized by immunofluorescence microscopy using rabbit antisera against both KLH-linked peptides on *B. bovis* in vitro cultures attached to glass slides and permeabilized with methanol (Fig. 3).

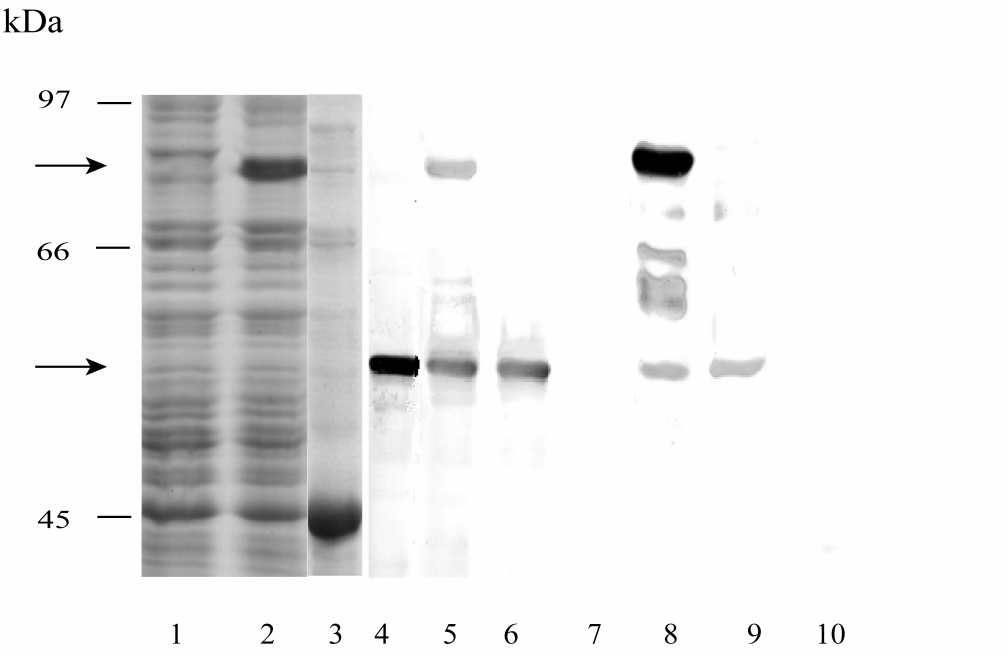

Fig. 2. Western blots of recombinant BbTRAP probed with polyclonal rabbit antisera against synthetic peptides. Expression of BbTRAP in *E. coli* is demonstrated on a Coomassie Blue-stained SDS-polyacrylamide gel containing *E. coli* total cell lysate of uninduced cells (lane 1) and induced (lane 2) expressing recombinant BbTRAP. As a control expression of *B. bovis* rab5 in the same vector is shown in lane 3. Blots of recombinant BbTRAP (lanes 4, 5, 7, 8, 10) were incubated with pre-immune serum (lane 4, 7), immune sera (lane 5, 8) raised against peptide 1 (lane 4, 5), peptide 2 (lane 7, 8) or with *B. bovis* rab5 antisera (lane 10). Lane 6 and 9 shows immunoblots of *B. bovis* recombinant protein rab5 with TRAP antisera against peptide 1 and peptide 2 respectively. Molecular weights are indicated.

Incubation with pre-immune sera (panels A and C) resulted in a very weak fluorescent signal of equal intensity at infected and non-infected erythrocytes. Immune serum directed against peptide 2, derived from the C-terminal region of the ectodomain of BbTRAP, resulted in intense staining of parasites in any microscope field examined (panels D) whereas incubation with the immune serum directed against peptide 1 stained parasites at a weaker intensity. The enlargement in panel E shows the staining pattern of a duplicated, double-pear shaped, intra-erythrocytic parasite (free merozoites are hardly observed in standard in vitro cultures) as observed at maximum magnification. Staining was present mainly on the apical end of the parasite and shows a somewhat dotted appearance (the faint background staining allows easy identification of the apical end as the parasites remain attached by a residual body at the opposite end after duplication).

Anti-BbTRAP-peptide antisera inhibit in vitro *invasion and detect secreted BbTRAP*

Several lines of evidence suggest that members of the TRAP family are directly involved in gliding motility and host cell invasion [134, 212, 235]. Fig. 4 displays the results of an in vitro invasion study in which free *B. bovis* merozoites were preincubated for 1 h at 20°C with the two anti-BbTRAP-peptide rabbit antisera described above. After preincubation the direct effect of the antisera on invasion of erythrocytes by free merozoites, performed for 1 h in a protein free buffer, was determined. Preincubation with pre-immuneserum or with a comparable control antiserum directed against a non-related peptide had no significant effect on invasion efficiency. Both immune sera caused a significant invasion reduction of 55% $\pm$ 11% for the serum directed against the TSP1 domain (anti-peptide 1) and of 36% $\pm$ 15% for anti-peptide 2 (C-terminal end of ectodomain). The direct addition of immune sera to the in vitro invasion reaction without prior preincubation did not cause a significant inhibition (result not shown).

Proteins secreted or shed into the surroundings during in vitro invasion, under serum-free conditions in a PBS-bicarbonate buffer, were concentrated and analysed by western blot (Fig. 5) using the BbTRAP peptide-specific antisera. Both antisera recognized a 75 kDa protein in total merozoite extracts (lanes 1 and 5) and a band of similar size in the supernatant of the invasion reaction (lanes 3 and 7). Controls using similarly cultured uninfected erythrocytes demonstrated some cross-reactivity with erythrocyte derived proteins in the invasion supernatant (lanes 4 and 8).

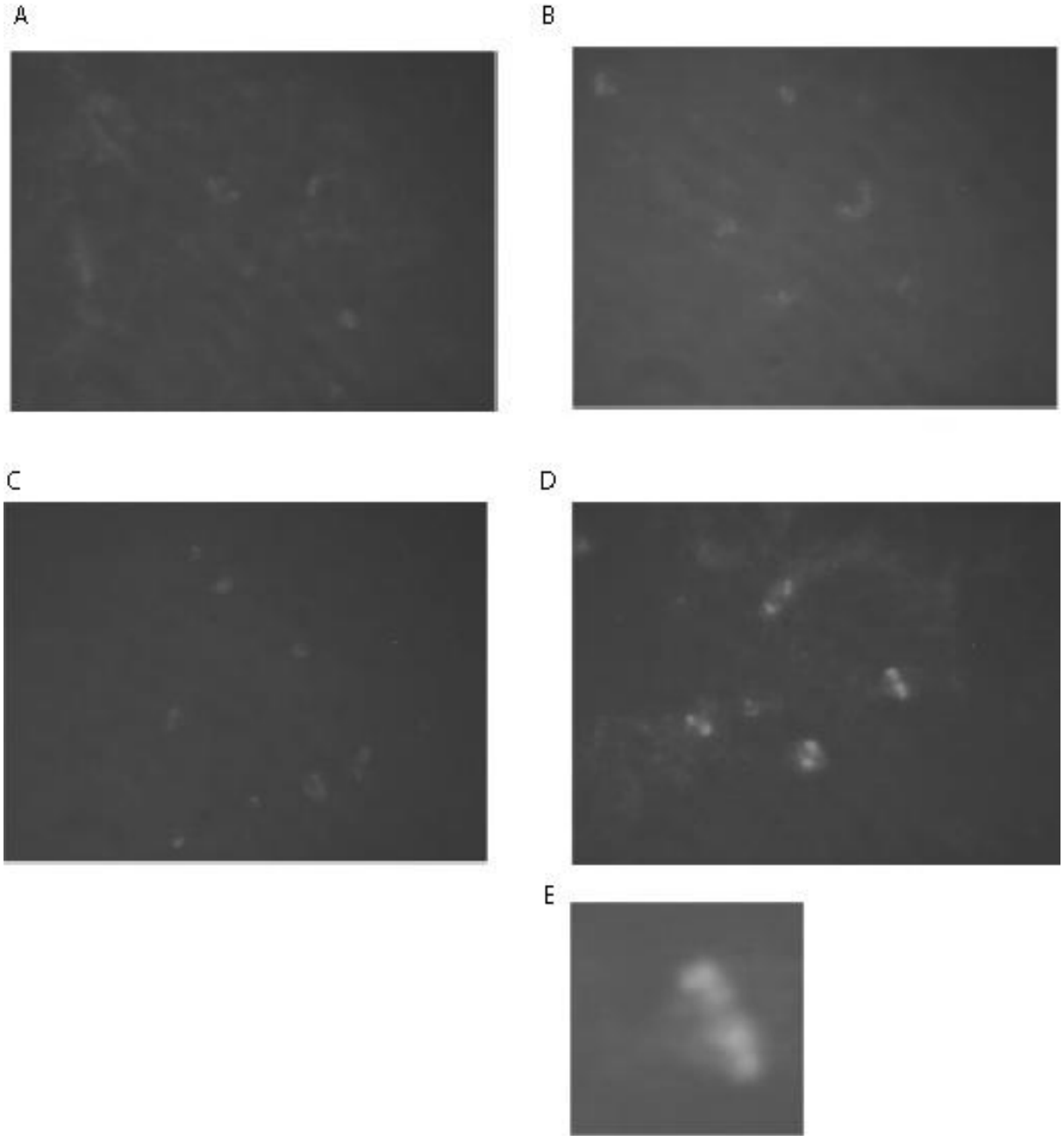

Fig 3. Immunofluorescence reactivity of *B. bovis* infected bovine erythrocytes with antisera against BbTRAP derived peptides. Slides were incubated with immune serum against peptide 1 (B), peptide 2 (D) and the corresponding preimmune sera (A, C). An enlargement of a duplicated *B. bovis* merozoite at maximum magnification (1000×), reacting with anti-peptide 2 serum, is shown in panel E. For coloured picture see page 97

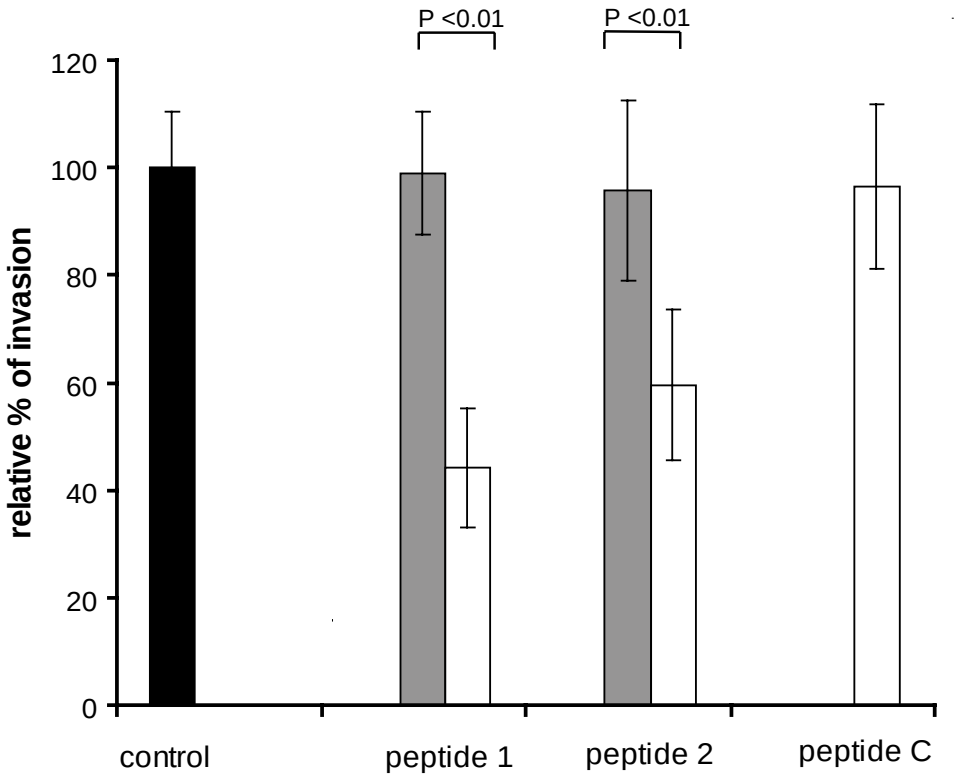

Fig. 4. Inhibition of erythrocyte invasion by *B. bovis* merozoites by antisera raised against three different synthetic peptides. The black bar shows invasion of *B. bovis* merozoites without preincubation and is taken as the 100% value against which the other incubations are plotted. White bars represent invasion of erythrocytes by *B. bovis* merozoites after pre-incubation with antisera against peptide 1, peptide 2 and peptide C (non-related peptide), whereas grey bars shows invasion of *B. bovis* merozoites after pre-incubation with pre-immune serum. Each bar represents the average value of six individual experiments and error bars indicate standard deviation. The groups were tested by the non-parametric test of Kruskal-Wallis and considered significant at $p<0.05$. Pairwise comparisons of the groups were calculated by post-hoc analysis as advised by Kruskall-Wallis.

For both antisera the band present in the supernatant was much weaker as the band in merozoite extracts. In both cases 15 µg was applied to gel which corresponded to 4.7 x 10^6 merozoites for the total merozoite extract and an amount of supernatant corresponding to the invasion of 7.6 x 10^6 merozoites into new erythrocytes (invasion efficiency was 25 % in this case) leading to the conclusion that only part of BbTRAP has been secreted.

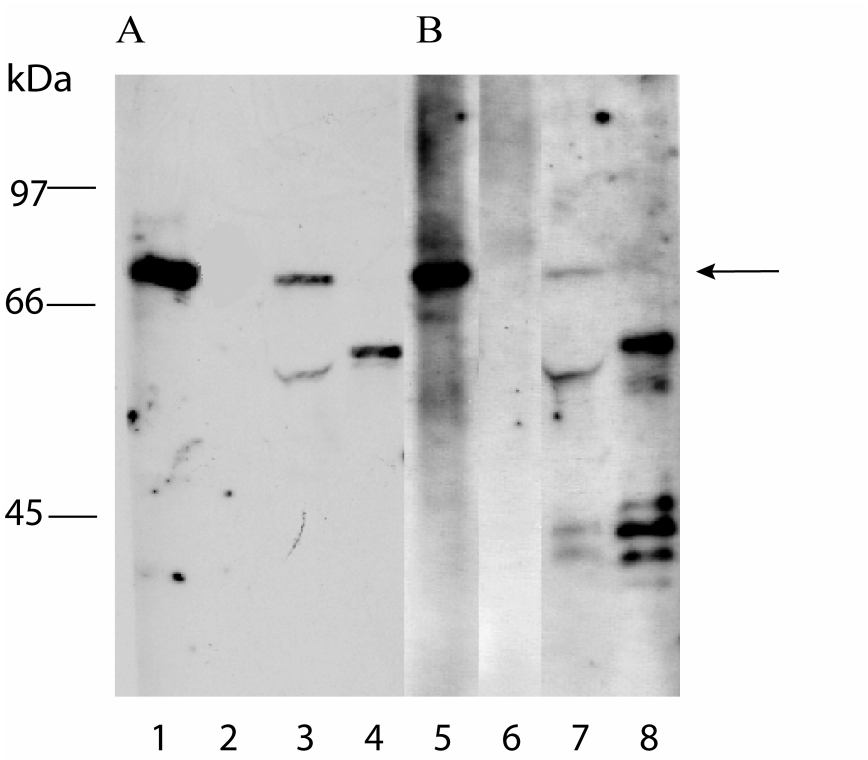

Fig. 5. Detection of BbTRAP in total merozoites extracts and invasion supernatant by western blotting. 15 µg of total merozoites extract (lane 1 and 5) or invasion supernatant (lane 3 and 7) were separated by SDS-PAGE (10%) and incubated after blotting with anti-peptide 1 (panel A) or anti-peptide 2 (panel B) antiserum. Control extract prepared by identical procedures but derived from non-infected cultures were included for total merozoite extract (lane 2 and 6) and invasion supernatant (lane 4 and 8).

DISCUSSION

The TRAP-family and apical membrane antigen 1 (AMA-1) [63, 221] represent the only transmembrane proteins involved in host cell invasion that are well conserved throughout the apicomplexa, indicating an important conserved function. The identification of BbTRAP presents the first example of a member of the TRAP-family of adhesins within the order of the Piroplasmida, formed by the genera *Theileria* and *Babesia*.

Despite the anticipated discovery of a TRAP-family protein in *B. bovis*, given the range of apicomplexan parasites that were previously shown to harbour one or several members of this family [127, 183, 206, 230], some remarkable findings were obtained. Phylogenetic analyses have not unambiguously grouped the Piroplasmida in a clade with either the Haemosporida or the Eimeriida. The modular composition of

BbTRAP is more similar to that of malaria parasites, in particular the rodent parasites *P. berghei* and *P. yoelli*. Like these, the predicted extracellular domain of BbTRAP has a region composed of simple imperfect repeats, in addition to a single vWFA-domain and a TSP1 domain. In *Plasmodium* simple repeat regions occur frequently, in particular in antigenic proteins [6]. A specific function for the tripeptide repeat regions in PbTRAP and PyTRAP has not been identified. A Blastp similarity search with the repeat region of BbTRAP (11 repeats; consensus MSSSHSD), only identified three hypothetical *P. falciparum* proteins (Acc. No. AAN36420, CAB62891 and AAC71881) carrying imperfect heptapeptide repeats. The highest score ($E = 2e^{-8}$, using a BLOSUM 45 scoring matrix and standard gap penalties for searching the Genebank nr database) was obtained with hypothetical protein PFL1670c (Acc. No AAN36420; consensus of repeat is KSSQHND) that was predicted to have a signalpeptide. Whether these repeats represent an evolutionary conserved apicomplexan feature, an acquired functional similarity or just coincidental sequence similarity remains speculative.

A highly similar modular architecture of BbTRAP and PbTRAP does not translate into involvement in exactly the same biological events. Whereas *B. bovis* and *Plasmodium* multiply by an asexual erythrocytic cycle involving a similar invasion process, TRAP has never clearly been demonstrated to be expressed during this stage in *Plasmodium*. Here, we have obtained proof for an apical location of BbTRAP in *B. bovis* merozoites by immunofluorescence, which is in accordance with the micronemal localization of other TRAP-family proteins, and have demonstrated that BbTRAP migrates as a 75 kDa protein when extracted from merozoites. Its possible presence in sporozoite stages, like for *Plasmodium* TRAP, has of course not been excluded.

