Aus dem Department für Veterinärwissenschaften der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München
Lehrstuhl für Vergleichende Tropenmedizin und Parasitologie
Vorstand: Prof. Dr. med. vet. Kurt Pfister

Angefertigt am
Forschungszentrum Borstel
Leibniz-Zentrum für Medizin und Biowissenschaften
Abteilung Immunologie und Zellbiologie
Laborgruppe Veterinär-Infektiologie und -Immunologie
(Prof. Dr. med. vet. Jabbar S. Ahmed)

Identification of Novel Genes for the Development of a Rapid Diagnostic Test for *Theileria uilenbergi* Infection by Screening of a Merozoite cDNA Library

Inaugural-Dissertation
zur Erlangung der tiermedizinischen Doktorwürde
(Dr. med. vet.)
der Tierärztlichen Fakultät der
Ludwig-Maximilians-Universität München

von

Zhijie Liu

aus
Gansu, VR China

München 2010

Gedruckt mit Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Braun

Berichterstatter: Univ.-Prof. Dr. Pfister

Korreferent: Univ.-Prof. Dr. Aigner

Tag der Promotion: 13. Februar 2010

Table of contents

1	**Introduction**	1
2	**Literature review**	3
2.1	*Theileria* taxonomy	3
2.2	*Theileria* life cycle	3
2.3	Pathology and pathogenesis	4
2.4	Important *Theileria* species of sheep and goats	5
2.4.1	*Theileria lestoquardi*	5
2.4.2	*Theileria luwenshuni and T. uilenbergi*	6
2.4.2.1	Brief history and economic importance	6
2.4.2.2	Phylogeny	7
2.4.2.3	Transmission	9
2.4.2.4	Morphology, pathogenicity and immune response	9
2.4.2.5	Distribution and epidemiology	11
2.4.2.6	Diagnostics	13
3	**Materials and methods**	17
3.1	Establishment and screening of a merozoite cDNA library of *T. uilenbergi*	17
3.1.1	Infection of experimental animals	17
3.1.2	Purification of merozoites	17
3.1.3	Preparation of total RNA and mRNA	18
3.1.4	Establishment of the cDNA library	19
3.1.4.1	cDNA synthesis	19
3.1.4.2	Modification of the cDNA termini and size fractionation	20
3.1.4.3	Ligation of the cDNA into the Uni-ZAP XR vector	21
3.1.4.4	Packaging of the ligation reaction products	22
3.1.4.5	Plating of bacterial strains	22
3.1.4.6	Preparing host bacteria	23
3.1.4.7	Titration of the packaging reactions	23
3.1.4.8	Determining background by blue-white color selection	24
3.1.4.9	Amplification, titration and storage of the library	24
3.1.5	Generating subtraction library by in vivo mass excision	25

3.1.6	Identifying the cDNA inserts from phagemid subtraction library	26
3.1.6.1	Isolation of plasmid from overnight bacteria culture	26
3.1.6.2	PCR identification of the cDNA inserts	26
3.1.7	Sequence analysis and characterization of the Clone 2 gene family	27
3.1.7.1	Identification of Clone 2 gene family heterologous genes from *T. luwenshuni*	27
3.1.7.2	Protein expression and purification of Clone 2	28
3.1.7.3	Western blot and silver staining	30
3.2	Practical protocol for loop-mediated isothermal amplification (LAMP) assay	32
4	**Results & publications**	34
4.1	Publication 1: Small-scale expressed sequence tag analysis of *Theileria uilenbergi*: Identification of a gene family encoding potential antigenic proteins	34
4.2	Publication 2: Development of loop-mediated isothermal amplification (LAMP) assay for rapid diagnosis of ovine theileriosis in China	41
5	**Discussion**	54
6	**References**	59
7	**Summary**	69
8	**Zusammenfassung**	71
9	**Abbreviations**	73
10	**Publications**	75
11	**Acknowledgements**	77

