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Development of two diagnostic tools (ELISA and ICT) for detection of antibodies against ovine and bovine theileriosis

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1 Introduction

Theileriosis is a tick transmitted-protozoan disease in cattle, sheep and goats as well as in wild and captive ungulates and is caused by several different pathogenic *Theileria* (*T.*) species (Mehlhorn et al., 1994). Thus, *Theileria annulata* (*T. annulata*) is a pathogenic species in cattle that is responsible for significant economic losses in animal husbandry and causes tropical theileriosis (also called mediterranean theileriosis). This species is transmitted by ticks of the genus *Hyalomma* (Uilenberg et al., 1981). The infection occurs over a wide geographic area ranging from Southern Europe to Southern Russia, the Middle East, Central Asia, China, India, Northern Africa and Sudan, Eritrea and Mauritania (McCosker, 1979; Dolan, 1989; Minjauw and McLeod, 2000).

Regarding *Theileria* of small ruminants, the highly pathogenic species *T .lestoquardi* causes a disease called malignant ovine theileriosis in sheep and goats (Hooshmand–Rad and Hawa, 1973a; Brown et al., 1998). Other pathogenic ovine *Theileria* include the newly identified *Theileria* parasites designated *T .luwenshuni* (previously referred to as *Theileria* sp. China 1) and *T. uilenbergi* (previously referred to as *Theileria* sp. China 2), which are the causative agents of ovine theileriosis in China (Ahmed et al., 2006; Yin et al., 2007). Both these species are transstadially transmitted by the three host ticks *Haemaphysalis qinghaiensis* and *H. longicornis* (Yin et al., 2002a, 2002b; Li et al., 2007; Li et al., 2009).

Generally, the diagnosis of infection by *Theileria* parasites in cattle and small ruminants is usually based on clinical signs, vector distribution and on the morphological examination of the piroplasm and schizont stages of the parasite in Giemsa-stained blood and lymph node smears (Hooshmand-Rad and Hawa, 1973a; Gao et al. 2002). Although these methods can be used for the detection of acute cases, they do however have limited value for chronic cases because of the low degree of parasitaemia in those animals and additionally, it is difficult to discriminate between piroplasm species according to morphology (Hooshmand-Rad, 1974; Friedhoff, 1997). Several molecular techniques for the specific detection of different *Theileria* parasites have been developed. Reverse line blotting (RLB) was established to detect and differentiate all known *Theileria* and *Babesia* (*B.*)species on the basis of differences in their 18S subunit rRNA gene sequences (Gubbels et al., 1999; Schnittger et al., 2004). However, the RLB technique requires equipped laboratories, is expensive and impractical for field diagnosis. In addition, molecular biology techniques have been developed as precise tools for the detection of parasite DNA and several diagnostic procedures based on PCR with high

sensitivity and specificity have been established to detect the parasite in large and small ruminants (d'Oliveira et al., 1995; Shayan et al., 1998; Kirvar et al., 2000; Habibi et al., 2007; Sun et al., 2008; Yin et al., 2008). However, these techniques are expensive, require a high degree of expertise and cannot be used to detect subclinical infected animals. Recently, loop-mediated isothermal amplification of DNA (LAMP) has been successfully developed for the detection of some *Theileria* species (Salih et al., 2008; Liu et al., 2008b; Thekisoe et al., 2010; Wang et al., 2010).

With respect to development of diagnostic tools for the detection of *Theileria* infection, the study conducted in this thesis consists of two parts. Firstly, the development of a recombinant protein indirect ELISA for the detection of specific antibodies against *T. uilenbergi* was aimed for, in order to achieve an improvement to existing serological methods for the detection of infection with this pathogen. Secondly, a rapid test for the detection of infection with *T. annulata* was developed based on existing components used for detection of this infection by ELISA. The aim was to provide a diagnostic tool suitable for use under field conditions. The study parts are described in more detail in the following:

First part: Development of an indirect test (ELISA) for detection of infection with *T. uilenbergi*

Detection of antibodies against *Theileria* causing ovine theileriosis in China using ELISA have been applied in epidemiological studies using two previously developed indirect ELISA methods: The first one was based on crude merozoite antigen (Gao et Al., 2002) and the second one on the partially expressed *T. lestoquardi* recombinant heat shock protein 70 (rTIHSP 70) (Miranda et al., 2006a). Nevertheless, these assays needed improvement. Thus, the crude antigen ELISA is difficult to standardize, requiring the preparation of antigen from experimentally infected animals. In addition, both methods bear the possibility of cross-reactivity with other related pathogens.

To meet this demand of an improved, specific indirect ELISA, in the first part of this study a *T. uilenbergi* antigenic and specific protein was utilized for the development of a serological diagnostic test (indirect ELISA). The gene of this protein was identified by random screening of a *T. uilenbergi* merozoite cDNA library (Liu et al., 2008a) by PCR followed by sequencing and bioinformatic analyses aimed at identifying potential antigenic parasite proteins suitable for developing diagnostic tools. Using this approach, a gene named clone-9 was discovered which was partially expressed as a His-tagged recombinant protein (c9b) and used to establish an indirect ELISA assay. The potential application of this test for

serological surveys of infection with *T. uilenbergi* was investigated by using serum from experimentally infected sheep and serum that was collected from sheep of endemic regions in China.

Second part: Development of an immunochromatographic strip test (ICT) for the detection of *T. annulata* infection

Regarding the serological methods for detecting *T. annulata* antibodies, many tests have been applied for epidemiological survey studies. Thus, the indirect fluorescence antibody test (IFAT) has been successfully used to detect theileriosis in cattle (Burridge and Kimber, 1973), whereby this test was reported to be more sensitive than examination of blood smears (Dhar and Gautam, 1977; Darghouth et al., 1996). However, this test has the major drawback of showing cross-reactivity between different *Theileria* species (Burridge et al., 1974).

In an effort to develop a *T. annulata* specific indirect ELISA, a number of *T. annulata* recombinant proteins have been evaluated for their use in ELISA, including TaSP, TaD, TaSE and TaHSP70 (Seitzer et al., 2008). Many publications confirmed the suitability of the TaSP protein for the detection of tropical theileriosis (Bakheit et al., 2004; Salih et al., 2005, 2007a; Seitzer et al., 2007, 2008). Furthermore, a competitive ELISA (cELISA) for the detection of circulating antibodies against the parasite based on the same antigen was recently established and validated (Renneker et al., 2008, 2009).

In the framework of the second part of this thesis, an immunochromatographic strip test (a lateral flow device or 'pen-side test') was developed for the detection of the infection with *T. annulata* based on the TaSP protein. The advantages of this assay in comparison to available tests are that it is very simple and easy to use, not requiring trained personnel, it delivers fast test results that are analyzable by the eye, and it exhibits long-term stability over a wide range of climates not requiring any cooling (refrigerator). These features make this immunochromatographic strip test ideal for individual testing, rapid point of care testing, testing in the field, and reliable testing that might otherwise not be available to third world countries.

2 Literature review

2.1 Genus *Theileria*

Theileria are tick-transmitted intracellular protozoan parasites which cause economic losses in domestic livestock involving large and small ruminants. *Theileria spp.* infect wild and domestic ruminants in tropical and subtropical regions of the world (Dolan, 1989; Shaw, 2002). Theileriosis occurs over a wide geographic area ranging from Southern Europe and extending to Southern Russia, the Middle East, Central Asia, China, India, Northern Africa and Sudan, Eritrea and Mauritania (McCosker, 1979; Dolan, 1989; Minjauw and McLeod, 2000). The species which infect cattle and small ruminants are transmitted by ixodid ticks of the genera *Rhipicephalus, Amblyomma*, *Hyalomma* and *Haemaphysalis*. *T. parva* and *T. annulata* are the most important species that infect cattle in sub-Saharan Africa and Europe, North Africa and South Asia (Spickler, 2009). Among known *Theileria* parasites of small ruminants, *T. lestoquardi* is highly pathogenic and causes malignant ovine theileriosis (Uilenberg, 1981; Friedhoff, 1997). Two newly identified *Theileria* species namely *T. luwenshuni* - previously referred to as *Theileria* sp. China 1 and *T. uilenbergi* - previously referred to as *Theileria* sp. China 2 are responsible for theileriosis in sheep in China (Ahmed et al., 2006; Yin et al., 2007).

2.2 Taxonomy

The taxonomy of *Theileria* has been a subject of controversy for many years (Uilenberg, 1981; Irvin, 1987; Stewart et al., 1996). Traditionally, taxonomy has been based on morphology, transmitting vectors, host specificity, epidemiological data, geographic origin and mammalian hosts and life cycle characteristics. The development of molecular methods, particularly sequence data, enhanced the classification outlines that were originally based on morphological and life history criteria (Barta, 2001) and did not allow the separation of different *Theileria species* (Gubbels et al., 2002; Schnittger et al., 2003; Yin et al., 2004). However, molecular data based on 18S rDNA sequencing subjected new knowledge into the phylogeny of *Theileria* species infecting both large and small ruminants (Chansiri et al., 1999).

2.3 Life cycle of *Theileria*

The life cycles of all *Theileria* species are generally similar (Shaw, 2002). The transmission of the disease involves two stages: the transmitting invertebrate tick vector and the vertebrate (Mehlhorn and Schein, 1984; Shaw, 2002; Bishop et al., 2004). *Theileria* parasites go through at least three phases during their life cycle: asexual reproduction by schizogony and merogony in the mammalian host followed by sexual reproduction and sporogony in the tick vector. The life cycle begins when the sporozoites are injected into the host with the saliva of the vector during its feeding process (Tait and Hall, 1990). After being injected into the mammalian host, sporozoites invade different leukocyte sub-types depending on the *Theileria* species. Inside the leukocyte, they develop into a macroschizont and induce transformation and proliferation of the host cell, leading to rapid clonal expansion of parasitized cells in the lymphoid tissues (William and Dobbelaere, 1993; Radostits et al., 2000; Ahmed et al., 2008). As the infection spreads, some schizonts will develop into merozoites which leave the lymphocytes and are released into the bloodstream. The merozoites will then enter erythrocytes and develop into piroplasms (Glascodine et al., 1990; Urquhart et al., 1996).

The transmitting invertebrate tick vectors begin with the ingestion of piroplasm-infected erythrocytes during their blood meal. Lysis of infected erythrocytes occurs in the gut of the tick and the ingested piroplasms begin to develop into ‘ray bodies’. By the fifth day of the tick feeding, the ray bodies give rise to uninucleate gametes (Schein, 1975). The zygote is formed by the fusion of two gametes and subsequently the zygote invades a gut epithelial cell and differentiates into a motile kinete (Mehlhorn and Schein, 1984). The motile kinetes finally enter the acinar cells of the salivary gland. Inside this gland, the kinetes develop into sporoblasts, which wait locally for coming blood triggering extensive multiplication into full grown and infective sporozoites, a process known as sporogony (Mehlhorn and Schein, 1984). When infested ticks attack animals, sporozoites are released into the mammalian host and a new life cycle starts (Fig.1).

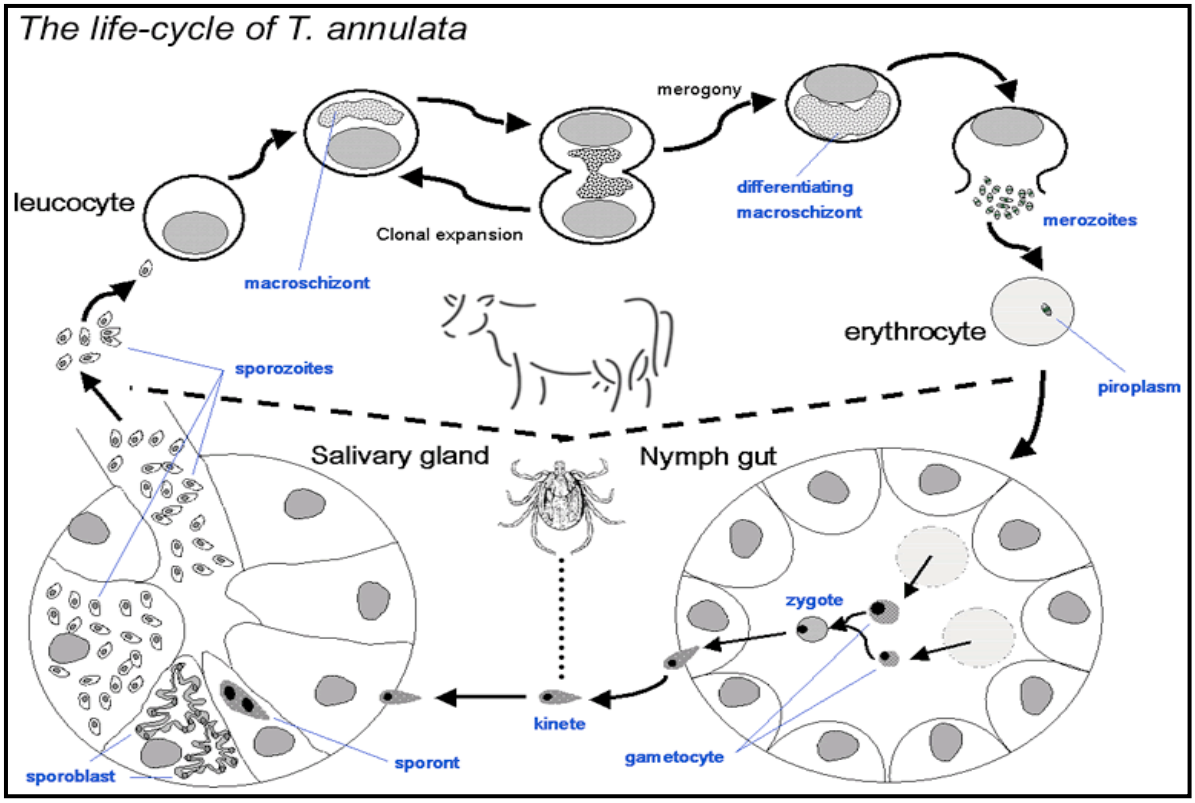

Fig. 1 Schematic representation of the life cycle of *T. annulata*
(taken from http://www.theileria.org/background.htm)

2.4 Clinical signs and pathogenesis

Clinical symptoms and the pathogenesis are related to the multiplication of parasites within transformed lymphoblastoid cells. The infections with *T. parva*, *T. annulata* and *T. lestoquardi* are characteristic for the lympho-destructive processes while *T. sergenti*, *T. mutans*, *T. uilenbergi* and *T. luwenshuni* infections are associated with invasion and destruction of erythrocytes resulting in anaemia (Yin et al., 2003).

The incubation period in infected animals varies from 9-25 days and the severity of the infection is dependent on susceptibility of the animal, virulence of the parasite and the number of sporozoites that were transmitted to the animal during infection (Preston et al., 1992). The course of infection may consequently vary from peracute, acute or sub acute to chronic depending on the interaction between the host and the parasite. The clinical signs of acute infected animals are enlargement of lymph nodes that drain the site or area of tick infestation, anorexia, high heart frequency, inappetence, weakness, ceasing of rumination, decreased milk production, conjunctivitis, nasal and ocular discharge, lacrimation, diarrhea and hemoglobinuria. In later stages, affected animals become markedly emaciated due to rapid weight loss. Terminally, severe dyspnea develops with an increasing respiratory rate, moist

cough and frothy nasal discharge followed by recumbency. In fatal cases, one to two weeks after the onset of clinical signs, death usually occurs. The most common post-mortem signs are enlargement of lymph nodes, a clearly enlarged spleen and pulmonary edema (Levine, 1985; Aiello and Mays, 1998; Radostits et al., 2000; Mehlhorn, 2008). Infected animals that suffer from the peracute form of the disease may die in 3-4 days after the first symptoms are noticed. In the chronic form, irregular fever, clear emaciation, anemia and icterus may persist for one to two months before the animal recovers to normal (Levine, 1985).

The pathogenesis of theileriosis is dependent on the stage of the parasite and type of affected cells. The stages of *Theileria* are found in erythrocytes, lymphocytes and Histiocytes (Soulsby, 1982). Regarding infection with *T. annulata*, the schizonts are considered to be the most pathogenic parasitic stage. Thus, cells infected with this species express high amounts of mRNA for cytokines, particularly IFN-γ (Preston et al., 1993; Ahmed et al ., 2008).The main causes of parasite induced pathology are an apparent proliferation of naive T-cells leading to an enhanced IFN-γ production on one hand and an excessive production of pro-inflammatory cytokines including IL-1α, IL-1β, IL-6 and TNF-α on the other hand (Brown et al., 1995; Glass et al., 2005). Moreover, schizont-infected cells are rapidly disseminated through the lymphoid tissues (Forsyth et al., 1999). Piroplasms become manifest after the transient schizont stage and simultaneously a transient fever may be noticed with developing anemia. Anemia is likely caused by the removal of parasitized or even uninfected erythrocytes from the circulation system (Yagi et al., 1991). Animals that recover carry a persistent infection. Occasionally, a relapse may occur when the animal is under condition of stress (Sugimoto and Fujisaki, 2002).

2.5 *Theileria* in small ruminants

For a long time *T. lestoquardi* (syn. *T. hirci*) has been considered to be the only highly pathogenic species infecting sheep and goats which cause malignant ovine theileriosis (Uilenberg, 1981; Friedhoff, 1997). Recently, two newly identified parasites were described as pathogenic *Theileria* species of sheep and goats in Northern China. These species have been currently designated as *T. luwenshuni* and *T. uilenbergi* (Ahmed et al., 2006; Yin et al., 2007). More recently a newly isolated *Theileria* sp. was identified in Xinjiang Province of China, whereby the study inferred that this parasite belongs to the cluster of *T. ovis* (Li et al., 2010).

2.5.1 Non pathogenic *Theileria* species of sheep and goats

T. ovis has been described as a non pathogenic parasite of sheep and goats in Africa, Europe and Asia and is transmitted by *Rhipicephalus evertsi* (Neitz, 1957; Uilenberg, 1981).

T. recondita (Lestoquard, 1929) was described as a non pathogenic parasite of sheep in Germany and Wales (Alani and Herbert, 1988) and it has been shown that this parasite was transmitted by adults of *Haemaphysalis punctata* .

T. separata which was described as a non pathogenic parasite of sheep in Eastern and Southern Africa is transstadially transmitted by *Rhipicephalus evertsi* (Uilenberg and Andreasen, 1974.)

2.5.2 Pathogenic *Theileria* species in sheep and goats

2.5.2.1 *Theileria lestoquardi*

T. lestoquardi is a tick-borne protozoan parasite highly pathogenic for sheep. The disease caused by the pathogen is known as malignant theileriosis of sheep and goats, the disease was first described by a team of veterinarians in Egypt. Later on, the parasite has been reported from sheep and goats in other countries such as Algeria, Iraq (Khayyat and Gilder, 1947), India (Raghvachari and Reddy, 1959), Serbia (Dschunkovsky and Urodschevich, 1924) and the infection was reported to be common in Iran and Iraq (Hooshmand-Rad, 1974; Hawa et al., 1981). *T. lestoquardi* has been shown to be transmitted by *Hyalomma anatolicum anatolicum* ticks (Hooshmand-Rad and Hawa, 1973b).

Phylogenetically *T. lestoquardi* is closely related to *T. annulata* (Katzer et al., 1998; Schnittger et al., 2003). Both parasites share the same vector, are transmitted transstadially and parasitize the same cell phenotype of their respective host (Leemans et al., 1999). Serological studies using IFAT showed a certain degree of cossreactivity between both parasites, indicating that common antigens do exist between these two parasites (Leemans et al., 1997). The clinical symptoms of an infection with *T. lestoquardi* are similar to those observed for tropical theileriosis particularly in the acute stage of these infections. The symptoms include high fever, anorexia, listlessness, emaciation, diarrhea or constipation, enlargement of draining lymph nodes and icteric mucous membranes (Neitz, 1957, Hooshmand-Rad and Hawa, 1973a). The pathological characteristics of malignant ovine theileriosis in sheep and goats are also very similar to those described for tropical theileriosis of cattle and have been described above under chapter 2.4 (Neitz, 1957; Hooshmand-Rad and Hawa, 1973a; Tageldin et al., 1992).

2.5.2.2. Ovine theileriosis in China

Infection with *T. uilenbergi* and *T. luwenshuni* causes theileriosis in sheep and goats in Northwest China. In Gannan Tibet region of Gansu Province, this disease has existed for more than 100 years. The disease was reported in several other regions of China such as Qinghai in 1956, Sichuan in 1958, Gansu in 1974, Inner Mongolia in 1980, and Lianoing in 1981 (Luo and Yin, 1997; Guo et al., 2002) (Fig. 3).

Concerning morphology and biology *T. uilenbergi* and *T. luwenshuni* are very similar but the complete pathogenic mechanism of the Chinese *Theileria* parasites remains to be resolved. However, it seems that the life cycle is similar to the one of other *Theileria* species, although exhibiting a less marked leukocytic phase (Schnittger et al., 2000c). The described Chinese *Theileria* are most closely related to the *T. buffeli/T. sergenti* group, and it is assumed that the pathogencity seems to be closely associated with the proliferation of the intraerythrocytic piroplasms. Furthermore, these parasites, like *T. buffeli* and *T. sergenti*, are not able to transform their host cells (Schnittger et al. 2000b). Schizonts were observed in smears prepared from many organs and tissues (liver, spleen, lung, kidney, lymph node and peripheral blood) and most of these schizonts were found outside the host cell cytoplasm, indicating that perhaps the schizonts play a role in the pathogenicity of the disease (Yin et al., 2003)

The infection results in great economical losses in the Northwest part of China. The infection in China was first reported to be caused by *T. lestoquardi* (Luo and Yin, 1997) but further findings indicated that it is caused by a different *Theileria* species since several biological features of this parasite differ from *T. lestoquardi*. Firstly, the latter is transmitted by ticks of the genus *Hyalomma* (Hooshmand-Rad and Hawa, 1973b) while the pathogens in Chinese sheep and goats are transmitted by *Haemaphysalis qinghaiensis* (by both nymph and adult) (Li et al., 2007; Li et al., 2009). Secondly, culture of *T. lestoquardi* schizonts *in vitro* has been successfully established (Hooshmand-Rad and Hawa, 1975; Brown et al.,1998), whereas the attempt to cultivate the schizont stage of the Chinese parasites failed. Ultimately, the analysis of the 18S rRNA gene sequence indicated that *T. lestoquardi* and *Theileria* sp. (China) belong to different clades of the phylogenetic tree. The established phylogenetic tree of *Theileria* divided into branches including one with *T. annulata*, *T. parva, T. taurotragi* and *T. lestoquardi* while the other branch included *T. sergenti, T. buffeli* and the Chinese isolates. The parasites of the first group are marked by an intra-leukocyte phase, while the second group belong to the non-lymphoproliferative *Theileria* species (Schnittger et al., 2000b; 2000c).