Inhibition of erythrocyte invasion by the two antisera raised against peptides derived from the ectodomain of BbTRAP suggests an essential function in invasion. The inhibitory effect might be due to specific interference with a crucial mechanistic step, like adhesion to a specific host receptor, or to more aspecific effects like the immuno-aggregation of merozoites (although this was not observed under the microscope). This observation is remarkable as antibodies directed against *Plasmodium* TRAP or *T. gondii* MIC2 have been reported to be unable to achieve invasion inhibition [80]. A preincubation of free merozoites with antisera against BbTRAP was required (as compared to direct addition of the antiserum to in vitro invasion) to obtain the inhibitory effect. This implies that BbTRAP, the antibody target, is already exposed on the membrane before contact with the host cell takes place. In *Plasmodium*, TRAP was shown to be constitutively present on the sporozoite membrane in only small amounts whereas massive secretion of TRAP stored in the

micronemes only takes place upon contact with the host cell or after application of artificial stimuli resulting in microneme secretion [80]. TRAP located on the *Plasmodium* sporozoite membrane is involved in gliding motility over solid substrates and becomes capped from the apical end, where it is presumably constitutively secreted, to the posterior end, where it is liberated from the membrane leaving a trail of TRAP protein on the substrate [134]. Membrane localization of TRAP is required for gliding motility which is however insensitive to mutations in the extracellular adhesive domains [134]. These mutations do impair invasion of sporozoites but not the preceding step of host cell adhesion [134], suggesting a tight interaction of the host cell with TRAP molecules that become secreted upon initial contact with the host cell. The moving junction, attaching the invading sporozoite to the host cell, is reminescent of capping and may contain the TRAP molecules. Merozoites do not possess gliding motility (nor express TRAP in *Plasmodium*) but do invade by a moving junction mechanism. In *Plasmodium* the role of TRAP in sporozoites is suggested to be performed in merozoite invasion by highly specific binding ligands like DBL, EBA-175 or EBA-140 [1, 81, 217]. A role for TRAP in the invasion of erythrocytes thus presents a novelty. One difference between invasion by *B. bovis* and *Plasmodium* merozoites is the more relaxed specificity observed in vitro for *B. bovis* merozoites which were shown to be able to invade erythrocytes from a wide range of host species [78]. This could make the use of BbTRAP in *B. bovis* erythrocyte invasion plausible as TRAP does not seem to be involved in highly specific sequence interactions. Also MIC2 of *T.gondii* is a TRAP family member which is clearly involved in invasion of host cells by tachyzoites [22, 103] and it may contribute to the efficient invasion of almost any nucleated vertebrate cell type.

The release of soluble BbTRAP into the medium was demonstrated to take place during in vitro invasion. TRAP has been shown to be released from *Plasmodium* sporozoites after capping to the posterior end [80]. Also, PbTRAP has been shown to be secreted into the culture supernatant by *P. berghei* sporozoites incubated at 37^{o}C [16]. Although proteolytic processing is held responsible for secretion, an identical doublet of protein bands, of which the upper one was recognized by antiserum directed against the cytoplasmic domain, was detected on western blots from total cell lysates and from culture supernatant [16]. This indicates that a processed form of PbTRAP was present in total cell lysates and corresponds to our observation that the secreted form of BbTRAP has a similar size as the form detected in total merozoite extracts.

Release of the extracellular domain of *T. gondii* MIC2 by proteolysis has been shown to be essential to the proceeding of invasion [22, 35, 164]. Proteases involved in this process have been indicated [22, 35, 164] and several reports described the mapping of the proteolytic cleavage site in MIC2 and related proteins [35]. The extraordinary

sequence conservation of the transmembrane region observed for a number of micronemal proteins including MIC2 and TRAP was also noted here for BbTRAP. Apicomplexan homologues of a protease called rhomboid are likely to recognize this sequence resulting in intra-membrane cleavage [224]. Another cleavage site was mapped to a lysine residue of MIC2 located 11 amino acids N-terminal to the transmembrane region [22], which might be conserved in BbTRAP (K576). The cytoplasmic tail of BbTRAP is also conserved at a number of critical residues. One conserved residue is a sub-terminal tryptophane (W651) that has been implicated in the binding of aldolase which probably serves as a link between transmembrane proteins and the actin/myosin motor that drives invasion and gliding motility [26, 106]. Another conserved motif, involved in micronemal targeting of proteins [16], consists of a tyrosine at position one and a bulky hydrophobic residue at position four and is possibly represented by the sequence YAMY (607-610) in BbTRAP. In conclusion, BbTRAP displays conserved sequence features shown to be involved in other TRAP family members with micronemal targeting, adhesion to host cells, attachment to an actin/myosin motor and cleavage of the ecto-domain by conserved proteases.

ACKNOWLEDGEMENT

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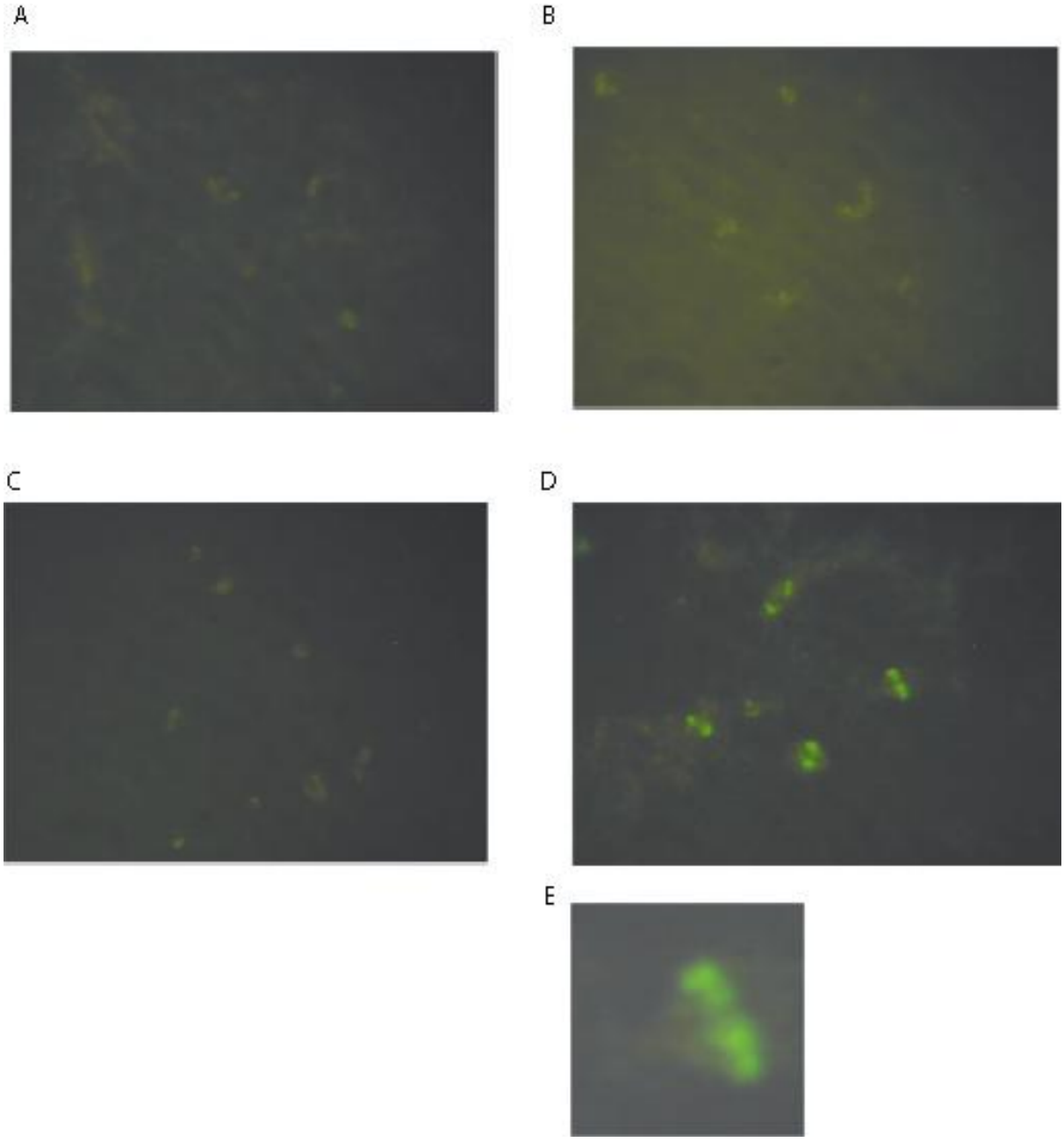

Fig 3. Immunofluorescence reactivity of *B. bovis* infected bovine erythrocytes with antisera against BbTRAP derived peptides. Slides were incubated with immune serum against peptide 1 (B), peptide 2 (D) and the corresponding preimmune sera (A, C). An enlargement of a duplicated *B. bovis* merozoite at maximum magnification (1000×), reacting with anti-peptide 2 serum, is shown in panel E.

CHAPTER 6

A STUDY ON THE POSSIBILITIES FOR OBTAINING GENETIC TRANSFORMATION OF *BABESIA BOVIS*

Fasila R. Gaffar, Karlijn Wilschut, Sietze Brandes, Frits F. J. Franssen and Erik de Vries

Submitted for publication

ABSTRACT

A first attempt was undertaken to establish a transfection system for *B. bovis*. Plasmids containing as selectable markers *Toxoplasma gondii dhfr-ts*m2m3 and human DHFR under control of *B. bovis dhfr-ts* promotor and termination region were constructed. Reporter genes for transient and stable transfection were introduced in a head to tail position in both plasmids. Different combinations of electroporation conditions, plasmid introduction (linear or circular), concentration of plasmid and target cells (infected erythrocytes or free merozoites) were tested. Although culture lines moderately resistant against pyrimethamine and WR99210 drugs were selected, none of these cultures were shown to contain plasmid constructs by southern blotting. Expression of the reporter genes YFP, GFP or β-galactosidase was not detected. Sofar, we have not been able to establish a stable or transient transfection for *B. bovis.*

INTRODUCTION

The ability to alter the encoded amino acid sequences of apicomplexan genes or to manipulate their levels or timing of expression in their natural context has in recent years become feasible for *Toxoplasma gondii* and several *Plasmodium* species [51, 65, 130, 141, 239]. Although the versatility of the various transfection systems that have been established is not yet comparable to eukaryotic model systems like yeast, it has greatly expanded the knowledge on the biology of this group of important parasites. With increasing numbers of *Babesia bovis* gene and protein sequences becoming available from an EST project (www.sanger.ac.uk) and, more specifically, from studies aiming at the identification of potential vaccine components [49, 161] or delineating the mechanism of erythrocyte invasion (this thesis), transfection is becoming a desirable tool for this species too.

Here we briefly describe the first steps that were undertaken to transfect *B. bovis* by aiming either at establishing stable transformation by using a drug resistance gene as selectable marker, or at obtaining the transient expression of a reporter gene. From a number of different selectable markers that have been successfully applied for apicomplexan transfection [51, 65, 130] we have selected here the human dihydrofolate reductase (*dhfr*) gene as a first option. Human DHFR is highly resistant to the antimalarial drug pyrimethamine and another dihydrofolate analogue denoted WR99210. Pilot experiments indicated that especially WR99210 was lethal to *B. bovis* in vitro cultures in the nanomolar range (chapter 7). In apicomplexans dihydrofolate reductase (DHFR, EC 1.5.1.3) is encoded as part of a single bi-functional protein in

combination with thymidylate synthase (TS, EC 2.1.1.45) and its inhibition eventually leads to an arrest of DNA replication by depletion of the precursors required for pyrimidine de novo synthesis on which apicomplexans are fully dependent. Expression of a selectable marker requires the addition of control elements. Hardly anything is known for *B. bovis* about transcriptional regulation and the promoter and termination sequences involved. Therefore, the expression of the human *dhfr* gene under the control of the flanking regions of the *B. bovis dhfr-ts* gene, of which the cloning is described in chapter 7, seems a logic choice for an attempt to obtain levels and timing of expression of human DHFR similar to the natural situation. As reporters the genes encoding green fluorescent protein (GFP), yellow fluorescent protein (YFP) and β-galactosidase have been incorporated in the transfection plasmid constructs. Electroporation was chosen as the method for introduction of the plasmid DNA.

MATERIALS AND METHODS

Transfection plasmids

Transfection constructs were designed as schematically depicted in Fig. 1. All oligonucleotides used to amplify the PCR fragments described below are listed in table II. The *T. gondii dhfr-ts*m2m3 gene (TgD) or human *dhfr* (HsD) gene were cloned under control of the flanking regions of *B. bovis* DHFR which functioned as promoter and termination regions of transfection plasmids. The HsD fragment was excised from plasmid pD+WT by *Bam*HI and *Nco*I restriction sites digestion, whereas the TgD fragment was digested from plasmid pExpress1 with *Hin*dIII and *Nsi*I [51, 226]. Both fragments were ligated in pUCPCR [55] plasmid digested by the same restriction enzymes giving plasmids pUC-HsD and pUC-TgD. The promoter region of the *B. bovis* DHFR-TS (d_5) was ligated upstream of the HsD or TgD gene. To create plasmids pUC-d_5HsD and pUC-d_5TgD, the promoter region of *B. bovis dhfr-ts* was amplified by PCR using primers 5 UTRse and 5UTRnco or primers 5UTRkpn and 5UTRas, respectively. PCR was performed in a 50 µl volume containing 0.2 mM dNTP, 20 pmolµl^{-1} of each primer, 100 ng *B. bovis* genomic clone g6 (chapter 7) and 0.5 U Taq DNA polymerase in standard buffer (Promega). Amplification was performed for 30 cycles (92°C for 30 s, 55°C for 30 s and 72°C for 30 s) preceded by initial denaturation for 3 min at 95°C and a final elongation at 72°C for 10 min. The 2.83 kb amplified promotor fragment was cloned into pUC-HsD digested with *Nco*I and *Pst*I or into pUC-TgD digested with *Age*I and *Kpn*I. The 3'UTR of *B. bovis* DHFR-TS was amplified from clone g2 with primers 3UTRse and 3UTRkpn or primers 3UTRse and 3UTRas giving 1.35 kb products that were introduced after cleavage with respectively *Kpn*I and *Bam*HI or *Hin*dIII in plasmids pUC-d_5HsD and pUC-d_5TgD digested by the same enzymes creating pUC-d_5HsDd_3 and pUC-d_5TgDd_3. For introduction of a reporter gene into the plasmids, a terminator and promoter region were

ligated. The intergenic region between ABC and 12D3 ($a_3 12 d_5$) gene of *B. bovis* was amplified for genomic DNA with primers ABCse and 12D3as. The amplified fragment was used for nested PCR with primers 3UTRabc and 5UTR12d3. The resulting 450 bp fragment was digested with *Bgl*II and *Bam*HI and ligated into pUC-d_5HsDd_3 and pUC-d_5TgDd_3 (vectors were digested with *Bam*HI) creating pUC-d_5HsD$a_3 12 d_5 d_3$ and pUC-d_5TgD$a_3 12 d_5 d_3$. Next the reporter genes GFP, YFP and β-galactosidase were amplified. GFP was amplified from plasmid pdhfrCAT-GFP with primers GFPxho and GFPnsi or primers GFPxba and GFPnsi. The 730 bp amplified fragments were digested (with *Bam*HI and *Nsi*I or *Xba*I and *Nsi*I) and ligated into pUC-d_5HsD$a_3 12 d_5 d_3$ or pUC-d_5TgD$a_3 12 d_5 d_3$ plasmids (vectors were digested with the same restriction enzymes) resulting in pUC-d_5HsD$a_3 12 d_5$GFd_3 and pUC-d_5TgD$a_3 12 d_5$GFd_3. YFP was amplified from tubYFPYFP-sagCAT with primers YFPxho and YFPnsi or primers YFPxba and YFPnsi. The 740 bp amplified fragments were digested (with *Bam*HI and *Nsi*I or *Xba*I and *Nsi*I) and ligated into pUC-d_5HsD$a_3 12 d_5 d_3$ or d_5TgD$a_3 12 d_5 d_3$ plasmids (plasmids digested with the same enzymes) creating pUC-d_5HsD$a_3 12 d_5$YFd_3 and pUC-d_5TgD$a_3 12 d_5$YFd_3. Finally β-galactosidase was amplified from χgt11 with primers βLACnsi and βLACxho. The 3.0 kb amplified fragment was digested with *Xho*I and *Nsi*I and ligated into pUC-d_5HsD$a_3 12 d_5 d_3$ plasmid (plasmid digested with the same enzymes) resulting in pUC-d_5HsD$a_3 12 d_5$GALd_3. All constructs described above were partially sequenced in order to confirm the sequences of PCR primers and proper ligation of restriction sites.

In vitro *cultivation and electroporation of* B. bovis

B. bovis Israël strain (clonal line C61411), was maintained in vitro by serial passage of bovine red blood cells (RBCs) as described in chapter 5. Medium changes were carried out every 24 h and parasitaemia was kept between 1 and 12 % by dilution. The parasitaemia was measured by counting 3000 RBC in giemsa stained smears.

For transfection, ~2.10^9 *B. bovis* infected red blood cells (50% packed cell volume, 7% to 10% parasitaemia) were electroporated in a Gene Pulser II system (Biorad) with 100µg DNA construct in a final volume of 500 µl in cytomix (120mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/ KH_2PO_4 pH 7.6, 25mM Hepes pH 7.6). Besides directly electroporating *B. bovis* red blood cells with DNA we also liberated the merozoites first with electroshock (5 times under the same conditions described below) before adding plasmid DNA. Cells were cooled on ice for 5 min before pulsing at high voltage (1 kV, 200Ω, 25 µF). 120 µl of electroporated cells were immediately added to 1020 µl pre-warmed M199 culture media containing 60 µl bovine red blood cells. For 72 h in vitro cultivation was performed in 24 well plates at 1.25 µM pyrimethamine or 2.5 nM WR99210 in 5% CO_2 in air. After three days the concentration of pyrimethamine was increased to 5.0-10.0 µM and of WR99210 to 5.0-20 nM.

Table Ia: Nomenclature used for coding and non-coding regions

Flanking regions		**coding regions**	
DHFR promotor	d_5	Human DHFR	HsD
DHFR terminator	d_3	*T. gondii* DHFRTSm2m3	TgD
12D3 promotor	$_{12}d_5$	YFP	YF
ABC terminator	a_3	GFP	GF
		β-galactosidase	GAL

Table Ib: Conditions and test methods used for transfection

Transfection plasmids	**µg**	**Plasmid form** Linear or circulair	**Target cell** Free merozoites (F) or Intra-erythrocytic (I)	**Stable transfection** [Pyrimethamine] (PYR) or [WR99210] (WR**)** allowing normal growth rate	**Resistant lines** Number tested on southern blot	**Transient transfection** Number of lines tested
d_5TgDd_3	20 50 100	C	F, I	5-10 µM PYR	3	
d_5HsDd_3	20 50 100	C	F, I	5-20 nM WR	3	
d_5HsD$a_3 12 d_5$YFd_3	20 100	L, C	F	5-20 nM WR	2	5
d_5TgD$a_3 12 d_5$YFd_3	20 100	L, C	F	5-10 µM PYR	2	5
d_5HsD$a_3 12 d_5$GFd_3	20 100	L, C	F	5-20 nM WR	2	5
d_5HsD$a_3 12 d_5$GALd_3	20 100	L, C	F	N.D.	N.D.	10

Table II: Oligonucleotides used in this study with restriction sites being underlined

Name	*Nucleotide sequence*
3UTRse	5'- gatcacaagcttttggatcctacagtcttatttggtagaacc
3UTRas	3'- gctcttggtaacgttccatggatatcgatcgaagcttgaattgagg
3UTRabc	5'- tgaagatctagcgcgcctgtatagattatgacagtagttac
3UTRkpn	5'- cggggtaccaacgttaccaagagcaactcc
12D3as	5'- gcttacccattctgatcgtacat
5 UTRse	5'- aaactgcagaccggtcatactagttattga gatacagtgccaaattc
5UTRnco	3 - agtagccatggttaatcatatatg cacttgat ataa
5UTRkpn	5'- cggggtacccatactagttattgagatacagtgccaaattc
5UTRas	3'- tgattaaccaagtgcatataatgcagaaaccggtgtgtc
5UTR12d3	5'- cgggatcctatctagaagcatgcatcttgtatgtcgaatagtatt
ABCse	5'- gaaacagtggtgaaaaataaggc
GFPxba	5'- gctctagatagttcatccatgccatgtgta
GFPnsi	5'- ccaatgcatgctgccggcgctgctgccgtgagtaaaggagaagaactt
GFPxho	5'- gcggatccttactcgagtagttcatccatgccatgtgtaa
HsDse	5'- tatgattaaccatggttggttcgcta
HsDas	5'- atcattcttctcatatacttcaaatttgt
LACnsi	5'- gggatgcatattacggattcactgg
LACxho	5'- gggctcgagttatttttgacaccagac
TgDse	5'- ggtcgtcgcgatgacccccaagaggggc
TgDas	5'- cggtagagggtagacctaagcaggcacgc
YFPxba	5'- gctctagacagctcgtccatgccgaga
YFPnsi	5'- ccaatgcatgctgccggcgctgctgccgtgagccaagggcgaggagc
YFPxho	5'- gcggatccttactcgagcagctcgtccatgccgaga

Cultures were three-fold diluted every day with non-infected RBCs in drug containing medium from day 3 onwards. After parasitaemia declined below detection level cultures were transferred to 96 well plates and maintained under 5% CO_2, 2% O_2. In about six weeks the parasitaemia increases from undetectable level to more than 1.0 % in ~2% of the wells. For examining transient expression of the reporter gene the cultures were harvested 0, 4, 8, 24 and 40 hr after transfection.