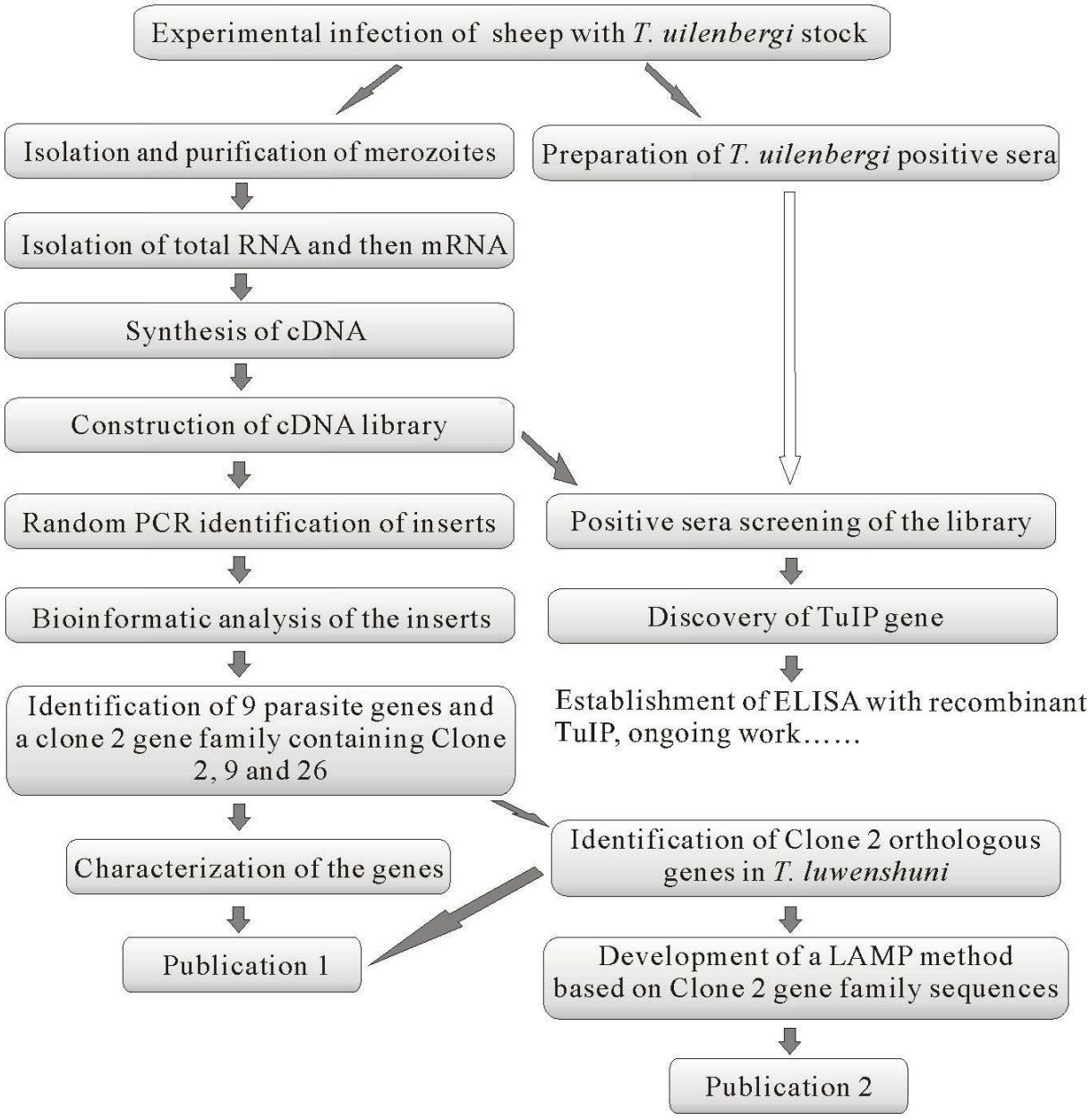
1 Introduction

Theileria is a tick-borne protozoan parasite belonging to the phylum Apicomplexa (Boulter and Hall, 1999). Theileriosis of small ruminants in China is caused by *Theileria luwenshuni* (previously referred to as *Theileria* sp China 1) and *T. uilenbergi* (previously referred to as *Theileria* sp China 2), which are newly identified and designated *Theileria* parasites (Ahmed et al., 2006; Yin et al., 2007). *T. luwenshuni* and *T. uilenbergi* are transstadially transmitted by the three host ticks *Haemaphysalis qinghaiensis* and *H. longicornis* (Li et al., 2009; Li et al., 2007; Yin et al., 2002a). The early symptoms of *T. luwenshuni* or *T. uilenbergi* infection are fever, inappetence, cessation of rumination, dyspnea, weakness, listlessness and swelling of the superficial lymph nodes, followed by development of marked icterus and anemia (Luo and Yin, 1997). The pathogenesis of the disease is believed to be closely associated with not only schizonts but also the proliferation of the intraerythrocytic piroplasms (Yin et al., 2003). The disease is widely distributed in northwest China with an infection rate of 64.8-91.7% (Guo et al., 2002) and economically it constitutes a major constraint on the small ruminant husbandry industry in endemic areas. Control of the disease mainly depends on eradicating the transmitting ticks by acaricides, while treatment of sick animals is performed with anti-parasitic compounds such as Primaquin, Berenil and Chloroquine (Luo and Yin, 1997).

Traditionally, the diagnosis of the disease is based on observation of the clinical signs or examination of piroplasms from blood smears using light microscopy, which needs expertise and is inefficient for epidemiological studies. In recent years, reverse line blot (RLB) (Schnittger et al., 2004) and polymerase chain reaction (PCR) (Yin et al., 2008) have been developed for detection and differentiation of *T. uilenbergi* and *T. luwenshuni* infections. These methods are sensitive, specific and have great value for epidemiological studies; however they are expensive and laborious, thus difficult to apply in the disease endemic regions by local veterinarians. In fact, these methods are currently only in use as laboratory reference diagnostic methods. Regarding serological tests, two enzyme-linked immunosorbent assays (ELISAs) have been developed, one of which is based on crude merozoite antigen (lysate of the whole merozoite) (Gao et al., 2002) and the other is based on recombinant protein TIHSP 70 originally identified from *T. lestoquardi* (Miranda et al., 2006a). However, the crude antigen ELISA is difficult to standardize, requires infection of animals for antigen preparation and is potentially cross-reactive with other related pathogens; the rTIHSP 70

antigen ELISA has not yet been evaluated and its potential cross reactions with many other pathogens cannot be excluded. Therefore, the development of novel, simple, rapid and more reliable diagnostic tools is still a prerequisite and a key element for devising integrated control measures against Chinese *Theileria* infections.

In this study, the goal was to identify novel genes of *T. uilenbergi* for development of a rapid diagnostic tool through establishment and screening of a merozoite cDNA library. The strategy is illustrated by a flow chart as follows:



2 Literature review

Theileria are tick-transmitted, obligate intracellular protozoan parasites that are important pathogens of livestock in the tropical and subtropical regions of the Old World (Dolan, 1989; Shaw, 2002). They are transmitted by two or three host ixodid ticks. The main bovine pathogens are *T. parva*, *T. annulata*, *T. mutans* and the *T. orientalis*/*T. sergenti*/*T. buffeli* complex. The present review will emphasize on current knowledge covering *Theileria* classification, life cycle and pathogenesis in general, with special attention to the progress of ovine theileriosis in China.

2.1 *Theileria* taxonomy

Traditionally, *Theileria* taxonomy has been mainly based on morphology, life cycle characteristics, transmitting ticks and some other biological parameters as the presence of macroschizonts, geographic origin and mammalian host (Cavalier-Smith, 1993; Irvin, 1987; Mehlhorn et al., 1987; Levine et al., 1980; Levine, 1988). With the development of molecular phylogenetic analysis, the comparison of small ribosomal RNA gene sequences has been introduced for deducing phylogenetic relationships of the Piroplasmidia and other Apicomplexa (Allsopp et al., 1993; Chansiri et al., 1999; Ellis et al., 1992; Gajadhar et al., 1991; Marsh et al., 1995). Phylogenetic analysis based on this method is unambiguous in separation of *T. parva* and *T. annulata*, *T. lestoquardi* and *Theileria* species in China (Gubbels et al., 2002; Schnittger et al., 2003; Yin et al., 2004a). As an additional approach, the major piroplasm surface protein (MPSP) gene sequences and ribosomal RNA internal transcribed spacers (ITS1 and ITS2) have been used for taxonomy of the ‘benign’ *T. orientalis*/ *T. sergenti*/*T. buffeli* complex (Aktas et al., 2007; Allsopp et al., 1994; Gubbels et al., 2000; Kakuda et al., 1998). However, neither of these methods have been able to define conclusively taxonomic status of the benign *Theileria* group (Chae et al., 1999; Kawazu et al., 1999), highlighting the need for additional studies using complementary approaches.