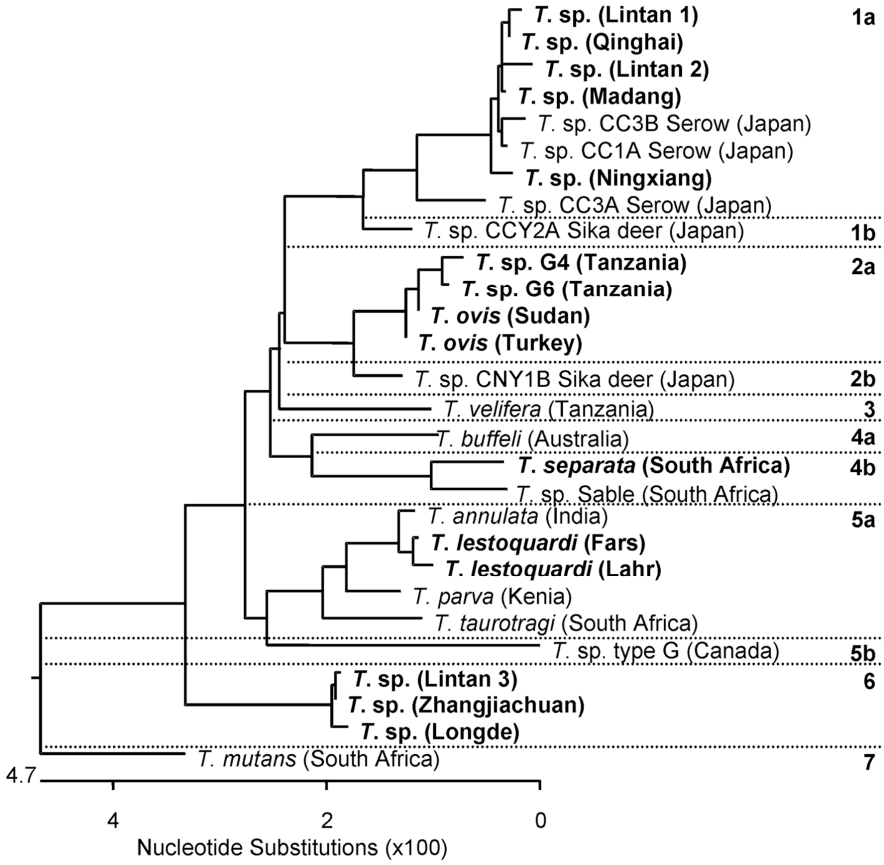

Fig. 2 Phylogenetic tree based on the sequence of the 18S ssrRNA gene of several *Theileria* parasites infecting small ruminants .The Chinese isolates are found in cluster 1a and 6. Cited from Yin et al., 2007.

The occurrence of ovine theileriosis in China is related to the distribution and activity of the transmitting vector. In the Tibetan region in Gansu Province, *H. qinghaiensis* ticks are most active from March until May. In late August, some ticks can be found again but in November they are unobservable. If the temperature is high in spring, the ticks appear in late February but when the temperature is low, they appear in the middle of March. Sheep and goats that recover from an infection throughout spring do not show any clinical symptoms when again being exposed to an infection in autumn. Usually, naturally infected hosts are found two weeks after tick infestation. In experimental infection studies, the incubation period was 4-12 days (Guo et al., 2002).

As mentioned above, the relative incidence of the disease is related to the distribution of the transmitting vector. The investigations of ticks collected in Gannan Tibet region (Gansu Province) led to the identification of the following tick species: *Ixodes ovatus*, *I. persulcatus*, *I. pomerantzevi*, *I. crenulatus*, *Dermacentor abaensis*, *D. silvarum*, *D. sinicus*, *D. nuttalli*, *D. coreus*, *Haemaphysalis qinghaiensis*, *H. aponommoides*, *H. concinna*, *H. bispinosa* and *Rhipicephalus sanguineus*. Experimental transmission research has shown that *T. uilenbergi*

and *T. luwenshuni* could be transmitted transstadially by *Haemaphysalis* sp ticks, *H. qinghaiensis* and *H. longicornis* (Li et al., 2007; Li et al., 2009).

The prevalence of infection rates varies substantially with age and breed of sheep and goats as well as the region. The incidence of the disease in sheep was higher (27.63 %) than it was in goats (13.12 %) and the incidence in young animals was higher than in adults (lambs 60.81 %, young goats 40 %, adult sheep 17.12 %, and adult goats 8.06 %). The mortality rate of young animals was also higher than that of adult animals: lambs 49.55 %, adult sheep 12.17 %, young goats 34.29 % and adult goats 5.91 %. The incidence rate in cross-bred animals was almost the same compared to that of local herds. However, the incidence rate of animals from *Theileria*-free areas brought into endemic areas was higher (77.8-100 %) than that of local herds (Guo et al., 2002).

Fig. 3 Distribution of ovine theileriosis in Northwest China.
The endemic regions are indicated by filled circles (Liu, 2010)

2.6 Identification of the parasite

Several approaches are used in the diagnosis of ovine theileriosis, including clinical signs, postmortem findings, vector distribution and finding of *Theileria* parasites in Giemsa-stained blood smears and lymph node needle biopsy smears. The development of molecular biology methods made several tools available that are able to detect parasite-specific DNA. These are polymerase chain reaction (PCR); reverse line blotting (RLB) and loop mediated isothermal amplification (LAMP). In addition, for the detection of circulating antibodies against *Theileria* serological tests like immunofluorescent antibody test (IFAT) and Enzyme-linked immunosorbent assay (ELISA) are available.

2.6.1 Diagnosis of ovine theileriosis

2.6.1.1 Diagnosis of *T. lestoquardi* infection

The diagnosis of malignant ovine theileriosis has been based on the detection of parasite stages by Giemsa-staining of blood or organ smears and the observation of clinical symptoms (Ahmed et al., 2002). Specific primers have been established to amplify *T. lestoquardi* gene fragment coding for a 30-kDa merozoite surface protein (Kirvar et al., 1998). The benefit of this PCR is that it can be used to differentiate between *T. lestoquardi* and *T. annulata* in the transmitting *Hyalomma* vector and in sheep and goats (Leemans et al., 1999). Other PCR-based techniques have been developed such as reverse line blotting which is able to differentiate the *Theileria* and *Babesia* parasites infecting small ruminants (Schnittger et al., 2004).

Several serological tests have been established and used either to follow up immunization or in epidemiological surveys on malignant ovine theileriosis of sheep or goats. Among these tests, the indirect fluorescent antibody test (IFAT) was used to detect antibodies against *T. lestoquardi* (Hawa et al., 1981; Leemans et al., 1997; Salih et al., 2003; Taha et al., 2003). However, false positive and negative results due to cross-reactivity or weak specific immune response have been observed in this test (Leemans et al., 1997).

To minimize the chance for cross-reactivity an enzyme-linked immunosorbent assay (ELISA) has been developed and evaluated for the serological detection of *T. lestoquardi* based on the newly discovered recombinant clone 5 surface protein of *T. lestoquardi* (Bakheit et al., 2006).

2.6.1.2 Diagnosis of *T. uilenbergi* and *T. luwenshuni*

The routine diagnosis of *T. uilenbergi* and *T. luwenshuni* infection depends on a combination of clinical signs and microscopic examination of blood and/or biopsy smears. However, these methods require experienced personnel and are not practical for epidemiological studies. A polymerase chain reaction (PCR) using species-specific primers, which were designed based on the hypervariable region of the small subunit ribosomal RNA gene sequences, has been established to detect *T. luwenshuni* and *T. uilenbergi* in both transmitting ticks and in the ovine host (Sun et al., 2008; Yin et al., 2008). A reverse line blot (RLB) was also developed which specifically identifies different ovine *Theileria* and *Babesia* parasites (Schnittger et al., 2004) but these diagnostic tests are expensive and require a complex protocol and experienced laboratory personnel. Recently, a loop-mediated isothermal

amplification (LAMP) method was developed that is able to detect *T. uilenbergi* and *T. luwenshuni* parasite DNA at high sensitivity and specificity (Liu et al. 2008b), which still needs to be validated in the field.

Efforts have been made to identify antigenic proteins of the parasites being suitable for the development of serological assays (Miranda et al., 2004, 2006b). Two indirect ELISAs for the detection of ovine theileriosis in China have been established, one based on crude merozoite material (Gao et al., 2002) and another based on the partially recombinantly expressed *T. lestoquardi* heat shock protein 70 (rTIHSP 70; Miranda et al., 2006a). These assays still need improvement since on the one hand the crude antigen ELISA is difficult to standardize, requires infection of animals for antigen preparation and is potentially cross-reactive with other related pathogens. On the other hand the rTIHSP 70 gene used for the recombinant protein ELISA originated from *T. lestoquardi* and bears the risk of cross-reactivity with other piroplasms infecting small ruminants.

To identify specific *T. uilenbergi* antigens in order to establish specific serologic tests pooled *T. uilenbergi* positive sera was used to screen a merozoite cDNA expression library (Liu et al., 2008a), leading to the identification of an antigen suitable for indirect ELISA (Liu et al., 2010). A second approach involving the application of random sequencing of selected clones followed by bioinformatic analyses identified potential immunogenic parasite antigens, leading to the identification of a gene family (clone-2 family) suitable for the development of serological tools (Liu et al., 2008a).

2.6.2 Diagnosis of bovine Theileriosis

Generally, the diagnosis of an infection with *T. annulata* is also based on clinical signs and the demonstration of parasite stages in blood or organ smears. But as mentioned before, these methods are only suitable for the detection of acute cases but have limited value for the detection of chronic and long-lasting carrier cases because of the degree of parasitaemia in those animals. Furthermore, it is difficult to differentiate between piroplasm species according to morphology (Hooshmand-Rad, 1974; Friedhoff, 1997). The clinical signs involve enlargement of lymph nodes, increased body temperature, diarrhea, frothy exudates, dyspnea and in severe cases recumbency and death (Uilenberg, 1981; Robinson, 1982; Norval et al., 1992) as mentioned in 2.4.

Research in molecular biology has delivered precise tools for the detection of parasite DNA. Many molecular tests with high sensitivity and specificity have been developed for the diagnosis of *T. annulata* in the bovine host including PCR (d'Oliveira et al., 1995; Shayan et

al., 1998; Kirvar et al., 2000; Habibi et al., 2007) and reverse line blotting (RLB) to detect and differentiate all known *Theileria* and *Babesia* species on the basis of differences in their 18S subunit rRNA gene sequences (Gubbels et al., 1999; Schnittger et al., 2004). However, these techniques require equipped laboratories, are expensive and impractical for field diagnosis.

Recently, a loop-mediated isothermal amplification (LAMP) assay was developed and evaluated for the diagnosis of tropical theileriosis, which operated at high specificity, efficiency and rapidity (Salih et al., 2008) but which has not been validated in the field yet.

Serological tests are considered to be suitable for epidemiological studies, as they detect antibodies against *Theileria* parasites. They include indirect fluorescent antibody test (IFAT) which was developed using either the piroplasm or cultured macroschizont as antigen (Pipano and Cahana, 1969; Burridge et al., 1973) and which has been reported to be more sensitive than examination of blood smears (Dhar and Gautam, 1977; Darghouth et al., 1996). However, it has the major drawback of cross-reactivity between different *Theileria* species and thus limits the specificity of the IFAT (Burridge et al., 1974).

Several enzyme-linked immunosorbent assays (ELISA) were established for the detection of tropical theileriosis. An ELISA was developed and applied for epidemiological field studies using purified schizont or piroplasm antigen for detecting antibodies against schizont and piroplasm stages of *T. annulata*, respectively (Manuja et al., 2000, 2001). As a point of fact, it is impossible to standardize antigen purified from crude parasite material and in addition, experimental infection of animals for parasite production is required. To evade this trouble, several assays based on recombinant parasite antigens, such as the merozoit rhoptry antigen Tams-1 and the sporozoite antigen SPAG-1, have been developed and used (Gubbels et al., 2000). Although Tams-1 was found to be suitable for the detection of antibodies, some cross reactivity has been observed (Williamson et al., 1989; Ilhan et al., 1998; Gubbels et al., 2000). Recently, *T. annulata* surface protein (TaSP) (Schnittger et al., 2002) has been proven to be highly suitable for the detection of *T. annulata* specific antibodies in comparison with several other *T. annulata* proteins including TaD, TaSE and TamtHSP70 (Seitzer et al., 2008). Different publications documented the suitability of the recombinant TaSP for application in the diagnosis of tropical theileriosis (Bakheit et al., 2004; Seitzer et al., 2007, 2008). An indirect ELISA based on TaSP has been established (Bakheit et al., 2004), validated in the field (Salih et al., 2005) and used for epidemiological studies (Salih et al., 2007b). Furthermore, to increase specificity, a competitive ELISA (cELISA) based on the same antigen has been established and validated for the detection of circulating antibodies against *T. annulata* (Renneker et al., 2008, 2009). ELISAs are the method of choice for

epidemiological studies and large scale investigations but the procedures are time-consuming, labor-intensive and also require professional personnel, special laboratory materials and equipment. Hence, a convenient, rapid and sensitive diagnostic test, such as lateral flow device that does not require instrumentation or specially trained personnel, would be extremely valuable for the use in both, clinical and field applications for the diagnosis of tropical theileriosis. Given the high suitability of the TaSP antigen for serodiagnosis of *T. annulata* infection, the establishment of a lateral flow device (LFD) on the basis of this protein for use as a rapid point of care assay was aimed for in this thesis.

2.7. Lateral flow device

Lateral flow assays (LFAs) employ carrier material which contains dry reagents attached to prefabricated strips that are activated by applying the fluid sample. Lateral flow devices are also known as immunochromatographic strip tests (ICT), an extension of latex agglutination tests which were developed in 1956 (Singer and Plotz, 1956). The assay strip has been firmly established for rapid immunoassays since the mid-1980s, when the first test was established for the detection of human chorionic gonadotropin (HCG). Nowadays, many classes of analytes, like antigen, antibody, hapten and even oligonucleotides have been used for specific qualitative and quantitative detection. In recent years, the technology has been used in different application as a device system, for example in human clinical diagnostics, veterinary diagnostics, agriculture, environmental health, food safety and industrial diagnostics as depicted in Fig. 4 (Geertruida et al., 2009; O'Farrell, 2009). Many lateral flow immunoassays are non-instrumental and rely on visual interpretation of the results, allowing easy portability and testing at any time and any place by non-technical personnel. Hence, many lateral flow immunoassays have been developed for ''point-of-care'' use and field application outside the laboratory (O'Farrell, 2009).

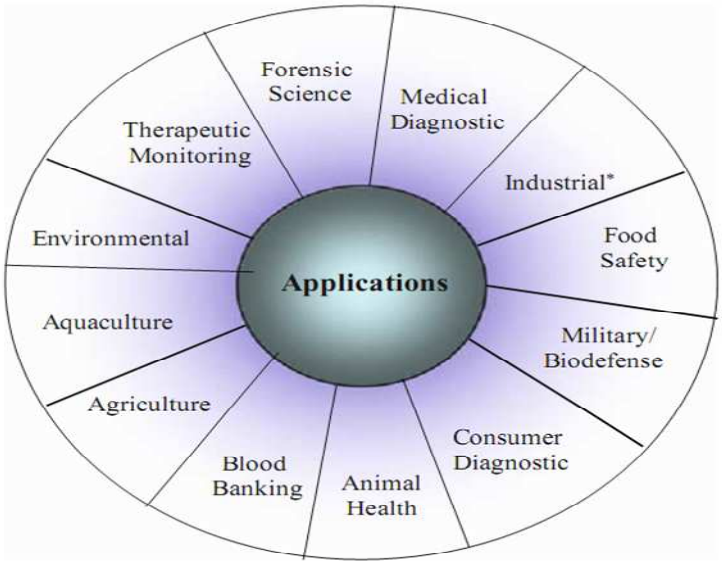

Fig. 4 Utility of the Lateral Flow Immunoassay Technology that is already in production or is known to be in development (O'Farrell, 2009)

2.7.1 Principle of the lateral flow immune assay

To produce a functional test strip a large number of critical components are brought together, therefore developing an LFD is complex. A typical LFD format consists of several zones: a sample pad that is closely associated with a conjugate pad, which in turn touches a membrane onto which test and control reagents have been immobilized. An absorbent pad wicks fluid away from the membrane (Fig. 5). The colloidal gold particles are the most common detector reagents that are used for the visualization of a positive reaction; usually these particles are available commercially. Other possibilities to visualize the specific interaction of antigen and antibody include latex beads, enzyme conjugates, other colloidal metals, dye sacs, fluorescent particles and magnetic particles.

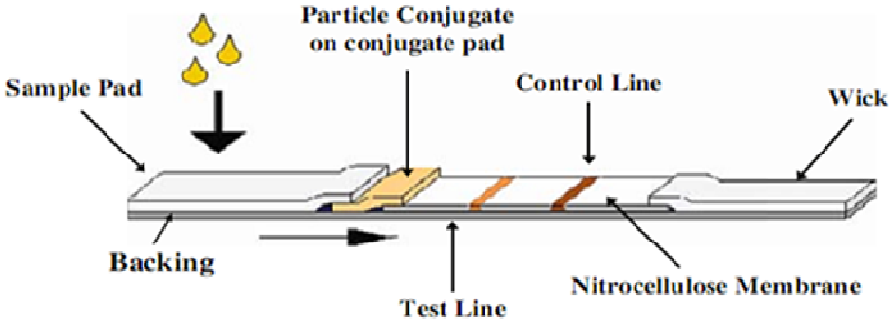

Fig. 5: Configuraton of a lateral flow device (O'Farrell, 2009)

To perform the test, firstly the sample must be mixed with a specific test-dependant buffer, which may simply be a diluent or a running buffer. The treated sample which is used as specimen (e.g. whole blood, urine, saliva, plasma or serum) is added to the proximal end of the strip (sample pad). Driven by capillary forces the fluid migrates towards the conjugate pad, where a particulate conjugate has been immobilized. These particles have been conjugated either to an antigen or to an antibody depending on the assay format. The sample rehydrates the gold conjugate and the analyte contained within interacts with the conjugate. The complex of gold conjugate and analyte then move into the next section of the strip, which is the reaction matrix, where other specific biological components of the assay have been immobilized. The complex will react with the immobilized antigen or antibody on the test line to form the coloured band. The excess conjugate or the free conjugate, if the sample does not contain antibody or antigen, will migrate along the membrane towards the control line, where it will interact with another immobilized reagent. This control line typically comprises a species-specific anti-immunoglobulin antibody. The rest of the solution is entrapped in the wick / absorbent pad (O'Farrell., 2009) and the test results can usually be read in 2 to 15 min through the viewing window. A sample is considered to be positive if two colored lines appear in the viewing window, one at the test line and another one at the control line (Fig. 6 B). A sample is considered to be negative if a colored line appears at the control line only (Fig. 6 C).

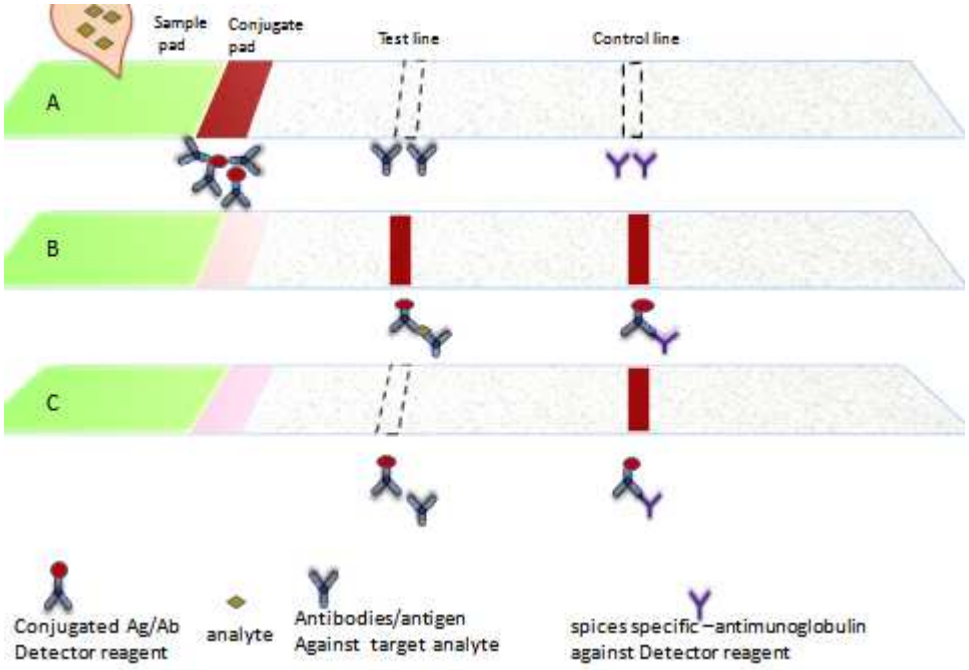

Fig. 6 Schematic illustration of positive and negative test results (Direct assay)

A pre-run assay

B a signal in the "Test line" and in the "Control line" indicates a positive result

C no signal in the "Test line" but one in the "Control line" indicates a negative result

3 Materials and methods

3.1 Ovine serum samples

Ovine sera collected from sheep experimentally infected with *T. uilenbergi* were kindly provided by Dr. Yin Hong, LVRI (Lanzhou Veterinary Research Institute), China. The 6-12 months old lambs were bought from *Theileria* and protozoa free areas, and were splenectomised 30 days before the experimental infection. The *T. uilenbergi* stock used in this study originated from Longde County of Ningxia Province, China and was verified in previous phylogenetic studies (Schnittger et al., 2003; Yin et al., 2004; Yin et al., 2008). The parasite stock was preserved in liquid nitrogen at Lanzhou Veterinary Research Institute. Eight animals were infected with *T.* sp. (China) in two groups. The ´tick infected´ group (animal no. 1229, 1207, 1240 and 1250) was infected by attraction of 200 *H. qinghaiensis* ticks collected from *T.* sp. (China) endemic areas. The other ´blood-infected´ group (animal no. 1219, 1236, 1237 and 2203) was experimentally infected by 8 ml of blood with 4 % parasitaemia (Seitzer et al., 2008). Serum samples were sequentially collected from all eight sheep post inoculation on day 14, 19, 26 and 30. Pre-infection sera from these animals as well as sera from a slaughterhouse in Germany (068, 903, 943, b1, b2, b3, b4 and S1-S20) were collected as negative controls. *T. lestoquardi* positive sera were collected from endemic regions in Sudan (Bakheit et al., 2006).

3.2 Identification, characterization and recombinant expression of clone-9 antigenic protein of *T. uilenbergi*

3.2.1 Identification of clone-9 antigenic protein of *T. uilenbergi*

To identify immunodominant proteins of *T. uilenbergi,* a previously established merozoite cDNA library was screened by random amplification of the insert with vector PCR primers. Searches for sequence identities were performed using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) provided at NCBI. The SignalP 3.0 prediction server was used to analyze for the presence of potential signal sequences (Bendtsen et al., 2004) (http://www.cbs.dtu.dk/services/SignalP/). Prediction of transmembrane helices in proteins was performed using the TopPred server (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred) and the TMHMM Server v. 2.0

(http://www.cbs.dtu.dk/services/TMHMM/). Prediction of antigenic peptides was performed using the method of Kolaskar and Tongaonkar (1990) provided online by the Cancer Vaccine Center, Dana-Farber Cancer Institute, Harvard Medical School (http://bio.dfci.harvard.edu/Tools/antigenic.pl).

Three potentially antigenic highly conserved mRNA gene sequences (clone 2, clone 26 and clone 9) were obtained which were found to be part of a gene family and termed Clone 2 gene family (Liu et al., 2008a).