Purification of B. bovis *genomic DNA and transfection plasmids*

B. bovis infected red blood cells (2×10^9 parasites) were washed two times in PBS and lysed in hypotonic buffer (PBS diluted with water to 70mM NaCl concentration and re-adjusted to 150 mM NaCl) for 2 min. The lysate was washed four times and resuspended in lysis buffer ((10 mM Tris, 1mM EDTA, 50 mM sodium chloride, 0.1% SDS) pH 7,4) with 10 µg/ml Proteinase K and incubated at 37°C for 2 h. Genomic DNA was extracted by phenol-chloroform (1:1) mixture and precipitated with 0.5 M NaCl and 2 volume of cold ethanol and resuspended in TE pH 7.4 (1mM TrisCl, 0,1 mM EDTA pH 7.4).

Transfection plasmids were transformed into competent *E. coli* XL1-blue MRF cells and selected on ampicillin (100 µg/ml) medium. The clones containing the plasmids were grown to large volumes till OD_{600} was equal to 0.7. Plasmids were purified on a column according to manufacturer's instructions using Giga prep kit (Qiagen). After column purification plasmids were precipitated with 0.5 M sodium chloride and 2 volume of cold ethanol and resuspended in TE pH 7.4 (1mM TrisCl, 0,1 mM EDTA pH 7.4).

Screening for GFP and YFP fluorescence and β-galactosidase activity

B. bovis cultures harvested at 0, 4, 8, 24 and 40 h after transfection, were assayed for fluorescence or β-galactosidase activity in merozoites. The transfected cells were washed once in PBS and 10µl merozoite suspension (5% PCV) was examined on a microscope slide under a cover slide. For detecting GFP and YFP standard FITC excitation emission filters (488/507 nm) under a light fluorescence microscope (Olympus) at X 100 magnification was used. At least 1000 cells per sample were screened. For β-galactosidase activity, transfected parasites were pelleted by centrifugation at 500 g and resuspended in lysis buffer (100 mM HEPES, pH 8.0, 1mM $MgSO_4$, 1% Triton X-100, 5 mM dithiotreitol) for 1 h at 50°C. As positive control β-galactosidase of pUC19 was induced with IPTG and lysed by sonification. The lysate was centrifuged and supernatant was frozen at -20 °C. One volume of cleared lysate was diluted with one volume of lysis buffer (total volume 100 µl) and mixed with an equal volume of lysis buffer containing 2 mM chlorophenol red-β-D-galactopyranoside (CPRG) (Boehringer-Mannheim) as substrate. Reactions were performed in 96-well plates for 30 min at 37°C . Absorbtion at 570 nm was measured in a microplate reader [50, 138].

Selection of resistant lines and detection of DNA integration by southern blot

Pyrimethamine and WR99210 were dissolved at 10 mg/ml in DMSO. To determine IC_{50} values for both drugs, wildtype *B. bovis* culture was incubated in M199 culture medium in 24-microwell plates under standard culture conditions in presence of different concentrations of pyrimethamine and WR99210 for a period of 96 h. Serial dilutions of the drug were made in culture medium and the concentration of drug tested ranged from 0 nM to 5 nM WR99210 and 0 to 2.5 µM pyrimethamine. Cultures were diluted 1:3 every day with 5% fresh RBCs. Giemsa stained smears were made every 24 h. IC_{50} values were calculated at

96 h after counting 3000 RBCs. Drug-resistant cultures were tested by the same method at drug concentrations of 5 nM, 10 nM and 20 nM WR99210 or 2.5 μM, 5 μM and 10 μM pyrimethamine. All drug-resistant cultures were growing at wild-type rate in 10 nM WR99210 and 5 μM pyrimethamine whereas cultures eventually died at 20 nM WR99210 and 10 μM pyrimethamine prohibiting a more precise determination of IC_{50}.

Genomic DNA obtained from pyrimethamine or WR99210 resistant lines was examined by PCR amplification (PCR conditions as used above) using specific oligonucleotides (Table II).

The presence of HsD or TgD plasmid in resistant culture lines was tested by southern blotting. Ten μg of genomic DNA of the resistant lines were single or double-digested with *Kpn*I, *Eco*RI or *Hind*III, separated by electrophoresis on a 1% agarose gel, and transferred to positively-charged nylon membrane (Hybond N+, Amersham-Pharmacia Biotech). Plasmid containing the *T. gondii dhfr-ts*m2m3 or human *dhfr* was used as positive control. A human *dhfr*-specific 500 bp fragment and a *T. gondii dhfr-ts*m2m3 -specific 1800bp fragment were obtained by PCR using primer combinations HsDse and HsDas or TgDse and TgDas respectively. PCR reactions were performed under the same conditions as described above. Both fragments were purified from agarose gel and labelled with 50 μCi ^{32}P-dATP (3000 $Cimmol^{-1}$), using a Random Primer labelling kit (Roche). The membranes were incubated overnight with the labelled probe in 6×SSC at 65°C, and washed at a final stringency in 1× SSC at 65°C.

RESULTS

Selection of transfected parasite cultures resistant to pyrimethamine and WR99210

Transfection plasmids carrying the human *dhfr* gene (insensitive to WR99210 and pyrimethamine) or the *T. gondii dhfr-ts*m2m3 gene (a mutant that is insensitive to pyrimethamine) as a selectable marker, in addition to a YFP reporter gene, were constructed as described in the material and methods section and schematically outlined in Fig. 1. The flanking regions of the *B. bovis dhfr-ts* gene (chapter 7) were used as control regions for initiation of transcription of the selectable marker genes and transcription termination of the reporter genes respectively. The complete intergenic region containing the putative termination signal of a *B. bovis* ABC transporter gene and the putative promoter region of the highly expressed 12D3 antigen [55] was used as a control region separating the selectable marker and reporter genes.

Electroporation has been a successful method to introduce plasmid DNA into *Plasmodium* [130, 202] and *T. gondii* [198, 209] parasites. *B. bovis* parasites were demonstrated to remain viable at voltages as high as 2.5 kV (chapter 2). A number of variations in the experimental transfection conditions were examined in combination

with the different plasmid constructs as indicated in Table Ib. Variables tested included the amount of plasmid DNA used (20 µg, 50 µg, 100 µg), the state of the plasmid (linear or circular) and the state of the culture to be transfected (infected erythrocytes or free merozoites liberated prior to transfection by electroshock). Upon electroporation parasites were transferred to 24-well plates and cultivated for three days at 5% CO_2 in air followed by a transfer to 5% CO_2 and 2% O_2 in 96-well plates after nine days. Cultures were diluted on a daily basis. Drug concentrations were increased at day three as indicated in Fig. 2 which shows a growth curve typical for the small percentage of electroporated cultures surviving on the long term.

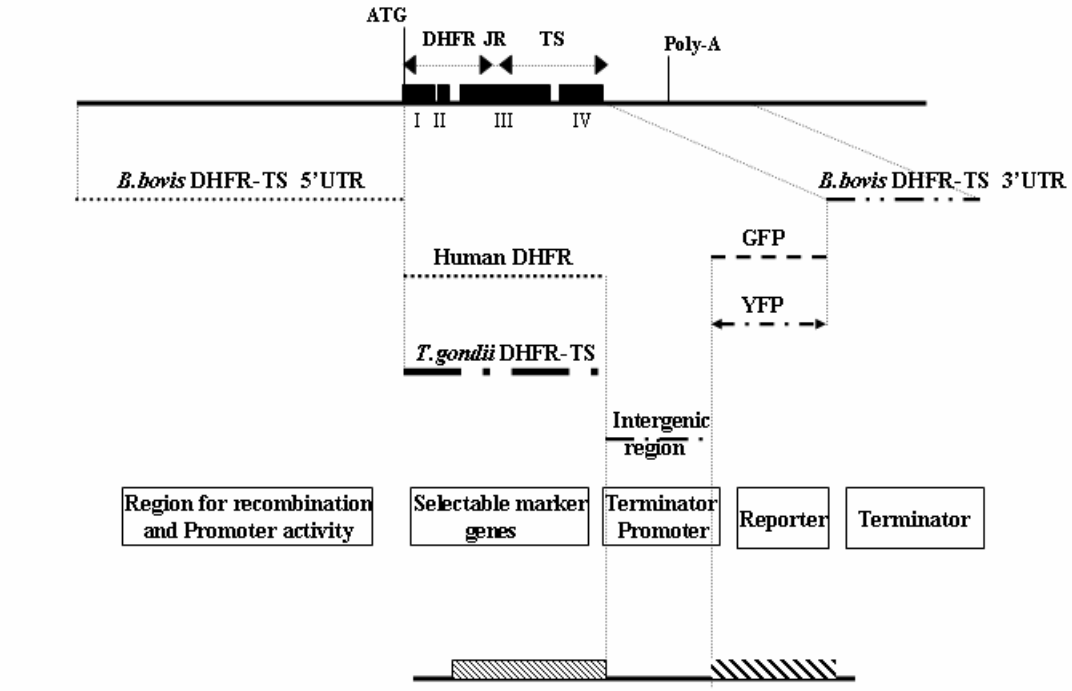

Fig. 1. A schematic representation of plasmid contructs used for transfection of *B. bovis*. The two selectable markers genes (Human *dhfr* and *T. gondii dhfr-ts*m2m3) are placed under promotor and terminal regions of *B. bovis* DHFR-TS gene. The intergenic region of an ABC transporter gene and the 12D3 antigen gene of *B. bovis* was incorporated as a control region in plasmids containing reporter genes (YFP, GFP and β-galactosidase).

After an initial phase of growth parasitaemia always decreased below detection level after 9 to 17 days. About 2% of the cultures displayed an increase in parasitaemia after 17 to 24 days of continued in vitro culture. Of these a number were selected and propagated for another 4 to 6 weeks under daily dilution at drug concentrations increased from 5 to 10 µM pyrimethamine or 5 to 20 nM WR99210. A further increase of drug concentrations always resulted in rapid decrease in parasitaemia.

Cultures transfected with the constructs containing a YFP reporter gene were examined for fluorescence at 488/507 nm under the immunofluorescence microscope. No increase above background fluorescence as compared to control cultures was observed in any of the selected lines. Total DNA was isolated of parasite lines with decreased drug sensitivity that were electroporated with plasmids without a reporter gene. Southern blots of the DNA were hybridized with probes derived from the human DHFR gene or the *T. gondii* DHFR-TS gene and compared to positive control lanes containing the corresponding plasmids used for transfection. No signal for the human *dhfr* gene or the *T. gondii dhfr-ts*m2m3 gene was detected in the DNA samples of any of the transfected and drug-resistant cultures indicating that no stable integration of the plasmid DNA in to the genome has taken place nor that detectable amounts of extrachromosomal plasmid are present.

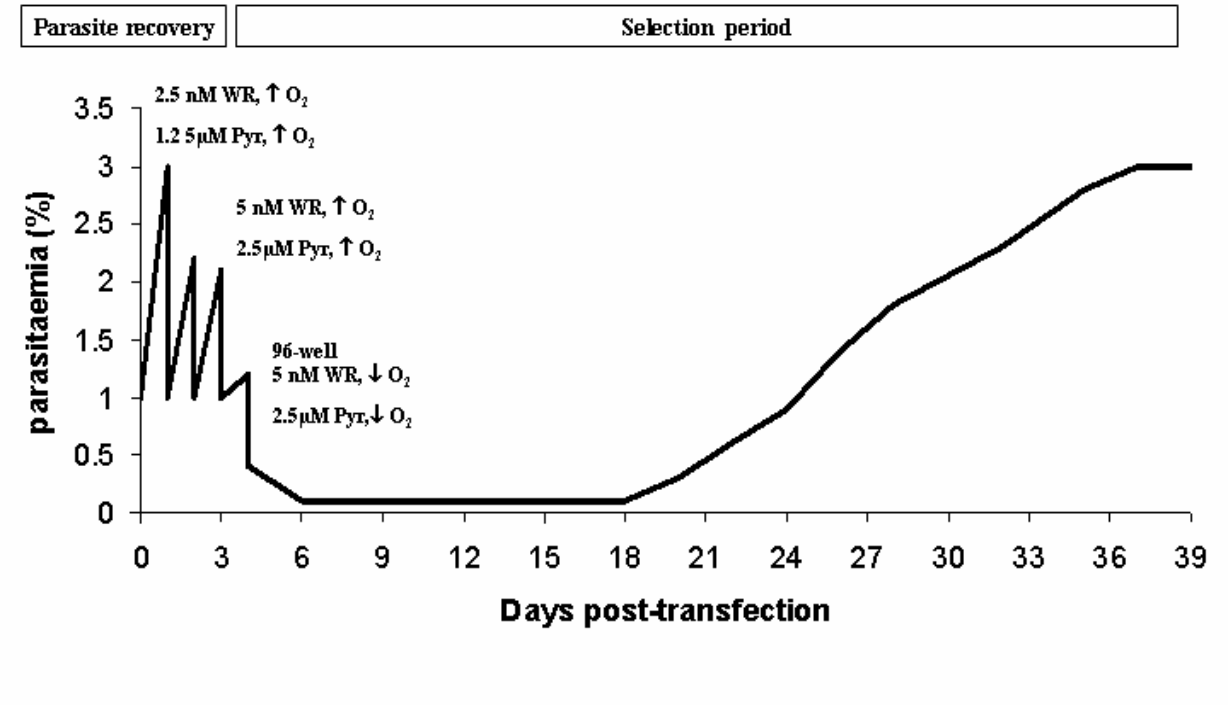

Fig. 2. Typical parasite growth curve of parasites transfected with one of the different plasmids (Table II). Immediately after electroporation parasites were cultured in medium containing 2.5 nM WR99210 or 1.25 µM pyrimethamine. After three days drug concentrations were increased to 5 nM WR99210 and 2.5 µM pyrimethamine. After parasitaemia decreases till undetectable levels the culture conditions are changed to 96-well plates and low oxygen level (2% O_2). In the case shown here, an increase in parasitaemia occurred after three weeks (variation from 3 to six weeks for other culture lines) upon which drug concentrations were further increased.

Screening for transient expression of reporter genes shortly after electroporation

In view of the negative results obtained after selection for parasites displaying decreased inhibitor sensitivity a new construct was made carrying β-galactosidase (Table II) as a reporter gene. Colorimetric enzyme assays for β-galactosidase activity in extracts from transfected parasites have been reported as a very sensitive method for detecting low levels of expression. In addition, the constructs carrying a YFP reporter gene were used for examination of transient expression. Upon electroporation parasites were transferred to 24-well plates and cultivated for 0 to 40 h at 5% CO2 in air. Wells were harvested at 0, 4, 8 ,24 and 48 h. Microscopic slides were made and examined under the fluorescence microscope for YFP expression. Extracts were made and assayed for β-galactosidase activity. Negative and positive controls were examined as indicated in Material and methods. None of the samples tested gave a signal that indicated the occurrence of transient transfection at any of the timepoints examined.

DISCUSSION

In this chapter we describe the construction of plasmid vectors designed for stable or transient transfection of *B. bovis* parasites. The combinations of two *dhfr*-based selectable marker genes and two receptor genes have not yet resulted in the generation of such transfectants. Initially our attempts were directed at the establishment of stable transfection by making use of WR99210 and/or pyrimethamine resistant *dhfr* selectable marker genes. It was a slightly opportunistic approach, aiming to establish most rapidly the type of transfection system that is most needed. The success of using electroporation in combination with these markers in obtaining stable transfection in *T. gondii* and *Plasmodium* inspired this approach.

Initial tests on experimental conditions for *B. bovis* electroporation were promising, especially when compared with the problems still associated with *Plasmodium* transfection. It appeared to be possible to obtain free merozoites, by application of a high-voltage pulse prior to addition of the plasmid constructs, that were still highly viable and capable of efficient reinvasion of freshly added red blood cells (see Chapter 2 for a discussion on invasion efficiency of merozoites liberated by this method). In this way plasmids only have to pass the merozoite membrane and the nuclear envelope and avoid the long journey that has to be taken by crossing also the erythrocyte membrane and the parasitophorous vacuole membrane when parasitized erythrocytes are electroporated directly. Considering the applicability of the chosen

selectable markers it appeared that the IC_{50} value for WR99210 of *B. bovis* cultures was very low and in the same range as observed for *T. gondii* and *Plasmodium*, but for pyrimethamine the selection window is certainly narrower as *B. bovis* is relatively resistant to this drug (IC_{50} is 1.25 μM). Despite these positive indications, only parasite lines with moderately increased resistance to either WR99210 or pyrimethamine, but without any evidence for having taken up plasmid DNA, were obtained. After doing more extensive controls in the absence of any transfection plasmids it appeared that such resistance was easily induced. This has been analysed in more detail in chapter 7. After this, the initial aim was lowered and an attempt was done to achieve transient expression using a sensitive reporter gene in the form of *E. coli* β-galactosidase, sofar without success.

Which factors could currently be involved in prohibiting the establishment of transfection for *B. bovis*?

Regarding the transient expression system that was used it cannot be excluded that β-galactosidase is expressed but not in an enzymatically active form, despite positive results with this system in other apicomplexans [87, 138]. Other reporters could be tested in future like luciferase or chloramphenicol transferase. Most likely, there has been no or only very low expression of the reporters used. The chosen promoter (from the 12D3 antigen gene that is highly expressed in *B. bovis* merozoites) may not have been functional in the context in which it was cloned. Moreover, the constructs that were used for examining transient expression were complex in that they, in addition to the reporter gene contained a DHFR gene and 5' and 3' flanking regions of the *B. bovis dhfr-ts* gene that were later shown to contain (parts of) other genes (see Chapter 7). As a consequence, other gene promoters are likely to be present in the constructs. They may direct transcription of the genes (of unknown function) behind them, which are also cloned in the construct but in a truncated form, with unpredictable consequences. Therefore it might be necessary to employ simple constructs only expressing a reporter gene, possibly under the control of randomly inserted *B. bovis* DNA as a kind of promoter trap in order to isolate functional promoter sequences.