2.2 *Theileria* life cycle

Theileria life-cycle stages in the tick vector and bovine host have been intensively reviewed previously (Mehlhorn and Schein, 1984; Shaw, 2002; Bishop et al., 2004) and the reader is referred to these articles for additional details. The life cycles of all *Theileria* species are generally similar (Shaw, 2002). Infection of vertebrate hosts with *Theileria* starts with the

release of *Theileria* sporozoites by infested ticks taking a blood meal. Infective sporozoites are released from about day 3 to day 7 of tick feeding (Mehlhorn and Schein, 1984; Shaw, 2003). When introduced into the host, *Theileria* sporozoites invade a restricted range of lymphocytes (Baldwin et al., 1988; Shaw, 1997; Ahmed et al., 1984; Glass et al., 1989; Spooner et al., 1989; Dobbelaere and Heussler, 1999). The sporozoites differentiate to the schizont stage; and then the schizont undergoes differentiation and cellularization process to produce uninucleate merozoites (Shaw and Tilney, 1992). The merozoite invasion of erythrocytes occurs in a similar manner to sporozoite entry (Shaw and Tilney, 1995).

The life cycle of *Theileria* in ticks begins with the ingestion of piroplasm-infected erythrocytes during the blood meal. The ingested piroplasms are rapidly destroyed in the gut lumen and then 'ray bodies' form (Bishop et al., 2004). By the fifth day after 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 Shein, 1984). The motile kinetes finally enter into the acinar cells of the salivary gland in which sporogony occurs. When infested ticks attack animals, sporozoites are released into the mammalian host and a new life cycle starts.

2.3 Pathology and pathogenesis

The schizont of *T. parva* and *T. annulata* initially causes a lymphoproliferative, and later a lymphodestructive disease. The infected animal shows enlargement of the lymph nodes, fever, a gradually increasing ventilation rate, dyspnea and/or diarrhea. The most common post-mortem lesions are enlarged lymph nodes, a markedly enlarged spleen, and pulmonary edema (Irvin and Morrison, 1987; Mehlhorn, 2008). Studies on *T. annulata* infection have shown that the overproduction of cytokines (in particular TNF-alpha) by schizont-infected cells and uninfected cells underlies many of the clinical symptoms and pathological reactions (Preston et al., 1993). The schizont-infected cells become disseminated rapidly through the lymphoid tissues (Forsyth et al., 1999). Tissue responses throughout the lymphoid organs are dominated by macrophages and there are no signs of extensive populations of large lymphoid cells as found in the lymphoid tissues of cattle infected with *T. parva* (Preston et al., 1999; Uilenberg, 1999). As infection progresses, the schizonts differentiate into merozoites. By the

late stages of disease, the cells containing merozoites greatly out-numbered schizont-infected cells. The parasitized mononuclear cells were observed containing erythrocytes parasitized by piroplasms, suggesting that the parasitized mononuclear cells themselves play a role in the development of clinical disease and in tissue damage (Forsyth et al., 1999).

For the *T. orientalis* complex, the schizonts do not develop in leukocytes and therefore do not induce transformation and fatal lymphoproliferation (Sugimoto and Fujisaki, 2002). The infected cells in these organs show an enlarged cytoplasm and giant nuclei, subsequently merogony appears (Kawazu et al., 1991; Sato et al., 1993; Sato et al., 1994). After the transient schizont stage, piroplasms appear in erythrocytes and at the same time a transient fever may be observed with developing anemia. Anemia is caused likely by the extravascular removal of parasitized or even uninfected erythrocytes from the circulation system (Yagi et al., 1991). Animals that recover from infection carry a persistent infection. Occasionally, a relapse may occur when the animal is under condition of stress (Sugimoto and Fujisaki, 2002).

2.4 Important *Theileria* species of sheep and goats

T. lestoquardi, *T. uilenbergi*, *T. luwenshuni*, *T. separata*, *T. ovis* and *T. recondita* are currently recognized species of *Theileria* infecting sheep and goats (Ahmed et al., 2006, Uilenberg, 1981). The first three species are pathogenic; the last three are non-pathogenic. The available knowledge on the last three species is limited. It is known that *T. separata* is distributed in eastern and southern Africa and transmitted transtadially by *Rhipicephalus evertsi* (Uilenberg and Andreasen, 1974). *T. ovis* is distributed in Africa, Europe and Asia and transmitted by *R. evertsi* (Uilenberg, 1981).

2.4.1 *Theileria lestoquardi*

T. lestoquardi (syn *T. hirci*) as the causative agent of malignant theileriosis of sheep and goats was first described by a veterinary inspector in Egypt. It was recorded that the pathogen of two fatal cases in sheep in Sudan was a small, polymorphic intraerythrocytic piroplasm with exoerythrocytic macroschizonts (as ‘Koch’s blue bodies’) in the spleen and lymph nodes. The pathology, smallness of the piroplasms and the exoerythrocytic schizonts led them to believe that this parasite was a *Theileria*. Later it was known as *T. hirci* (Dchunkowsky and Urodschevich, 1924) and eventually as *T. lestoquardi* (Morel and Uilenberg, 1981).

T. lestoquardi exhibits many similarities to *T. annulata* in terms of biology, serology and

morphology. For example, they share the same transmitting vector *Hyalomma anatolicum anatolicum* and the way of transmission is transstadial (Hooshmand-Rad and Hawa, 1973). *In vivo* experiments have shown that *T. annulata* can infect both cattle and sheep, but is not pathogenic to sheep, while *T. lestoquardi* is only infective to sheep and goats (Brown et al., 1998). Recently, molecular phylogenetic studies based on 18S rRNA gene comparison confirmed that these species are genetically close to each other (Schnittger et al., 2000a). The symptoms of *T. lestoquardi* infection consist of high fever, listlessness, anorexia and emaciation, diarrhea or constipation, enlarged superficial lymph nodes and pale and icteric mucous membranes, which are similar to those of cattle undergoing acute tropical theileriosis. The pathological findings recorded for malignant theileriosis of sheep and goats are also very similar to those described for tropical theileriosis (Neitz, 1957, Hooshmand-Rad and Hawa, 1973). Sporozoites of *T. lestoquardi* can transform mononuclear cells of sheep *in vitro*. This led to the successful cultivation of the schizonts of *T. lestoquardi in vitro* (Hooshmand-Rad and Hawa, 1975). The mechanism of pathogenesis and immune response of *T. lestoquardi* are not well studied. However, it is known that animals that survive infection are resistant to challenge and goats show a more significant resistance to the disease compared to sheep (Brown et al., 1998).