3.2.2 Polymerase chain reaction (PCR) and agarose gel electrophoresis

PCR reactions were performed in a final volume of 35 µl which contained 25.5 µl water, 2 µl template (20 ng - 400 ng cDNA, genomic DNA), 3.5 µl 10x PCR buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 0.1 % Tween 20 and 15 mM $MgCl_2$), 0.7 µl dNTPs (final concentration 200 µM each), 1.6 µl each primer (final concentration 450 nM each primer) and 0.15 µl (75 units) of Taq DNA polymerase. Amplified PCR products were run on agarose gels of concentrations ranging between 1-1.5 %. The agarose gels were prepared by heat-dissolving agarose (Invitrogen, Karlsruhe, Germany) in Tris/boric acid/EDTA (TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) using a microwave oven. After cooling the agarose to 50°C, 0.5 g/ml ethidium bromide (Merck, Darmstadt, Germany) were added, the solution gently swirled and poured into a small gel casting tray (Agagel Mini; Biometra, Goettingen, Germany) fitted with a 12 well comb. After the agarose gel was polymerized and transferred to the electrophoresis machine, samples were loaded at volumes of 6 µl containing 5 µl PCR product plus 1 µl 6x loading dye (10 mM Tris-HCl pH 7.6, 0.03 % (v/v) bromophenol blue, 0.03 % (v/v) xylene xyanol FF, 60 % (v/v) glycerol, 60 mM EDTA). As a size standard 5 µl (0.1 µg) molecular weight marker was used (10-kb MassRuler TM DNA Ladder mix, Fermentas, St. Leon-Rot, Germany; 1-kb and 100-bp peqGOLD DNA Ladder Mix, Peqlab, Erlangen, Germany). The electrophoresis was carried out at 10 V/cm gel with a voltage source (80 V, 400 mM, Model 200 / 2.0 power supply, Biorad, Munich, Germany) in 1 x TBE buffer for 1 h (1 % agarose gel) or 90 min (1.5 % agarose gel). Visualization and photography of the gel were done using a transilluminator equipped with a digital camera (Biometra, Goettingen, Germany).

3.2.3 Cloning of the clone 9 b PCR product (c9b)

A 480 bp sequence of clone 9b, omitting the predicted signal peptide, was amplified from clone 9 (Genbank accession EU016504) by PCR using the primers forward (5'-caggatccCTGTGTTTGCTCATTTTGA-3') and reverse (5'-GGTCATTACTGGAGTCTTG-3). PCR cycling was performed using a thermocycler (T3; Biometra, Goettingen, Germany). The cycling conditions of the PCR were 3 min at 94 ˚C for denaturing followed by 35 cycles with denaturing at 94 ˚C for 30s, annealing for 60s at 57 ˚C and extension for 1 min at 72 ˚C. The final extension step was 7 min at 72 ˚C. The PCR products were ligated into the pDrive vector and then transformed into M13 *E. coli* according to the pDrive cloning manual (Qiagen, Hilden, Germany). Briefly, a ligation reaction mixture was prepared by adding 1 µl pDrive Cloning Vector (50 ng/µl), 2 µl PCR product, 2 µl distilled water and 5 µl Ligation Master Mix and then incubated at 16 ˚C for 3-16 h. Two microliters of ligation-reaction mixture were added to a tube of pre-thawed Qiagen EZ Competent Cells, gently mixed and incubated on ice for 5 min, followed by heating in a heating block at 42°C for 30 s without shaking and subsequently transferred on ice for 2 min. After this incubation 250 µl SOC medium (2 % tryptone, 0.5 % yeast extract, 0.05 % NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20mM glucose) were added at room temperature. Then 50 µl and 100 µl of transformation mixture were directly plated onto two LB agar plates containing ampicillin (100 µg/ml), IPTG (50 µM Isopropyl-beta-D-thiogalactopyranoside in ddH_2O, Roth, Karlsruhe, Germany) and X-gal (80 µg/ml) at 37°C overnight. The white colonies were individually grown overnight in 5 ml LB broth (Gibco/BRL, Eggenstein, Germany) containing 100 µg/ml ampicillin. They were then tested for the correct insert in an M13 PCR reaction utilizing the M13 forward (5'-TGTAAAACGACGGCCAGT-3') and the M13 reverse (5'-CGAGAAACAGCTATGACC-3') primers. The conditions of the PCR were as mentioned except that 2 µl of the overnight cultures of each clone were taken as template for the PCR without prior treatment. The cycling program was as follows: a 94°C initial denaturing step for 2 min and 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1-3 min depending on the size of the product. The correct product size was determined as the original PCR product size plus 267 bp representing the total distance of the M13 primers annealing sites from the PCR insert in the pDrive derived sequence. The plasmids were then sequenced by automated sequencing (MWG, Ebersberg, Germany).

If required, an aliquot of the overnight culture was stored as follows: 750 µl of an overnight culture were combined with 250 µl glycerol (Sigma, Deisenhofen, Germany) in a

labelled 1.5 ml screw capped tube. The tube was vortexed and left for 2 h in a refrigerator to equilibrate and was then snap-frozen in liquid nitrogen and stored at -70° C.

3.2.4 Isolation of plasmid DNA

The main steps to isolate plasmid DNA are growing of a culture, harvesting the cells, analysis of bacteria and finally the amplification and purification of the plasmid DNA. Twenty eight single and separate colonies were picked from the LB plates prepared in section 3.2.3 and inoculated in 28 tubes each containing 5 ml of bacterial culture (LB medium with 100 µg/ml kanamycin). Plasmid isolation was carried out by centrifuging 5-10 ml of the overnight bacterial culture at room temperature.

The extraction of plasmid DNA from bacterial cultures was carried out on a small scale. The plasmid isolation was performed with the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer. All subsequent centrifugation steps were carried out at 16,000×g (Eppendorf Centrifuge 5418AG, Hamburg, Germany). Briefly, each of the bacterial pellets was resuspended in 250 µl P1 buffer (RNase A had been added) and then 250 µl P2 buffer were added and mixed thoroughly by inverting the tube 4-6 times. This was followed by adding 350 µl N3 buffer and mixing immediately and thoroughly by inverting the tube 4-6 times; the tubes were then centrifuged for 10 min and the supernatant was loaded to the QIAprep spin columns. The spin columns were centrifuged for 1 min and washed once with 0.75 ml PE buffer by centrifugation for 1 min. After an additional centrifugation step for 1 min to remove residual washing buffer, the QIAprep columns were placed in a clean 1.5 ml microcentrifuge tube for elution of DNA by addition of 50 µl water to the center of each QIAprep spin column. After incubation for 1 min the column was centrifuged for 1 min.

3.2.5 Restriction digestion of plasmid DNA and pQE32 vector

The pDrive vector containing the c9b insert and the pQE32 vector were restriction digested with the enzymes *Hind* III and *Bam* H1 (New England Biolabs, Frankfurt, Germany). The final volume of 20 µl contained 17 µl (0.4-5 µg) plasmid DNA, 2 µl of 10x NE Buffer 2 and 1 µl (20 U) *Hind* III. This was incubated for 4 h at 37° C followed by heat inactivation for 30 min at 65 ˚C. The subsequent digestion with *Bam* H1 enzyme was performed in a final volume of 30 µl which contained 20 µl plasmid DNA, 3 µl of 10x NE Buffer 2 and 1 µl (20 U) enzyme, 2 µl BSA and 4 µl distilled water. This was again incubated at 37 ˚C for 6 h and the reaction mixture was finally stored at 4 ˚C until purification. The DNA fragments containing the c9b insert and the restricted pQE32 vector were cut out from an agarose gel

and then purified as described in section 3.2.6.

3.2.6 Purification of DNA fragments

Purification was performed using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. To solubilize the gel fragment, 300 µl of buffer QG were added to each 100 mg of gel, then incubated for 10 min at 50°C. After adding 1 gel volume of isopropanol, the solution was applied onto a labeled QIAquick spin column in a 2 ml collection tube. To bind the DNA to the column matrix, the column was centrifuged at 15000xg for 30 s. The flow-through was discarded and the column was placed back into the same collection tube. The column was then washed by addition of 0.75 ml of the ethanol containing PE buffer and centrifugation as above. The flow-through was discarded again and the column was placed back in the same tube and centrifuged again for another minute. Thereafter, the column was placed into a clean 1.5 ml microcentrifuge tube and the DNA was eluted by addition of 50 µl H_2O to the center of the QIAquick membrane and centrifugation for 1 min.

3.2.7 Ligation of c9b into digested pQE 32 vector

After purification, the c9b DNA fragment was ligated into the vector and used to transform *E. coli* M15 cells. The ligation was done by adding 8 µl double digested pQE32 (10-100ng), 8µl double digested Clone 9b plasmid, 1 µl ligase (40 U/µl), 2 µl ligase buffer (New England Biolabs, Frankfurt, Germany) and 1 µl distilled water. Ligation reactions were incubated in a PCR machine at 16 ˚C overnight.

3.2.8 Transformation of M15 (pREP4) competent cells

Five microliters of ligation-mix were added by tipping to 100 µl aliquots of the competent *E.coli* M15 (pREP4) cells previously thawed on ice and gently re-suspended. This solution was kept on ice for 10 min then heat-shocked at 42°C for 90s. After a 2 min incubation step on ice, 400 µl of Psi broth (LB medium, 4mM $MgSO_4$, 10 mM KCl) were added, followed by incubation of the cells at 37°C for 60 min in a shaker. Aliquots of 50, 100, and 200 µl from each transformation mix were plated on LB-agar plates containing 25 µg/ml kanamycin (Carl Roth, Karlsruhe, Germany) and 100 µg/ml carbenicillin (Carl Roth, Karlsruhe, Germany); the plates were then incubated at 37°C overnight. As controls, competent cells were transformed with 10 ng of the intact undigested pQE32 and the 1/100 and 1/10 dilutions of the transformation mix were plated. Similarly, 200 µl of a transformation mix omitting the

plasmid was plated onto a single plate containing antibiotics. Twenty single and separate colonies from LB plates were picked each into 2 ml LB broth containing kanamycin (25 µg/ml) and carbenicillin (100 µg/ml) and cultured for 4-6 hrs. Colonies were verified to contain the insert in a PCR reaction using the pQE primers (Type III/IV forward: 5'-CGGATAACAATTTCACACAG-3' and reverse: 5'-GTTCTGAGGTCATACTGG-3') and 2 µl overnight culture as template. PCR conditions were as described before with an annealing temperature of 52 °C for 30s. Aliquots of all positive clones were frozen at -70°C. One microgram plasmid DNA isolated from a positive clone was sequenced using the pQE Type III/IV primer (MWG, Ebersberg, Germany).

3.2.9 Recombinant protein expression

Five ml of overnight cultures from the pQE-clone was used to seed 100 ml freshly prepared LB medium containing 100 µg/ml carbenicillin and 25 µg/ml kanamycin and grown at 37 ˚C and with gentle shaking (100 rpm) in a water bath (Julabo SW-20C, Mickley Diagnostics GmbH, Berlin, Germany) until an OD_{600} value of 0.6 was reached (measured by a spectrophotometer; Eppendorf, Hamburg, Germany). IPTG (isopropyl-β-D-thiogalactopyranoside in ddH_2O, Roth, Karlsruhe, Germany) was added to a final concentration of 1 mM. Induction was allowed to continue for 4 h, after which the cells were harvested and the pellet was stored at -25 ˚C until resuspension of the usable pellets in 1 ml of a buffer containing 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (buffer B, pH 8.0). Cells were left to lyses under rotation at room temperature for 1 h or overnight at 4°C. Lysates were cleared from debris by centrifugation at 20,000xg for 20 min at 4 ˚C. The supernatants were then applied to the Ni-NTA spin columns previously equilibrated with 600 µl buffer B and centrifuged at 700xg for 2 min. The flow through was discarded. This was followed by three washing steps each with 600 µl of buffer C (the same as buffer B but with pH 6.3) and finally, the proteins were eluted twice each with 200 µl buffer E (the same as buffer B but with pH 4.5). To produce large quantities of recombinant proteins for use in ELISA, a volume of 500 ml LB medium with antibiotics was inoculated with the 10 ml bacterial culture and grown at 37°C until an OD_{600} value of 0.6 was reached. IPTG was added to a final concentration of 1 mM and the culture was further incubated with vigorous shaking for 4 h. Cells were then harvested by centrifugation at 3300xg for 15 min and the pellets were stored at -20°C till used.

3.2.10 Protein quantification

A protein assay using the BioRad Micro-DC Assay kit (BioRad, Munich, Germany) was used to estimate the protein concentration, in which bovine serum albumin (BSA) was used as reference. It was serially diluted to concentrations ranging between 2.0 and 0.2 mg/ml as shown in Table 1. The protein samples were diluted 1:1 and 1:2 in water. Volumes of 5 µl of the BSA and the samples were pipetted into a microtiter plate (Greiner, Frickenhausen, Germany) and 25 µl of reagent A and 200 µl of reagent B were added. The plate was incubated at room temperature for 30 min then the optical density values were read at 550 nm using an ELISA reader (Expert 96, Asys Hitech GmbH, Eugendorf, Austria). The results were processed automatically using a computer program (Microwin, Ver. 4.2), where the concentrations of the samples were given in mg/ml.

Table 1 Preparation of BSA dilutions

Tubes No.	1	2	3	4	5	6	7	8	9	10
Concentration (mg/ml)	2.0	...								
BSA (µl)	50	45	40	35	30	25	20	15	10	5
ddH2O (µl)	0	5	10	15	20	25	30	35	40	45

3.3 Protein analysis

3.3.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out to separate proteins due to their molecular size. Different concentrations of the resolving gel (10 % and 12.5 %) were used according to the expected molecular size of the separated protein. The SDS-PAGE technique used here was similar to the originally described method by Laemmli in 1970 in a gel electrophoresis chamber (Mini/Maxi gel tank vertical, Harnischmacher, Schauenburg, Germany). Firstly, two glass plates (10 cm x 11 cm) or (10 cm x 20 cm) were washed and wiped with 70 % ethanol which was allowed to evaporate and then assembled onto a setting rig. Sterile water or 70 % ethanol was then applied to check that the set-up was tightly sealed. Two separate gels were prepared freshly, namely a stacking gel and a resolving (running) gel, whereby the running gel solution was prepared according to Table 2 and was then transferred to the assembled chamber using a pipette. Approximately 1.5 cm space was left for the stacking gel. The gel was covered with 70 % ethanol and allowed to

polymerize for at least 30 min. Alcohol covering the running gel was removed by inverting the chamber and draining the residual drops with a filter paper. The 3 % stacking gel solution was prepared according to Table 2 simultaneously with the running gel, but the ammonium persulphate solution (10 % (w/v) APS; Merck, Darmstadt, Germany) was added only shortly before transferring the stacking gel solution into the chamber. Combs with the desired number of wells were placed between the glass into the stacking gel and the gel was left to polymerize for at least 30 min. The set gels were removed from the setting rig and placed into the electrophoresis chamber (Mini/Maxi gel tank vertical, Harnischmacher, Schauenburg, Germany). 1x running buffer (SDS buffer; 25 mM Tris HCl, pH 8.3, 192 mM glycerol (VWR, East Grinstead, UK), 0.1 % (w/v) SDS) was poured into the rig ensuring the plates were covered. Once the gel was set, the comb was taken out and the wells cleaned out with sterile water or running buffer to guarantee the removal of all acrylamide and air bubbles.

The samples were prepared in duplicate by adding a 4x sample loading buffer (180 mM Tris/HCl, pH 6.8, 40 % glycerol (v/v), 4 % SDS (w/v), 0.04 % bromphenol blue (w/v) and 100 mM DTT). One of the duplicates was set aside for silver staining and one for western blot and the samples were heated at 98°C for 5 min. Up to 15 µl of each sample was then loaded into the corresponding well along with a PageRuler™ prestained protein ladder (Fermentas, St. Leon-Rot, Germany) and /or the 6 x His protein Ladder (Qiagen, Hilden, Germany). The prestained broad range protein was added to the first well and samples were added to the other wells. The amount of sample or marker added to each well was adjusted to the same volume by adding the appropriate amount of sample reducing buffer plus bromphenol blue (BPB). Electrophoresis was carried out in sequence as follows: 50 volts for 5 min, 100 volts for 10 min and 200 volts until the dye had run to the bottom of the gel (approximately 45- 60 min).

Table 2 (A) Composition of the resolving gel

Component	10 % running gel	12.5 % running gel
Distilled autoclaved H_2O	5 ml	4 ml
Acrylamide/bisacrylamide solution	4 ml	5 ml
1.5 M Tris-HCl (pH 8.8), 0.4 % SDS	3 ml	3 ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	10 µl	10 µl
Ammonium persulphate (APS) (10 % solution)	100 µl	100 µl

Table 2 (B).Composition of the stacking gel

Component	3 % stacking gel
Distilled autoclaved H2O	3 ml
Acrylamide/bisacrylamide solution	0.5 ml
0.5 M Tris-HCl (pH 6.8), 0.4 % SDS	1.25 ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	10 µl
Pyronin Y buffer (0.5 M Tris-HCl, 10 % Glycerol, 0.4 % SDS, 0.01 % Pyronin Y)	10 µl
Ammonium persulphate (APS) (10 % solution)	20 µl

3.3.2 Transfer of protein to nitrocellulose membrane

Once the gels have finished running they were removed from the running rig and the glass plates. The gels were soaked in anode buffer II (25 mM Tris, 20 % methanol, pH 10.4) for 15 min. During this time nitrocellulose membranes (0.2 mm, BA 85, Schleicher and Schuell, Dassel, Germany) were also equilibrated in the same buffer for at least 15 min. Blot papers were wetted in cathode buffer (300 mM Tris, 20 mM 6-aminohexan acid, 10 % methanol, pH 9.4), anode buffer I (300 mM Tris, 20 % methanol, pH 10) and anode buffer II and the system was assembled in the following order: two pieces of anode buffer I wetted blotting papers were placed at the bottom of the BioRad Transblot-SD semi-dry blotter (BioRad, Munich, Germany) and then one piece of anode buffer II wetted blotting paper, the equilibrated NC membrane, the SDS-PAGE gel and three pieces of cathode buffer wetted blotting papers were placed on top of each other sequentially. After driving out the air bubbles trapped between the blotting papers, the cover of the device was assembled and the separated proteins were transferred to nitrocellulose membranes using the BioRad Transblot-SD semi-dry blotter (BioRad, Munich, Germany). Transfer was carried out at 25 V and 120 mA for 60 min for 2 gels or at 25 V and 200 mA for 60 min for 4 gels (approximately 2.5 mA/cm^2 of the gel).

3.3.3 Western blot analysis

Protein transfer was checked with Ponceau S red stain (Eltest GmbH, Bonn, Germany; 0.5 % Ponceau S in 1 % acetic acid). Membranes were blocked with 3 % skim milk in phosphate buffered saline (PBS: 137 mM NaCl, 2.67 mM KCl, 3.2 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) for 2 h at room temperature. The blocking solution was discarded and then the membrane was washed with PBST (PBS containing 0.05 % Tween-20) three times for 10 min each. The membrane was dried and cut into 3 mm strips which were further incubated for

1 hour at room temperature or at 4 ˚C overnight in *T. uilenbergi* positive and negative serum at a dilution of 1:200 and in RGS-His antibody (mouse anti His-tag antibody, Qiagen, Hilden, Germany) at a dilution of 1:2000 in dilution buffer (1 % skim milk, 0.1 % Tween-20 in PBS). After washing three times with PBS containing 0.05 % Tween-20, immuno-detection was performed with alkaline phosphatase (AP)-conjugated rabbit anti-sheep IgG (Dianova, Hamburg, Germany) or AP-conjugated rabbit anti-mouse antibody (Dianova, Hamburg, Germany) at dilutions of 1:5000 and 1:20000, respectively in dilution buffer. Then the membranes were incubated for 1 h at room temperature with platform shaking washed as described above. After the last washing step in PBST, the membranes were washed once in PBS for 10 min then equilibrated in alkaline phosphatase (AP) buffer (100 mM Tris HCl pH 9.5, 5 mM $MgCl_2$, 100 mM NaCl) for 5 min. Freshly prepared substrate solution was added, which contained 0.33 mg/ml Nitroblue tetrazolium (NBT; Roth, Karlsruhe, Germany) and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roth, Karlsruhe, Germany) in AP buffer. After bands were visible color development was stopped by rinsing the membranes in a stop solution containing 20 mM EDTA.

3.3.4 Silver staining of SDS gels

Silver staining was performed using the BioRad silver staining kit (BioRad, Munich, Germany). The gels were first fixed for at least 30 min or overnight in 100 ml of fixative (40 % methanol, 10 % acetic acid), then placed in 50 ml of freshly prepared oxidizer solution for 5 min, followed by rinsing with 200 ml distilled water for 15 min until the gels became almost colorless. Distilled water was changed during the first 5 min. Thereafter, the gels were placed in 100 ml silver stain reagent (Bio-Rad) for 20 min and then rinsed for a maximum of 30 s in 200 ml H_2O, which was also frequently changed. The gels were then placed in 50 ml developing solution ((BioRad, Munich, Germany) until they turned yellowish brown (approximately 1 min). The solution was poured off and replaced with 50 ml fresh developing reagent ((BioRad, Munich, Germany). Development continued until the required degree of staining was obtained (5-15 min). The reaction was finally stopped in a stopping solution (5 % acetic acid) for 15 min.

3.4 Enzyme-linked immunosorbent assay (ELISA)

The classical antigen-antibody reaction of the immune system represents the basis for an ELISA. Depending on the setup of the detection system an ELISA is able to detect either antigen or antibodies whereby, due to the objective of this study, only the setup for the detection of antibodies is described here.

Serial dilution of different components necessary for the indirect enzyme linked immunosorbent assay (iELISA) was performed. In order to avoid discrepancies due to different titers of the antiserum, the optimum dilution was considered to be the highest dilution of antigen/conjugate that still saturated the plate and gave maximum contrast in terms of optical density (OD) between known positive and known negative serum samples.

The amount of coated antigen that will successfully bind to antibodies and that in turn can be detected with an optimal optical density was determined. To obtain the maximum differences in the OD values between the positive and negative controls, various titration protocols were checked on a 96-well ELISA plate. Different antigen concentrations (10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml and 0.625 µg/ml) in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6) were titrated against different serum dilutions (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600) and different conjugate dilutions (1:10,000, 1:7500, 1:5000). All incubations were performed in 100 µl per well. Other variable conditions included different coating times, different blocking agents (3 % BSA, 5 % fish gelatin, 2 % casein, 20 % rabbit serum) and different Tween-20 concentrations (0.05 %, 0.1 %, 0.5 %) in the washing buffer. The final protocol which gave the maximum OD differences between positive and negative serum controls and at the same time the lowest background is summarized as follows:

3.4.1 Coating of the plate

ELISA plates (Nunc Maxisorp, Glostrup, Denmark) were labeled and noted in the right position of letters A-H and numbers 1-12. The plate was placed with A at the top left hand corner and the 8 wells labeled by letters A-H were referred to as rows. The 12 wells labeled by numbers 1-12 were referred to as columns. The antigen was diluted in special coating buffer at a high pH of 9.6 not containing other proteins that might compete with the target antigen for attachment to the plastic solid phase. Recombinant protein was diluted in carbonate/bicarbonate buffer (pH 9.6) (Sigma, Deisenhofen, Germany) to a final concentration of 4 µg/ml. The total amount of 100 µl of diluted antigen was pipetted with an 8

channel pipette from a clean trough or a fresh Petri dish to prevent cross contamination. The plates were sealed to prevent evaporation of the coating solution and then incubated at room temperature overnight.