Obviously, several of the arguments raised above can also be involved in the inability to obtain stable transfectants. The arrangement of genes within the construct deserves further attention. It has been reported that putting genes in a head to head orientation gives better results as compared to a head to tail orientation that was also employed here [48, 234]. Aiming at stable transfection is usually synonymous to achieving integration of (part of) the plasmid constructs into the genome, although maintenance of an extrachromosomal plasmid under selective drug pressure is still an

option. Intergration can be accomplished by homologous or heterologous recombination. For homologous intergration the size of the target sequence available for recombination certainly plays a role[47, 64, 234, 239]. We, therefore, made a choice for incorporation of long flanking sequences of the *B. bovis dhfr-ts* gene but the subsequent discovery of genes within these sequences (see above) may corrupt this choice by having a profound effect on the viability of transfectants that have recombinated in these regions.

Transfection efficiency is certainly an important issue in establishing stable or transient transfection. Both are reported to be much lower in *Plasmodium* as compared to *T. gondii* but the reason for this remains speculative. The high AT content of *P. falciparum* has been mentioned as a possible factor [143, 222, 231] in this but on the other hand transfection efficiency of *P. berghei* is also much lower as reported for *T. gondii* despite its more normal GC content [52, 64]. For the moment there seems to be no obvious rational approach in selecting methods for optimal transformation efficiency of *B. bovis,* other than trial and error, until the moment that an initial transformation level is obtained from which optimization can take a start.

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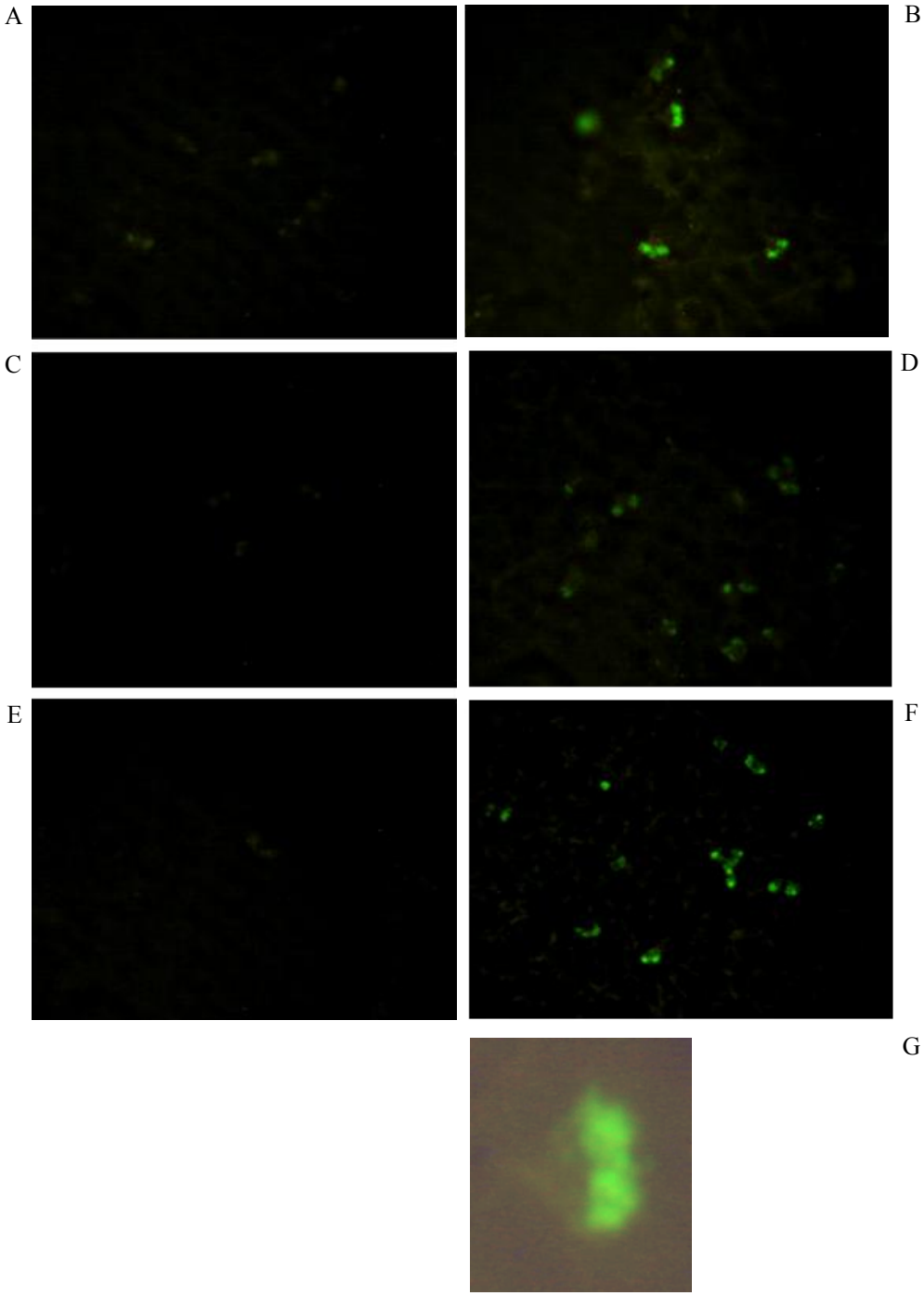

Fig. 3. Immunofluorescence reactivity of antiserum against *B. bovis* AMA-1 incubated with methanol-fixed *B. bovis* infected bovine erythrocytes. Slides were incubated with immune serum against peptide 1 (B), peptide 2 (D) and peptide 3 (F) and the corresponding preimmune sera (A, C, E). An enlargement of duplicated *B. bovis* merozoites showing immunofluorescence is shown (G).

CHAPTER 7

AN AMINO ACID SUBSTITUTION IN *BABESIA BOVIS* DIHYDROFOLATE REDUCTASE-THYMIDYLATE SYNTHASE IS CORRELATED TO CROSS-RESISTANCE AGAINST PYRIMETHAMINE AND WR99210

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Molecular and Biochemical Parasitology in press

"Voor iedere ziekte is er een genezing" "God liet zowel ziekte als medicijn ontstaan en heeft voor iedere ziekte een medicijn gegeven, gebruik het medicijn zolang dat medicijn niet haraam (verboden) is."
(Abu Dawud)

ABSTRACT

The genomic locus and cDNA encoding *Babesia bovis* dihydrofolate reductase-thymidylate synthase (DHFR-TS) were cloned and sequenced. A single *dhfr-ts* gene, composed of four exons, encodes a 511 aa protein that is most closely related to *Plasmodium falciparum* DHFR-TS. The genomic locus is characterized by the presence of four other genes of which at least three are expressed during the erythrocytic cycle. Three of the genes were highly conserved in closely related *Theileria* species and for two of the genes and *dhfr-ts*, gene synteny was observed between *B. bovis* and *T. parva*. *B. bovis* in vitro cultures displaying ~10 to 20-fold decreased sensitivity towards the antimalarial drugs WR99210 and pyrimethamine were selected repeatedly after prolonged growth in presence of drugs. Five cultures examined in detail were shown to encode a DHFR-TS carrying amino acid substitution S125F. 3D-modelling, using the *P. falciparum* DHFR structure as a template, suggests that substitution S125F protrudes into the binding site of NADPH. The S125F mutant could be isolated by growth under pyrimethamine or WR99210 pressure conferring cross-resistance to both drugs. Although opposing selection for pyrimethamine or WR99210 resistance was reported recently using *P. falciparum* or *P. vivax* strains carrying wildtype *dhfr*, the results obtained here are reminiscent of a quadruple mutant of *P. falciparum dhfr* displaying strong resistance to pyrimethamine and 10-fold enhanced resistance against WR99210. Wildtype *B. bovis* DHFR carries three mutations present in this mutant possibly explaining the low sensitivity to pyrimethamine and the ease by which moderately WR99210 resistant mutants could be isolated.

INTRODUCTION

In apicomplexans dihydrofolate reductase (DHFR, EC 1.5.1.3) and thymidylate synthase (TS, EC 2.1.1.45) are part of a single protein (DHFR-TS) that is subject of research in important pathogens like *Plasmodium falciparum* [28] and *Toxoplasma gondii* [184] for several reasons. At first, *dhfr* is the target of inhibition for widely applicated antimalarial drugs like pyrimethamine, a substrate analogue of dihydrofolate. Inhibition of *dhfr* depletes the reduced folate pool thus blocking de novo thymidylate biosynthesis by TS and eventually preventing DNA replication [232]. Secondly, *dhfr* has been used as selectable marker for transfection of apicomplexan parasites [51, 65, 234, 240]. The recent development of systems for stable transformation

offers the prospect of genetic approaches to understand the biology of apicomplexan parasites. Genetic transformation of *Babesia bovis*, a tick-transmitted intraerythrocytic apicomplexan parasite of cattle causing high mortality rates in many (sub-) tropical countries [24, 54, 98], would expand opportunities for research on this important pathogen of cattle.

A range of plasmid vectors designed for accomplishing stable transfection of *T. gondii* or *Plasmodium* species carry a human *dhfr* or altered *T. gondii dhfr* gene conferring resistance against the antifolates pyrimethamine and WR99210. The in vitro isolation of strains with decreased sensitivity for pyrimethamine, due to mutations in the *dhfr* gene, has been frequently described [105, 167]. In contrast, *dhfr* mutants with decreased sensitivity to WR99210 were only obtained after deliberate mutagenic procedures followed by selection in a heterologous yeast expression system [73].

We determined the sequence of the *B. bovis dhfr-ts* gene as a step towards the establishment of a transfection system based on *dhfr* as a selectable marker. Pilot experiments, aiming at the establishment of experimental conditions suitable for transfecting *B. bovis*, resulted in the selection of parasites with decreased sensitivity for pyrimethamine and WR99210. Such resistant parasite lines were further analysed, resulting in the detection of an amino acid substitution in the DHFR-TS enzyme. Resistance against antifolates is an important issue regarding anti-malarial chemotherapy. Understanding of the molecular interactions of antifolate drugs with wild type and mutant DHFR has come from studies on *P. falciparum dhfr-ts* as well as from *T. gondii dhfr-ts* [38, 92, 119, 177, 180, 237]. The recent description of the crystal-structure of *P. falciparum* DHFR in a ternary complex with NADPH and WR99210 [243] provided and excellent template for model building and was used here as a basis for comparing the *dhfr* sequence of *B. bovis* with those of other apicomplexans and for studying the potential effects of the observed amino acid substitution.

MATERIALS AND METHODS

B. bovis *cultures*

B. bovis cultures (Israel strain, clonal line C 61411 [195] were maintained in vitro by serial passage in bovine red blood cells (RBCs) according the previously described MASP method with slight modifications [121]. Bovine red blood cells used for the culture were collected every two weeks. RBCs were separated from serum and washed three times with VYMS buffer [227] in presence of 50 $\mu g ml^{-1}$ gentamycin and stored at 4ºC. *B. bovis* was cultured at 37°C in a mixture of 5% CO_2 in air in 24 wells plates, 25 cm^2 flasks or 80 cm^2 flasks containing respectively 1200 µl, 15 ml or 50 ml medium composed of M199 with Hanks salt

supplemented with 25 mM sodium bicarbonate, 40% normal bovine serum from a selected donor and 50 μgml^{-1} gentamycin. Medium was replaced every 24 h and the culture was maintained at a parasitaemia between 1 and 10 %. The parasitaemia was measured by counting 3000 RBCs in giemsa-stained smears.

DNA isolation of B. bovis

B. bovis infected red blood cells (2.10^9 parasites) were washed 2 times in PBS and lysed in hypotonic buffer (2 min. in PBS diluted with water to a final NaCl concentration of 70 mM after which the buffer was re-adjusted to 150 mM NaCl). After 4 washes to remove haemoglobin, merozoites were resuspended in lysis buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl, 0.1% SDS) pH 7.4 with 10 μgml^{-1} Proteinase K and the lysate was incubated at 37°C for 2 h. *B. bovis* genomic DNA was extracted with phenol-chloroform (1:1) and precipitated with 0.5 M NaCl and 2 volumes of cold ethanol [187] and resuspended in TE pH 7.4 (10 mM Tris, 0.1 mM EDTA pH 7.4).

mRNA isolation of B. bovis *and Northern Blot*

2.10^9 merozoites were obtained as described above. After washing twice with PBS, total RNA of *B. bovis* merozoites was extracted by using RNAzol^{TM} according the manufacturer's instructions (Campro). mRNA was isolated using the Poly(A)-Pure^{TM} kit according the manufacturer's instructions (Ambion).

7 μg of mRNA from *B. bovis* merozoites was separated by electrophoresis on a 1% agarose gel, containing 2.2 M formaldehyde and blotted to nitrocellulose membrane (Nytran N, Schleicher and Schuell) by capillary transfer following standard techniques [187]. For size calibration mRNA was run in parallel with a RNA ladder (New England Biolabs). The mRNA was fixed on the membrane by baking it for 2 h at 80°C. Probes were labelled and hybridised according standard procedures [187]. Filters were washed under moderate stringency conditions (3 times 15 min in 2×SSPE /0.1% SDS at 65 °C).

Construction of B. bovis *genomic and cDNA library*

A cDNA library was constructed from 5 μg *B. bovis* mRNA using the λZAP-cDNA® Synthesis Kit (Stratagene) according to the manufacturer's instructions. cDNA fragments of 0.5 to 4 kb were collected by gel filtration on a sepharose CL-4B column and ligated into the *Eco*RI/*Xho*I site of λ uniZAP-XR Express vector. Giga pack III Gold was used for packaging into phage particles followed by transformation of *Escherichia coli* XL-1 Blue MRF'cells. 1.2×10^6 plaques were obtained of which an amplified library was made.

For constructing the genomic library, 300 μg *of B. bovis* DNA was digested with 150 units *Eco*RI and another 300 μg *of B. bovis* DNA was digested with 250 units *Eco*RI for 1 h at 37°C. The digested DNA was pooled and size fractionated on a Sepharose CL-4B column. Fragments of 0.5 kb to 8 kb were ligated into the *Eco*RI site of λ-ZAPII-Express, packaged using Gigapack III Gold Packaging extract and transformed in *E. coli* XL1-Blue

MRF'competent cells. 2.5×10^6 plaques were obtained of which an amplified library was made.

Cloning of dhfr-ts gene locus and encoded cDNA

Genomic and cDNA libraries were screened with probes F4 and F3 (Fig. 1A). Oligonucleotides P1 (5'–ta[tc]ggitt[tc]ca[ag]tgg[ac]gica[tc]t-3') and P2 (5'-[ag]tg[ag]caigg [tc]a[ag]igccat-3') derived from conserved *ts* domain of *P. falciparum,* were used to amplify probe F4 by PCR in a 50 µl volume containing 0.2 mM dNTP, 20 pmolµl^{-1} of each primer, 100 ng *B. bovis* genomic DNA and 0.5 U Taq DNA polymerase in standard buffer (Promega). Amplification was performed for 30 cycles (92°C for 30 s, 55°C for 30 s and 72°C for 30 s) preceded by initial denaturation for 3 min at 95°C and a final elongation at 72°C for 10 min. Probe F3 is the 5'-end *Eco*RI fragment of cDNA clone C1.2 (one *Eco*RI site derived from the vector). Both fragments were purified from agarose gel and labelled with 50 µCi ^{32}P-dATP (3000 Cimmol^{-1}), using a Random Primer labelling kit (Roche). In total 2.10^6 cDNA and 2.10^5 genomic DNA library plaques were screened by standard procedures [187] for cloning the genomic locus and *dhfr-ts* cDNAs. Subsequently, 3.10^5 plaques of the cDNA library were screened by PCR probes F1 (primers 1se, 5'-ggtagtccactaggttactga-3' and 1as, 5'-cccactgcttaagtggcaat-3'), F2 (primers 4se, 5'-cttgacatggaggtactgcg-3' and 4se, 5'-gatgatcacgtttatacggttt-3'), F5 (primers 2as, 5'-ggcccgtgttaaatcgctaa-3' and 2as, 5'-gattcaagcttccgttcttcaaccctgaaacgttacca-3') and F6 (primers 3 se, 5'-gggaatgccgccaagatgttggatcgt-3' and 3as, 5'-caagcgttcatagcctccaa-3'). After 2 cycles of plaque purification all clones were in vivo excised for isolation of the phagemids inserts as described in the manufacturer's instructions (Stratagene) and sequenced on both strands, using automated cycle sequencing with the dye terminator method (ABI PRISM dye terminator kit, Pharmacia).

Analysis of sequence data

DNA sequences or conceptual translations of orf-1 and the cDNAs 1 to 3 were submitted for BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/) using blastx and blastp for screening the nr protein database and tblastn for screening the EST database (dbEST). BLOSUM 45 was used as scoring matrix. The Pfam motif database (www.sanger.ac.uk/Software/Pfam/) was screened with the conceptual translations of the genes. Preliminary sequence data of the *Theileria parva* genome sequencing project (available from The Institute for Genomic Research at www.tigr.org) and the *T. annulata* genome sequencing project (produced by the *T. annulata* sequencing group at the Sanger Institute and available from www.sanger.ac.uk/Projects/T_annulata/blast_server) were screened by tblastx. SignalP 2.0 was accessed at www.cbs.dtu.dk/services/SignalP using conceptual translations and TMHMM v.2.0 was accessed at www.cbs.dtu.dk/services/TMHMM. Sequence alignments of DHFR and TS domains were made using CLUSTALX [216] using the default alignment parameters. The DHFR alignment was adjusted manually for optimal alignment of conserved secondary structure elements (see Fig. 2). Unrooted phenograms were constructed by the

Neighbor Joining algorithm excluding gap regions from the analysis. Bootstrap analyses were performed using 1000 replicates. Phenograms were drawn using Njplot [172]

Selection of B. bovis *drug resistant culture lines*

Approximately 2.10^9 *B. bovis* infected red blood cells (50% packed cell volume, 7% to 10% parasitaemia) were electroporated in a Gene Pulser II system (Biorad) in absence of any DNA construct in a final volume of 500 µl in cytomix (120mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/ KH_2PO_4 pH 7.6, 25mM Hepes pH 7.6). Cells were incubated on ice for 5 min. before pulsing at high voltage (1 kV, 200Ω, 25 µF). 120 µl of electroporated cells were immediately added to 1020 µl pre-warmed M199 culture media containing 60 µl bovine red blood cells. During the first three days the culture was kept in 24 well plates at 1.25 µM pyrimethamine or 2.5 nM WR99210 in 5% CO_2 in air. After three days the concentration of pyrimethamine was increased to 5.0-10.0 µM and of WR99210 to 5.0-20 nM. Cultures were three-fold diluted every day with non-infected RBCs in drug containing medium from day 3 onwards. After parasitaemia declined below detection level cultures were transferred to 96 well plates and maintained under 5% CO_2 and 2% O_2. In about six weeks the parasitaemia increases from undetectable level to more than 1.0 % in ~2% of the wells.