The diagnosis of *T. lestoquardi* infection is mainly based on the clinical signs and the demonstration of the schizont stage in lymph nodes or organ smears, or/and piroplasms in blood smears. The available serological diagnostic tools are the indirect fluorescent antibody test (IFAT) (Leemans et al., 1997) and the enzyme-linked immunosorbent assays (ELISA) (Bakheit et al., 2006b). The molecular diagnostic tools are the polymerase chain reaction (PCR) (Kirvar et al., 1998) and the reverse line blot (RLB) (Schnittger et al., 2004). These methods provide important means for monitoring the disease or for epidemiological surveys. At present, control of the disease is based on chemical therapy involving buparvaquone or immunization with attenuated *T. lestoquardi* schizonts (Hashemi-Fesharki, 1997).

2.4.2 *T. luwenshuni* and *T. uilenbergi*

2.4.2.1 Brief history and economic importance

Ovine theileriosis is an important disease of sheep and goats in northwest China. It is said that the disease has existed in the Gannan Tibet region of Gansu Province for more than 100

years (Guo et al., 2002). However, the disease was not discovered until 1958 when it was first reported in Sichuan Province by a local veterinarian (Yang, et al., 1958). Subsequently, the disease was found to be widely distributed in the other neighboring provinces, which include Ningxia, Inner Mongolia, Qinghai and Shaanxi (Luo and Yin, 1997). The pathogen causing this disease was firstly recognized as *T. lestoquardi* (syn *T. hirci*) owing to its pathogenicity and high morbidity and mortality. However, investigations based on biological findings and molecular taxonomic approaches revealed that ovine theileriosis was not caused by one but two *Theileria* species, named *T. luwenshuni* and *T. uilenbergi* in 2007 (Ahmed et al., 2006; Yin et al., 2007).

Theileriosis is considered an important disease because of its severe economic impact on the sheep and goat production. It has been crudely estimated that approximately 35 million small ruminants in northwest China are suffering from tick-borne diseases including anaplasmosis, babesiosis and theileriosis, and among these diseases, theileriosis causes the most economic damage. As the economic loss per capita of the sheep or goats infected by these tick-borne pathogens is at least 2 USD, the total annual economic loss in the small ruminant husbandry due to tick-borne diseases is estimated to be around 70 million USD (Yin and Luo, 2007).

2.4.2.2 Phylogeny

As mentioned above, *Theileria* species of small ruminants include the non-pathogenic species *T. ovis*, *T. separata* and *T. recondita* (Alani and Herbert, 1988; van Vorstenbosch et al., 1978; Uilenberg, 1981); the pathogenic species *T. lestoquardi* (Morel and Uilenberg, 1981) and the two newly identified Chinese *Theileria* species (Ahmed et al., 2006; Yin et al., 2007). *Theileriosis* in China was first considered to be caused by *T. lestoquardi* due to its high pathogenicity and since *T. lestoquardi* was the only known pathogenic *Theileria* species of small ruminants at that time (Luo and Yin, 1997). However, findings contradicting this supposition were that, firstly, *T. lestoquardi* is transmitted by ticks of the genus *Hyalomma* (Hooshmand-Rad and Hawa, 1973), while the infectious agents in China are transmitted by ticks of the genus *Haemaphysalis* (Li et al., 2009; Li et al., 2007). Secondly, *in vitro* culture of *T. lestoquardi* schizonts can be established with relative ease, whereas *in vitro* transformation of leukocytes by the Chinese *Theileria* species has so far been unsuccessful. Finally, investigations using comparison of the 18S rRNA gene sequence of the Chinese *Theileria*

with the sequences of *T. lestoquardi*, *T. annulata*, *T. parva*, *T. taurotragi*, *T. sergenti* and *T. buffeli* as well as *Babesia* species, was carried out (Schnittger et al., 2000a). This analysis showed that the Chinese *Theileria* was most closely related to the *T. buffeli* and *T. sergenti* group and was clearly divergent from *T. lestoquardi*, suggesting a new *Theileria* species (Schnittger et al., 2000b). This discovery helped initiate the taxonomic study of Chinese *Theileria* species. Later, alignment of the complete 18S rRNA gene sequences of more Chinese *Theileria* species by using the neighbor joining method revealed that besides the previously described *Theileria* species (China), another distinct pathogenic *Theileria* parasite occurs in the same region, falling into a separate taxonomic cluster (Schnittger et al., 2003; Yin et al., 2004) as shown in Fig 1. Accordingly, these two parasites were designated as *Theileria* sp 1 (China) and *Theileria* sp 2 (China), and eventually they were renamed as *T. luwenshuni* and *T. uilenbergi* respectively (Ahmed et al., 2006; Yin et al., 2007).