3.4.2 Washing the plate

Washing was used to separate bound and unbound (free/unwanted) reagents. After incubation, any excess antigen was removed by flooding the wells with PBS (pH 7.4) and emptying them using a Bio-Tek 405 automated ELISA washer (Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany). Three cycles of pouring and immediate aspirating of 250 µl washing buffer were performed. In all subsequent washing steps the plates were washed by a buffer composed of 0.05 M PBS, pH 7.4 and 0.5 % v/v Tween 20 (Roth, Karlsruhe, Germany). Every washing step consisted of three flooding/aspiration cycles and residual wash buffer was removed by inversion and tapping the plate on paper towels.

3.4.3 Blocking of the non-specific binding sites

The remaining protein-binding sites of the plates were blocked after the first washing step using a blocking buffer containing 3 %(w/v) bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany) in PBS (pH 7.4). Blocking buffer was prepared, added to a clean trough and 200 µl were transferred to each well of the microtiter plate using the multichannel pipette fitted with 8 tips. The plates were then covered and incubated for 2 h at room temperature on an orbital shaker (Janke and Kunkel IKA-Werk, Staufen, Germany).

3.4.4 Addition of serum (Primary Antibody)

After the blocking step, the plates were washed as described above. Test serum samples were diluted 1:400 in dilution buffer composed of PBS containing 0.1 % Tween 20, 1 % BSA (pH 7.4) and 50 µg/ml of *E. coli* lysate prior to application to the plate. The lysate was prepared from *E. coli* M15 (pREPL4) strain containing an intact pQE vector without any insert after induction with 1 mM IPTG and growth for 4 hrs. The pH of the buffer was adjusted to 7.4. Diluted sera were incubated at room temperature under orbital shaking for at least 30 min for the absorption of non-specific antibodies. Aliquots of 100 µl of the diluted and absorbed sera were added per each well. Test sera were applied in duplicates and control positive (C+) and control negative (C-) sera were always applied in 4 replicates. Four wells were used for conjugate control (CC), which received no serum but only the second antibody. The plates were covered and incubated for 2 h at room temperature on an orbital shaker, followed by a

washing step consisting of two steps with soaking cycles for 10 min in between.

3.4.5 Addition of the conjugate

The conjugate (peroxidase-labeled rabbit anti-sheep antibody; Dianova, Hamburg, Germany) was diluted 1:10000 in dilution buffer similar to the serum dilution buffer (PBS containing 0.1 % Tween 20 and 1 % BSA; pH 7.4) but omitting the *E. coli* lysate. The conjugate was added to all wells in an amount of 100 µl per well. The plates were again covered and incubated at room temperature for 2 h under orbital shaking conditions. After incubation, a washing step which consisted of three single washing steps with soaking cycles of 10 min in between was performed and after the last soaking step, the plate was washed another time as described.

3.4.6 Addition of substrate/chromogen solution

The chromogen solution was prepared by dissolving 480 mg of TMB (3,3',5,5'-tetramethylbenzidine; Sigma, Deisenhofen, Germany) in 10 ml of acetone, then adding 90 ml of absolute ethanol. This solution was stored in a dark place at room temperature.

The substrate buffer was prepared by dissolving 6.3 g citric acid in 1 liter H_2O and H_2O_2 was added to a final concentration of 0.003 %.

The substrate/chromogen solution was prepared for one plate by adding 500 µl TMB solution to 10 ml substrate buffer containing H_2O_2. After carefully mixing the solution, 100µl were added to each plate and color development was allowed to take place for 10 min before 100 µl of 1M (phosphoric acid) were added to each well to stop the reaction. The stop solution was applied to the wells in the same order like the substrate/chromogen solution to ensure the maximum similarity in color development between the wells.

3.4.7 Expression of results

The absorbance at 450 nm was measured using an ELISA reader (Asys Hitech GmbH, Eugendorf, Austria). The OD values of twenty five known negative serum samples which were collected from a slaughterhouse in Bad Bramstedt, Germany were expressed as percent positivity (PP) of the internal positive control by dividing the average OD value of the two replicates by the average OD values of the four replicates of the positive control and multiplying by one hundred. The ELISA cut-off value, which would serve as the threshold between positive and negative sera was determined as the mean PP value obtained when testing negative control sera samples plus two standard deviations. The variation between the

results were estimated by repetition of six plates in two days (three plates every day), measuring the standard deviation under the same conditions and procedures of ten duplicates of C++PP, C+PP, C-PP and CcPP (percentage positivity of a highly positive, a positive and a negative serum as well as the conjugate control). To find suitable internal positive and negative controls, sera collected from experimentally infected animals at different time points post infection were tested in indirect ELISA. Of the eight experimentally infected animals four sheep (no 1207, 1250, 1240 and 1229) were infected by ticks collected from Lintan, China and four sheep (no 2203, 1219, 1237 and 1236) were inoculated with infected blood. The sera were collected from all sheep pre-infection as negative controls. In addition, controls including blank control (PBS only), known negative sera (negative control), serum plus conjugate without antigen (blocking control) and antigen plus conjugate without serum (conjugate control) were tested.

3.5 Development of a Lateral flow device for detection of *T. annulata* infection

3.5.1 Components of the *T. annulata* lateral flow device (Ta-LFD)

The complete lateral flow device was constructed of four main elements containing different reagents. The analytical region consists of the detection membrane with a band of absorbed protein (rTaSP) as test line and a band with rabbit-anti-TaSP antibody as control line. Furthermore, the sample application pad, the conjugate pad containing colloidal gold conjugated to TaSP and the absorbent pad (wick) are part of the analytical region (Fig. 7).

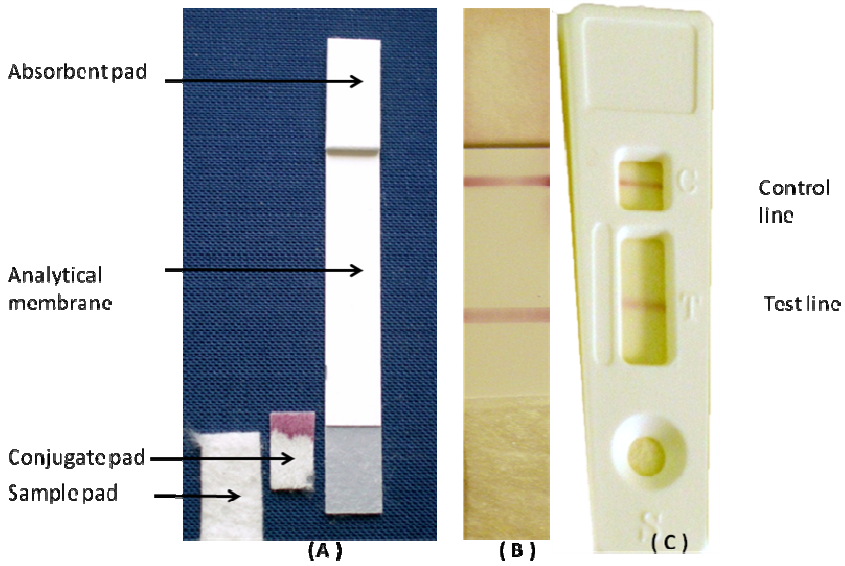

Fig. 7 Structure of a lateral flow strip: (A, B) Schematic representation of the lateral flow strip. A lateral flow strip typically consists of four elements: a sample pad, a conjugate pad, a membrane containing test and control lines and an absorbent pad. C) Lateral flow device after assembling and packaging.

The chromatography strips were prepared separately, whereby the general manufacturing processes for production of the strips include the following steps: preparation of colloidal gold conjugate, application of reagents onto the analytical membrane and pads, assembly of membrane, conjugate pad, sample pad, and absorbent pad onto a support backing, then cutting the prepared master cards into strips of determined length and width and strip packaging (Fig. 8).

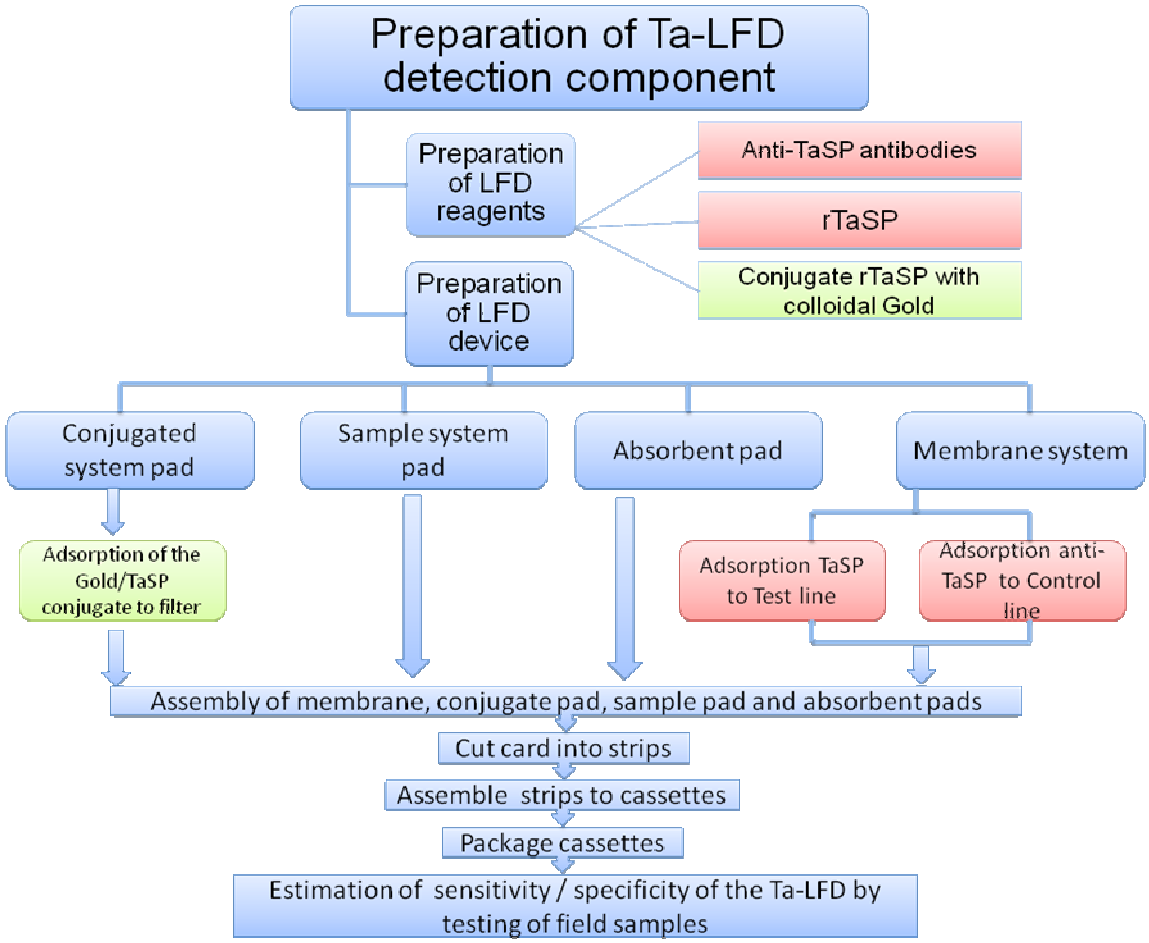

Fig. 9 Outline of the procedure for the development of a lateral flow device for the detection of *T. annulata* infection (TaLFD)

3.5.2 Identification of TaSP

The TaSP protein was previously identified by screening of a *T. annulata* complementary DNA library with anti-schizont antiserum. The TaSP-gene is present as a single copy gene within the parasite genome and is expressed in both, the sporozoite and schizont stage of the parasite. The N-terminal part contains a predicted signal peptide and the C-terminal section encodes membrane spanning regions. The comparison of a number of cDNA clones showed that both these sequence regions are conserved while the central region shows both size and amino acid sequence polymorphism (Schnittger et al., 2002).

3.5.3 Purification and Repurification

Recombinant expression and purification of the predicted extracellular domain of TaSP (rTaSP, aa 25-156) was performed as described before (Schnittger et al. 2002). Histidine tagged recombinant protein was expressed in M15 *E. coli* and purified using Ni-NTA agarose beads (QiaExpressionist protocols; Qiagen, Hilden, Germany). Briefly, the cells were

harvested and pelleted and the pelleted cells resuspended in 5 ml of sonication buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8) and sonicated for 25 cycles (Branson Sonifier 250 with Microtip, Branson, Schwaebisch Gmuend, Germany) with a duty cycle of 30, a control output of 3, one min burst and 30 seconds cooling. The lysate was centrifuged at 10,000xg for 40 min at 4°C and the supernatant was transferred into a new 50 ml tube to which 6 ml of 50 % Ni-NTA agarose (Qiagen, Hilden, Germany) previously equilibrated with sonication buffer were added. The tube was incubated overnight at 4°C under rotation and the contents were added into a column and allowed to drain. The Ni-NTA agarose in the column was then once washed with 20 ml of sonication buffer, twice with 10 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM Imidazole, pH 8) and then the protein was eluted from the column using 8 ml of an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM Imidazole, pH 8). The purified recombinant protein was re-purified using the Äkta prime high system (Amersham Bioscience, Uppsala, Sweden) with 5 ml HiTrap columns packed with Ni-Sepharose 6 Fast Flow (GE Healthcare Europe GmbH, Freiburg, Germany). The Ni-chromatography was carried out according to the manufacturer's instructions using the following chromatography program: (i) washing the column with 5-10 column volume (cv) H_2O with a flow rate of 5 ml/min; (ii) equilibration with 10 cv application buffer (20 mM Tris-HCl, pH 8.0, 8 M urea, 0.5 M NaCl and 5 mM Imidazole); (iii) application of the sample to the column with a flowrate of 1 ml/min; (iv) washing with 20 cv washing buffer (20 mM Tris-HCl, pH 8.0, 8 M urea, 0.5 M NaCl and 5 mM Imidazole) at a flow rate of 3 ml/min until the absorbance at OD280 is constant; (v) collection of 1 ml fractions after application of 1 cv elution buffer (20 mM Tris-HCl, pH 8.0, 8 M urea, 0.5 M NaCl, 1 M Imidazole) with a linear gradient of 500 mM to 1 M Imidazole until the protein peak appears at a flow rate of 1 ml/min.

3.5.4 Dialysis and concentration of protein samples

In order to obtain salt-free protein the purified and pooled fractions were dialyzed at 4°C for 24 h against PBS using high retention seamless cellulose tubing (Sigma-Aldrich, Steinheim, Germany) with a wide cut off of 12.4 kDa. The average or maximum pore size of a dialysis membrane determines the molecular-weight cutoff (MWCO) of the molecules that can diffuse through it. The dialysis procedure was performed according to the manufacturer's instructions. Briefly, pre-wet cellulose tubes were washed in water for 3-4 hour to remove the glycerol, then treated with a 0.3 % (w/v) solution of sodium sulfide at 80 ˚C for 1 min followed by acidification with a 0.2 % (v/v) solution of sulfuric acid. Afterwards they were

rinsed with hot water. Five ml of protein fraction were loaded in a treated tube and then dialysed against 5 liters of dialysis buffer (PBS) at 4 ˚C overnight. After 24 hours dialysis buffer was changed for a second turn. For concentration or exchanging buffer of protein solutions ultra centrifugation was applied. For this purpose, the Ultracel Centricon-YM-30 column with a cut off of 30 kDa or the Amicon Ultra-4 column with a cut off of 10 kDa (both from Millipore, Schwalbach, Germany) were used according to a protocol of the manufacturer. The dialyzed and concentrated protein bands were analyzed for the presence of protein in Western blot using anti-histidine antibody (RGS-His^{TM} mouse anti-histidine antibody, Qiagen, Hilden, Germany), *T. annulata*-positive serum and anti-TaSP antiserum as described previously (section 3.3.3).

3.6 Conception of the *T. annulata* Lateral flow device (Ta-LFD)

3.6.1 Equipment used for constructing and assembling the lateral flow device

The XY1164 TK platform with BioJet Quanti 3000 and AirJet Quanti 3000 dispensers (Bio-Dot Ltd., West Sussex, UK) were used for membrane blotting and conjugate dispensing.

An air drying oven (Weiss-Gallenkamp, United Kingdom) was used for drying the blotted membrane and conjugate pad. The BioDot cutter (Bio-Dot Ltd., West Sussex, UK) was used to cut the assembled master cards into strips. A packaging machine (Weiss-Gallenkamp, United Kingdom) was used to assemble the device.

3.6.2 Materials used for making the test strips

Colloid gold particles with a diameter of 40 nm were purchased from DCN (Diagnostic Consulting Network, Carlsbad, CA, USA). Hi-flow nitrocellulose membranes (Cat no. R90N93558-Hf1350, Millipore Corp., Bedford, MA, USA) were used. Cellulose filters (Millipore Corp., Bedford, MA, USA) were used as sample pads and filters from Whatman, UK were used as conjugate pads. Filter paper (Millipore Corp., Bedford, MA, USA) was used for the absorbent pads. Double-sided adhesive tapes (G&L Precision Die Cutting, Inc. San Jose, CA, USA) were used for making adhesive layers on the backing plate.

3.6.3 Buffers

200 mM borate buffer, pH 9

0.1 M sodium carbonate (Na_2CO_3)

10 % BSA (1 mg + 10 ml distilled water)

Conjugate buffer (0.05M TBS, 1 % BSA, 0.02 % NaCl, 0.01 % Triton-X100)

3.6.4. Reagents

As conjugate reagent served rTaSP which was conjugated with colloidal gold. It was expressed, purified and dialyzed against PBS as mentioned before and stored at -20 ˚C till used. The test line reagent was also rTaSP which is recognized by antibodies against *T. annulata* in serum from infected animals. For the control line, rabbit anti-TaSP antibodies were used. These antibodies were prepared by immunization of a rabbit with recombinant TaSP (Schnittger et al., 2002) and subsequent purification of specific IgG antibodies by affinity chromatography (HYDRA®-gelmatrix, Charles River Laboratories, Kisslegg, Germany). The specific antibodies were applied to the control line at a concentration of 2 mg/ml parallel to the rTaSP test line.

3.6.5 The procedure to set up the Ta-LFD

Designing and development of a lateral flow test strip is complicated and requires the attentive analysis on the selection of the optimal conditions and material.

3.6.5.1 Conjugation of rTaSP to colloidal gold particles

Purified rTaSP was used as the mobile phase by coupling it to 40 nm colloidal gold particles using a proprietary method and was stored at 4°C until use.

3.6.5.2 Membrane blotting

Nitrocellulose membranes were cut into 2.5 x 30 cm^2 strips and put on a XY1164 TK platform (Bio-Dot Ltd., West Sussex, UK) (Fig. 10). Fifty µl of rTaSP solution were dispensed linearly to 30 cm of the membrane at a concentration of 2 mg/ml for the test line and the same amount and concentration of rabbit anti-TaSP antibody was dispensed to the control line. The blotted membrane was then dried at 37 ˚C for 45 min in the Forced Air Drying Oven (Weiss-Gallenkamp, United Kingdom) and the blotted membrane was finally sealed in a plastic bag and stored at 2-8 ˚C until use.

Fig. 10 The XY1164 TK platform with Bio-Dot air-brush equipment (Bio-Dot Ltd., West Sussex, UK)

3.6.5.3 Preparation of the conjugate pads

To prepare the conjugate pads, special filters (Whatman, UK) were cut into 1.5 x 30 cm strips. The antigen/gold conjugate was sprayed onto the conjugate filter using the Bio-Dot air-brush equipment (Bio-Dot Ltd., West Sussex, UK) at a volume of 1 µl/mm filter. The filters were dried at room temperature for 45 min and then stored in sealed foil sachets together with desiccants at 2-8 ˚C until required.

3.6.5.4 Assembly of the master card

The present material was assembled manually to the lateral flow strip master card. The blotted membrane was stamped to the middle of the adhesive backing card and then the conjugated and the sample pads were affixed sequentially next to the membrane with a 1-2 mm overlap at the end of the sample pad. The absorbent pad was fixed to the distal end of the membrane with a 1-2 mm overlap.

3.5.6.5 Slitting and cutting the master card

To form individual final test strips the assembled master cards were cut into 0.8 cm wide strips using the BioDot cutter (Bio-Dot Ltd., West Sussex, UK).

3.5.6.6 Cassette (device) assembly

The test strips were placed onto the bottom part of the cassette manually. Then the cassette cover was welded or snapped to the bottom part to complete the assembly.

3.7 Detection of *T. annulata* infection

During the assay performance, 20 µl of bovine sera mixed with 160 µl of chase buffer were applied to the sample pad. The strips were then placed horizontally for 8 to 10 min to observe the results. When a test is run, the sample is added to the the sample pad at the proximal end of the strip. The treated sample then migrates through this region towards the conjugate pad, where the TaSP antigen bound to colloidal cold has been immobilized. The sample re-mobilizes the dried conjugate and the antibodies in the sample interact with the conjugate. A complex is formed which migrates to the next section of the strip, which is the reaction matrix. The complex will react with the immobilized TaSP antigen on the test line to form the coloured band. The excess complex or, if the sample does not contain *T. annulata* antibodies, the free conjugate will migrate along the membrane towards the control line where it will interact with immobilized anti-TaSP antibodies. The rest of the solution is entrapped in the absorbent pad/wick. If both, the test and the control line, show colour, the sample is considered as positive. If only the control line but not the test line is coloured the test serum is considered to be negative (Fig. 11).

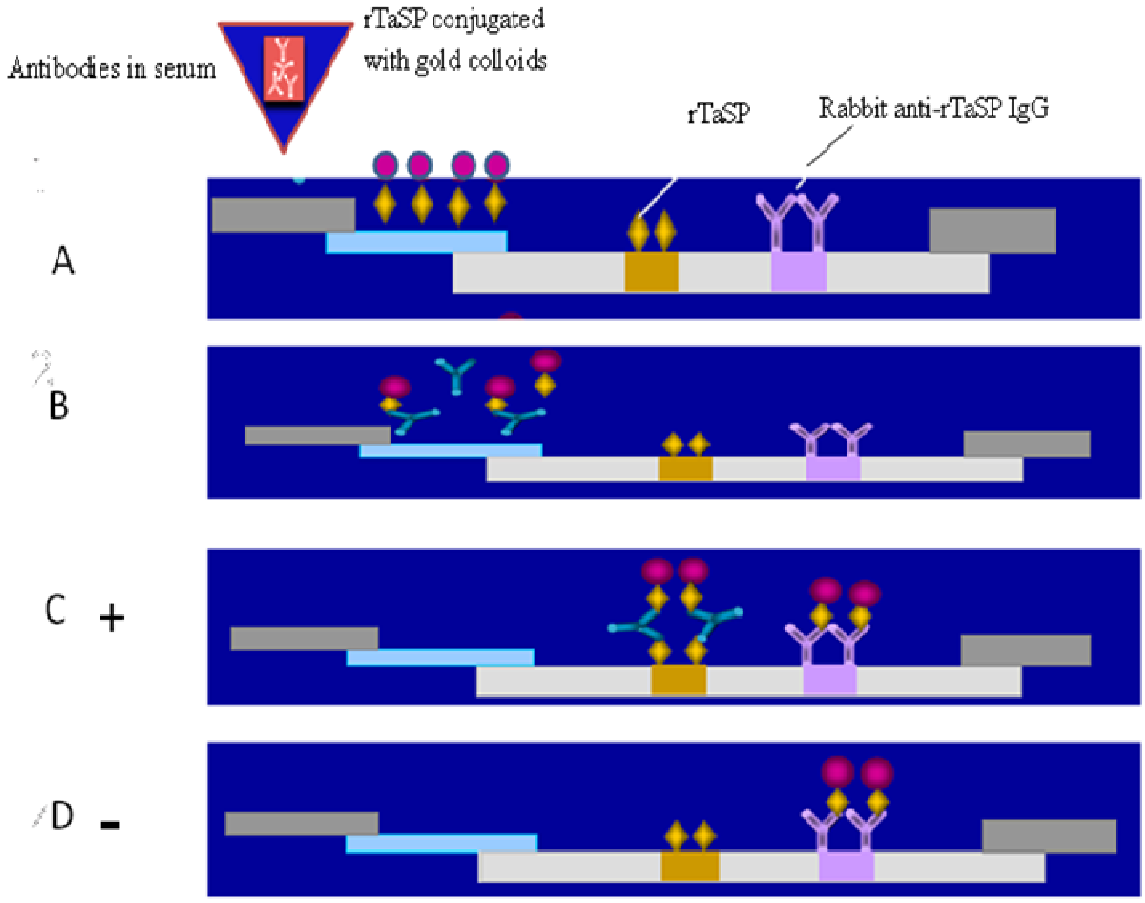

Fig. 11 The diagram illustrates the mechanism of the Ta-LFD. (A) LFD prior to addition of serum to the strip. (B) Addition of sample (in chase buffer). The sample re-mobilizes the dried conjugate and the *T. annulata* antibodies in the sample interact with the conjugate antigen forming a complex. (C) Formation of two colored lines indicates a positive result. (D) Formation of one colored line indicates a negative result.