Drug sensitivity assay

Pyrimethamine and WR99201 (provided by Jacobus Pharmaceuticals, Princeton, NJ) were made as a 10 $mgml^{-1}$ solution in DMSO. Wild type *B. bovis* culture was incubated in M199 culture medium in 24-microwell plates under standard culture conditions in presence of different concentrations of pyrimethamine and WR99210 for a period of 96 h. Serial dilutions of the drug were made in culture medium and the concentrations of drug tested ranged from 0 nM to 5 nM WR99210 and 0 to 2.5 µM pyrimethamine. Cultures were diluted three-fold every 24 h with 5% fresh RBCs. Giemsa stained smears were made every 24 h for a period of three days. IC_{50} values were calculated at 96 h after counting 3000 RBCs. Drug-resistant cultures were tested by the same method at drug concentrations of 5 nM, 10 nM and 20 nM WR99210 or 2.5 µM, 5 µM and 10 µM pyrimethamine. All drug-resistant cultures were growing at wild-type rate in 10 nM WR99210 and 5 µM pyrimethamine whereas cultures eventually died at 20 nM WR99210 and 10 µM pyrimethamine prohibiting a more precise determination of IC_{50}.

Homology modelling of B. bovis *DHFR*

DHFR amino acid sequences and 3D-structures of *P. falciparum* (PDB Accession number 1J3KA) [243], *E. coli* (1RX2) [189], *Gallus gallus* (1DR1) [229], *M. tuberculosis* (1DG7) [124]and *Pneumocystis carinii* (2CD2) [43] were downloaded from the Brookhaven protein database (PDB) and used for constructing the alignment of Fig.2. *B. bovis* DHFR was modelled on the ternary complex of *P. falciparum* DHFR with NADPH and WR99210 (1J3KA) using Swiss-

Model [83] (http://swissmodel.expasy.org) on the basis of the alignment of Fig.2. The DeepView program [83] was used for refinement and examination of the model. Energy was minimized by 25 steps of steepest descent and 15 steps of conjugate gradient using GROMOS from within the Deep View package thus finding a balance between minimal energy (-6335 kJ/mol) and limited structural drift. The Cα-backbone atoms of residues making direct contacts with NADPH or WR99210 of *P. falciparum* 1J3KA (indicated in Fig. 2) were superpositioned with corresponding residues in the modelled *B. bovis* structure. The RMS deviation of the all backbone atoms of the superpositioned residues was 0.14 Å. Mutation S125F was introduced and inspected for conformational problems using the Deep View package. Only one rotamer conformation (rotamer 6) of F125 did not result in incompatible interactions with other residues. Very limited energy minimization (-5738 kJ/mol) was needed (3 steps of conjugate gradient). In brief, modelling on the *M. tuberculosis* template was done as described above using 25 steps of steepest descent and 15 steps of conjugate gradient for energy minimization (-4673 kJ/mol). Superpositioning of residues in contact with NADP and Br-WR99210 [83] resulted in a RMS deviation of 0.76 Å of backbone atoms of the superpositioned residues. Following mutagenesis 20 steps of steepest descent were used for energy minimization (-4329 kJ/mol).

RESULTS AND DISCUSSION

Cloning and sequencing of the B. bovis *dhfr-ts genomic locus*

Although functional characteristics of both parts of the bifunctional apicomplexan DHFR-TS enzyme are well conserved across species boundaries, only TS is highly conserved at the primary amino acid sequence level. PCR primers P1 and P2, designed from a conserved region of the TS domain, amplified a 150 bp fragment (probe F4, Fig. 1A) that was used to screen a genomic DNA library of *B. bovis*. Hybridising plaques were purified and clones g1.2 and g2.4, containing inserts of 1660 bp and 3430 bp, were sequenced completely on both strands. Conceptual translation and comparison to other apicomplexan *dhfr-ts* sequences indicated that most of the *ts* domain and 2800 bp of downstream sequence were obtained. Extended library screening with a cDNA derived probe (F3, see below), encoding part of the *dhfr* region, resulted in isolation of clones g2, g6 and g7 which, by sequence similarity with other *dhfr-ts* sequences, encode the putative N-terminus of the DHFR domain and 2830 bp of upstream sequence (see Fig. 1A for a map). Southern blot results indicated that *dhfr-ts* is encoded by a single copy gene (data not shown).

Cloning and sequencing of cDNAs derived from the dhfr-ts genomic locus

The genomic sequence encoded several regions homologous to *dhfr-ts* interrupted by putative introns containing stopcodons in all reading frames. To obtain the coding region of *dhfr-ts* a *B. bovis* cDNA library was screened with probe F4. Several cDNA clones were obtained of which clones C1.2 and C1.3 were sequenced. C1.3 contained 3 deletions (40 bp, 134 bp and 40 bp) in comparison with the genomic sequence that all conformed to the GT/AG rule for splice donor and acceptor sites of introns. The 3 introns are located at identical positions as introns I, II and VIII in the *T. gondii dhfr-ts* gene [(184)].

Conceptual translation of the cDNA revealed a 1431 bp open reading frame starting at the first nucleotide followed by a 3'-untranslated region of 592 bp ending with a poly A-tail. The first methionine codon in the 1431 bp orf was located at position 678 in a region encoding the TS domain indicating that the cDNA was truncated at the 5'-end lacking the start codon. Despite several efforts no longer cDNAs were isolated. A comparison with the genomic sequence suggested the presence of a start codon 102 bp upstream of position 1 of cDNA clone C1.3 resulting in an open reading frame encoding 511 amino acids. The proximity of another expressed gene only 121 bp upstream of this putative startcodon (see below) excludes with near certainty the possibility that any other ATG codon can be the actual initiation codon of *dhfr-ts*. A northern blot using purified mRNA of in vitro cultured *B. bovis* showed a single band of ~2500 nucleotides (Fig. 1B). Without the introns and assuming a polyA-tail of ~150 nucleotides this size just fits in between the upstream and downstream located genes of *dhfr-ts* that have been mapped below.

In addition to the *dhfr-ts* coding sequence, the 7.4 kb genomic locus contains several long open reading frames, none of which is characterized by a BLAST hit of high probability within the NCBI nr protein database (see below). Four probes (F1, F2, F5, F6) were used to screen for cDNA clones mapping to the flanking regions of the *dhfr-ts* gene, resulting in the cloning and sequencing of 3 cDNAs indicated in Fig.1A. Table 1 gives an overview of the location of cDNAs and putative start and stop codons on the genomic locus. All cDNAs encoded a putative stop codon and terminated in a poly-A tail. cDNA-2 appears to be encoded by a gene without introns and a putative startcodon is present at position 20 of the cDNA. The 129 bp genomic region present between the poly-adenylation site of *dhfr-ts* and the start of cDNA-2 does not appear to contain additional exons indicating that a cDNA encoding the full-length protein has been obtained. Open reading frames encoded by cDNA-1 and cDNA-3 initiate at their very 5'-end and alignment with the genomic sequence (not shown) indicates the presence of putative startcodons at respectively 20 and 181 nucleotides upstream of their 5'-end. Both genes contain 2 introns but the presence of

more introns in the cDNA-3 clone cannot be excluded as the cDNA extends 1326 nucleotides beyond the genomic sequence (Fig.1A).

Comparison of the 5'-splice donor sequences of the seven introns of *dhfr-ts*, cDNA-1 and cDNA-3 with the 20 *B. bovis* intron sequences that have been obtained to date (accession numbers AJ289247, U70130, U34076, AAC27389, AF027149, AAC2788, AF331455) indicate that *B. bovis* 5'-splice sites have an extended consensus in comparison to universal GT consensus at positions 1 and 2. All seven introns reported here have a consensus GTNNGT, whereas 23 out of all 27 introns conform to this consensus. Such a feature is potentially useful for a more reliable prediction of *B. bovis* 5'-splice donor sites in genomic sequence data. The 3'-splice acceptor sites of the *B. bovis* introns all have the dinucleotide motif AG at the last two nucleotides of the intron but the preceding pyrimidine-rich tract seen in most eukaryotic 3'-splice sites is rarely present in *B. bovis* introns.

The above findings demonstrate a dense head-to-tail packing of genes in the *dhfr-ts* genomic locus, all of which are expressed during the asexual erythrocytic cycle of the parasite. Transcription start sites have not been mapped but the three intergenic regions have sizes of respectively 121 bp, 148 bp and 108 bp, measuring from the poly-adenylation site to the ATG startcodon of the adjoining downstream gene. This includes the 5'-untranslated regions containing the ribosome binding sites and therefore the basal promoter regions of the *dhfr-ts*, cDNA-2 and cDNA-3 genes will have a maximum size of about ~90 bp, ~120 bp and ~80 bp respectively. Using probe F1, no cDNA clones (out of 2.10^6 plaques) were obtained for the 1383 bp orf-1 (Fig. 1A) arranged in a head-to-head orientation with cDNA-1 at an intergenic distance of 486 bp. If a gene at all (see below), orf-1 may not be expressed, or only at a very low level, in the erythrocytic stage of which the cDNA library was made.

Three genomic loci of *B. bovis* have been examined in some detail to date, all encoding surface antigens and additional genes of unknown function [74, 210, 211]. The 11 intergenic regions of the *msa*-1 locus (4 genes), the *msa*-2 locus (6 genes) and the *rap*-1 locus (4 genes) have an average size of 926 bp and only two are smaller than 650 bp (322 bp and 324 bp). The intergenic regions of the *dhfr-ts* locus have an average AT-content of 61.3% which is comparable to the average AT-content of the seven introns (62.9%) and not significantly higher than the average AT-content of the coding regions (55.6%). The latter is similar to the 54.8% AT-content which we observed for 44,331 bp of coding region derived from 30 *B. bovis* coding regions available from NCBI genbank.

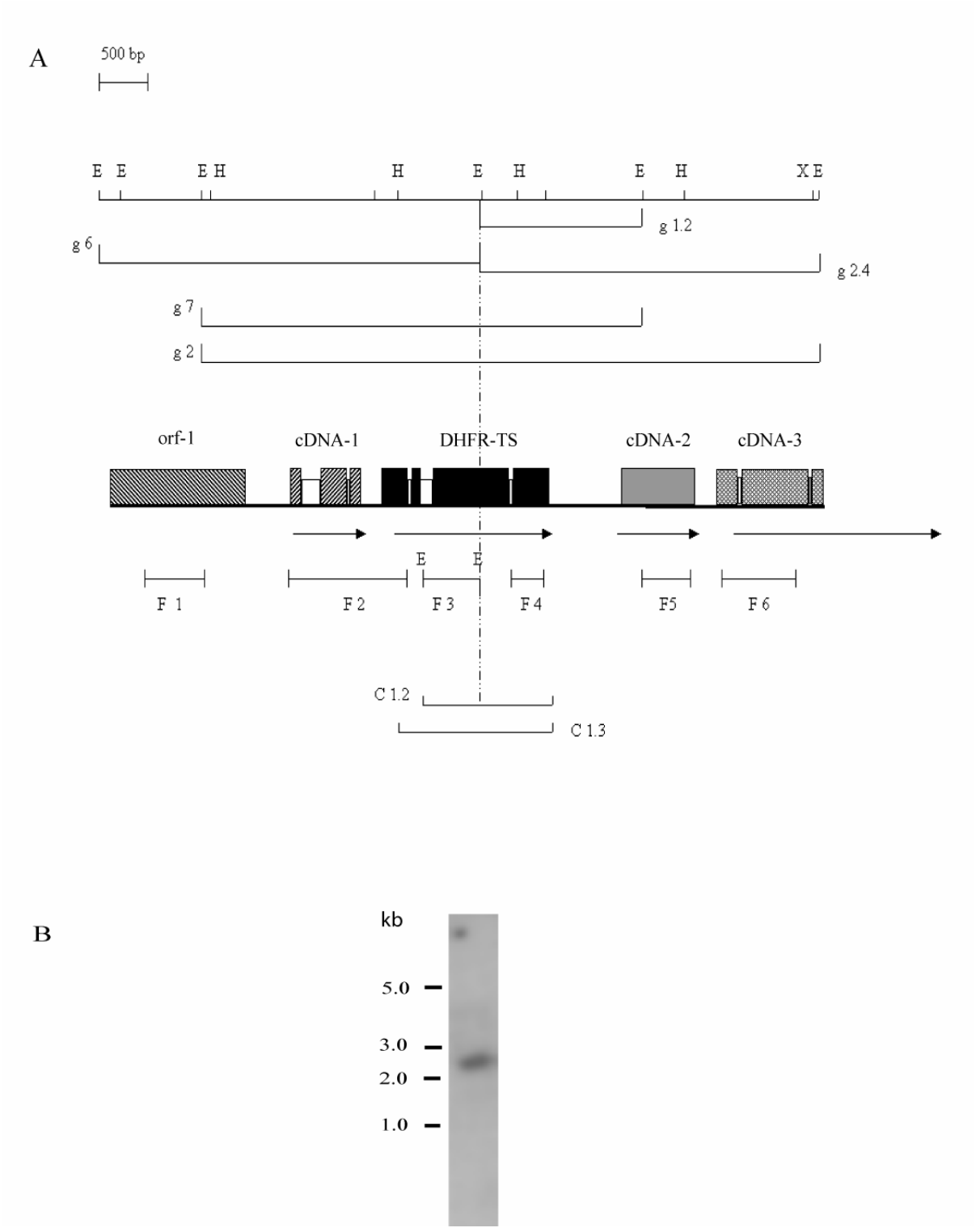

Fig. 1. Genomic and transcriptional organization of the *dhfr-ts* locus. (A) Restriction map and organization of the *dhfr-ts* gene. Positions of genomic (lines) and cDNA (arrows) clones used for sequencing are indicated. Probes used for library screening are also indicated (F1 to F6). E=*Eco*RI, H=*Hin*dIII, X=*Xho*I. (B) Northern blot of 7 µg *B .bovis* mRNA isolated, run on 1% gel and hybridized with TS probe F4 as described in materials and methods.

Comparison of B. bovis *DHFR-TS with other apicomplexan DHFR-TS proteins*

B. bovis DHFR-TS contains a moderately conserved 5'end DHFR domain (190 aa), a non-conserved linker region (33 aa) and a highly conserved 3'end TS domain (288 aa). A sequence alignment of the *B. bovis* DHFR domain with three apicomplexan species (*P. falciparum, T. gondii* and *C. parvum*), another unicellular eukaryote (*Pneumocystis carinii*), a vertebrate (*Gallus gallus*) and two prokaryotes (*Mycobacterium tuberculosis, Escherichia coli*) is shown in Fig.2. Only 16 amino acids are identical among all species. At five positions (shaded in blue) the *B. bovis* sequence deviates from a highly conserved consensus sequence as defined by the other seven sequences in the alignment (see next section for detailed discussion of several individual amino acids in the context of their potential effects on co-factor and substrate binding). In comparison to other DHFR sequences, *T. gondii* and *P. falciparum* DHFR have insertions of several stretches of amino acids. These are lacking from the 20% to 25% smaller *B. bovis* DHFR domain that is similar in size to vertebrate and *C. parvum* DHFR. A simple pair wise analysis of aa identities or similarities of the DHFR domain indicates that *B. bovis dhfr* is most closely related to *P. falciparum dhfr* although conservation between apicomplexan species (e.g. *B. bovis* and *P. falciparum*: 28% identity, 45% similarity) or between prokaryotic species (e.g. *E. coli* and *M. tuberculosis*: 33% identity and 48% similarity) is hardly larger than between an apicomplexan and a prokaryote (e.g. *B. bovis* and *E. coli*: 25% identity, 41 % similarity). A more accurate phylogenetic comparison, either by neighbor joining or maximum parsimony methods (results not shown) resulted in trees in which eukaryotes and prokaryotes cluster in separate branches with good bootstrap values. Also apicomplexans cluster together within the eukaryote branch but a further resolution of the apicomplexan branch is not supported by strong bootstrap values.

Analysis of the other proteins encoded by the dhfr-ts genomic locus

orf-1, cDNA-1, cDNA-2 and cDNA-3 were submitted to BLAST analysis (GenBank nr, dbEST and *T. parva* and *T. annulata* genomic sequence databases) and motif searches (Pfam, SignalP, TMHMM). High similarities were only observed with putative proteins of *T. parva* or *T. annulata* and orf-1 ($8.2e^{-20}$), cDNA-2 ($3.7e^{-23}$) and cDNA-3 ($2.7e^{-103}$) indicating that these genes might encode piroplasm-specific proteins. orf-1, *dhfr-ts* and cDNA-2 homologues of *T. parva* were all located within a ~5 kb region in equal relative orientation and position as their counterparts on the *B. bovis dhfr-ts* genomic locus. Gene synteny was restricted to these three genes. In *T. annulata* a cDNA-2 homologue is also located directly downstream of *dhfr-ts*. orf-1 was found to encode a conserved Ser/Thr protein kinase motif by screening the Pfam motif database (PF00069; $1e^{-4}$). cDNA-1 encodes a putative single-pass

transmembrane protein of which the short C-terminal domain is predicted to have a cytoplasmic location.

Selection and analysis of drug-resistant mutants

Pilot studies, aiming at determination of suitable conditions for the establishment of in vitro transfection procedures, led to the isolation of *B. bovis* culture lines with decreased sensitivity to anti-folate drugs. For *B. bovis* an IC^{50} value for pyrimethamine of 1.2 μM has been reported [(162)] and was determined here at 1.25 μM for *B. bovis* clonal line C61411 whereas an IC^{50} of 1.0 nM for WR99210 was obtained (described in materials and methods, results not shown). For both drugs steep inhibition curves were observed with no inhibition by 0.5 μM pyrimethamine or 0.5 nM WR99210 and culture death within 48 h by 2.5 μM pyrimethamine or 2.5 nM WR99210. In vitro cultures, growing at normal rate under increased concentrations of pyrimethamine or WR99210, were selected repeatedly (about 1 out of every 50 cultures) in about six weeks after electroporation in absence of DNA constructs. Culture lines selected for continuous growth (in different experiments) in presence of 5 μM pyrimethamine (lines R1 and R2) or 10 nM WR99210 (lines R3, R4 and R5) were studied in more detail. Growth, 96 h, experiments showed that all five lines grew well at 5 μM pyrimethamine and 10 nM WR99210, no matter by which drug they were initially selected. Fig. 3 displays the growth curves of a typical experiment. All lines were tested in parallel several times (with comparable outcome) after re-initiation of cultures from stabilates stored under liquid nitrogen. These repeated experiments demonstrated that differences in multiplication factors between the drug-resistant lines within a typical experiment, as observed in Fig. 3 after 96 h, are not significant. Continuous growth requires a standard 3-fold dilution every 24 h. This procedure contributes to a variation in growth rate comparable to the variation observed for triplicates of wild-type cultures grown in absence of inhibitors. Prolonged growth curves are required as during the first 24 h the parasitaemia of the wild-type culture increases as well, suggesting that pyrimidine levels are sufficient to complete several ounds of multiplication. After 48 h wild-type cultures consistently die. Resistant lines display growth at levels of 10 μM pyrimethamine or 20 nM WR99210 for 3 to 6 days but eventually die at these concentrations (not shown). In conclusion, *B. bovis* lines that are cross-resistant against ~10 to 20-fold increased levels of pyrimethamine and WR99210 can be selected under selective pressure of either one of the drugs.