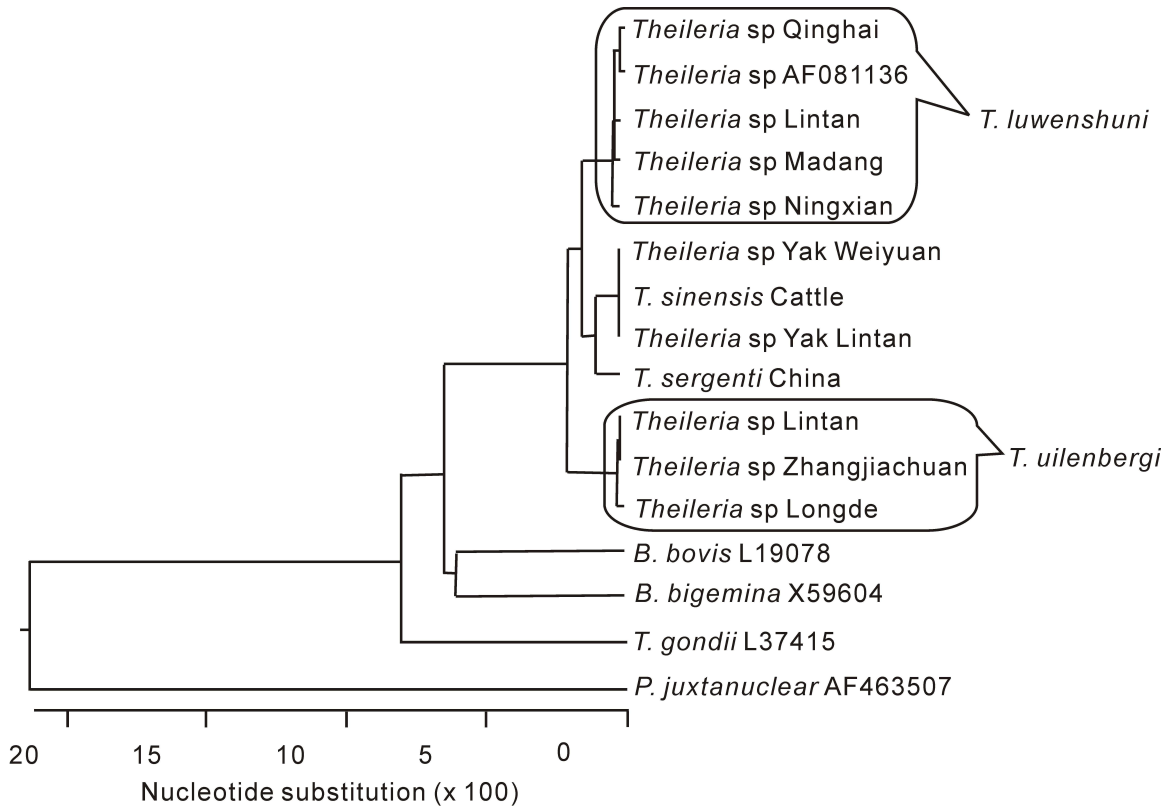


Fig. 1 Rooted neighbor-joining phylogenetic tree of the 18S ssrRNA gene of Chinese *Theileria* spp. Cited from Yin et al., 2004.

Despite molecular differentiation, the two parasites are morphologically indistinguishable sharing identical biological characteristics with regard to tick vectors, specificity of animal hosts and pathogenicity. Therefore, further phylogenetic analysis of these species with new genetic markers is still needed and essential.

2.4.2.3 Transmission

Transmission of ovine *Theileria* to sheep by adult *H. qinghaiensis* ticks collected in Ningxia Province was confirmed in the 1980's. Transmission experiments showed that *Dermacentor silvarum* was unable to transmit the disease although this tick widely co-exists in the theileriosis endemic regions (Li et al., 1983; 1986). Transmission experiments suggested that the adult ticks moving on sheep or goats, the nymphal and adult ticks from grassland and even the partially engorged ticks of *H. qinghaiensis* could transmit the disease to sheep and goats (Yin et al., 2002b). More recently, well designed transmission experiments showed that the nymphs developing from larvae engorged on sheep infected with the parasite transmitted the pathogen to splenectomized sheep in 30 days; but the subsequent adult ticks of *H. qinghaiensis* derived from these nymphs did not transmit the pathogen to sheep. Similarly, adults developed from nymphs engorged on sheep infected with the parasite transmitted the pathogen to sheep in 24–27 days. The larvae, nymphs and adult ticks derived from female *H. qinghaiensis* ticks engorged on infected sheep were unable to transmit the parasite, suggesting the absence of a transovarian route of transmission. The same experimental design was used for investigation with *Hy. a. anatolicum*, showing that this tick could not transmit the pathogens to sheep and goats (Yin et al., 2002c).

Besides *H. qinghaiensis*, the nymphs and adults of *H. longicornis* developed from larvae and nymphs engorged on infected sheep have been shown to transmit *T. uilenbergi* and *T. luwenshuni* transstadially (Li et al., 2007; Li et al., 2009).

2.4.2.4 Morphology, pathogenicity and immune response

T. uilenbergi and *T. luwenshuni* are morphologically indistinguishable. Both parasites are pleomorphic in the piroplasm stage as shown in Fig. 2 and 3. They appear typically as single piriform, ring, parachute, semicolon, nail needle, rod, round, oval, three-leafs, cross-shaped or amoeboid forms. Round, oval and piriform are the dominant shapes (Luo and Yin, 1997). The observation of experimentally infected sheep demonstrated that the ring form constituted 32.7%; parachute 29.2%; semicolon 15.3%; oval 10.2%; nail 5.5%; rod 5.0% with other forms 2.1% (Guo et al., 2002). Similar forms are seen usually in naturally infected sheep. In experimentally infected sheep, the piriform and needle forms were found first, then rod, round and oval forms. Three-leafed and cross-shaped parasites appeared in the later period of

infection. The dimensions of piriform parasites were 0.8-1.8 × 1.2-2.1 μm; needle-shaped parasites 0.5-0.9 × 1.8-2.5 μm; rod-shaped parasites 0.4-1.0 × 1.6-2.3 μm; round or oval-shaped parasites 1.0-2.5 μm. The ratio of the round, the piriform and the needle was 11:8:5 when the parasitemia reached the peak (Li et al., 2009).

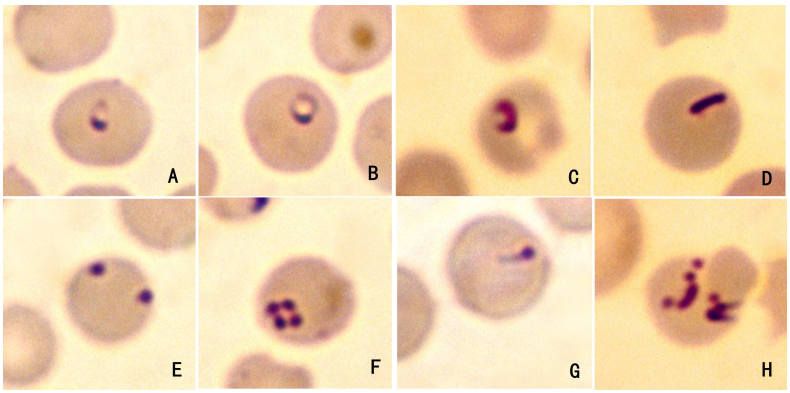


Fig. 2 Morphological shapes of *T. luwenshuni* piroplasm in erythrocytes.

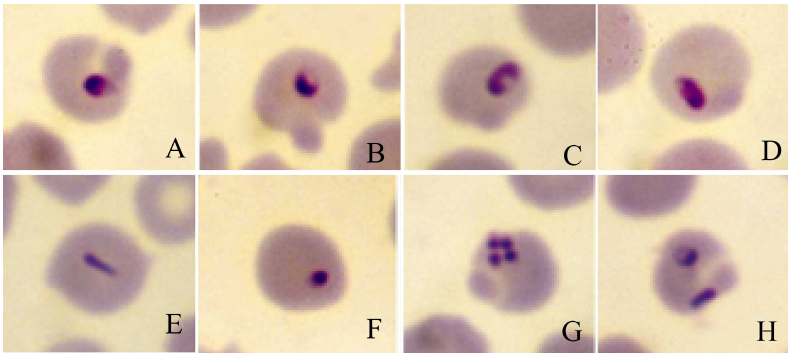


Fig. 3 Morphological shapes of *T. uilenbergi* piroplasm in erythrocytes.