3.8 Evaluation of the Ta-LFD by testing sera from experimentally infected animals and field sera

To assess the strip test positive serum samples from animals experimentally infected with *T. annulata*, previously generated at the Free University Berlin, Germany, were tested by the Ta-LFD. Furthermore, the Ta-LFD assay was applied to diagnose *T. annulata* infection in a total of 90 field serum samples collected at random from cattle in Sudan in areas where tropical theileriosis is known to be endemic. The results using the Ta-LFD were compared to results obtained by other serological tests, in order to calculate the sensitivity and specificity of the test. The tests used for comparison were IFAT as the gold standard for serological detection of *T. annulata* infection, and an indirect ELISA and a competitive ELISA based on the same recombinantly expressed TaSP protein as used for the Ta-LFD. The specificity and sensitivity were calculated using the following formulas:

Specificity (Sp= (# of samples negative in both tests/total numbers of negative samples in the reference test) x 100)

Sensitivity (Se= (# of samples positive in both tests/total numbers of positive samples in the reference test) x 100)

To investigate potential cross-reactivity of the TaLFD, sera from animals experimentally infected with *B. bovis*, *B. bigemina*, *Trypanosoma brucei*, *Anaplasma marginale*, *T. mutans* and *T. parva* were tested.

To calculate the degree of agreement between the Ta-LFD and compared tests using Intercooled Stata 6.0 (StataCorp LP, College Station, TX, USA) and SPSS 11.5.0 for Windows (SPSS Inc., Chicago, IL, USA), the Kappa test (Cohen's kappa measure of association) was applied (Thomas et al. 1988). Interpretation of kappa can be rated as follows: kappa less than 0.0: "poor" agreement; between 0.0 and 0.20: "slight" agreement; between 0.21 and 0.40: "fair" agreement; between 0.41 and 0.60: "moderate" agreement; between 0.61 and 0.80: "substantial" agreement; and between 0.81 and 1.00: "almost perfect" agreement (Landis and Koch 1977). Hypothesis tests of whether kappa is significantly greater than 0 are based on standard errors derived from approximations to its variance (Bishop et al. 1975).

4 Results

4.1 Publication 1

PARASITOLOGY RESEARCH (2010) 107: 517-524

Identification of clone-9 antigenic protein of *Theileria uilenbergi* and evaluation of its application for serodiagnosis

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Abstract

The pathogenic protozoan parasite *T. uilenbergi* is one of the causative agents of theileriosis in small ruminants in China. The infection results in great economical losses in the northwest part of China. Efforts are underway to establish an ELISA based on a *T. uilenbergi* immunodominant recombinantly expressed protein using different approaches in order to perform epidemiological studies in the area. In this study we describe the potential use of the clone-9 protein for this purpose, which was identified as a potential immunogenic piroplasm protein by random sequencing of cDNA library clones followed by bioinformatic analyses. The clone-9 gene was partially recombinantly expressed and used for the development of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of circulating antibodies in sera of *T. uilenbergi*-infected sheep. No cross reactivity was observed in serum from animals infected with *T. lestoquardi*. The cut-off was calculated at 48.6 % percent positivity using 25 serum samples from uninfected animals. A total of 101 field samples collected from an endemic area in China were used to evaluate the clone-9 ELISA for its use in the field.

Introduction

T. uilenbergi and *T. luwenshuni* are highly pathogenic protozoan parasites for small ruminants in China (Ahmed et al. 2006). The disease caused by these parasites is one of the major limitations for the development of the sheep and goat industry, mainly concerning exotic animals in northwestern China (Ahmed et al. 2002; Luo and Yin 1997). Both parasites are transmitted by the three host tick *Haemaphysalis qinghaiensis* and by *Haemaphysalis longicornis* (Yin et al. 2002; Li et al. 2007; Li et al. 2009). *T. luwenshuni* and *T.uilenbergi* were previously described as *Theileria sp*. 1 (China) and *Theileria sp. 2* (China.), respectively (Yin et al. 2007). Molecular phylogenetic studies indicated that they are most closely related to the *T. buffeli* and *T. sergenti* group (Schnittger et al. 2000; Schnittger et al. 2003).

Different methods to detect infection with *T. uilenbergi* or *T. luwenshuni* are used and have been developed, ranging from conventional methods depending on the detection of the merozoites in Giemsa stained thin blood smears by light microscopy and clinical symptoms to molecular based technologies. Although the application of Giemsa stained blood smears are suitable for the detection of acute cases, it requires experienced personnnel and furthermore is not practical for epidemiological studies. A polymerase chain reaction (PCR) is

a recently described molecular technique to detect *T. luwenshuni* and *T. uilembergi* in both

transmitting ticks and in the ovine host (Sun et al. 2008; Yin et al. 2008), however it is expensive, requires a high degree of expertise and cannot be used to detect subclinical infection. A reverse line blot (RLB) assay was also developed which specifically identifies different ovine *Theileria* and *Babesia* parasites (Schnittger et al. 2004; Altay et al. 2008). However, this method requires an expensive and complex protocol requiring experienced laboratory personnel. Recently, a loop mediated isothermal amplification (LAMP) method was developed with high sensitivity and specific detection of *T. uilenbergi* and T. *luwenshuni* parasite DNA (Liu et al. 2008a), which still needs to be validated in the field. In comparison to DNA-based methods, serological methods such as the enzyme-linked immunosorbent assay (ELISA) have the advantage of being less expensive, easier to perform, and are of high applicability in large scale epidemiological studies. Several efforts have been made to identify antigenic proteins of the parasites (Miranda et al. 2006b, Miranda et al. 2004) suitable for development of serological assays. Indirect ELISAs for the detection of ovine theileriosis in China have been established, one based on crude merozoite material (Gao et al. 2002) and another on the partially recombinantly expressed *T. lestoquardi* heat shock protein 70 (rTIHSP 70) (Miranda et al. 2006a). There is still, however, a need for improvement, since on the one hand, the crude antigen ELISA is difficult to standardize, requires infection of animals for antigen preparation and is potentially cross-reactive with other related pathogens. On the other hand, the rTIHSP 70 gene used for the recombinant protein ELISA originated from *T. lestoquardi* and bears the risk of cross reactivity with other piroplasms infecting small ruminants. In an attempt to identify *T. uilenbergi* specific antigens, a cDNA library of the piroplasm stage of this parasite was recently established (Liu et al. 2008b). The availability of this source of information was exploited in two ways. First, immunoscreening using serum from infected animals led to the identification of a *T. uilenbergi* specific protein (*Tu*IP) which has been applied for ELISA development (Liu et al. 2010). Second, application of random sequencing of selected clones followed by bioinformatic analyses was performed to identify potential immunogenic parasite antigens. The latter approach led to the identification of potential antigenic proteins belonging to a gene family (clone-2 family) suitable for development of serological tools (Liu et al. 2008b). In this study, we describe the application of the expressed predicted 173 amino acid antigenic region of clone-9, which is a member of the clone-2 gene family, in the development of an indirect ELISA for the serological detection of infection with *T. uilenbergi*.

Materials and methods

Origin of serum samples

Sera were collected from sheep experimentally infected with *T. uilenbergi* in the experimental animal facility of the Lanzhou Veterinary Research Institute, Lanzhou, China. Sheep were bought from a theileria and other protozoa free area. Two groups of 4 animals each were either infected by attraction with 200 *H. qinghaiensis* ticks collected from a *T. uilenbergi* endemic area (tick-infected group, animals # 1229, #1207, #1240, #1250) or by innoculation with 8 ml of blood with 4 % parasitemia (blood-infected group, animals #1219, #1236, #1237, #2203) (Seitzer et al. 2008). Sequential sera were collected post infection on days 14, 19, 26, and 30. Sera collected before experimental infection were used as negative sera. Furthermore, 25 sera collected from a slaughterhous in Germany were used as negative sera and *T. lestoquardi* positive sera were from an endemic region in Sudan (Bakheit et al., 2006). One hundred and one field samples were collected from an endemic area in China.

Bioinformatic analyses and subcloning of clone-9 predicted antigenic region

Searches for sequence identities were performed using the Basic Local Alignment Search Tool (BLAST) provided at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). The SignalP 3.0 prediction server was used to analyze for the presence of potential signal sequences (Bendtsen et al. 2004) (http://www.cbs.dtu.dk/services/SignalP/). Prediction of transmembrane helices in proteins was performed using the TopPred server (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred) and the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Prediction of antigenic peptides was performed using the method of Kolaskar and Tongaonkar (1990) provided online by the Cancer Vaccine Center, Dana-Farber Cancer Institute, Harvard Medical School (http://bio.dfci.harvard.edu/Tools/antigenic.pl).

The clone-9 gene (Genbank accession: EU016504) is a *T. uilenbergi* gene identified in complementary DNA and gDNA by PCR amplification (Liu et al. 2008b). The region corresponding to aa 14-162 excluding the predicted signal sequence was subcloned into the pQE32 expression vector (Qiagen, Hilden, Germany) using the Bam HI and Hind III restriction sites resulting in an open reading frame of 519 nucleotides and yielding a histidine-tagged recombinant protein of 172 amino acids with a predicted molecular weight of 18 kDa.

Expression and purification of clone-9b

For purification under denaturing conditions the Ni-NTA Spin kit (Qiagen, Hilden, Germany) was used. Overnight cultures (5 ml) of clone-9b were used to inoculate 100 ml of LB/Car/Kan (LB medium containing 100 µg/ml carbenicillin and 25 µg/ml kanamycin). At an OD600 value of 0.6, induction of expression was made by adding IPTG (final concentration of 1 mM) and the cultures incubated at 37 ºC for another 4-5h, under shaking. Cells were harvested by centrifugation at 4000 x g for 20 min and the pellet was stored at -20°C. The cell pellet was thawed for 15 min on ice and resuspended in lysis buffer (B) (100 mM NaH2PO4, 10 mM Tris-HCl, 8 M urea, pH 8.0) at 5 ml per gram wet weight. Cells were left to lyse under rotation or stirring for 15-60 min at room temperature. Lysate was cleared from debris by centrifugation at 20000 x g for 20 min at 4°C.The supernatant was mixed gently with 50 % Ni-NTA (1 ml of 50 % slurry + 4ml of cleared lysate) and shaken on a rotary shaker at 200 rpm for 1h .The mixture was applied to the Ni-NTA spin columns previously equilibrated with buffer B. This was followed by three washing steps with 600 µl of wash buffer C (100 mM NaH2PO4, 10 mM Tris HCl, 8 M urea, pH 6.3) and the protein was eluted from the column twice with 200 µl of elution buffer (100 mM NaH2PO4, 10 mM TrisHCl, 8 M urea, pH 4.5). Protein content was determined using the BioRad Micro-DC assay kit (Biorad, Munich, Germany).

SDS-PAGE and Western-blot

Expression of recombinant protein was verified by Western-blots. Briefly, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12 % polyacrylamide gels under reducing conditions. The samples were prepared by adding a 4x sample loading buffer (180 mM TrisHCl, pH 6.8, 40 % glycerol (v/v), 4 % SDS (w/v), 0.04 % bromphenol blue (w/v), 100 mM DTT) and heating at 98°C for 5 min. The gels were placed into the running chamber filled with running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS). Electrophoresis was carried out as follows: 50 Volts for 5 min, 100 Volts for 10 min and 200 Volts for 45 min. For specific detection of protein expression, gels were blotted onto nitrocellulose membranes (Schleicher und Schüll, Dassel, Germany) and blocked by incubation with 3 % skim milk in phosphate buffered saline (PBS: 137 mM NaCl, 2.67 mM KCl, 3.2 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4) for 2 h. The membrane was dried and cut into 3 mm strips. *T. uilenbergi* positive serum and negative sheep serum were added at a dilution of 1:200, RGS-His antibody (mouse anti His-tag antibody, Qiagen, Hilden, Germany) at a dilution of 1:2000 in dilution buffer (1 % skim milk, 0.1 % Tween-20 in PBS) and

incubated overnight at 4ºC or 1 h at room temperature. After washing 3 times with a 0.05 % Tween-20 in PBS solution, immuno-detection was performed with alkaline phosphatase (AP) conjugated rabbit anti-sheep IgG (Dianova, Hamburg, Germany) or AP conjugated rabbit anti-mouse antibody (Dianova, Hamburg, Germany), at dilutions of 1:5000 and 1:20,000, respectively, in dilution buffer and incubated for 1h at room temperature with platform shaking. Bands were detected after washing and application of BCIP/NBT substrate (Biomol, Hamburg, Germany). To test for specificity, *T. lestoquardi* and *Babesia* sp. positive sera (1:200) were used.

Silver staining

Silver staining of gels was performed to assess the quality of purified recombinant protein, staining using the BioRad silver staining kit (BioRad, Munich, Germany). Gels were first fixed for at least 30 min or overnight in 100 ml of a fixative solution (40 % methanol, 10 % acetic acid). The gels were then placed in 50 ml freshly prepared oxidizer solution for 5 min and then rinsed in 200 ml distilled H2O for 15 min until they became almost colourless. Distilled H2O was changed during the first 5 min. Thereafter, the gels were placed in 100 ml silver stain reagent for 20 min, and then rinsed for a maximum of 30 s in 200 ml H2O, while H2O was also frequently changed. The gels were then placed in 50 ml developing solution until they turned yellowish brown (approximately 1 min). The solution was poured off and replaced with 50 ml fresh developing reagent. Development continued until the required degree of staining was obtained (5-15 min.). The reaction was finally stopped in a stopping solution (5 % acetic acid) for 15 min.

Enzyme-linked immunosorbent assay (ELISA)

To obtain the maximum differences in the OD values between positive and negative control sera (positive serum 1229P and negative serum 1229N), antigen was diluted two-fold from a concentration of 10 µg/ml to 0.625 µg/ml in coating buffer (0.05 M carbonate/bicarbonate buffer, pH9.6). Serum diluted two-fold from 1:50 to 1:1600 and different conjugate dilutions (1:10000,1:7500,1:5000) were used. All incubations were performed in 100 µl/well. Other variable conditions tested were coating time, blocking agents (3 % BSA, 5 % fish gelatine, 2 % casein, 20 % rabbit serum) and different Tween-20 concentrations in the wash buffer (0.05 %, 0.1 %, 0.5 %). The final protocol which gave the maximum positive negative OD differences and simultaneously the lowest background is summarized as follows:

Recombinant protein (4 µg/ml) in carbonate bicarbonate buffer (pH 9.6) (Sigma, Deisenhofen, Germany) was added 100 µl each into the well of a 96-well plate (Nunc Maxisorp, Nunc, Glostrup, Denmark), the plate was covered and incubated at room temperature overnight. Plates were then washed in 0.05 M PBS (pH 7.4) in 3 dispensing/aspiration cycles using a Bio-Tek 405 automated ELISA plate washer (Biotek instruments, Bad Friedrichshall, Germany) and then blocked with 3 % w/v BSA (bovine serum albumin, Sigma, Deisenhofen, Germany) in PBS (pH 7.4) for 2 h under orbital shaking. Subsequent washing steps were performed using PBS containing 0.05 % (v/v) Tween 20 (PBST, pH 7.4). Serum was diluted 1:400 in dilution buffer (PBS containing 0.1 % Tween 20 and 1 % BSA, pH 7.4) with 50 µg/ml of *E. coli* lysate prior to application to the plate. Absorption with lysate prepared from *E. coli* M15 (pREPL4) strain transfected with the pQE32 vector (Qiagen, Hilden, Germany) and induced with IPTG was performed to minimize reactivity of the sera with *E.coli* antigens present in the coated recombinant antigen. Plates were incubated for 2 h at room temperature on an orbital shaker, followed by a washing step consisting of two steps with soaking in between for 10 min. The conjugate (horse radish peroxidase (HRPO)-labeled rabbit anti-sheep antibody (Dianova, Hamburg, Germany) was diluted 1:10,000 in a buffer similar to the serum diluent buffer but omitting the *E. coli* lysate. After incubation, a washing consisting of three washing steps with soaking cycles in between, where 250 µl/well of the washing buffer were dispensed and left for 10 min on the orbital shaker, was performed. After the last soaking step, the plate was again washed as described. Colour development was induced by adding a substrate composed of TMB and H_2O_2 in citric acid buffer (pH 4.0) (Sigma, Deisenhofen, Germany). Colour was allowed to develop for 10 min before 100 µl/well of 1 M phosphoric acid were added to stop the reaction. The absorbance at 450 nm was measured using an ELISA reader (Asys Hitech GmbH, Eugendorf, Austria). Controls included blanks (PBS only), known negative sera (negative control), serum plus conjugate without antigen (blocking control), antigen plus conjugate without serum (conjugate control).

The OD values of twenty five sera samples of uninfected sheep were expressed as percent positivity (PP) of the internal positive control by dividing the average OD value of the two replicates by the median value of OD values of the four replicates of the positive control, and multiplying by one hundred. The ELISA cut-off value, which would serve as a threshold between the positive and the negative sera was determined as the mean PP value obtained when testing negative control sera samples plus two-fold standard deviation or as the mean PP obtained from the negative sera multiplied by two.

Results

Bioinformatic analysis of clone-9 gene

Clone-9 was obtained by random PCR amplification and sequencing from a cDNA expression library constructed from the piroplasm stage of *T. uilenbergi* (GenBank Accession EU16504) (Liu et al. 2008b). The gene fragment was 699 bp in size with an apparent complete open reading frame (ORF) of 519 nucleotides encoding a protein of 172 amino acids with a predicted mol. wt. of 22 kDa. Searching of the GenBank database using BLASTP algorithm showed highest identities with the ToLocg 1 gene of *T. orientalis* (GenBank accession BAD08223.1; Identities = 69/145 (47 %), Positives = 88/145 (60 %), Gaps = 9/145 (6 %) and with a hypothetical protein TP03_0363 of *T. parva* strain Muguga (GenBank accession EAN31100.1; Identities = 63/179 (35 %), Positives = 82/179 (45 %), Gaps = 21/179 (11 %), indicating a gene of *Theileria* origin.

Analysis using Signal P 3.0 server prediction showed that the gene contains a signal peptide with a most likely cleavage site between position 17 and 18 using both neural networks (NN) and hidden Markov models (HMM) (Bendtsen et al. 2004). Prediction of transmembrane regions indicated a high probability between aa region 126-146 using both prediction servers. Antigenic peptide prediction indicated that the protein contained 6 antigenic determinants, including the signal peptide, from amino acid (aa) positions 4-19, 21-45, 48-67, 76-109, 111-147 and 150-167.

The predicted signal peptide was excluded for recombinant protein expression (aa 14-162) using the PCR approach described in the methods section.

Expression and purification of clone-9b recombinant protein (rc9b)

After induction of expression, a protein band of approximately 18 kD was observed in Coomassie-stained SDS-gels (Fig. 1). Before and after purification, a band of also approx. 18 kDa was detected in Western blots using the anti-his-antibody for detection of the expressed protein (Fig. 1).

Immunoreactivity of sera from three *T. uilenbergi* infected sheep was investigated by Western blotting (Fig. 2), resulting in observation of a band corresponding in size to the his-tagged rc9b of approx. 18 kD. No reactivity was observed with serum from an animal infected with *T. lestoquardi* nor from uninfected sheep (Fig. 2). In addition, no cross reactivity was observed with *Babesia* sp. (data not shown).

Establishment of the rc9b indirect ELISA

The optimal dilutions of antigen coating, serum samples and conjugate were checkerboard-titrated using known positive and negative serum samples diluted twofold from 1:150 to 1:600, while conjugate was diluted at 1:7,500, 1:5,000 and 1:10,000, and antigen was tested at concentrations diluted two-fold from 10 µg/ml to 0.625 µg/ml. The optimal antigen concentration was determined at 4 µg/ml, and since the serum sample dilution at 1:600 and conjugate dilution at 1:10,000 gave the maximum contrast of the OD value between positive and negative samples, these conditions were used in all subsequent experiments (data not shown).

The internal quality control was assessed by testing two positive samples 1229P (C+(1)) and 1236P (C+(2)) and negative sample 1229N (C-) in five different plates on two different days (Fig. 3). Similar values for the samples were obtained throughout for PP values of positive controls (C+(1) and C+(2)), conjugate control (Cc) and negative control (C-). The average PP value of the two positive controls C+(1) and C+(2) was 88.42±2.77 with a maximum of 91.09 PP and a minimum of 84.40 PP and average of 66.20±2.23 with a maximum of 68.70 PP and a minimum of 62.67 PP, respectively. The negative control C- had an average of 3.88±1.00 PP with a maximum of 4.8 PP and a minimum of 3.10 PP.

The cut-off of the rc9b ELISA was defined as the mean PP value of 25 negative serum samples plus two times the standard deviation or as the mean PP obtained from the negative sera multiplied by two. The average PP value obtained from these samples was 26.40, while the calculated standard deviation was 9.01, resulting accordingly in a value of 44.4 PP and 52.8 PP, respectively. A mean value of 48.61 PP cut-off value was used for subsequent analyses.Regarding the specificity of the ELISA, sera from animals infected with *T. lestoquardi*, *Babesia* sp. or *T. annulata* analyzed by the rc9b ELISA proved to be negative (data not shown).