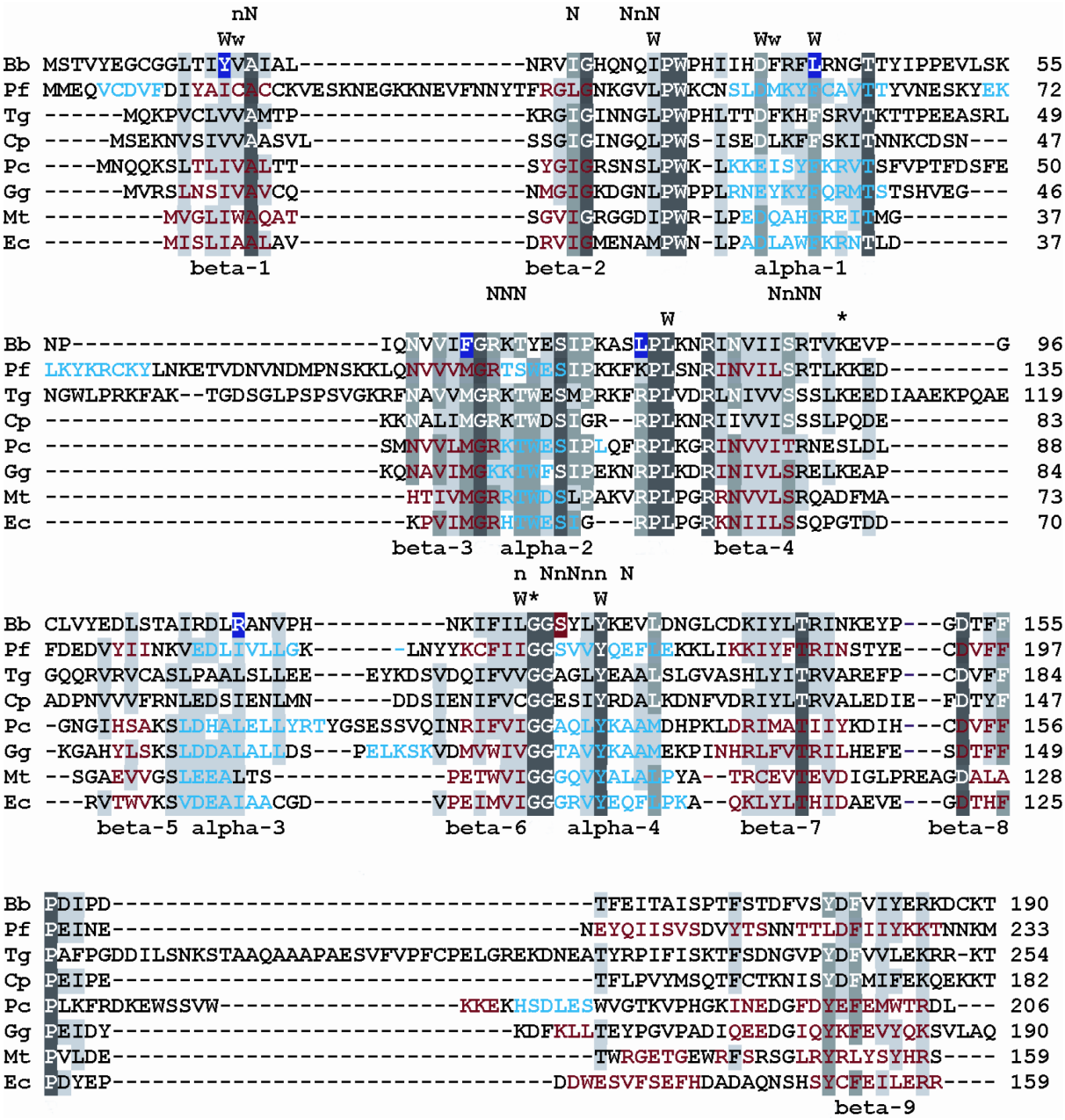

Fig. 2. Amino acid sequence alignment of *B. bovis* DHFR.The *B. bovis* (Bb) sequence was aligned with those of *P. falciparum* 3D7 (Pf, GenBank Accession No. NP_702821), *T. gondii* (Tg, A46005) and *C. parvum* (Cp, AAC47230). In addition the *dhfr* sequences and structural elements of 3D-structures (from the PDB database) were aligned from *P. falciparum* (1J3KA), *Pn. carinii* (Pc, 2CD2), *G. gallus* (Gg, 1DR1), *M. tuberculosis* (Mt, 1DG7) and *E. coli* (Ec, 1RX2). The *P. falciparum* structure was determined from the quadruple mutant (N51I, C59R, S108N and I164L; boxed in red on the 3D7 sequence that is otherwise identical to 1J3KA) that gives rise to high levels of pyrimethamine resistance. Alpha-helices (blue letters) and beta-strands (red letters) are indicated. The S125F mutation in *B. bovis* is shaded in red whereas five positions of *B. bovis* that deviate from the consensus shared by the other seven species are shaded in blue. Absolutely conserved positions are shaded in black, positions at which six or seven sequences are identical are indicated in white and

shaded gray. Positions with at least five similar amino acids are shaded in light gray (similarity groups: ILMV; KR; DE; ST; YF). Positions at which the *P. falciparum* 3D-structure revealed hydrogen bonds or hydrophobic interactions between NADPH (N) or WR99210 (W) and DHFR are indicated above the sequence (upper case characters indicate positions of contact also observed in the 1DG7 structure of *M. tuberculosis* whereas lower case indicates contacts unique for *P. falciparum*). An asterix indicates the position of the two introns.

Table 1. Summary of genes localised in the up- and downstream region of *dhfr-ts* gene

Gene	cDNA start	Positions		Introns	poly-A	product
		ATG	stop			
	(bp)	(bp)			(bp)	(kDa)
orf-1	1383	<1	0 [a]			
cDNA-1	1889	1869 [c]	2614	2	2714	19.2
dhfr-ts	2933	2831 [c]	4577	3	5171	58.3
cDNA-2	5300	5319	6078		6097	27.7
cDNA-3	6486	6305 [c]	8695	2 [b]	8727 [b]	87.6

Legend

Genes localised in the upstream and downstream region of *dhfr*-ts gene. Start position of the cDNA and genomic clones (also terminal position) are presented here. Furthermore introns, position of poly-A and molecular weight of the genes are indicated.

[a] no cDNA clone of orf- 1 was isolated. From genomic DNA sequence of orf 1 it was predicted that there are no introns in the sequenced region of orf-1.

[b] cDNA clone of cDNA-3 extended beyond the known genomic sequence. Therefore cDNA-3 contains at least two introns but can contain more.

[c] start methionine from these genes predicted from genomic sequence.

Pyrimethamine resistance in *P. falciparum* and other apicomplexan parasites have been shown to be related to point mutations in the *dhfr-ts* gene that affect critical interactions in the ternary complex [38, 42, 56, 73, 92, 105, 119, 120, 167, 177, 180, 188, 201, 237, 243]. In the current experiment such mutations could have arisen *de novo* and been selected for during six weeks of propagation under drug pressure (an estimated multiplication factor of $\sim 3^{40}$ has occurred) but, alternatively, drug-resistant mutants may have been present in the starting culture (a clonal line being clonal for only few generations). In both cases extensive selection for drug-resistance must have taken place, therefore yielding equally informative results. The *dhfr* domain of the five resistant *B. bovis* lines was amplified by PCR and sequenced. In all cases a single point mutation at

position 3381 (a C to T transition), giving rise to substitution of serine[125] by phenylalanine, was identified. Southern blotting detected no rearrangements (single and double-digestions with *Eco*RI, *Hin*dIII and *Xho*I on DNA from resistant cultures using probes F3, F4 and C1.2) within the *dhfr-ts* region whereas the intensity of the hybridisation signals remained equal, excluding gene amplification as a cause of drug resistance (not shown). Changes in steady-state DHFR-TS protein levels cannot be ruled out completely however.

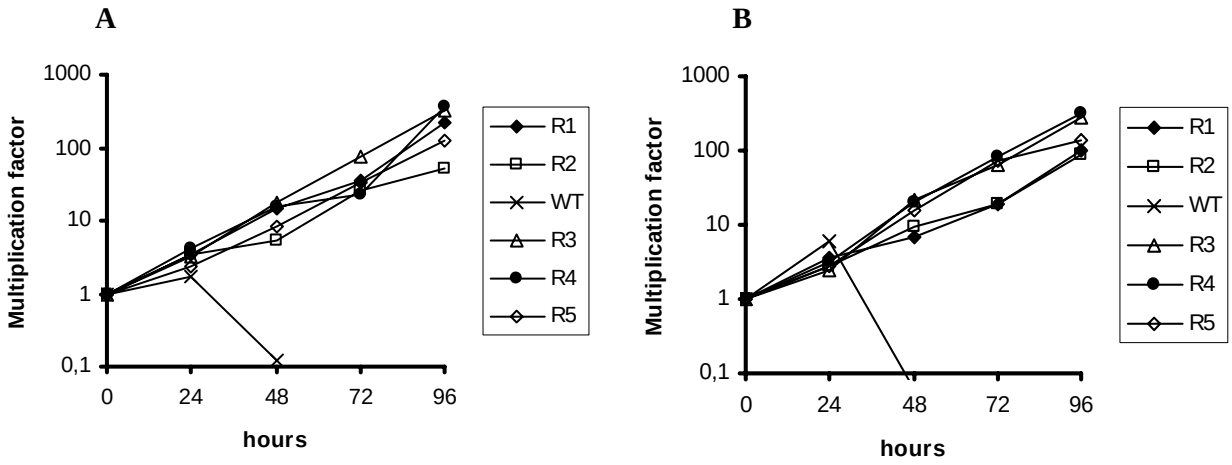

Fig. 3. Inhibition curves for WR99210 and pyrimethamine of wild-type (WT) and resistant culture lines (R1-R5). After electroporation, cultured lines were in medium that contained either pyrimethamine or WR99210. Parasitaemia was determined every 24 h. Cultures were diluted 1:3 with 5% RBCs in drug-containing medium every 24 h. Panel A: 5 μM pyrimethamine, panel B: 10 nm WR99210.

Understanding the molecular interactions by which the S125F substitution could lead to increased resistance against pyrimethamine and WR99210 requires 3D structural information about the ternary complex of DHFR, NADPH and the inhibitors. The recently resolved crystallographic structure of *P. falciparum* DHFR [(243)] provided the first apicomplexan DHFR structure and, together with the 3D-structure of *M. tuberculosis* DHFR [(124)], the only examples of a ternary complex including NADPH and WR99210. Comparative modelling of *B. bovis* DHFR using Swiss-Model [(83)] was based on the alignment of Fig. 2. Five sequences have been included for which a high-resolution 3D-structure of a ternary complex between DHFR, NADPH and folate or an antifolate inhibitor has been resolved by crystallography. Despite low conservation at the primary sequence level, all five 3D-structures are highly similar [(43, 124, 189, 229, 243)]. Conserved aa residues were associated with all of the conserved secondary structure elements (9 β-strands and 4 α-helices), thus guiding an accurate positioning of loop-regions. As could be expected,

considering phylogenetic relationships, modelling on the *P. falciparum* template gave a better fit (rms of superpositioned atoms) and required less adaptations than modelling on a *M. tuberculosis* template. Details of modelling on the *M. tuberculosis* template are therefore given in the material and methods section. After energy minimization (see material and methods), the model based on *P. falciparum* DHFR has left 4 residues located just outside tolerated areas of a Ramachandran plot. Subsequently, *in silico* substitution and inspection of mutant S125F showed that only one rotamer conformation of the phenylalanine side chain required very little energy minimization to give tolerable interactions with surrounding residues.

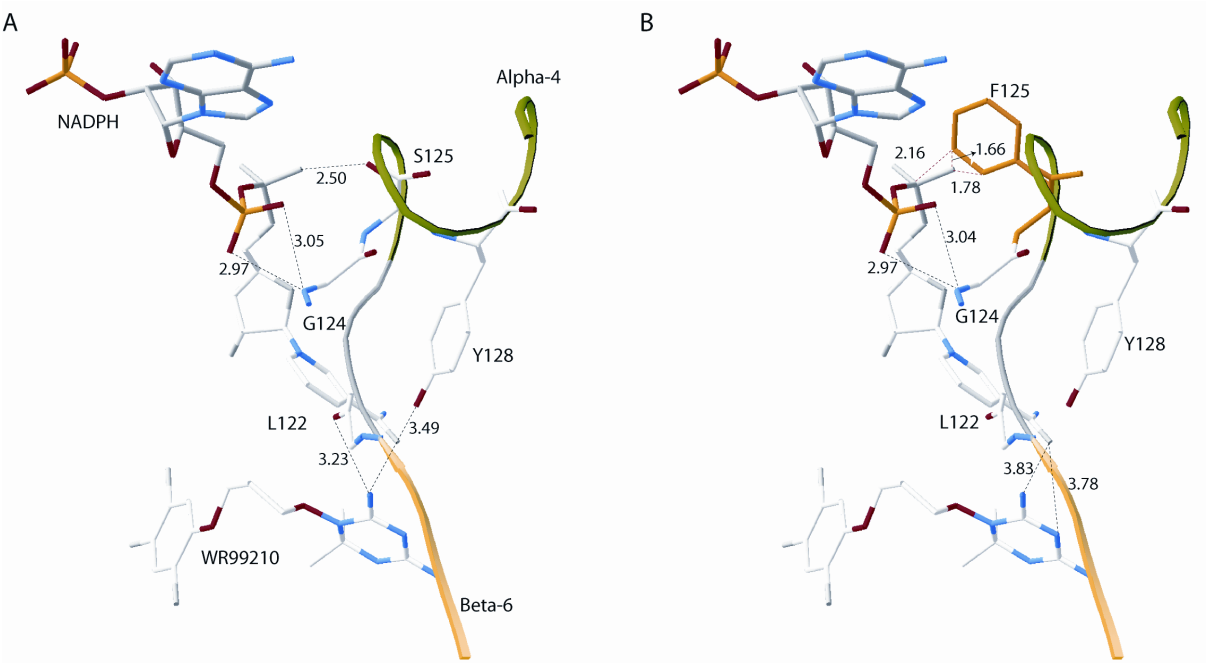

Fig 4. 3D-model of *B. bovis* DHFR. (A) Wildtype DHFR. (B) Mutant S125F DHFR. Modeling using the *P. falciparum* ternary complex with NADPH and WR99210 as a template has been described in materials and methods. NADPH and WR99210 are displayed, together with a ribbon representation of the backbone of beta strand-6, alpha helix-4 and the sharp bend connecting them. Phenylalanine 125 is shown in orange in B. The other sidechains of interest are shown in yellow for the wildtype in A and in white for the mutant in B. Some relevant atoms have been indicated in colour with blue for Nitrogen, red for Oxygen, orange for Phosphorus. Interactions described in the text between G124 and NADPH, Y128 with WR99210, L122 with WR99210 are indicated by dotted lines labelled with the distance in Å (distances are almost identical in A and B). The short distances between Phenylalanine 125 and NADPH are indicated by red dotted lines in B. Positions where NADPH and WR99210 are closely opposed (and where hydrogen transfer takes place when dihydrofolate is bound instead of WR99210) are also indicated in B.

Twenty five aa residues of *P. falciparum* interacting (distance < 3.50 Å) with NADPH or WR99210 in the ternary complex (see Fig. 2) were superpositioned with the *B.*

bovis DHFR wild-type and mutant models, followed by merging NADPH and WR99210 into their binding sites and comparison of wild type and mutant. In the wild type model the side chain hydroxyl of S125 interacts with O1 of the adenine β-phosphate of NADPH. S125 is located amidst conserved residues (e.g. L122, G124 and Y128 of *B. bovis*), all making H-bonds with NADPH and WR99210 (Fig. 2 and 4). The conserved dipeptide G123G124 has a cis peptide linkage in all resolved structures, giving a sharp bend between β-strand 6 and α-helix 4 that causes insertion of the backbone nitrogen of G124 between O1 and O2 of the adenine α-phosphate of NADP to form H-bonds. In *B. bovis* DHFR modelled upon the *M. tuberculosis* template, a clash between the aromatic side-chains of mutant S125F and Y126 induced a translocation of several adjoining residues by 0.2 to 0.3 Å resulting in altered contact distances with NADPH and WR99210 (not shown). When modelled on the *P. falciparum* DHFR template, residue Y126 adopts a different conformation (like in the *P. falciparum* structure itself), due to the close proximity of conserved residue F155 (corresponding to F197 in *P. falciparum* and A128 in *M. tuberculosis*), preventing a clash with mutant residue S125F. Interactions of the aromatic ring of S125F with F154 or F155 only allow for a rotamer conformation in which the side chain is projected towards the surface of the molecule, bending over the shallow cleft that binds the NADPH co-factor. This resulted in intolerably short distances between the side chain and P and O1 of the adenine β-phosphate of NADPH (Fig. 4). Consequently, the binding conformation of NADPH must be altered which can in addition lead to induced changes in the local DHFR structure of the S125F mutant. Thus, although S125F does not directly interact with WR99210, kinetic parameters and the binding of WR99210 or dihydrofolate could be influenced indirectly as the hydrogen donation site of NADPH may shift position (see Fig. 4).

The kinetic effects of numerous single and multiple amino acid substitutions in DHFR have been studied in in vitro cultures of *Plasmodium*, in assays using purified native or recombinant enzyme and in assays where mutant *dhfr* genes are expressed and studied in heterologous organisms [42, 53, 88, 201]. In general, pyrimethamine resistant mutants are easily obtained but sofar the amino acid corresponding to *B. bovis* S125 has not been found to be involved in resistance against any inhibitor. The IC_{50} that we and others [162] observed for pyrimethamine on in vitro *B. bovis* cultures is at least 1000-fold higher than observed for the most pyrimethamine sensitive *P. falciparum* strains [40] whereas sensitivity towards WR99210 is still in the low nM range for the *B. bovis* wild type as well as the mutant. Promise for WR99210 as a lead for further development of anti-folate drugs comes from the observation that a quadruple mutant (N51I, C59R, S108N and I164L), displaying a synergistically acting combination of four of the most frequently observed mutations [85] has a thousand-fold reduced

sensitivity towards pyrimethamine but a only 10-fold reduced sensitivity towards WR99210. Three of these mutations (N51I, C59R and I164L, see Fig. 2) are present in wild type *B. bovis dhfr* and might thus explain the comparatively low sensitivity towards pyrimethamine. Moreover *P. falciparum* mutant S108T, having a threonine at this position like *B. bovis dhfr*, displays resistance against the anti-folate drug cycloguanil. Possibly, in such a background of natural resistance against pyrimethamine, single amino acid substitutions conferring resistance to WR99210 are more easily selected in the way demonstrated here for *B. bovis* in vitro cultures but even than WR99210 is still efficient in the lower nM range. For *P. falciparum* only expression in yeast of mutagenized *dhfr* genes, already carrying two or three of the above mentioned resistance mutations, allowed the isolation of a few single amino acid substitution mutants displaying a 5 to 10-fold increased resistance against WR99210. Interestingly, this study [73] and a similar study on *P. vivax dhfr* [88]indicate that there is a mechanism of opposing selection. Mutants selected for decreased sensitivity towards pyrimethamine did become hypersensitive for WR99210, giving rise to the hope that a combination-therapy of such drugs will prevent the spread of drug-resistant mutants. We conclude that cross-resistance against pyrimethamine and WR99210, for *P. falciparum* or *B. bovis*, sofar has only been demonstrated to occur in a genetic background that already confers high levels of resistance against pyrimethamine. Such mutants could easily be obtained from *B. bovis* in vitro cultures but in all cases resistance against WR99210 does not exceed the therapeutically useful lower nM range.