Like the schizonts of *T. parva*, *T. annulata* and the benign *Theileria* species, two types of schizont, large and small were demonstrated in smears prepared from liver, spleen, lung, kidney and peripheral lymph nodes of the sheep infected with Chinese *Theileria* species. The dominant form was microschizonts, whereas macroschizonts were rarely seen (Yin et al., 2003). Furthermore, it was noted that most schizonts were outside the lymphocytes, either at the early period of infection or at the late stage of theileriosis (Luo and Yin, 1997; Yin et al., 2003).

Little is known about the basis for the pathogenicity of the Chinese *Theileria* parasites. It seems that their life cycle is similar to other *Theileria* species (Schnittger et al. 2000a). The evidence that the infected animals often die before the piroplasm can be seen in the blood smears and the widespread occurrence of the schizonts in many organs and tissues indicate that the schizonts play a role in the pathogenicity of the disease (Yin et al., 2003). Since

Chinese *Theileria* are phylogenetically close to the *T. buffeli* complex, it is assumed that the pathogenicity seems to be also closely associated with the proliferation of the intraerythrocytic piroplasms (Schnittger et al. 2000a), which is indicated by remarkable anemia and icterus in animals infected by these parasites (Yin et al., 2003).

Regarding the immune response, it is assumed that piroplasm antigens may play the major role in the induction of a protective immune response inducing $CD4^+$ T cells and opsonizing antibodies. Specific immune responses to the two parasite proteins, TaD and TcSP, were investigated independently in both tick-infested and parasitized-blood-infected animals. The presence of specific IgG antibodies directed against these two antigens strongly indicated that $CD4^+$ T cells are involved. Moreover, the PBMC from some animals infected by ticks exhibited a markedly higher *in vitro* proliferative response to each of the two proteins compared to blood-infected animals. This may reflect the exposure to the immune system of the different parasite stages in tick infected animals, leading to a booster effect during the infection process (Seitzer et al., 2008).

2.4.2.5 Distribution and epidemiology

It is said that ovine theileriosis has existed in Gannan Tibet region of Gansu Province for more than 100 years. However, the disease was not reported by a local veterinarian in Ganzi County of Sichuan Province until 1958 (Yang, et al., 1958). Thereafter, microscopic examination of blood smears has shown that the disease was widely distributed in northwest China including Gansu (Qiu and Yuan, 1982), Ningxia (Li, et al., 1985), Inner Mongolia (Yu, et al., 1982), Qinghai in 1963 (Wang, et al., 1980), Sichuan (Yang, et al., 1958) and Shanxi (Li, et al., 1985) Province as shown in Fig 4. All of these provinces are adjacent to Gansu Province, comprising the major ovine theileriosis endemic region. In 1993, a systematic survey was carried out to investigate ovine theileriosis via microscopic examination of blood smears in 45 counties in Gansu Province. The results showed that the disease was endemic in 35 counties (Lu, et al., 1994).

The disease distribution was always closely associated with the distribution of its transmitting vectors. In a tick survey in Gannan Tibet region in Gansu Province, the following ticks have been found: *Dermacentor abaensis*, *D. silvarum*, *D. sinicus*, *D. nuttalli*, *D. coreus*, *Ixodes ovatus*, *I. persulcatus*, *I. pomerantzevi*, *I. crenulatus*, *H. qinghaiensis*, *H. aponommoides*, *H. concinna*, *H. bispinosa and Rhipicephalus sanguineus*. Among these

ticks, *H. qinghaiensis* and *D. silvarum* are the dominant species. Experiments have shown that *H. qinghaiensis* transmits the disease while *D. silvarum* and *D. abaensis* do not (Li, et al., 1986; Guo, et al., 2002). Whether or not the rest of the ticks existing in the area can transmit the disease is unknown. Indeed, in all provinces mentioned above, *H. qinghaiensis* is always found together with the disease and was considered as the only tick species responsible for transmission (Luo and Yin, 1997). Exceptionally, ovine theileriosis was also found in Hebei and Liaoning Province which are not neighbors of the major ovine theileriosis endemic regions (Zhu, et al., 1990; Ren, et al., 1982) and *H. qinghaiensis* does not exist in these provinces. Recent experiments have demonstrated that *H. longicornis* is able to transstadially transmit *T. uilenbergi* and *T. luwenshuni*, and is believed to be responsible for disease transmission in these regions (Li et al., 2009; Li et al., 2007). Apart from China, the occurrence and distribution of this disease in other parts of the world is unclear, however, recently a parasite showing close identity on the 18 S RNA gene level was described in Spain (Nagore et al., 2004).

Fig. 4 Distribution of ovine theileriosis in northwest China.
The endemic regions are indicated by filled circles.

As stated previously, the presence of ovine theileriosis is closely correlated with the activity of *H. qinghaiensis*. In the Tibetan Region in Gansu Province, the ticks appear in early March, through March, April and May, which are the months of greatest activity. The ticks are no longer active in June. In late August, some ticks can again be found, but are undetectable again in November. In the spring, when the temperature is high, the ticks appear in late February; however, when the temperature is low, they appear in mid-March. Naturally infected hosts are usually found 2 weeks after tick infestation. In experimental infections, the

incubation period was 4-12 days (Guo et al., 2002). Sickness and death rates vary considerably with age and breed of animals. Studies on 1144 sheep and goats in the mountain districts in the south of Ningxia Province showed that the incidence and lethality were 28.3% and 75.3% respectively. Most cases were lambs and animals aged 1-2 years (Li, et al., 1985). Imported animals are more susceptible to this disease. As recorded in 1971, of 320 sheep brought to Banma County in Qinghai Province, 200 sheep (62.5%) and 100 out of the 140 new-born lambs (71.4%) died (Zhou, et al., 1982). In Shaanxi Province, 65 out of 82 sheep (79.2%) imported from Shandong Province were sick and 21 died (Li and Niu, 1995). Both sheep and goats were affected by the disease with the incidence higher in sheep than in goats (27.63% and 13.12%, respectively). The incidence in young animals is higher than in adults (lambs 60.81%, young goats 40%, adult sheep 17.12%, and adult goats 8.06%). The death rate of young animals was also higher than that of adults (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 to that of local herds (Guo et al., 2002).