Analysis of the antibody response in experimentally infected animals using rc9b ELISA

To monitor the antibody response of animals experimentally infected with *T. uilenbergi* against rc9b, the serum samples from different time points during infection from eight individual sheep were investigated. Among these animals, four (No. 2203, 1236, 1237 and 1219) were infected by inoculation of blood infected with Lintan strain, and another four (No. 1250, 1240, 1229 and 1207) were infected by feeding 200 *H. qinghaiensis* ticks from an

endemic region (Seitzer et al., 2008). Infection of the animals was verified according to the observation of the parasites in blood smears. The results of the ELISA demonstrated that the infection with infected blood induced a steep rise in antibodies binding to rc9b in less than 14 days and remained on a high level from there on until at least 30 days post infection. Tick infestation on the other hand required up to 19 days to produce measurable antibodies reacting with rc9b, and titres rose continuously over the time period investigated but did not reach as high levels as in the other group (Fig. 4). Despite this difference in magnitude of response, in both infection models the rc9b ELISA was able to detect specific antibodies more than three months post infection. This indicates that the test is a suitable tool for monitoring of persistent infections with *T. uilenbergi*.

Detection of circulating anti-rc9b antibodies in field samples from theileriosis endemic regions

A preliminary assessment for the use of the rc9b indirect ELISA in the field was carried out by testing 101 samples collected in an endemic region in China. As shown in figure 5, a total of 82 serum samples showed PP above the calculated threshold, giving a prevalence rate of 81.18 %.

To further assess the diagnostic potential of the rc9b ELISA, 80 serum samples were also analyzed using the r*Tu*IP ELISA as previously described (Liu et al. 2010) and compared to the results obtained with the rc9b ELISA. The concordance of the two analyses is shown in Table 1. A total of 64 sera were positive and 4 were negative in both tests. Three samples positive using the rc9b ELISA were negative using the r*Tu*IP ELISA, and nine samples that were negative using the rc9b ELISA were negative using the other test. Accordingly, the sensitivity of the rc9b ELISA was calculated to be 87.7 % and the specificity 57.1 % in comparison with the r*Tu*IP ELISA. The agreement between the two tests was 85 %.

Discussion

Detection of infection with *Theilria uilenbergi* and *T. luwenshuni*, the causative pathogens for theileriosis of sheep and goats in northwestern China (Yang 1958), is conventionally performed based on clinical signs and the detection of piroplasms in Giemsa stained blood smears (Gao et al. 2002), both approaches being not reliable for specific pathogen detection or identification of infected animals not showing clinical signs. In the last years, efforts have been successfully made to establish diagnostic methods based on detection of parasite DNA (PCR (Yin et al, 2008), RLB (Schnittger et al, 2004) and LAMP (Liu et al, 2008a)) as well as

serological assays based on the identification of immunogenic parasite proteins to be used for development of recombinant protein based indirect ELISA (Miranda et al, 2004, 2006a, 2006b; Liu et al, 2008b, 2010). In the latter case, the establishment of an ELISA based on a *T. uilenbergi* or *T. luwenshuni* specific protein was largely hampered by a lack of knowledge of these two pathogens. A significant step forward to aid in the identification of suitable antigens was made through the establishment of a *T. uilenbergi* merozoite cDNA library (Liu et al, 2008b). The availability of this library was exploited in two ways, firstly by screening for immunodominant antigens using sera from *T. uilenbergi* infected sheep, which led to the identification of the *T. uilenbergi* immunodominant protein (*Tu*IP) and the successful establishment of an ELISA based on the partial recombinant expression of *Tu*IP (Liu et al, 2010). Secondly, randomly selected clones from the library were sequenced and analyzed using different bioinformatic servers for the presence of antigenic determinants, leading to the identification of the potentially antigenic clone-2 gene family (Liu et al, 2008b).

In this study, clone-9, which is a member of the clone-2 gene family, was studied for its suitability to be used in the establishment of an indirect ELISA. Interestingly, the sequence of clone-9 gave only a limited number of hits using Blast-searches, the highest identity being only moderate (47 %) with the ToLocg 1 gene of *T. orientalis* and 35 % with a hypothetical protein of *T. parva*. Since also no further data is available on the ToLocg 1 gene, information gained from these searches was minimal. Nonetheless, theilerial origin of the gene was confirmed, and the finding also fits well to previous investigations indicating that *T. uilenbergi* is most closely related to the *T. buffeli/orientalis/sergenti* complex (Schnittger et al, 2003).

Concerning the possible structure and function of clone-9, bioinformatic analyses predicted the presence of a signal peptide, transmembrane region and six antigenic determinants, indicating that the clone-9 protein may represent a parasite membrane protein exposed to the infected host and thus potentially inducing a humoral immune response. To verify this prediction, clone-9 was recombinantly expressed in *E. coli* in order to analyze sera from *T. uilenbergi* infected animals for the presence of anti-clone-9 antibodies. For expression, the signal peptide carrying one of the antigenic determinants was excluded to improve expression levels, the remaining determinants except the last 5 aa of the N-terminal 150-167 aa antigenic determinant were included.

Testing in Western blot indeed indicated that antibodies against rc9b were present in sera from *T. uilenbergi* infected animals, prompting the establishment of an indirect ELISA system, since this method has been used extensively for parasitic diseases and is the

serological method of choice for epidemioligcal surveys due to its practicability in examining large sample sizes and relative low cost (Bidwell et al. 1978; Ruitenberg et al. 1977). The optimized rc9b ELISA procedure effectively detected specific antibodies in *T. uilenbergi* experimentally infected sheep applying an established threshold calculated from values obtained from sera from uninfected sheep. Interestingly, experimental infection by innoculation with infected blood resulted in an earlier and steeper rise in anti-rc9b antibody titres than experimental infection with ticks carrying the pathogen. This perhaps reflects that clone-9 is an antigen expressed in later stages of the parasite life cycle in the host, possibly the merozoite stage, as the cDNA library from which the gene was isolated was generated from this stage. This assumption will have to be clarified in future investigations.

Although absence of cross reactivity with related piroplasms could be ascertained (*T. lestoquardi*, *Babesia* sp.), due to unavailability testing of sera from the most closely related and very similar *T. luwenshuni* was not performed. However, some cross-reactivity is to be expected, as a clone-2 family-like gene has been identified in *T. luwenshuni* (GenBank: ACE86412.1) with high identity to clone-9 (84 %). Future investigations will need to identify clone-2 family genes in *T. luwenshuni* and to perform comparative analyses of these sequences for regions likely to be specific to *T. uilenbergi* or *T. luwenshuni*, which may then be used for the development of specific serological assays for these two pathogens. It is highly likely that members of this family are antigenic and induce a humoral immune response, since besides clone-9 in this study, clone-2 was also shown to be reactive with sera from infected animals (Liu et al, 2008a). To date, no such two tests are available, although the indirect ELISA based on the *Tu*IP protein shows some promise of being able to differentiate between *T. uilenbergi* and *T. luwenshuni* (Liu et al, 2010).

The suitability of the rc9b ELISA for testing of field sera was established using field sera collected from an endemic region in China. The rc9b ELISA showed an agreement of 85 % in comparison with another recently established ELISA for detection of ovine theileriosis in China based on a different recombinantly expressed antigen (*Tu*IP). The reason for the observed differences is not clear to date, being possibly due to the levels of the respective specific antibodies present. This discrepancy will also be the subject of further studies aiming to continually improve the serodiagnostics of ovine theileriosis in China.

In summary, the approach of using random sequencing of clones and bioinformatic tools to predict antigenicity was successful in identifying an antigenic protein from a merozoite cDNA library of *T. uilenbergi* suitable for the establishment of an indirect ELISA to be used in serology. Moreover, the identification of a gene family with potential antigenicity holds the

possibility of identifying antigens suitable for the development of serological tools able to discriminate between infection with the closely related *T. uilenbergi* and *T. luwenshuni*, to which future studies will be aimed at.

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Figures

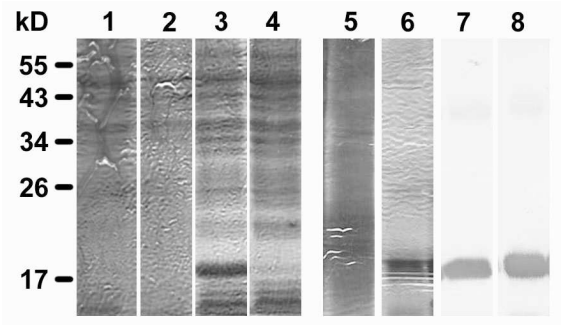

Figure 1 Confirmation of recombinant Clone 9b expression. Coomassie staining of lysate of transformed (lanes 1 & 3) and untransformed *E.coli* cells (lanes 2 & 4) before (lanes 1 & 2) and 3 h after induction of expression (lanes 3 & 4). A protein band of approx. 18 kDa is observed in lane 3. Silver stain (lanes 5 & 6) and Western blot (lanes 7 & 8) of lysate of *E. coli* cells expressing rc9b before (lanes 5 & 7) and after purification (lanes 6 & 8). Detection of the approx. 18 kDa his-tagged recombinant protein in Western blot was with anti-His-antibody.

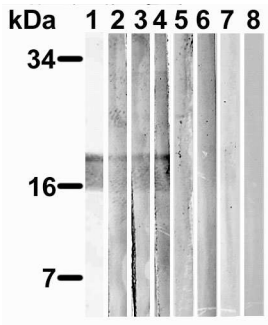

Figure 2 Specificity of immunoreactivity of sera from *T. uilenbergi* infected sheep with rc9b. Western blot with rc9b was incubated with serum from *T. uilenbergi* infected sheep (lanes 2-4), resulting in visualization of a band corresponding in size (~18 kD) to the his-tagged protein as detected by anti-his-tag antibody (lane 1). No immunoreactivity was observed neither with serum from uninfected sheep (lanes 5 & 6) nor from an animal infected with *T. lestoquardi* (lane 7). Lane 8 represents the secondary anit-sheep-IgG antibody control

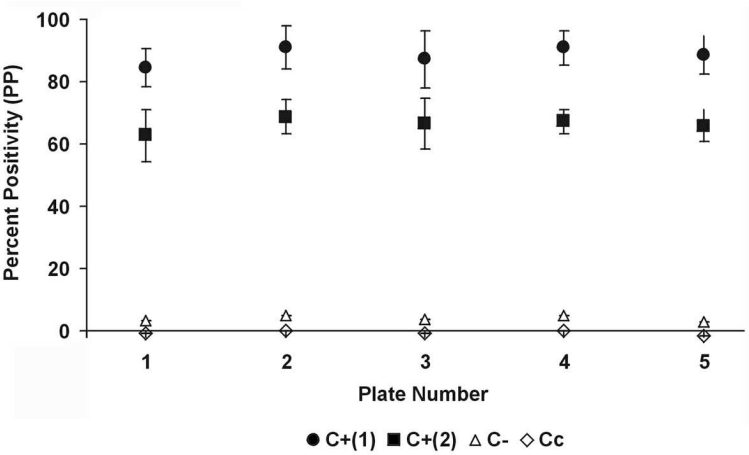

Figure 3 Internal quality control results of measuring five different plates on two different days. C+(1) and C+(2), mean PP value of the positive sera, C-, mean PP value of negative control, Cc, mean PP value of conjugate control. The standard deviations are indicated by vertical bars

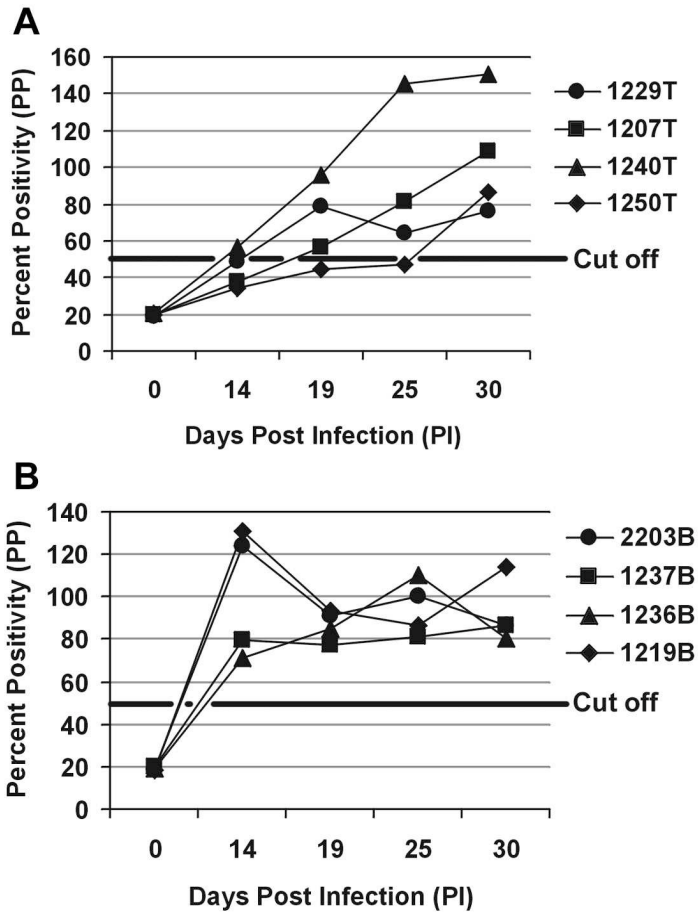

Figure 4 Humoral response of eight *T. uilenbergi* infected animals over time as assessed by the rc9b ELISA post infection. A) Sheep No. 1207, 1250, 1240 and 1229 were infected by infested ticks collected from Lintan County. B) Sheep No. 1219, 2203, 1236 and 1237 were infected by innoculation with 8 ml of blood with 4 % parasitemia (Lintan isolate).

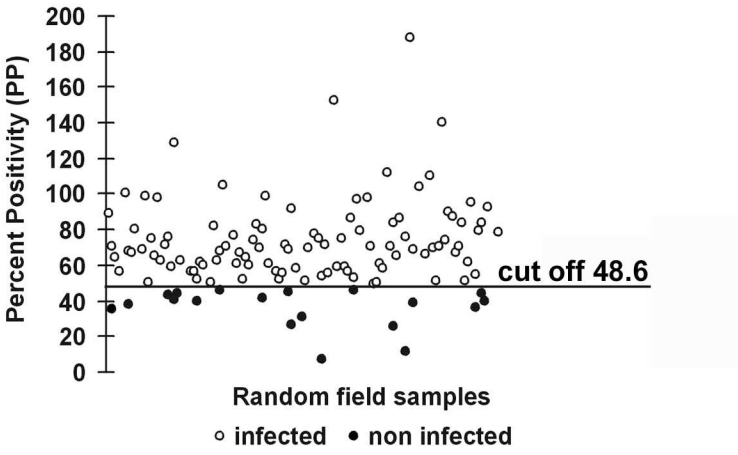

Figure 5 Percent positivity of 101 random field sera samples using the calculated threshold of 48.6 %.

Table 1 Comparison of the r*Tu*IP ELISA (Liu et al. 2010) and the rc9b ELISA using 80 serum samples from China

Test		r*Tu*IP ELISA		
		Positive	Negative	Total
rc9b ELISA	Positive	64	3	67
	Negative	9	4	13
	Total	73	7	80

4.2 Publication 2

PARASITOLOGY RESEARCH (2010) ONLINE FIRST

Development and laboratory evaluation of a lateral flow device (LFD) for the serodiagnosis of *Theileria annulata* infection

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Abstract

Several DNA-based and serological tests have been established for the detection of *T. annulata* infection, including polymerase chain reaction, reverse line blot and loop-mediated isothermal amplification, indirect ELISA and competitive ELISA. In this study we have applied knowledge from the development and application of a recombinant protein based indirect ELISA and competitive ELISA to establish a rapid test for point of care diagnosis of *T. annulata* infection in the field to be used by the veterinarian. For the development of a lateral flow test, the recombinantly expressed *T. annulata* surface protein (TaSP) was applied as the test antigen and anti-TaSP antiserum as the control line. TaSP antigen conjugated to colloidal gold particles was used as the detection system for visualization at the test line for the binding of anti-TaSP antibody present in the serum of infected animals. The developed test specifically detected antibodies in the serum of animals experimentally infected with *T. annulata* and showed no cross-reactivity with serum from animals infected with other tested bovine pathogens (*Trypanosoma brucei*, *Anaplasma marginale*, *B. bigemina*, *B. bovis*, and *T. parva*). Testing of field samples was compared to results obtained by other serological tests, resulting in a sensitivity and specificity of 96.3 % and 87.5 % compared to IFAT, of 98.7 % and 81.8 % compared to indirect ELISA and of 100 % and 47.6 % compared to competitive ELISA. In conclusion, a rapid test for the detection of *T. annulata* infection (*T. annulata* lateral flow device – Ta-LFD) has been developed which is easy to perform, delivers results to be read with the naked eye within 10 minutes, and is suitable for the detection of infection in field samples.

Introduction

T. annulata causes tropical theileriosis in cattle, which is transmitted by ticks of the genus *Hyalomma* (Uilenberg 1981). It is an intracellular protozoan parasite that induces a spectrum of disease symptoms and is highly pathogenic to cattle. The disease occurs over a wide geographic area ranging from Southern Europe and extends to Southern Russia, the Middle East, Central Asia, China, India, Northern Africa and Sudan, Eritrea and Mauritania. (McCosker 1979; Dolan 1989; Minjauw and McLeod 2000).

Several methods for the diagnosis of *T. annulata* infection are available which are however not well suited for direct testing in the field. Primarily, these include the routine clinical diagnosis for theileriosis and the microscopic detection of parasites from sampled blood smears. Although the microscopic examination of blood smears is uncomplicated and is

of value for the detection of acute cases, it has limited value for chronic cases because of the low number of parasitaemia in those animals and additionally, it is difficult to differentiate between piroplasm species according to morphology (Hooshmand-Rad 1974; Friedhoff 1997).

Research in molecular biology has delivered precise tools for the detection of parasite DNA and a number of PCR-based assays with high sensitivity and specificity for the diagnosis of *T. annulata* in the bovine host have been developed, including PCR (d'Oliveira et al. 1995; Shayan et al. 1998; Kirvar et al. 2000; Habibi et al. 2007) and reverse line blotting (RLB) to detect and differentiate all known *Theileria* and *Babesia* species on the basis of differences in their 18S subunit rRNA gene sequences (Gubbels et al. 1999; Schnittger et al. 2004). These techniques require equipped laboratories, are expensive and impractical for field diagnosis. Recently, a loop-mediated isothermal amplification (LAMP) assay was developed and evaluated for diagnosis of tropical theileriosis, which operated at high specificity, efficiency and rapidity (Salih et al. 2008), but which has not been validated in the field yet.

Serological tests are ideally suited for epidemiological studies. The indirect fluorescence antibody test (IFAT) has successfully been used to detect antibodies against *T. annulata* infection in cattle (Burridge and Kimber 1973) and has been reported to be more sensitive than examination of blood smears (Dhar and Gautam 1977; Darghouth et al. 1996). However, it has the major drawback of cross-reactivity between different *Theileria* species (Burridge et al. 1974). In recent years efforts have been made to develop and validate an indirect ELISA (iELISA) based on recombinantly expressed antigens, such as the Tams1 (Gubbels et al. 2000) and the TaSP antigen (Bakheit et al. 2004). Especially the immunodominant antigen TaSP (*T. annulata* surface protein) (Schnittger et al. 2002) has proved to be highly suitable for detection of *T. annulata* specific antibodies (Bakheit et al. 2004; Seitzer et al. 2007, 2008), and it has been validated in the field (Salih et al. 2005) and used for epidemiological studies (Salih et al. 2007a, b; Mohammad Al-Saeed et al. 2010). Furthermore, a competitive ELISA (cELISA) based on the TaSP antigen to increase specificity of the detection of circulating antibodies against TaSP was recently established and validated (Renneker et al. 2008, 2009).

ELISAs are the method of choice for epidemiological studies and large scale investigations, however the applications are time-consuming, labor-intensive and also require professional personnel, special laboratory materials and equipment. Hence, a convenient, rapid and sensitive diagnostic test, such as an immunochromatographic test that does not require instrumentation or specially trained personnel, would be extremely valuable for the use in both clinical and field applications for the diagnosis of tropical theileriosis. Given the

high suitability of the TaSP antigen for serodiagnosis of *T. annulata* infection, the following study was performed to establish an immunochromatographic strip test (lateral flow device; LFD) on the basis of this protein for use as a rapid point of care assay.

Materials and methods

Preparation of recombinant TaSP

Recombinant expression and purification of the predicted extracellular domain of TaSP (rTaSP, aa 25-156) was performed as described before (Schnittger et al. 2002). This first round of purified recombinant protein was applied to re-purification using the Äkta prime high system (Amersham Bioscience, Uppsala, Sweden) using 5 ml HiTrap columns packed with Ni Sepharose 6 Fast Flow (GE Healthcare Europe GmbH, Freiburg, Germany). The Ni-chromatography was carried out according to the manufacturer's instructions using the following chromatography programme: (1) washing of the column with 5-10 column volume (cv) H_2O with a flow rate of 5 ml/min; (2) equilibration with 10 cv application buffer (20 mM Tris-HCl pH 8.0, 8 M urea, 0.5 M NaCl, 5 mM imidazole); (3) application of the sample to the column with a flowrate of 1 ml/min; (4) washing with 20 cv washingbuffer (20 mM Tris-HCl pH 8.0, 8 M urea, 0.5 M NaCl, 5 mM imidazole) at a flow rate if 3 ml/min until the absorbance at A_{280} is constant; (5) collection of 1 ml fractions after application of 1 cv elution buffer (20 mM Tris-HCl pH 8.0, 8 M urea, 0.5 M NaCl, 1 M imidazole) with a linear gradient of 500 mM to 1 M imidazole until the protein peak appears at a flow rate of 1 ml/min. Collected fractions were analyzed for the presence of protein in Western blot using anti-histidine antibody (RGS-His^{TM} mouse anti-histidine antibody, Qiagen, Hilden, Germany), *T. annulata*-positive serum samples and anti-TaSP antiserum (Schnittger et al. 2002). A protein band of approximately 41 kDa was observed (Fig. 1). Purity was assessed by Coomassie gel staining, and protein concentration was determined using the BioRadMicro-DC Assay kit (BioRad, Munich, Germany). Fractions containing the protein were pooled, dialyzed against PBS (phosphate buffered saline, 137 mM NaCl, 10 mM Na2HPO4, 2.7 mM KCl) and adjusted to a concentration of 0.2 mg/ml. This re-purified protein was used in all experiments with Western blot and LFD.

Sera from experimentally infected animals and field sera

Serum samples from animals experimentally infected with *T. annulata* were previously

generated at the Free University Berlin, Germany (Ahmed et al. 1989) and the University of Utrecht (kindly provided by Frans Jongejan). Serum from animals experimentally infected with *B. bovis*, *B. bigemina* or *Anaplasma marginale* were kindly provided by Jiansan Wu, Qingdao, China.

Serum samples from animals experimentally infected with *Trypanosoma brucei* or *T. parva* were kindly provided by Dirk Geysen, Antwerp, Belgium. Serum containing antibodies against *T. mutans* was from Svanova Biotech, Uppsala, Sweden. Ninety field serum samples were collected at random from cattle in Sudan (Bakheit et al. 2004) in areas where tropical theileriosis is known to be endemic. Sera from *T. lestoquardi*-infected animals were from Sudan (Bakheit et al. 2006). Results obtained with the Ta-LFD were compared to previously generated data using IFAT, indirect ELISA or cELISA (Bakheit et al. 2004; Renneker et al. 2008*)*. The specificity and sensitivity were calculated using the following formulas: specificity (Sp=(#samples negative in both tests/total number of negative samples in the reference test)*100), sensitivity (Se=(#samples positive in both tests/total number of positive samples in the reference test)*100). The Kappa test (Cohen's kappa measure of association) was used to calculate the degree of agreement between the Ta-LFD and compared tests using Intercooled Stata 6.0 (StataCorp LP, College Station, Texas, USA), and SPSS 11.5.0 for Windows (SPSS Inc., Chicago, Illinois USA) (Thomas et al. 1988). Interpretation of kappa can be rated as follows: kappa less than 0.0 "poor" agreement; between 0.0 and 0.20 "slight" agreement; 0.21 and 0.40 "fair" agreement; between 0.41 and 0.60 "moderate" agreement; between 0.61 and 0.80 "substantial" agreement; and between 0.81 and 1.00 "almost perfect" agreement (Landis and Koch 1977). Hypothesis tests of whether kappa is significantly greater than 0 are based on standard errors derived from approximations to its variance (Bishop et al. 1975).