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CHAPTER 8

GENERAL DISCUSSION

The aim of this thesis was to obtain a better understanding of the molecular processes underlying the invasion of red blood cells by *B. bovis* merozoites. Inevitably, the design of the studies was influenced by the wealth of knowledge that has accumulated on the invasion processes of the two genera, *Toxoplasma* and *Plasmodium,* which have served as the most important model systems for studying the fundamental biology of apicomplexan parasites. From this, a generalized model of invasion has been deduced [18, 20, 32, 41, 60, 67, 91, 165, 173, 174, 176, 196, 197] (summarized in the introduction of this thesis) to which many species-specific deviations have been noted. Here it is discussed to what extent the observations that were made in the previous chapters fit into this model and contribute to it. Most attention is paid to a comparison with the malaria parasite *P. falciparum*, as it also infects red blood cells causing a disease with many features similar to bovine babesiosis caused by *B. bovis.*

CHARACTERIZING INVASION IN VITRO

We envisaged that the set up of a *B. bovis* in vitro invasion assay would be crucial to future studies and, considering the problems experienced with such assays for *P. falciparum,* would also make a valuable contribution to the study of invasion of erythrocytes by apicomplexan parasites. The assay described in chapter 2, evolved as a corollary of the electroporation experiments described in chapter 6, is marked by its capacity to study invasion in a short timespan, independently from the preceding (parasite egress from the red blood cell) and following (intracellular development) stages. Thus it allowed demonstrating that the free parasite itself and the red blood cell to be invaded carry all factors required for productive invasion without the need for any component from the extracellular milieu. Surprisingly, such a synchronized start is not followed up by synchronous intra-cellular development, in contrast to what is seen for *P. falciparum* after synchronization by different procedures [118]. Asynchronicity might be an intrinsic property of *B. bovis* growth. Inhibitor studies suggest that *B. bovis* invasion follows the general model in its requirement of intracellular calcium in signalling the secretory events leading to invasion, although we have not yet studied the secretory events itself. Moreover, the use of cytochalasin D, presumably inhibiting actin polymerization, arrested invasion and led to the observation of merozoites that were apically reorientated and attached to the red blood cell. This provided, in agreement with the few electron microscopy pictures of invading *Babesia* parasites that are available [104] evidence that red blood cell invasion by *B. bovis* is more similar to the process described for *P. falciparum* than to the mechanism used by much closer related piroplasm *T. annulata*. For the latter parasite

it has been suggested to zipper tightly into the membrane of the red blood cell without apical reorientation [193].

RECEPTORS AND LIGANDS

Whereas *T. gondii* can parasitize almost any nucleated cell in a wide range of hosts, *Plasmodium* species show the opposite. For instance, *P. vivax* exclusively invades human reticulocytes. In agreement with this, no specific receptor-ligand interactions governing host cell specificity have yet been discovered for *T. gondii* whereas for *P. vivax*, and to a somewhat lesser extent *P. falciparum*, host cell specificity is due to interaction between highly specific receptors and ligands [68, 81, 108, 135, 169, 200]. *B. bovis* probably represents an intermediate case (chapter 3). Red blood cells of a variety of species were shown to be invaded by *B. bovis* in vitro. This relaxed host specificity is actually observed in vivo for *B. divergens,* another *Babesia* species infecting cattle that occasionally infects other species including splenectomized humans. Possibly a highly sequence specific receptor-ligand interaction is not required for erythrocyte invasion by *B. bovis*. Like for several of the alternative *P. falciparum* receptor-ligand interactions [62, 68, 126], sialic acid residues are a requirement for invasion (chapter 3). However, in the case of *B. bovis,* aminoacid sequence motifs may not contribute to binding specificity. It seems unlikely that sequence variation of putative receptors in between individuals of the same species could lead to differences in invasion efficiency as large as the differences observed with red blood cells of different hosts. Intuitively, exclusive attachment to a probably essential post-translational modification, occurring on several proteins in various organisms, is an optimal evolutionary acquirement for counteracting genetic selection in a population for receptor negative host cells, as exemplified by the absence of the Duffy blood group receptor for *P. vivax* in the human population in several endemic areas. Nevertheless there is no reason to consider *B. bovis* as a more successful parasite than *P. vivax* or *P. falciparum.*

THE INVOLVEMENT OF MICRONEMAL PROTEINS

The specific host cell binding ligands of *Plasmodium* species involved in attachment upon apical reorientation have been shown to be secreted from the organelles called micronemes just prior to invasion. Whereas these proteins clearly contain several homologous domains across the different *Plasmodium* species, this

conservation is sofar constricted to this genus. No homologs of EBA175, EBA140, DBL, MAEBL [1, 62, 146, 217] have been identified in any other apicomplexan genus including the large EST dataset obtained of *B. bovis* (E. de Vries, unpublished). No *B. bovis* proteins have been localized to the microneme sofar although the organelles have been observed by electron microscopy [140]. In contrast, other apicomplexans have been shown to contain an extended range of micronemal proteins (e.g. denoted MIC1 to MIC12 in *T. gondii*). Some of these form specific complexes but for none of the complexes or individual proteins a specific host cell receptor and a specific function in invasion have been revealed. Many of the micronemal proteins are composed of one or more domains showing similarity to a number of different eukaryotic adhesion domains like EGF-domain, thrombospondin-domain, apple domain or van Willlebrand factor A-domain, interspersed by non-conserved regions [59, 205]. Searches within the *B. bovis* EST database mentioned above have led to the identification of only two clear homologous of micronemal proteins. Those were studied in more detail in chapters 4 and 5 and have been denoted BbAMA-1 and BbTRAP respectively in analogy to their most closely related counterparts in *P. falciparum*. Although both proteins were localized to the apical half of the merozoite this is not yet sufficient proof for micronemal localization. Nevertheless BbAMA-1 shared functional characteristics with PfAMA-1 like a reduction of invasion efficiency by anti-BbAMA-1 antisera and secretion of a smaller-sized BbAMA-1 molecule into the surroundings. The secondary structure of AMA-1 is likely to be conserved considering the almost complete conservation of cysteine residues, spread over the length of the ectodomain, that are involved in disulfide-bond formation in PfAMA-1. Overall this makes AMA-1 one of the few, and certainly the most conserved, proteins involved in invasion. Its detection in *Theileria* EST datasets (*T. parva* genome sequencing project available from The Institute for Genomic Research at www.tigr.org and the *T. annulata* genome sequencing project available from www.sanger.ac.uk) raises questions about its molecular function as these parasites seem to follow a mechanism of invasion drastically different from the general model (see above).

GLIDING MOTILITY AND INVASION

A *P. falciparum* TRAP homologue was found somewhat surprisingly in *B. bovis* merozoites. PfTRAP has not been detected in *Plasmodium* merozoites but is expressed in the mature sporozoite stages present in the mosquito salivary gland. PfTRAP plays a role during gliding motility, the locomotive process by which sporozoites travel on artificial substrates in vitro. *Plasmodium* TRAP proteins are

composed of two adhesive domains (a vWFA-domain and a TSP-domain), a transmembrane region and a cytoplasmic domain. Recently a TRAP related *Plasmodium* protein was described and called CS protein-TRAP-related protein (CTRP). This protein is composed of five vWFA-domains followed by seven TSP-domains [58]. In other apicomplexans the TRAP-family harbours micronemal proteins containing five or more TSP-domains combined with a vWFA domain or an EGF domain as in *E. tenella* MIC-4. Gliding motility of sporozoites and *T. gondii* merozoites is essential for their travel through the host in order to reach new host cells. This has been shown to be dependent on the extracellular as well as intracellular domains of proteins like PfTRAP and TgMIC2. Such proteins are capped from the apical end to the back of parasites. Previous studies have proposed two hypothetical actin/myosin models for gliding motility of *P. falciparum* sporozoites that have been described in the general introduction of this thesis. Recent reports have indicated that the second model is the most likely model, showing that aldolase is the intracellular ligand connecting TRAP via its cytoplasmic domain indirectly to the actin/myosin locomotive machinery [106]. Intracellular capping and decapping of actin filaments of the motor would support capping of the transmembrane proteins to the posterior end. Moving of a dense junction around the parasite as observed during host cell invasion could involve a similar gliding mechanism using the same machinery. However, results on direct inhibitory effect of antibodies against TRAP on host cell invasion are still contradictory [80, 192, 235]. The results described in chapter 5 do describe such an effect for *B. bovis* erythrocyte invasion. This would provide an example for the use of the TRAP-family of proteins exclusively for host cell invasion as merozoites are supposed to be devoid of gliding motility. It remains puzzling why PfTRAP does not seem to be involved in erythrocyte invasion.

THE WAY FORWARD TO IDENTIFICATION OF INVASION PROTEINS

Many proteins residing in *P. falciparum* and *T. gondii* micronemes, rhoptries and dense granules have been identified over the past fifteen years, usually by a one-by-one process of gene or antigen identification and subsequent sub-cellular localization studies. Many more as yet unidentified proteins are expected to be present in these three organelles on the basis of sub-cellular fractionation studies performed on several apicomplexan parasites [21, 176]. For *B. bovis* only the highly expressed rhoptry protein RAP-1 and spherical body proteins SBP-1 to SBP-3 have been described [66, 94, 185, 211]. A more global identification of such proteins would thus be very helpful in order to close the gap in knowledge with *P. falciparum* and *T. gondii*. One approach would be a thorough sub-cellular fractionation of organelles followed

by proteomic methods involving mass spectrometry (MS) for identification of the proteins present in these fractions. Such an attempt has recently been described for the secretory organelles of sporozoites of *Eimeria tenella* [21], an economically important apicomplexan parasite of poultry. Microneme enriched fractions from *E. tenella* parasites were obtained by sucrose-gradient centrifugation and analyzed by MS. The results were disappointing in that most of the identified proteins proved to be derived from the parasite cytoskeleton, the endoplasmatic reticulum, the dense granule or the plasma membrane. Only two proteins were most likely of micronemal origin. Thus, alternative methods are urgently required, in particular for cells like *B. bovis* merozoites that cannot be obtained free of host cell remnants in contrast to *E. tenella* sporozoites. The observation that many, possibly most, micronemal and rhoptry proteins (or processed parts of them) are released into the extracellular milieu during host cell invasion provides such an alternative method. Two-dimensional PAGE gels presented in chapter 2 and 4 that were loaded with invasion supernatant showed many parasite specific spots compared to the mock-infected erythrocyte control. The 2 D-gels presented in these chapters were the first examples of direct staining of proteins released during invasion without the need of immunoprecipitation of metabolically labeled proteins. Moreover, it circumvents the predominant selection of the most immunogenic proteins invoked by the latter method. The use of specific antibodies allowed us to demonstrate the presence of AMA-1 in this fraction (Chapter 4), albeit at a very low level in comparison to many contaminating proteins of bovine origin. At least 100 additional, often weak, spots are thought to be of *B. bovis* origin. The close proximity of many of these spots to much more abundant bovine proteins requires the use of an additional purification step in order be able to load enough sample on a gel for MS identification of all spots.

Another interesting group of proteins involved in invasion and likely to be present in the extracellular milieu are proteases. Their function is pivotal to the continuation of the invasion process. Inhibitor studies have indicated that the transmembrane micronemal adhesion proteins involved in invasion have to be shed into the medium by proteolytic cleavage as their interaction with the host cell might otherwise block further movement. This has been described in Chapter 1 in some more detail. Several serine proteases have been implied in shedding such proteins from the surface of *P. falciparum* merozoites [100] but their genes have not been identified yet. Protease inhibitors specific for different types of proteases will be a valuable tool in identifying such proteins. Their effect on in vitro invasion can be determined and they can be used for screening subsequent fractionation procedures in order to purify the proteases. Application of specific invasion inhibitors may result in

altered protein patterns on 2D gels and thus give indications about the potential protease substrates.

A third group of proteins that have been predicted to function as the specific switch for initiating the invasion process, are signal transduction proteins. Sofar nothing is known in detail about putative receptor-ligand interactions that take place upon initial contact between parasite and host cell for any apicomplexan species. *Plasmodium* host cell binding ligands like EBA-175, EBA-140 and DBL are secreted only seconds prior to the onset of invasion and obviously this must be regulated by some specific trigger. The first attachment of merozoites to erythrocytes is reversible and probably based on charge interactions as erythrocyte membranes are negatively charged because of the sialic acid residues whereas merozoites are positively charged. This might induce the rolling movement leading to apical reorientation upon which irreversible attachment by the just released ligands takes place. Structures (transmembrane proteins?) near the apical conoid may transduce the signal. Also the nature of the signalling process still remains to be identified. Obviously, interference with the hypothesized signal transduction process should have serious consequences for erythrocyte invasion and the use of specific inhibitors of signal transduction in the vitro invasion assay might give clues on the type of proteins that are involved.

HOST CELL INVASION AS A TARGET FOR VACCINE DEVELOPMENT

Besides the fundamental scientific interest for the molecular mechanisms of host cell invasion by parasites belonging to the most important and threatening parasites of man and animals worldwide, there is obviously a great deal of attention for these studies considering the urge for efficient recombinant vaccines. Good vaccine candidates would be proteins that are exposed to the immune system like proteins that are secreted from the apical organelles (micronemes and rhoptries) during invasion and proteins localized on the membrane of the parasite. However, the extracellular timespan of *B. bovis* outside its host cell is very short, requiring a high antibody titre for invasion inhibition. Inside erythrocytes *B. bovis* multiplies by binary fission resulting in two parasites. An effective vaccine for *B. bovis* only needs to reduce invasion about 50% in order to decrease the parasitaemia level in a host. For *P. falciparum* the vaccine needs to inhibit invasion for more than 95% to be able to reduce parasitaemia, because *P. falciparum* merozoites multiplies to 20-30 new parasites per cycle during schizogony. In *Plasmodium* several proteins have already been used in vaccination trials. Antibodies raised against AMA-1 protein have shown protection of monkeys against the pathological effect of *P. falciparum* [115, 215].

Antibodies against other proteins like RAP-1, TRAP or EBA-175 have shown to inhibit invasion in vitro and in vivo studies [152, 157, 192, 212].

Previous reports of *Babesia* exoantigens have shown to give protection. Exoantigens, also named soluble parasite antigens (SPA) are proteins found in the medium of *Babesia* cultures [107, 170]. Culture medium will probably contain secreted proteins as well as breakdown products of erythrocytes and parasites. In addition, this fraction will most likely also contain proteins that are released during lysis of infected erythrocytes during the maturation process of the *Babesia* parasites. Culture medium that was partially purified and concentrated has been developed to a vaccine for *B. canis* in dogs [190]. The vaccine protected dogs against the pathological effects of the *B. canis* infection without expelling the parasite, indicating a mode of action directed against pathogenic molecules that are not involved with parasite propagation anymore. A vaccine based on SPA-containing medium from a European *B. canis* and a South Africa *B. rossi* isolate induced protective immunity against heterologous *B. canis* infection. Dogs that were vaccinated with the European *B. canis* isolate showed only protection against a homologous challenge infection [190]. Whether the observed protection can be contributed to the apical organelle proteins secreted during invasion or is evoked by other types of proteins is not known yet.

THE NEED FOR TRANSGENETICS

The identification of parasitic proteins involved in the invasion process will raise questions on their function and their suitability for inclusion in vaccination trials. Transfection systems for *T. gondii* and *P. falciparum* have been developed employing several selectable markers [48, 51, 65, 130]. Transfection has been used to knockout specific proteins. However, elimination of some proteins has shown to be lethal for the parasite, prohibiting a functional analysis. To overcome this problem, an inducible system was set up for *T. gondii* [141] with the possibility for conditional knockout of proteins. For our initial attempts to set up a transfection system for *B. bovis* we have used the methodology that has worked for several *Plasmodium* species. With the constructs and conditions used, this has sofar only resulted in the generation of resistant parasites instead of transformants. The resulting high IC_{50} for pyrimethamine, which is already high for wildtype *B. bovis*, makes this drug probably unsuitable for selection. In contrast, even against the isolated mutants, WR99210 is still very efficient at a low concentration and therefore usable providing that we can select under sufficiently high concentrations of WR99210. Alternatives for these drugs in obtaining stable transfection are other selectable markers such as blasticidin S-deaminase, puromycin-N-acetyltransferase, hypoxanthineguanine-phosphoribosyl-

transferase or dihydropteroate synthase. These have been employed for other apicomplexans and could be evaluated for *B. bovis*.

The constructs that have been used to transfect the parasites were under control of a *B. bovis* DHFR-TS promoter and terminal region (see Chapter 7). As was shown in chapter 7 these constructs contain (parts of) several other genes. Whether these extra genes interfere with stable integration into the genome remains unknown. Sofar little is known about promoters in *B. bovis*. The selection of additional promoters, active at a defined period of the life cycle, would expand our options for transfections with plasmid constructs.

On basis of the present dataset, it may be recommendable to setup a transient transfection system for *B. bovis* in order to optimize transfection conditions. At first we used green-fluorescent protein and yellow-fluorescent protein to detect transformants. Secondly we tried β-galactosidase as a more sensitive reporter gene. However, none of the reporters were detected in *B. bovis* merozoites in the first 48 h after transfection. In other apicomplexan species other sensitive reporter genes have been used like chloramphenicol transferase and luciferase genes. These reporter genes could also help to find specific transfection conditions for *B. bovis*.

The observed transformation efficiency for *P. falciparum* and *T. gondii* differs enormously. For *T. gondii* it is normally 10^3 to 10^5 times higher as observed for *P. falciparum*. If *B. bovis* also has an efficiency as low as *P. falciparum* it will not be easy to obtain an initial transfection level from which optimization can start. Alternative methods to electroporation may induce higher levels of transfection and could be investigated. Successful transformation of *B. bovis* will in future be an indispensable tool for further studies.

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SUMMARY

SAMENVATTING

CURRICULUM VITAE

DANKWOORD

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SUMMARY

In this thesis we investigated the invasion of erythrocytes taking place during the asexual erythrocytic blood stage of the apicomplexan parasites *Babesia bovis* parasite.