Recently, a large scale investigation was carried out, in which 1,165 serum samples collected from Biandu and Yangyong districts of Lintan County, Kache, Azitan and Wanmao districts of Zhuoni County and Ganjia, Zhayou and Madang districts of Xiahe County from July 2004 to June 2005 in Gannan Tibet Autonomous Region, Gansu Province were tested by crude merozoite antigen ELISA. The results showed that seroprevalence of reactive antibodies varied from 27.8 to 83.3%, whereby the average was 70.1% in the disease-endemic areas (Guo et al., 2007). Later, the corresponding 1,117 DNA samples were tested by reverse line blot (RLB) and the results showed 80.3% positive samples, a value higher than that obtained by ELISA (Niu et al., 2009a).

2.4.2.6 Diagnostics

The conventional diagnostic methods for ovine theileriosis in China depend on clinical signs, microscopic examination of the blood smears and biopsy smears, and epidemiological history. When animals become infected, the first sign is fever. The body temperature rises to 41.0°C-42.3°C and the fever is continuous or intermittent persisting for 12-20 days. A few days after fever onset, other symptoms appear which include inappetence, cessation of rumination, rapid heartbeat, dyspnea, weakness, listlessness and swelling of the superficial lymph nodes. Marked anemia and icterus develop in a few days. The piroplasms in

erythrocytes are predominantly round, oval and piriform but can also be rod-shaped, needle-shaped. The parasitemia of naturally infected sheep range from 3.2% to 3.7%; those of experimentally infected sheep can be as high as 53.4%. In a single erythrocyte, 1-7 piroplasms could be found. The percentages of 1, 2, 3, 4, 5-7 piroplasms in one cell were 50.8%, 33.9%, 12.7%, 2.1%, and 0.5% respectively. The schizonts were distributed throughout the lymphocytes and during the later period of the disease, many schizonts were found outside the cells (Guo et al., 2002; Luo and Yin, 1997).

In contrast to conventional methods, the molecular based tests would allow direct, specific and sensitive detection of parasites infecting a given animal. Currently there are three DNA-based diagnostic tests established.

Reverse line blotting (RLB), a PCR based and a molecular genetic diagnostic assay, enables detection of ovine/caprine piroplasm species via the recognition of species-specific gene regions. Oligonucleotide probes for this test were designed based on the hypervariable region 4 (V4 region) of the 18S small ribosomal RNA gene of ovine/caprine piroplasm species, and RLB enables the direct, concurrent, highly specific and sensitive identification of virtually all presently known ovine/caprine piroplasm species, including *T. luwenshuni* and *T. uilenbergi*. The RLB could detect parasitemia as low as 10^{-12}%, which increases the possibility of identifying carrier animals (Schnittger et al., 2004). Due to the fact that this assay allows screening of a great number of field samples, it is helpful for professional epidemiological studies.

The polymerase chain reaction (PCR) has been used for detection and differentiation of *T. uilenbergi* and *T. luwenshuni* based on the V4 region of 18S rRNA gene (Yin et al., 2007). It is another molecular diagnostic tool that enables detection of mixed infections of *Theileria*. However, both PCR and RLB are quite expensive and laborious; they are inconvenient for practical application in field.

Loop mediated isothermal amplification (LAMP) is a novel DNA detection method with the great advantages of being fast, easy and cheap. It has been successfully applied in detection of viral, bacterial and parasitic infections (Dukes et al., 2006; Njiru et al., 2008; Seki et al., 2005). This method employs a DNA polymerase and a set of four (or six) specially designed primers that recognize a total of six (or eight) distinct sequences on the target DNA as shown in Fig. 5. The principle of the assay was written in detail by Notomi et al. (2000).

Briefly, an inner primer containing sequence of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis is primed by an outer primer that releases a single-stranded DNA. This serves as a template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, producing a stem-loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of the target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six (or eight) distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity (Notomi et al., 2000). LAMP was established to detect *T. uilenbergi* and *T. luwenshuni* simultaneously in this study and the details are described in a following chapter.

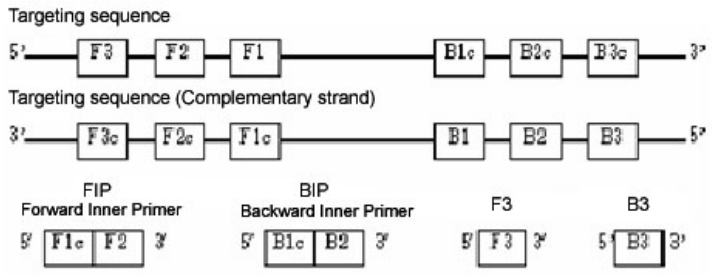


Fig. 5 Schematic illustration of LAMP primers and their binding sites.

Regarding indirect detection of infection using serological assays, two ELISA methods have been established for detection of circulating antibodies against the Chinese *Theileria* species:

For the merozoite crude antigen ELISA, antigen is prepared through ultrasonic lysis of purified *Theileria* merozoites and used for the detection of circulating antibodies against the Chinese *Theileria* infection (Gao et al., 2002) and epidemiological studies (Guo et al., 2007). However, the cross-reaction with other related pathogens such as ovine *Babesia* has impeded its application. Moreover, it is difficult to standardize antigens purified from parasite crude material, and there is also the requirement of experimental animals for parasite and antigen

production. Consequently, an ELISA based on a recombinant antigen would be a major achievement because it offers advantages such as negligible batch-to-batch variation in the antigen, absence of necessity to experimentally infect and sacrifice animals for preparation of the native antigen, and increased specificity.

An ELISA that employed a recombinant heatshock protein 70 originating from *T. lestoquardi* (rTIHSP 70 ELISA) was evaluated to detect *Theileria* species (China) infection in sheep (Miranda et al., 2006a). Although this ELISA showed no cross-reactivity with serum from *T. annulata* or *T. lestoquardi* infected animals, it was not tested against other related piroplasms of small ruminants to rule out cross-reactivity. Moreover, this ELISA has not been validated in the field yet. Therefore, the improvement of serological diagnostic methods for diagnosis of *T. uilenbergi* and *T. luwenshuni* infections is still a requirement.