Components of the *T. annulata* lateral flow device (Ta-LFD)

Adsorption of rTaSP and anti-TaSP antibody to the membrane

Recombinant *T. annulata* surface protein (rTaSP) was used as the solid-phase antigen in the test line by jetting it linearly onto the membrane (Hi-flow membrane no. R90N93558-Hf1350, Millipore, USA) at a concentration of 2 mg/ml using Bio-Dot air-brush equipment (Bio-Dot Ltd., West Sussex, UK). Fifty microliters of the TaSP solution were added per 30 cm of the membrane. The control line comprised rabbit anti-TaSP antibody, prepared by immunization of a rabbit with the recombinant TaSP (Schnittger et al. 2002) and subsequent

purification of specific IgG antibodies by affinity chromatography (HYDRA®-gelmatrix, Charles River Laboratories, Kisslegg, Germany), which were applied to the control line at a concentration of 2 mg/ml parallel to the rTaSP test line (test band). The membranes were then dried at 37°C for 45 min and stored in sealed foil sachets until use.

Conjugation of rTaSP to gold micro particles

Purified rTaSP labeled with colloidal gold was used as the mobile phase by coupling to 40 nm colloidal gold particles using a proprietary method and storing at 4°C until use.

Adsorption of gold/rTaSP conjugate to filters

The gold/Ab conjugate was sprayed onto the conjugate filter (Whatman, UK) using Bio-Dot air-brush equipment (Bio-Dot Ltd., West Sussex, UK) at a volume of 1 µl/mm filter. The filters were dried at room temperature for 45 min and then stored in sealed foil sachets until required.

Assembly of the strip test device

Sequentially, the membrane, absorbent pad, conjugate pad and sample pad were assembled on an adhesive card and cut into 0.8 cm wide strips with a BioDot cutter (Bio-Dot Ltd., West Sussex, UK). The membrane strips were assembled into a device as described previously (Brüning et al. 1999; Ferris et al. 2009).

Results

Optimum antigen concentration and sample buffer

The system for the lateral flow device was optimized by determining the physical and chemical conditions for maximum sensitivity of detecting a positive sample while assuring a negative signal for negative serum. These studies showed that the optimum concentration of rTaSP for binding to microspheres and the nitrocellulose was 0.2µg/µl and 2mg/ml, respectively. In addition, different sample volumes were tested and 180 µl were chosen as the optimal sample volume to solubilize the conjugate and to facilitate capillary flow.

***T. annulata* lateral flow device (Ta-LFD) test operation**

During the assay performance, 20 µl of bovine sera mixed with 160 µl of chase buffer

were applied to the sample pad. This resulted in rehydration of the air-dried rTaSP-conjugated and dyed gold particles and their migration by capillary action along the nitrocellulose membrane representing the reaction matrix. If anti-TaSP antibodies were present in the sample then the antibody-rTaSP-conjugate complex was captured by the immobilised rTaSP on the membrane at the 'T' (test) line and resulted in their accumulation, which could be visualised as a red line to signify a positive result. Excess (or unbound) rTaSP-labelled gold particles continued to migrate along the device until being captured by the immobilised rabbit anti-TaSP antibody and the formation of a red 'C' (control) line, to validate the test. The rest of the liquid is entrapped in the wick or absorbent pad. The results could be judged within 8-10 min and were recorded as shown in figure 2.

Sensitivity and specificity of the Ta-LFD

The sensitivity and specificity of the Ta-LFD were determined by detecting anti-TaSP specific antibodies in field sera using the optimized conditions and comparing the results with data obtained by investigation of the serum samples using IFAT, iELISA and cELISA (Bakheit et al. 2004; Renneker et al. 2008). In comparison to IFAT as the reference test, the sensitivity and specificity of the Ta-LFD were 96.34 % (79/82) and 87.5 % (7/8), respectively (Table 1). Seventy-nine samples were positive and 7 negative in both tests, whereas 1 was positive using the Ta-LFD but negative using IFAT and 3 were negative using Ta-LFD but positive using IFAT. The relative agreement was calculated to be 95.5 % (kappa = 0.75; substantial agreement; $p<0.0000$). Using the iELISA as the reference method, 78 samples were positive and 9 negative in both tests. Two samples were positive using the Ta-LFD and negative using iELISA, and only 1 sample was negative in the Ta-LFD test but positive in the iELISA, leading to a calculated sensitivity and specificity of the Ta-LFD of 98.7 % (78/79) and 81.1 % (9/11), respectively. The relative agreement was 96.6 % (kappa =0.83; almost perfect agreement; $p<0.0000$). Lastly, results using the Ta-LFD test were compared to results obtained with competitive ELISA as the reference test. Here, 69 samples were positive and 10 negative in both tests, whereas 11 samples were positive using the Ta-LFD and negative using cELISA, resulting in a sensitivity and specificity of 100 % (68/68) and 47.6 % (10/21), respectively with an agreement of 86.6 (kappa =0.56; moderate agreement; $p<0.0000$).

Serum from a cattle experimentally infected with *T. annulata* was collected at 2, 3, 4 and 5 weeks post infection and tested by indirect ELISA (Bakheit et al. 2004), competitive ELISA (Renneker et al. 2008) and Ta-LFD. Comparable results were obtained with the three tests, indicating that the Ta-LFD can detect infection 3 weeks post infection (Table 2).

Testing for possible cross-reactivity of the Ta-LFD was performed using sera from animals experimentally infected with *B. bovis, B. bigemina, Trypanosoma brucei, Anaplasma marginale, T. mutans* and *T. parva* as well as serum from an animal testing positive in an ELISA for detection of infection with *T. lestoquardi* (Bakheit et al. 2006). No cross-reactivity was observed in comparison to the *T. annulata* positive and negative controls (Fig. 3).

Discussion

Lateral flow immunoassays are well established as a valuable tool in medical, veterinary, food, agricultural, environmental and industrial diagnostics. Immunochromatographic tests have also been developed for the diagnosis of many protozoan diseases, such as babesiosis (Huang et al. 2004b; Kim et al. 2007, 2008), malaria (Mills et al. 1999), cryptosporidiosis (Chan et al. 2000), leishmaniasis (Reithinger et al. 2002), coccidiosis (Liao et al. 2005), toxoplasmosis (Huang et al. 2004a), and trypanosomosis (Houghton et al. 2009). Regarding the serodiagnosis of infection with *T. annulata*, there have been several reports in the last years, including IFAT (Burridge and Kimber 1973; Dhar and Gautam 1977; Darghouth et al. 1996), indirect ELISA (Gubbels et al. 2000; Bakheit et al. 2004) and competitive ELISA (Renneker et al. 2008). Besides having cross-reactivity problems for instance using IFAT (Burridge et al. 1974), these methods, involve complex procedures that require expensive laboratory materials, equipment and trained personnel. Thus, although the ELISA assays are specific and highly suitable for large scale epidemiological studies carried out in the laboratory, these methods are unsuited for rapid diagnosis for use by the veterinarian in the field. In the present study, we thus developed a new rapid immunochromatographic strip assay for the detection of infection with *T. annulata* (Ta-LFD) based on the same protein (TaSP) which was used in the previously established ELISA assays (Bakheit et al. 2004; Renneker et al. 2008). The application of this Ta-LFD is simpler, more convenient, and provides results very rapidly to be judged with the naked eye compared to the standard serodiagnostic tests, and can be applied by veterinarians in the field especially in countries where laboratories are less well equipped.

Several critical aspects in the establishment of an LFD need to be considered, such as the quality of antigen and/or antibody, pretreatment of the sample, and conjugate pad or membrane properties (Zhang et al. 2006). In the establishment of the Ta-LFD, the quality of TaSP used was crucial for the sensitivity and specificity of the assay, indicating that purification and consistency of supply are important to minimize non-specific binding effects. In addition, to maximize the adsorption of proteins, these should be applied to the membrane

in buffers that are preferably free of salt, surfactants and sugars (Mansfield et al. 2005; Millipore Technical Publication "Rapid Lateral Flow Test Strips: Considerations for Product Development" available at http://www.millipore.com/techpublications/tech1/tb500en00). Therefore, TaSP was re-purified and respective fractions were pooled and dialyzed against PBS, to ensure that it exhibits sufficient sensitivity, specificity, purity and stability to execute the performance requirements of the completed product.

For evaluation of the Ta-LFD, the results of testing field sera from a tropical theileriosis endemic region in Sudan using the Ta-LFD assay were compared to those obtained by indirect and competitive TaSP ELISA. These comparisons showed that the Ta-LFD had a significant correlation with all other tests (IFAT, iELISA, cELISA), the highest agreement existing between the Ta-LFD and iELISA (96.7 %), then with IFAT (95.5 %) and cELISA (86.6 %), indicating that the use of the Ta-LFD would be reliable for use in the field. The comparison also showed that the Ta-LFD had a high sensitivity compared to the respective reference tests, ranging from 100 % to 98.7 % and 96.34 % compared to the cELISA, iELISA and IFAT, respectively. Thus, only a minor number of samples detected as positive in the reference tests were tested negative with Ta-LFD. The specificity of the Ta-LFD compared to the reference tests was also high, ranging from 87.5 % for the IFAT to 81.8 % for the iELISA and 47.6 % for the cELISA. The latter value is due to the fact that 11 samples testing negative in the cELISA were positive using the Ta-LFD, a discrepancy which was also observed when comparing the cELISA to the iELISA (Renneker et al. 2008). This is perhaps attributable to the fact that detection of *T. annulata* infection using the cELISA relies on the inhibition of binding of a monoclonal antibody to an epitope of the TaSP protein by antibodies in the test serum, which may not be present in sufficient amounts in selected samples giving false negative results.

In conclusion, the developed Ta-LFD for serological detection of infection with *T. annulata* required no sophisticated equipment to be carried out and delivered easy to read and interpretable results with the naked eye after 10 minutes of application of the sample. The newly established test is sensitive and specific and may be regarded as a suitable diagnostic tool for the detection of theileriosis in cattle under field conditions. Considering also that the completely assembled Ta-LFD is stable when stored without refrigeration, this rapid test should be easily applicable by veterinarians as a point of care diagnostic assay.

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Figures

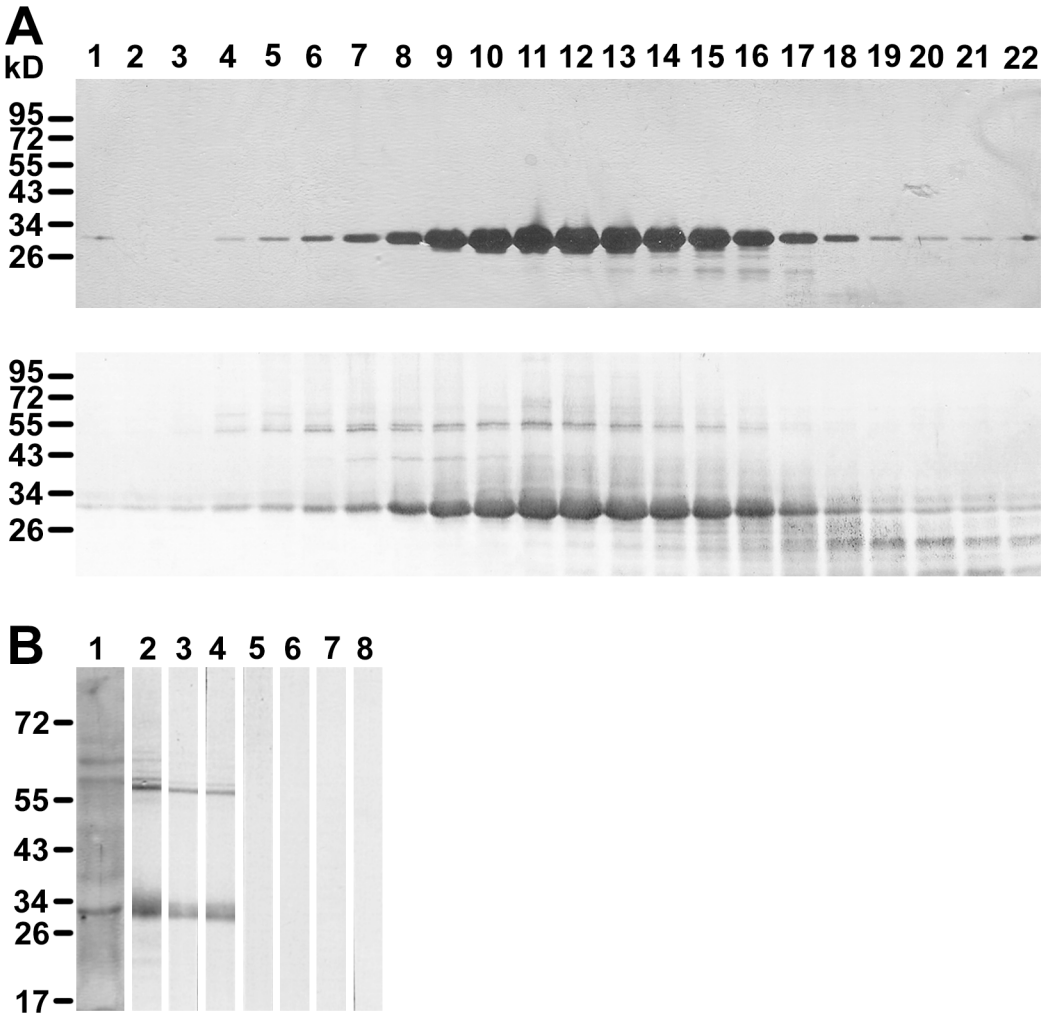

Figure 1 Purification of recombinant TaSP protein.

A) Recombinantly expressed and purified rTaSP was subjected to re-purification using column chromatography as described in the methods section. Collected fractions (lanes 1-22) were analyzed by silverstaining (top panel) and Western blot using anti-TaSP antiserum (bottom panel). Fractions 11 to 13 were pooled for further use.

B) The pooled fractions were analyzed in silver staining (lane 1) and were tested for reactivity with anti-TaSP antiserum (lane 2) and two sera from T. annulata infected cattle (lanes 3 and 4). Sera from uninfected cattle were tested in lanes 5 and 6. Lane 7: anti-bovine secondary antibody control. Lane 8: anti-rabbit secondary antibody control.

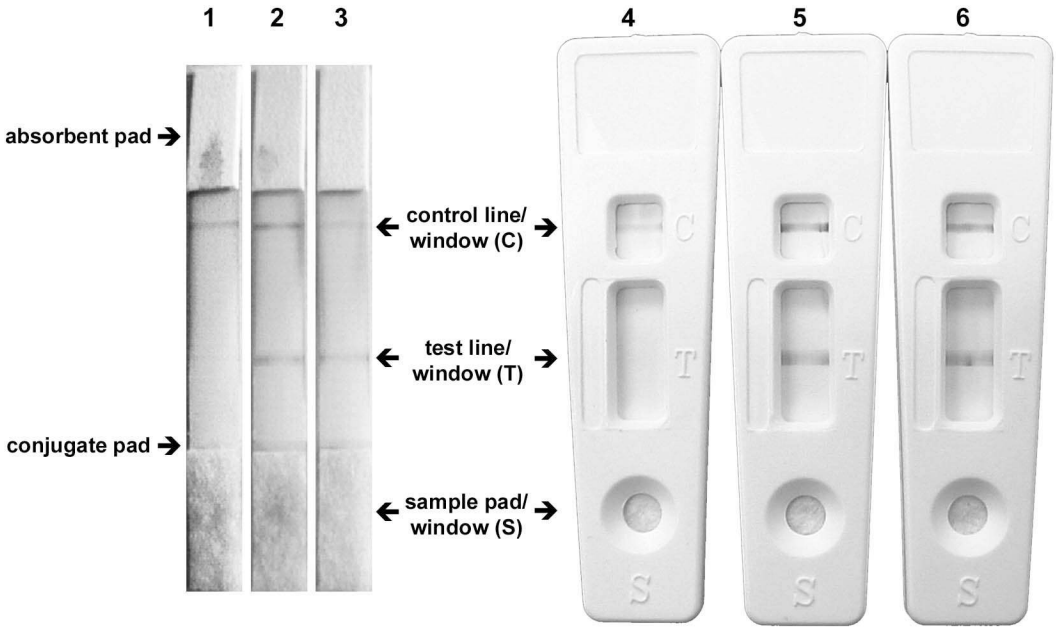

Figure 2 Test strip and assembled *T. annulata* lateral flow device (Ta-LFD)

Setup of the test strip (1-3) showing the nitrocellulose strip with the control line (C) and test line (T) and the lower and upper affixed sample, conjugate and absorbent pads. The assembled LFD device (4-6) correspondingly shows a sample window, test window and control window. Application of serum from an uninfected animal (1, 4) results in only the control line becoming visible; application of serum from an infected animal (2, 5) or anti-TaSP antiserum as control (3, 6) results in also the test line becoming visible.

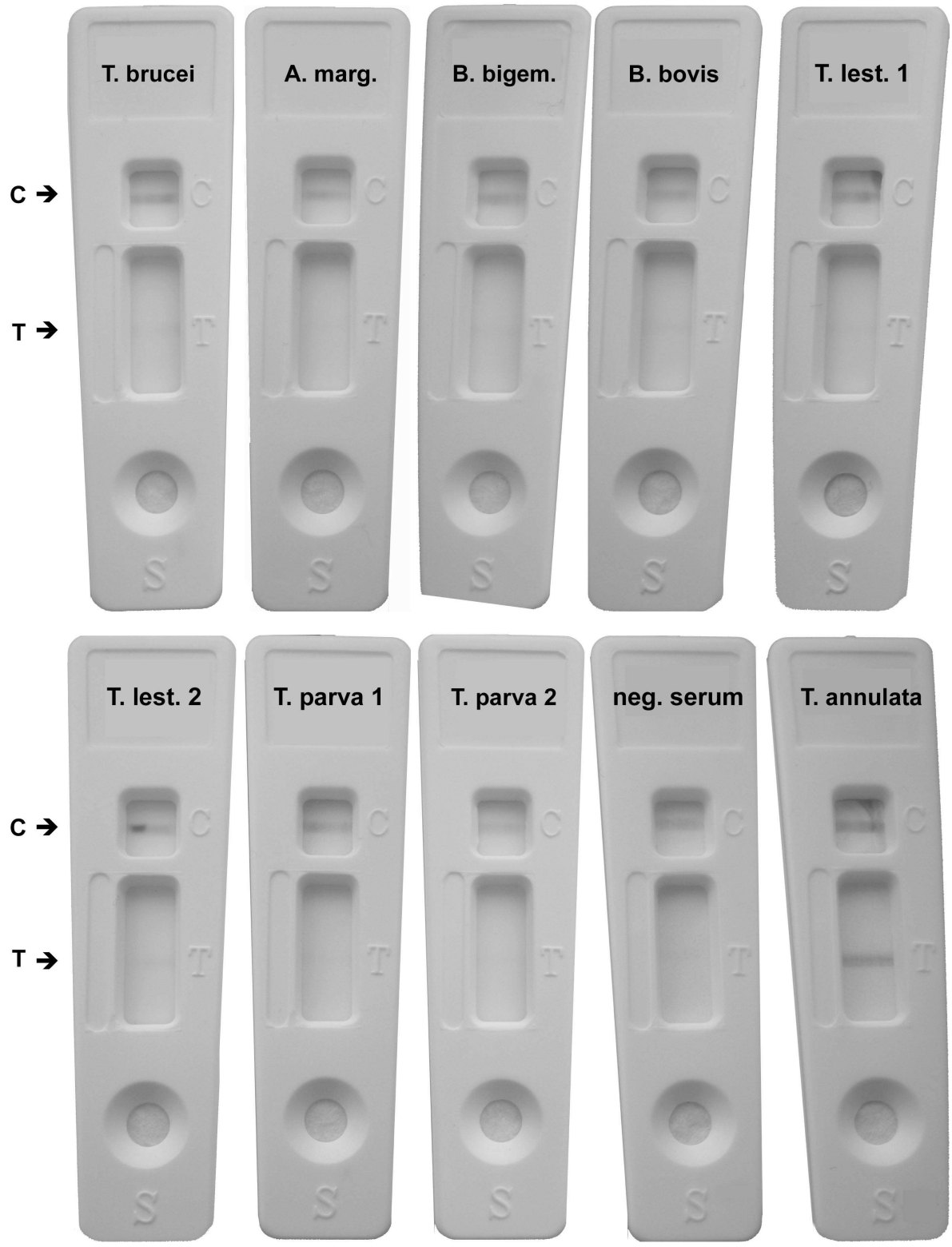

Figure 3 Testing for cross reactivity using the Ta-LFD

Serum from animals infected with *Trypanosoma brucei*, *Anaplasma marginale*, *B. bigemina*, *B. bovis*, *T. lestoquardi* and *T. parva* were tested in the Ta-LFD. Serum from an uninfected animal was used as the negative control and serum from an animal infected with *T. annulata* as the positive control. C: control line. T: test line.

Table 1 Comparison of *T. annulata* lateral flow device (Ta-LFD) with indirect fluorescent antibody assay (IFAT), indirect ELISA (iELISA) and competitive ELISA (cELISA) as the reference tests for detection of anti-*T. annulata* antibodies in 90 field samples collected from cattle in an endemic region in Sudan.

		Reference Test						
		IFAT		iELISA		cELISA		Total
		Positive	Negative	Positive	Negative	Positive	Negative	
Ta-LFD	Positive	79	1	78	2	69	11	80
	Negative	3	7	1	9	0	10	10
	Total	82	8	79	11	69	21	90
	Sensitivity	96.34%		98.7 %		100%		
	Specificity	87.5%		81.8%		47.6%		
	Agreement	95.5% (kappa = 7.2; p<0.0000)		96.7% (kappa = 0.83; p<0.0000)		86.6% (kappa = 0.56; p<0.0000)		

Table 2 Detection of anti-TaSP specific antibodies in one cattle experimentally infected with *T. annulata* in serum taken at weekly intervalls post infection (p. i.) using indirect ELISA (iELISA), competitive ELISA (cELISA) and Ta-LFD. + indicates a positive result, - a negative result.

	iELISA	cELISA	Ta-LFD
2 weeks p.i.	-	-	-
3 weeks p.i.	-	+	+
4 weeks p.i.	+	+	+
5 weeks p.i.	+	+	+

5 Discussion

In the frame of this work two diagnostic tests for the detection of antibodies were successfully developed: An indirect ELISA for the detection of antibodies against ovine Theileriosis (*T. uilenbergi*) and a rapid Lateral Flow Device for the detection of antibodies against Tropical Theileriosis (*T. annulata*). The recombinant protein based ELISA was achieved as an improvement to existing serological tests for the detection of infection with *T. uilenbergi* and was developed using a bioinformatics approach. Regarding the rapid test, the aim was to develop a diagnostic assay suitable for application in the field for diagnosis of tropical theileriosis. The achievement of this goal was reached by implementing components previously used for serological detection of *T. annulata* infection with an indirect ELISA. The developed diagnostic tools are discussed in two sections below.