Host cell invasion by apicomplexan parasites is a complex process requiring multiple receptor-ligand interactions, involving association of the merozoite with the erythrocyte surface, reorientation and attachment of the apical end of the merozoite. In parasites like *Toxoplasma gondii* and *Plasmodium falciparum* many proteins have been identified that are involved in invasion including proteins present on the merozoite surface and proteins present in secretory organelles located at the apical end of the merozoite. An in vitro invasion assay of *B. bovis* was established to be able to characterize the mechanisms and the proteins involved in invasion. Invasion of liberated merozoites is completed in 1 h in phosphate buffered saline solution. The manipulation of intracellular calcium concentration or actin polymerization in the merozoite inhibits invasion indicating that *B. bovis* invades erythrocytes in a similar way as *Plasmodium* merozoites. Proteins that are secreted into the environment during in vitro invasion can be detected directly by protein staining after 2D gel electrophoresis. This in vitro invasion assay was used to assess the host-specificity of *B. bovis*. In vivo, *B. bovis* infections have only been observed in bovine species. In vitro, human, ovine, porcine, equine and caprine erythrocytes were all invaded by *B. bovis*. Human erythrocytes were invaded more efficiently than bovine erythrocytes, whereas goat erythrocytes were invaded at very low level. Significant differences in invasion efficiency into erythrocytes from different individuals of the same species were observed. Besides invasion, intracellular duplication of *B. bovis* parasites also took place in all erythrocyte species, except for goats. Only in bovine erythrocytes subsequent rounds of invasion were observed. *B. bovis* merozoite invasion was shown to be dependent on sialic-acid residues present on the host cell. Pre-incubation of merozoites with n-acetylneuraminyl-lactose decreased invasion efficiency by ~45%, whereas addition prior to invasion had no significant effect. Thus invasion might be

dependent on the presence of merozoite membrane proteins already accessible during pre-incubation prior to invasion.

Only two proteins (AMA-1 and TRAP) involved in invasion of other apicomplexans were identified from a large *B. bovis* EST database and these were sequenced and characterized. *B. bovis* invasion was inhibited by antiserum raised against peptides from several domains of AMA-1. Antisera raised against domain III of AMA-1 give the strongest invasion inhibition. *B. bovis* TRAP was surprisingly found in merozoites. In *Plasmodium* species TRAP has been found in the sporozoite stage. Antibodies directed against BbTRAP inhibited invasion. Immunofluorescence showed that *B. bovis* AMA-1 and TRAP are localized at the apical region of the merozoites. *P. falciparum* AMA-1 and TRAP proteins are known to be cleaved off and secreted into environment during invasion. 1D and 2D-gel electrophoresis of total merozoites extract and invasion supernatant followed by immunoblotting with *B. bovis* AMA-1 antisera showed that this protein is cleaved from 82 kDa to a 69 kDa and secreted into the surrounding environment. *B. bovis* TRAP also was found in invasion supernatant.

Transfection of in malaria parasites provides a valuable tool for analyzing gene function. An attempt was done to establish a transfection system of *B. bovis* with *dhfr* as a selectable marker. The *B. bovis dhfr-ts* genomic locus and cDNA was cloned and sequenced. Beside the *dhfr-ts* gene of 2.83 kb, four other genes were identified in a 7.41 kb region of which at least three are expressed during the erythrocytic cycle. Three of the genes were highly conserved in closely related *Theileria* species and gene synteny was observed between *B. bovis* and *T. parva*. *B. bovis* merozoite cultures electroporated with plasmids containing *T. gondii dhfr-ts*m2m3 or human *dhfr* under control of *B. bovis dhfr* regions did not produce stable transfected parasites. Transient expression using GFP, YFP or β-galactosidase as a reporter gene was also not yet established. Nevertheless, parasite cultures with decreased sensitivity for pyrimethamine and WR99210 were repeatly isolated. The *dhfr* gene of five resistant cultures was sequenced and showed to contain a S125F amino acid substitution. Homology modelling of *B. bovis* DHFR by using the *P. falciparum* DHFR structure as a template, suggested that S125F affects the binding site of NADPH. The five

resistant cultures that were first selected on either pyrimethamine or WR99210 turned out to be cross-resistant for both drugs. Comparable drug sensitivities were only observed in a quadruple mutant of *P. falciparum dhfr* displaying strong resistance to pyrimethamine and 10-fold enhanced resistance against WR99210. Wildtype *B. bovis* was similar to this mutant at 3 of the 4 mutated positions possibly explaining its low sensitivity for pyrimethamine.

SAMENVATTING

Babesiosis is een veel voorkomende ziekte onder runderen in tropische en subtropische gebieden over de hele wereld. De ziekte wordt overgebracht door *Boophilus microplus* teken die geïnfecteerd zijn met de hemoparasiet, *Babesia bovis*. *B. bovis* behoort tot het phylum Apicomplexa, klasse Sporozoa, subklasse Piroplasmida. De replicatie van de parasiet vindt plaats in rode bloedcellen (erythrocyten). Dit resulteert meestal in anaemie en kan uiteindelijk organen aantasten, wat tot gewichtsverlies en zelfs tot de dood kan leiden. Ondanks de grote verliezen in de veestapel en wereldwijde verspreiding van de infecties is er nog geen effectieve controle op anaplasmosis en babesiosis in meeste betrokken gebieden.

B. bovis is ééncellige intraerythrocytaire parasiet die veel overeenkomsten vertoont met de malaria parasiet. *B. bovis* parasieten hebben een gemiddelde grootte van 2.5 bij 1.5 μm en behoren daarom tot de groep van kleine *Babesia*. Het meest opvallende morfologische kenmerk van de *B. bovis* parasiet zijn de apicale organellen, die waarschijnlijk gebruikt worden tijdens rode bloedcel invasie. Uit detail onderzoeken die gedaan zijn bij andere *Babesia* soorten, zoals *B. bovis* en *B. bigemina* is gebleken dat de parasieten behalve deze apicale organellen ook polaire ringen aan het apicale einde bevatten en twee binnen membranen die samen met de plasma membraan de pellicle genoemd worden. Nader onderzoek heeft aangetoond dat aan de buitenste membraan van het inner membraan complex myosine gebonden is terwijl actine met de plasma membraan geassocieerd is. Door hydrolyse van ATP dat bindt aan myosine komt energie en kracht vrij dat aanzet tot beweging. Een micropore is betrokken bij de voedselopname tijdens de intraerythrocytaire fase van het parasiet. De apicale organellen spelen een belangrijke rol bij de invasie van rode bloedcel door de parasiet. De apicale organellen worden rhoptries, micronemes en dense granules genoemd.

De vermenigvuldiging en verspreiding van *B. bovis* vindt plaats door opeenvolgende sexuele multiplicatie in de teek, via transovariële transmissie en asexuele multiplicatie van merozoieten in de gastheer. De levenscyclus van de *B. bovis* parasiet begint door een beet van een geïnfecteerde teek. Sporozoieten die zich

in de speekselklier bevinden van de teek komen door de beet in de bloedbaan van het rund terecht. De sporozoieten dringen rode bloedcellen binnen en bij lysis van de rode bloedcellen komen de merozoieten vrij. De vrijgekomen merozoieten kunnen direct rode bloedcellen binnendringen. Binnen de rode bloedcel, deelt de merozoiet zich tot twee peervormige merozoieten en komt door middel van lysis vrij uit de erythrocyten. Deze dringen dan weer nieuwe erythrocyten binnen. Naast het herhaaldelijk delen van de merozoieten kunnen deze zich ontwikkelen tot mannelijke of vrouwelijke geslachtcellen (gametocyten). De gametocyten kunnen door een teek worden opgenomen tijdens hun voeding op een gastheer. In de darm van de teek smelten de mannelijke en vrouwelijke gametocyt samen tot een zygote. Van zygote transformeert de parasiet tot kineet en kan nu verschillende organen infecteren waaronder de eierstokken van de vrouwelijke teek en komt zo in de eieren van de teek terecht. Ookineten infecteren de speekselklier van de teek en zullen bij de volgende voeding van de teek op een gastheer weer ingespoten worden in de bloedbaan.

Dit proefschrift beschrijft invasie van de *B. bovis* merozoiet in de rode bloedcel van de gastheer (rund). Het eerste contact van de parasiet met de rode bloedcel is omkeerbaar. De parasiet reorienteert zich en maakt opnieuw contact met de rode bloedcel alleen nu met zijn apicale pool, zodat de rhoptries en micronemes in contact komen met het celoppervlak van de erythrocyt. Een zogenaamde tight junction wordt gevormd en hierdoorheen zal de parasiet de erythrocyt binnen dringen en stulpt daarbij erythrocyt membraan naar binnen die uiteindelijk de vacuole (parasitofore vacuole) zal vormen waarin de parasiet zich zal bevinden als hij eenmaal binnen is. Bij andere apicomplexa zoals *Plasmodium falciparum* en *Toxoplasma gondii* is uit onderzoek gebleken dat de drie apicale organellen eiwitten bevatten die verschillende functies blijken te vervullen tijdens rode bloedcel invasie. Micronemes zijn de eerste organellen die tijdens invasie hun eiwitten uitscheiden en spelen waarschijnlijk een functie bij herkenning en aanhechting van de rode bloedcel en waarschijnlijk ook bij beweging in de rode bloedcel. Rhoptries zorgen mogelijk voor de vorming de parasitofore vacuole en dense granules voor transformatie van de vacuole in een metabolisch actief compartiment.

Om te voorkomen dat de veestapel besmet raakt met babesiosis kan men of de vector (de teek) uitschakelen of ingrijpen in de levenscyclus van de parasiet. Om de teek uit te schakelen zouden alle koeien behandeld moeten worden met acariciden. Dit is een dure en tijdrovende methode. Voor het stopzetten van invasie in de rode bloedcel gebruikt men vaccins met verzwakte levende parasieten. Nadelen die gepaard gaan met deze methode zijn de mogelijkheid van contaminatie met andere niet bekende ziekteverwekkers. Om deze problemen te omzeilen wordt er onderzoek gedaan om een effectief recombinant vaccin te maken.

In dit proefschrift zijn er onderzoeken beschreven die betrekking hebben op de asexuele erythrocytaire fase van de *B. bovis* parasiet. Allereerst is er een in vitro invasie assay opgezet voor *B. bovis*. Behalve dat de assay snel en efficient is, kan de intracellulaire groei en invasie van de parasiet ook gescheiden worden. Daarna is er een poging gedaan om receptor typen en liganden die nodig zijn bij invasie nader te onderzoeken. Verder zijn er twee *P. falciparum* homologe eiwittten die betrokken zijn bij invasie geidentificeerd, gekloneerd, gesequenced en geanalyseerd in de *B. bovis* parasiet. Als laatste is er een poging gedaan een transfectie systeem voor *B. bovis* te ontwikkelen, maar in plaats daarvan zijn er alleen maar resistente *B. bovis* cultures gekregen die geen plasmide hadden opgenomen.

De invasie van de merozoiet in de rode bloedcel is een complex proces waarbij verschillende receptor-ligand interacties aangegaan worden tijdens reorientatie en binding van de apicale pool aan de rode bloedcel. Verschillende eiwitten zijn betrokken bij merozoiet invasie die zowel op het merozoiet oppervlak aanwezig zijn als gelokaliseerd zijn in de apicale organellen. Een in vitro invasie assay is opgezet om het mechanisme van erythrocyt invasie nader te kunnen onderzoeken. Deze assay geeft de mogelijkheid om eiwitten die betrokken zijn bij de invasie direct te kunnen detecteren en analyseren m.b.v. 2D-electrophorese gevolgd door eiwitkleuring, omdat invasie in fosfaatbuffer plaatsvindt. Er is gekeken naar het effect van de intracellulaire calcium concentratie door gebruik te maken van verscheidene drugs (BAPTA-AM). Uit de resultaten is gebleken dat BAPTA-AM en cytochalasin B (een actine polymerisatie remmer) beiden de invasie afremmen. Naast dat de invasie assay snel en

efficient is, kan intracellular groei en invasie van de parasiet afzonderlijk bestudeerd worden.

De invasie assay is gebruikt om de gastheer specificiteit van de *B. bovis* parasiet te bestuderen. In de natuur blijkt dat *B. bovis* alleen runderen infecteert. Echter invasie experimenten met erythrocyten van mensen, schapen, varkens, paarden en geiten tonen aan dat deze erythrocyten ook geïnvadeerd worden door *B. bovis* parasieten. De humane erythrocyten worden het best geïnvadeerd en de minste parasieten werden gevonden in geiten erythrocyten. Verder blijkt er een significant verschil in invasie efficiency tussen verschillende individuen van dezelfde diersoort te zijn. Behalve invaderen, kunnen de parasieten ook verder uitgroeien in de andere rode bloedcel soorten, met uitzondering van geiten erythrocyten. Alleen in runder erythocyten kunnen de parasieten na deling nieuwe rode bloedcellen binnendringen en zo een verhoogde parasitaemia geven. De resultaten die hier gepresenteerd zijn geven aan dat de specificiteit van *B. bovis* parasieten later in de levenscyclus plaatsvindt en niet al tijdens de invasie van de parasiet. *B. bovis* merozoieten blijken bij erythrocyt invasie sialic acid bevattende receptoren te gebruiken. Neuraminidase behandeling van de erythrocyt geeft een invasie inhibitie van ongeveer 80% en preincubatie van *B. bovis* merozoieten met n-acetylneuraminyl-lactose ligand geeft een afname van de invasie met ongeveer 45%, maar toevoeging van dit ligand direct voor invasie gaf geen significant effect. Dus invasie van de *B. bovis* merozoiet in de rode bloedcel is afhankelijk van een ligand met affiniteit voor sialicacid-bevattende erythrocyt membraanreceptoren dat al aanwezig is op de membraan van *B. bovis* merozoieten alvorens invasie optreedt.

Twee invasie eiwitten die bij *P. falciparum* bekend staan als vaccin kandidaten zijn gesequenced en gekarakteriseerd in *B. bovis* merozoieten. *B. bovis* AMA-1 (Apicale membraan antigen 1) en TRAP (thrombospondin gerelateerd anoniem eiwit) komen tot expressie in de erythrocyt fase, terwijl TRAP bij *P. falciparum* alleen in het sporozoiet stadium tot expressie komt en betrokken is bij de beweging van de sporozoiet. *B. bovis* invasie werd geremd door serum dat tegen peptiden van pre-domein, domein II en domein III van AMA-1 was opgewekt. Serum tegen domein III gaf de meeste inhibitie. Invasie van *B. bovis* werd ook afgeremd door serum tegen de

twee verschillende peptiden van *B. bovis* TRAP eiwit (serum tegen peptiden van het TSP-1 domein of van het C-terminale eind van het ectodomein), alhoewel er met serum dat tegen TSP-1 domain de meeste inhibitie gaf. Een immunofluorescentie test geeft aan dat *B. bovis* AMA-1 en TRAP allebei gelokaliseerd zijn rond de apicale pool. Echter er kan niets gezegd worden over lokalisatie in een van de apicale organellen. Verder is er in *P. falciparum* bekend dat deze twee eiwitten bij invasie geknipt worden en zo terecht komen in het milieu. Door 2-D gel electroforese toe te passen op totaal merozoieten extract en invasie supernatant gevolgd door immunoblotten met *B. bovis* AMA-1 antisera hebben we kunnen aan tonen dat dit eiwit wordt geknipt en uitgescheiden in het milieu.

Om de functie te kunnen bestuderen van de eiwitten die een belangrijke rol spelen bij invasie is een transfectie systeem nodig. Een poging werd gedaan om een transfectiesysteem op te zetten voor *B. bovis* met als selectie marker het dihydrofolate reductase thymidylate synthase gen. De *B. bovis* dhfr genomische locus en cDNA werden gecloneerd en gesequenced. Naast het dhfr-ts gen van 2.83 kb zijn er in een regio van 7.41 kb ook vier andere genen aanwezig van welke er minstens drie tot expressie komen tijdens de erythrocytaire cyclus. Drie van de genen hebben een hoge conservering met genen van *Theileria* soorten en de ordening van een aantal van de geconserveerde genen is ook tussen *B. bovis* en *Theileria* gelijk. *B. bovis* merozoiet culturen die geëelectroporeerd zijn met een plasmied dat *T. gondii dhfr-ts*m2m3 of het humane *dhfr* bevat onder controle van *B. bovis* dhfr flankerende gebieden gaf niet getransformeerde resistente culturen. Het DHFR gen van vijf resistente culturen werd gesequenced en wees uit dat ze een S125F aminozuur substitutie hadden. Modellering van *B. bovis* DHFR met de *P. falciparum* DHFR 3D-structuur als matrijs, suggereerde dat substitutie S125F de binding met NADPH zou kunnen beinvloeden. De vijf resistente culturen die geselecteerd waren op pyrimethamine of WR99210 toonden kruisresistentie voor beide drugs. Een vergelijkbare resultaat werd ook gevonden bij een viervoudige mutant van *P. falciparum dhfr* dat een sterk verhoogde resistentie voor pyrimethamine had en slechts een 10 keer verhoogde resistentie voor WR99210 gaf. Wild type *B. bovis* had al drie van de vier mutaties die de *P. falciparum* mutant had. Waarschijnlijk draagt dit bij aan de lage pyrimethamine sensitiviteit van *B. bovis.*

CURRICULUM VITAE

Op 19 oktober 1973 werd Fasila Razzia Gaffar geboren. Na het volgen van de Mulo en verhuizing naar Nederland, ging ze verder met de MAVO waar zij op 20 juni 1989 aan het Augustinus College te Amsterdam haar diploma behaalde. Op 18 juni 1991 behaalde zij haar HAVO diploma en op 1 juli 1994 het VWO diploma. Na de doctoraalstudie Medische Biologie; specialisatie Immunologie en Moleculaire Biologie werd aan de Vrije Universiteit Amsterdam Faculteit Biologie op 17 februari 1999 de drs. titel behaald. Tijdens deze studie werd tevens een stage van 6 januari tot 10 juli 1997 aan het AMC Amsterdam/Vrije Universiteit Amsterdam gelopen. Met een onderzoek naar effect van de twee meest voorkomende mutaties in de lipoprotein lipase (LPL) gen van het lipoproteine metabolisme bij Diabetes-Mellitus type II patiënten. Daarnaast was er nog een stage van 2 maart 1998 tot 19 juni 1998 bij het AMC Amsterdam inzake een onderzoek naar het effect van parainfluenza virus typeIV op de IL-6 productie van H292 cellen . De afstudeerscriptie had als titel : "AIDS Dementia Complex in adults & children". Hierbij werd aandacht geschonken aan het AIDS Dementia complex waarin vooral werd gekeken naar pathogenese en neuropathogenese (veranderingen in de hersenen). Ten slotte de antiretrovirale medicijnen die momenteel aanwezig zijn om de ziekte in bedwang te houden. Na het behalen van haar diploma is zij gestart als Onderzoeker in opleiding aan de Universiteit van Utrecht vakgroep Parasitologie en Tropische Diergeneeskunde alwaar ter verkrijging van de graad van doctor eind januari 2004 het werk zoals beschreven in dit proefschrift werd uitgevoerd.

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Na mijn opleiding Medische Biologie aan de Vrije Universiteit kwam ik na niet lang zoeken terecht bij Diergeneeskunde in Utrecht, het vakgebied sprak mij erg aan, en ik besloot daarom de uitdaging aan te gaan. Ik woon in Amsterdam, dus dat werd op en neer pendelen, door regen en wind, met de trein, carpoolen met mijn grote broer en later ging ik met eigen vervoer. Met groot enthousiasme begon ik aan het onderzoek, veel lezen, overleggen en discussiëren met zowel lotgenoten als collega's.

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فسل