5.1 Development of a recombinant protein based indirect ELISA for the detection of antibodies against *T. uilenbergi*

Theileria species infective to small ruminants in China have been previously classified as two species, namely *T. luwenshuni* and *T. uilenbergi*, utilizing phylogenetic studies on 18S ribosomal RNA genes (Ahmed et al., 2006; Yin et al., 2007). More recently a new isolate of *Theileria* sp. was identified in Xinjiang Province of China, and studies of phylogenetic relationship of this isolate based on the 18S rRNAgene inferred that this species belongs to the cluster of *T. ovis* (Li et al., 2010). *T. luwenshuni* and *T. uilenbergi* cause the most severe tick-borne disease of small ruminants in China (Luo and Yin 1997; Schnittger *et al.,* 2000b, 2000c). In the past, ovine theileriosis in China was reported to be caused by infection with *T. lestoquardi*, which was widely known as the only species that causes malignant theileriosis of sheep and goats (Friedhoff et al., 1997). Regarding serology and morphology, *T. lestoquardi* exhibits many similarities with *T. annulata* (Brown et al., 1998) and their close phylogenetic relation was confirmed by investigation of the 18S rRNA (Katzer et al., 1998). Furthermore, phylogenetic analysis of the determined nucleotide sequences of small subunit ribosomal RNA (srRNA) of *T. lestoquardi* and the Chinese *Theileria* parasites showed that the latter were clearly divergent from *T. lestoquardi* and *T. annulata* and that they belong to different clades within the phylogenetic tree (Schnittger et al., 2000b). The Chinese parasites were most closely related to the *T. buffeli/orientalis/sergenti* complex (Schnittger et al., 2003)

which has the common characteristic to display a less marked leukocytic phase without being able to transform their host cells. Moreover, it was noticed that animals infected with *T. uilenbergi* often die before piroplasms could be observed in blood smears (Ahmed et al., 2006). In fact, sheep and goats infected with *T. luwenshuni* and *T. uilenbergi* exhibit a short schizont stage and pathogenesis is related to tissue injuries and anemia caused by the merozoites that infect erythrocytes. This fact implies that the piroplasm stage of the parasite seems to be the most pathogenic to the host animal and accordingly play an essential role in the pathogenesis of the disease (Yin et al., 2003). It is also thought that this stage has the most effect on the host's immune system leading to production of antibodies as well as activation of T-cells. Since to date all attempts to establish schizont infected cell lines of *T. uilenbergi* and *T. luwenshuni* as a source of biological material have failed (Yin et al., 2007), the construction of a cDNA library from the merozoite stage was performed by isolation and purification of mRNA from blood of animals experimentally infected with *T. uilenbergi* (Liu et al., 2008a). This library was used as a tool to identify genes expressed in the merozoite stage which may represent promising candidates for the development of specific diagnostic tests and vaccines.

Different strategies were used to identify specific antigens of *T. uilenbergi* using this cDNA library. The first strategy was to screen using sera from *T. uilenbergi*-infected sheep, leading to the identification of the *T. uilenbergi* immunodominant protein (*Tu*IP) which was successfully used to establish an ELISA for the detection of antibodies (Liu et al., 2010). The second approach consisted of random selection and sequencing of clones from the library followed by sequence analysis for the presence of antigenic determinants with the help of different bioinformatic servers, leading to the identification of the potentially antigenic clone-2 gene family (Liu et al. 2008a).

To this date, little is known about the sequences contained in the genome of *T. luwenshuni* and *T. uilenbergi* parasites. Research has displayed high identity between most known heterologous genes of *T. uilenbergi* and other *Theileria* species. These comprise TcD, which shows 88 % identity to a putative *T. annulata* membrane protein (TaD), TcSP partial cDNA, showing 94 % identity to *T. annulata* surface protein (TaSP), TcSE partial cDNA, showing 99 % identity to a secretory protein of *T. annulata* (TaSE) and Tc Clone-5, showing 100 % identity to *T. lestoquardi* Clone-5 on the genomic level (Miranda et al., 2006b). Additionally, TcHSP70 was identified as a homologue of *T. lestoquardi* TlHSP70, which was obtained through immunological screening of a *T. lestoquardi* schizont cDNA library (Miranda et al., 2006a, 2006b). Thus, the *Tu*IP and the clone-2 gene family (including clone-9) identified in

T. uilenbergi using the merozoite cDNA library represented proteins appearing to be unique to *T. uilenbergi* using BLAST analysis of known sequences. Taking into consideration that bioinformatic analyses indicated that clone-9 may be an antigenic protein, this supported the notion to further characterize the clone 9b recombinant protein and use it for the development of a serological test.

Interestingly, the sequence of clone-9 gave only a limited number of hits using BLAST-searches, the highest identity being only moderate (47 %) with the ToLocg 1 gene of *T. orientalis* and 35 % with a hypothetical protein of *T. parva*. Since also no further data are available on the ToLocg 1 gene, information gained from these searches was minimal. Nonetheless, *Theilerial* origin of the gene was confirmed and the finding also fits well to previous investigations indicating that *T. uilenbergi* is most closely related to the *T. buffeli/orientalis/sergenti complex* (Schnittger et al., 2003).

Bioinformatic analyses showed that members of the clone-2 gene family (clone 2, clone 9 and clone 26) could encode potential immunodominant proteins (Liu et al., 2008a). In this study, further bioinformatic analyses of clone 9 were performed and revealed the presence of a signal peptide, a transmembrane region and six antigenic determinants indicating that this protein may represent a parasite membrane antigen which induces a humoral immune response.

To verify this prediction, clone-9 was recombinantly expressed in *E. coli* in order to analyze sera from *T. uilenbergi*-infected animals for the presence of antibodies which can recognize recombinant expressed clone 9. For expression, the signal peptide carrying one of the antigenic determinants was excluded to improve expression levels. The remaining determinants except the last 5 aa of the N-terminal 150-167 aa antigenic determinant were included.

Immunoreactivity of recombinant clone 9b (rc9b) was assessed by Western blot. The gene fragment encoded a protein of 172 amino acids with a predicted molecular weight of 22 kDa. Investigation in Western blot indeed indicated that antibodies against rc9b were present in sera from *T. uilenbergi*-infected animals, suggesting that the infected animals produce antibodies against this parasite protein. Furthermore, cross-reactivity with positive sera of *T. lestoquardi* and *Babesia sp.* infected animals was not observed.

These results suggested that this clone has the potential to be used for the development of a serological diagnostic test, enabling the establishment of an indirect ELISA system, since this method has been used extensively for the detection of parasitic diseases and is the serological method of choice for epidemiological surveys due to its practicability in

examining large sample sizes at relatively low cost (Ruitenberg et al. 1977; Bidwell et al., 1978). Moreover, in contrast to crude antigen, the use of a recombinantly expressed protein allows to develop and perform a diagnostic test under standardized conditions.

Although the absence of cross-reactivity with related piroplasms could be ascertained (*T. lestoquardi, Babesia sp.*), testing of sera from the most closely related and very similar *T. luwenshuni* was not performed due to unavailability of positive serum samples. It will be interesting in the future to find out whether the protein can be used to differentiate between the two parasites infecting small ruminants in China. Some cross-reactivity is to be expected, as a clone-2 family-like gene has been identified in *T. luwenshuni* (Genbank ACE86412.1) with high identity to clone-9 (84 %). Future investigations will be needed to identify clone-2 family genes in *T. luwenshuni* and to perform comparative analyses of these sequences for regions likely to be specific to *T. uilenbergi* or *T. luwenshuni*, which may then be used for the development of specific serological assays for these two pathogens.

In this study, the optimized rc9b ELISA procedure effectively detected specific antibodies against *T. uilenbergi* in experimentally infected sheep applying an established threshold calculated from values obtained from sera from uninfected sheep. Interestingly, experimental infection by inoculation with infected blood resulted in an earlier and steeper rise in anti-rc9b antibody titers than experimental infection with ticks carrying the pathogen. This perhaps reflects that clone-9 is an antigen expressed in later stages of the parasite life cycle in the host, possibly the merozoite stage, as the cDNA library from which the gene was isolated was generated from this stage. This assumption will have to be clarified in future investigations.

The suitability of the rc9b ELISA for testing of field sera was established using field sera collected from an endemic region in China. The rc9b ELISA showed an agreement of 85 % in comparison with another recently established ELISA for detection of ovine theileriosis in China based on a different recombinantly expressed antigen (*Tu*IP). The reason for the observed differences is not clear to date, being possibly due to the levels of the respective specific antibodies present. This discrepancy will also be the subject of further studies aiming to continually improve the serodiagnostics of ovine theileriosis in China.

5.2 Development of an LFD for the detection of antibodies against *T. annulata*

In the last decades, lateral flow immunoassays have gained a wide approval. Many features made these assays popular such as simplicity of the test design, quick and easy detection of the results usually without the help of any instrument, rapid identification of analytes at the point of care and the low-cost powerful technology. The lateral flow immunoassays are valuable tools in medical, food, agricultural, environmental, industrial diagnostics as well as in veterinary medicine (O'Farrell, 2009). For the diagnosis of many protozoan diseases several assays have been developed, e.g. for babesiosis (Huang et al., 2004a; Kim et al., 2007, 2008), malaria (Mills et al., 1999), cryptosporidiosis (Chan et al., 2000), leishmaniasis (Reithinger et al., 2002), coccidiosis (Liao et al., 2005), toxoplasmosis (Huang et al., 2004b) and trypanosomosis (Houghton et al., 2009).

The diagnosis of different tick-borne protozoan diseases is similar. For clinical apparent cases of animals infected with *T. annulata*, demonstration of parasites by blood or lymph node biopsy smears stained with Romanowsky stain remains the quickest and most frequently executed method of diagnosis. However, this method is limited to the detection of acute cases and has restricted value for chronic cases of long-lasting carriers because of the low number of parasitaemia and in addition, it is difficult to differentiate between piroplasm species according to morphology (Hooshmand-Rad, 1974; Friedhoff, 1997).

In recent years several methods have been developed and used for the direct diagnosis of tropical theileriosis such as PCR (d'Oliveira et al., 1995), RLB (Gubbels et al. 1999), and loop-mediated isothermal amplification (LAMP) (Salih et al., 2008). Regarding the serodiagnosis, several approaches have been reported, including IFAT (Burridge and Kimber, 1973; Dhar and Gautam, 1977; Darghouth et al., 1996), indirect ELISA (Gubbels et al., 2000; Bakheit et al., 2004) and competitive ELISA (Renneker et al., 2008).

All these tests allow the evidence of infection with *T. annulata* but have different disadvantages in terms of costs, feasibility, low throughput or cross-reactivity, complex procedures and the requirement of trained personnel.

A number of *T. annulata* genes have been cloned, sequenced and expressed including TaSP (Schnittger et al., 2002), TaD (Schneider et al., 2004), TaSE (Schneider et al., 2003) and TamtHSP70 (Schnittger et al., 2000b,2000c). Several recent publications verify that among these genes TaSP is highly antigenic and thus suitable for the development of diagnostic tools for tropical theileriosis (Seitzer et al., 2007; 2008)

The main goal of the second part of this work was the development of a new rapid lateral flow immunoassay for the detection of infection with *T. annulata* (Ta-LFD) based on the same protein (TaSP) that was used in previously established ELISA assays (Bakheit et al. 2004; Renneker et al. 2008). The application of this Ta-LFD is simpler and more convenient, provides results that can very rapidly be judged by the naked eye compared to the standard serodiagnostic tests, and can be applied by veterinarians in the field especially in countries where laboratories are less well equipped.

Recombinant TaSP was produced as described (Schnittger et al. 2002) and expression was verified and quality assessed by Western blots and silver staining, respectively. In fact, the quality of TaSP is very important for the sensitivity and specificity of the assay.

To minimize nonspecific binding effects and at the same time maximize the adsorption of proteins, they should be applied to the membrane in buffers that are preferably free of salt, surfactants, and sugars (Mansfield, 2005). Regarding the recombinantly expressed TaSP, it was repurified and respective fractions were pooled and dialyzed against PBS to ensure that it exhibited sufficient sensitivity, specificity, purity and stability to execute the performance requirements of the completed product.

For evaluation of clinical and field utility of the Ta-LFD, the results of the Ta-LFD were compared with those obtained by IFAT and indirect as well as competitive TaSP-ELISA. Field sera from a region endemic for tropical theileriosis in Sudan were been used for this purpose and the comparisons showed that the Ta-LFD had a significant correlation with all other tests (IFAT, iELISA, cELISA). The highest agreement was obtained between the Ta-LFD and iELISA (96.7 %), followed by IFAT (95.5 %) and cELISA (86.6 %), indicating that the Ta-LFD would be reliable when using it in the field. It could also be shown that the Ta-LFD had a high sensitivity compared to the respective reference tests, ranging from 100 % to 98.7 % and 96.34 % compared to the cELISA, iELISA and IFAT, respectively. Thus, only a minor number of samples detected as positive in the reference tests were tested negative with Ta-LFD. The specificity of the Ta-LFD compared to the reference tests was also high, ranging from 87.5 % for the IFAT to 81.8 % for the iELISA and 47.6 % for the cELISA. The latter value is due to the fact that 11 samples that were classified as negative in the cELISA were positive using the Ta-LFD, a discrepancy which was also observed when comparing the cELISA to the iELISA (Renneker et al. 2008). It can be speculated that since detection of *T. annulata* infection using the cELISA relies on the inhibition of binding of a monoclonal antibody to an epitope of the TaSP protein by antibodies in the test serum, there may not be sufficient amounts present in selected samples, thus giving false-negative results.

Investigation of serum samples of animals infected with closely related and other parasites such as *T. parva*, *Babesia bigemina*, *Babesia bovis*, *Anaplasma marginale* and *Trypanosoma brucei* did not show any positive result meaning that there is no cross-reactivity observable.

Taken together, the Ta-LFD is applicable as a diagnostic tool for the detection of *T. annulata* infection in the field but further validation with serum samples from different regions endemic for tropical theileriosis should be taken into consideration to ascertain its reliability under different conditions.

5.3 Conclusion

On the basis of the novel protein clone-9b which has been described in the frame of this study an indirect ELISA was developed for the successful detection of antibodies against *T. uilenbergi*. In future studies, this protein might be applicable for the development of a rapid test such as a Lateral Flow Device for the detection of *T. uilenbergi* infection.

For the detection of antibodies against *T. annulata* a Lateral Flow Device named Ta-LFD has been successfully developed on the basis of the recombinant protein TaSP. This diagnostic test has been validated by evaluation of field sera and is an assay which may be used by veterinarians for the diagnosis of tropical theileriosis in the field.

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7 Summary

In the frame of this study, an indirect recombinant protein based ELISA to detect antibodies against *T. uilenbergi* and an immunochromatographic strip test as a rapid point of care test for the diagnosis of tropical theileriosis caused by *T. annulata* have been developed.

The pathogenic protozoan parasite *T. uilenbergi* is one of the causative agents of theileriosis in small ruminants in China and the infection results in great economical losses in the northwest part of China. Efforts have been undertaken to establish an ELISA based on a *T. uilenbergi* immunodominant recombinantly expressed protein using different approaches in order to perform epidemiological studies in the area. In this study the potential use of the clone-9b protein for this purpose is described. The protein was identified as a potential immunogenic piroplasm protein specific to *T. uilenbergi* by random sequencing of cDNA library clones followed by bioinformatic analyses. The clone-9 gene was partially recombinantly expressed and used for the development of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of circulating antibodies in sera of *T. uilenbergi*-infected sheep. No cross reactivity was observed in serum from animals infected with *T. lestoquardi*. The cut-off was calculated at 48.6 % percent positivity using 25 serum samples from uninfected animals. The suitability of the rc9b ELISA for testing of field sera was established using field sera collected from an endemic region in China. The rc9b ELISA showed an agreement of 85 % in comparison with another recently established ELISA for detection of ovine theileriosis in China based on a different recombinantly expressed antigen (*Tu*IP).

Regarding the second part of this thesis, several DNA-based and serological tests have been established for the diagnosis of infection with *T. annulata*. These include polymerase chain reaction, reverse line blot and loop-mediated isothermal amplification as methods for the direct detection of parasite DNA, and indirect and competitive ELISA. In this study knowledge from the development and application of a recombinant protein based indirect ELISA and competitive ELISA using the *T. annulata* surface protein (TaSP) was applied to set up a rapid test for point of care diagnosis of *T. annulata* infection in the field to be used by the veterinarian. The developed test specifically detected antibodies in the serum of animals experimentally infected with *T. annulata* and showed no cross-reactivity with serum from animals infected with other tested bovine pathogens (*Trypanosoma brucei*, *Anaplasma*

marginale, *Babesia bigemina*, *Babesia bovis* and *T. parva*). Testing of field samples was compared to results obtained by other serological tests, resulting in a sensitivity and specificity of 96.3 % and 87.5 % compared to IFAT, of 98.7 % and 81.8 % compared to indirect ELISA and of 100 % and 47.6 % compared to competitive ELISA. In conclusion, a rapid test for the detection of *T. annulata* infection has been developed which is easy to perform, delivers results to be read with the naked eye within 10 minutes, and is suitable for the detection of *T. annulata* infections in field samples.

8 Zusammenfassung

Im Rahmen dieser Arbeit wurde zum einen ein indirekter ELISA für die Detektion von Antikörpern gegen *T. uilenbergi* entwickelt, welcher auf einem rekombinant exprimierten Parasitenantigen basiert. Zum anderen wurde ein immun-chromatographischer 'Strip-Test' als Schnelltest für die Diagnose der tropischen Theileriose produziert.

Die Infektion mit dem pathogenen Protozoon *T. uilenbergi* ist eine Ursache von Theileriose bei kleinen Wiederkäuern in China. Die Infektion resultiert in großen ökonomischen Verlusten im nordwestlichen Teil von China. Daher gibt es Bemühungen, einen ELISA auf der Basis eines immundominanten, rekombinant exprimierten Proteins von *T. uilenbergi* zu etablieren um epidemiologische Studien in diesem Gebiet durchzuführen. Im Rahmen der vorliegenden Arbeit wurde der Einsatz des Klon-9b Proteins für diesen Zweck untersucht. Das Protein wurde als ein potentielles, immunogenes Protein des Piroplasmenstadiums des Parasiten durch Sequenzieren von Stichproben von Klonen einer cDNA Bibliothek und bioinformatischen Analysen identifiziert. Das Klon-9 Gen wurde partiell rekombinant exprimiert und für die Entwicklung eines indirekten Enzym-gekoppelten Immunadsorptionstests (ELISA) zum Nachweis von zirkulierenden Antikörpern in Seren von *T. uilenbergi*-infizierten Schafen verwendet (rc9b ELISA). Dabei wurde keine Kreuzreaktivität mit Seren von Tieren, die mit *T. lestoquardi* infiziert waren beobachtet. Der Schwellenwert (cut-off) von 48,6 % Positivität wurde mit Hilfe von 25 Serumproben von nicht-infizierten Tieren ermittelt. Die Eignung des rc9b ELISA für die Untersuchung von Feldseren wurde mittels der Analyse von Proben, die aus einem Endemiegebiet in China stammten, etabliert. Dabei zeigte der rc9b ELISA eine Übereinstimmung von 85 % mit einem anderen ELISA für die Detektion von oviner Theileriose in China.

Der zweite Teil dieser Arbeit beschäftigt sich mit der Diagnostik der Tropischen Theileriose. Verschiedene DNA-basierte Tests wie die Polymerase-Ketten-Reaktion, der Reverse-line Blot und die 'loop mediated isothermal amplification' (LAMP) sind für die Detektion einer Infektion mit *T. annulata* etabliert worden. Ebenso serologische Tests wie der indirekte und der kompetitive ELISA. In dieser Arbeit ist das Wissen über die Entwicklung eines indirekten bzw. kompetitven ELISA, die auf rekombinant hergestelltem *T. annulata* surface protein' (TaSP) basieren, angewendet worden, um einen Schnelltest für den Einsatz unter Feldbedingungen durch den Tierarzt für die Diagnose einer Infektion mit *T. annulata* zu entwickeln. Der Test ermöglichte die spezifische Detektion von Antikörpern im Serum von

experimentell mit *T. annulata* infizierten Tieren und zeigte keine Kreuzreaktivität mit Serum von Tieren, die mit anderen Erregern experimentell infiziert waren (*Trypanosoma brucei*, *Anaplasma marginale*, *Babesia bigemina*, *Babesia bovis* und *T. parva*). Die Untersuchung von Feldseren wurde mit Ergebnissen verglichen, die durch andere serologische Tests ermittelt wurden. Dieser Vergleich resultierte in einer Sensitivität bzw. einer Spezifität von 96,3 % bzw. 87,5 % mit dem IFAT als Referenztest, von 98,7 % bzw. 81,8 % mit dem indirekten ELISA als Referenztest und 100 % bzw. 47,6 % mit dem kompetitiven ELISA als Referenztest. Es wurde also ein Schnelltest für den Nachweis einer Infektion mit *T. annulata* entwickelt, der einfach durchzuführen ist und Ergebnisse liefert, die mit dem bloßen Auge innerhalb von zehn Minuten zu lesen sind und der geeignet ist, die Infektion in Feldseren zu detektieren.

9 Abbreviations

AP	Alkaline phosphatase
APS	Ammoniumperoxide sulfate
BCIP	5- Bromo-4-chloro-3-indolyl phosphate
BLAST	The Basic Local Alignment Search Tool
Bp	Base pair
BSA	Bovine serum albumin
C9	Clone 9
cDNA	Complimentary deoxyribonucleic acid
cELISA	Competitive enzyme linked immmunosorbent assay
DNA	Deoxyribonucleic acid
DNase	Deoxynuclease
dNTP	Deoxynucleotide triphosphates
DTT	Dithiotreitol
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immmunosorbent assay
HCG	Human chorionic gonadotropin
HRP	Horseradish peroxidase
HSP	Heat shock protein
ICT	Immunochromatographic test
IFAT	Indirect fluorescent antibody test
IFN-γ	Interferon gamma
IL	Interleukin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kDa	Kilo Dalton
LAMP	Loop-mediated isothermal amplification
LB medium	Luria Bertani medium (broth)
LFA	Lateral flow assay
LFD	Lateral flow device
LVRI	Lanzhou Veterinary Research Institute
MW	Molecular weight
NBT	Nitroblue tetrazolium

NC	Nitrocellulose
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
ORF	Open reading frame
P.i.	Post infection
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween20
PCR	Polymerase chain reaction
PP	Percent positivity
RLB	Reverse line blot
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SPAG-1	sporozoite antigen
TaD	*T. annulata* membrane protein
Tams-1	*Theileria annulata* merozoite surface protein
TaSE	*T. annulata* secretory protein
TaSP	*Theileria annulata* Surface protein
TBE	Tris/Boric acid/EDTA
TBS	Tris buffered saline
TE	Tris EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)amino-methane
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
WB	Western-blot
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
x g	Gravity force

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