3 Materials and methods

3.1 Establishment and screening of a merozoite cDNA library of *T. uilenbergi*

3.1.1 Infection of experimental animals

Five sheep (6-12 months old) purchased from a *Theileria*-free area, Jingtai County, China, were splenectomized 30 days before the experimental infection. Ten days prior to *T. uilenbergi* infected blood inoculation, blood films were made using a drop of blood from the ear vein, fixed with methanol and stained with Giemsa. They were examined under the light microscope for presence of hemoprotozoan parasites. Only animals negative for hemoprotozoan parasites were used for preparation of merozoites.

The *T. uilenbergi* stock used in this study was originated from Longde County of Ningxia Province and was verified in previous phylogenetic and other studies (Schnittger et al., 2003; Yin et al., 2004a; Yin et al., 2008). The parasite stock was preserved in liquid nitrogen at the Lanzhou Veterinary Research Institute.

A vial containing 3 ml *T. uilenbergi* infected blood with 22% parasitemia was taken from the liquid nitrogen and immediately thawed at 37°C. The infected blood was injected into the jugular vein of an experimental animal. The animal was monitored by daily observation of the clinical signs and daily blood smears. When the parasitemia reached approximately 20%, the animal was sacrificed to collect infected blood in 1% heparin. The blood was stored at 4°C until merozoite purification.

3.1.2 Purification of merozoites

The method for purification of the merozoites was as described previously (Miranda et al., 2006b; Sugimoto et al., 1991). Heparinized blood was centrifuged at 1,000× *g* at 4°C for 10 min. The upper phase containing plasma and the buffy coat was discarded and the erythrocyte pellet was resuspended in Tris-NaC1 buffer (10 mM Tris-HC1, 150 mM NaC1 buffer, pH 7.4). This wash step was repeated three times, and the final erythrocyte pellet was diluted 1:5 (v/v) in Tris-NaC1 buffer and applied to a column packed with cellulose powder (200-300 mesh; Advantec, Tokyo, Japan) to remove remaining white blood cells. The erythrocytes eluted from the column were washed once and resuspended at a concentration of

50% (v/v), and lysed with α-hemolysin (Sigma-Aldrich, St. Louis, Missouri, USA) at 37°C for 30 min. Percoll solutions of 40% and 60% (v/v) (Amersham, Munich, Germany) were prepared in Tris-NaC1 buffer. The erythrocyte lysate containing merozoite material was subjected to Percoll gradient centrifugation at 5000× *g* for 30 min where the merozoite-containing band was localized between the Percoll layers. After collection, merozoites were washed three times with Tris-NaC1 buffer at 5000× *g* for 10 min at 4°C and stored at -70°C.

3.1.3 Preparation of total RNA and mRNA

Total RNA was isolated from *T. uilenbergi* merozoite pellets using TRIzol Reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturers' instructions. Briefly, merozoite pellets (approximately 10^6-10^7 cells) were resuspended in 1 ml of TRIzol Reagent. The homogenized samples were incubated at 16°C for 5 min to permit the complete dissociation of nucleoprotein complexes, and then 0.2 ml of chloroform was added. The tube was shaken vigorously by hand for 15 seconds. This was followed by incubation of the tubes at 16°C for 3 min. The samples were centrifuged at 12,000× *g* for 15 min at 4°C. Following centrifugation, the colorless upper aqueous phase was collected and transferred to a fresh tube. The RNA from the aqueous phase was precipitated by mixing with 0.5 ml of isopropyl alcohol. The samples were incubated at 16°C for 10 min followed by centrifugation at 12,000× *g* for 10 min at 4°C. The resulting RNA precipitate formed a gel-like pellet, which was observed on the side at the bottom of the tube. The RNA pellet was washed once with 1 ml of 75% ethanol and centrifuged at 7,500× *g* for 5 min at 4°C. At the end of the procedure, the RNA pellet was air-dried for 5 min, dissolved in 300 µl of elution buffer (TE buffer, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) by heating at 65°C for 5 min and the yield was measured by a spectrophotometer.

Messenger RNA was purified from total RNA using an mRNA Purification Kit (Amersham Bioscience, Munich, Germany) following the manufacturer's instructions. Briefly, approximately 300 µg total RNA was applied directly to the pre-equilibrated oligo(dT)-cellulose spin column, which was previously washed 2× with 1 ml high salt buffer (10 mM Tris-HCI pH 7.4, 1 mM EDTA, 0.5 M NaCl). The column was left to drain through by gravity. The samples were applied to the top of the cellulose bed in the column and allowed to flow

into the cellulose bed by gravity. The column was centrifuged at 350× *g* for 2 min and then washed 3× by adding 0.25 ml high salt buffer via centrifugation at 350× *g* for 2 min followed by 3× washing with 0.25 ml low salt buffer (10 mM Tris-HCI, pH 7.4; 1 mM EDTA, 0.1 M NaCl) using the same procedure. The mRNA was eluted 4× with 0.25 ml of elution buffer by centrifugation at 350× *g* for 2 min. The successful obtainment of total RNA and mRNA was detected on agarose gels by electrophoresis as showed in Fig. 6. The concentration of the mRNA was measured by a spectrophotometer and stored at -70°C until used.

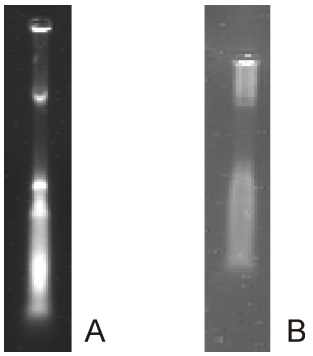


Fig. 6 Electrophoresis of the isolated total RNA and mRNA in agarose gels.
A, Total RNA;
B, Messenger RNA.

3.1.4 Establishment of a merozoite cDNA library

For establishment of the merozoite cDNA library, the ZAP Express cDNA Synthesis Kit and ZAP Express cDNA Gigapack Gold Cloning Kit (Stratagene, La Jolla, CA, USA) were used. Brief details about the full protocol, with some modifications, are given below:

3.1.4.1 cDNA synthesis

Briefly, *T. uilenbergi* merozoite mRNA was primed in the first-strand synthesis with the linker-primer and was transcribed using StrataScript^{TM} reverse transcriptase and a nucleotide mixture containing dATP, dGTP and dTTP plus the analog 5-methyl dCTP. The first strand cDNA made by this procedure has a methyl group on each cytosine base, which protects the cDNA from restriction enzymes used in subsequent cloning steps. For second-strand synthesis, RNase H was added to nick the RNA bound to the first-strand cDNAs, which served as primer for DNA polymerase to synthesize second-strand cDNA. In an RNase-free microcentrifuge tube, the following reagents were added in this order: 5 μl of 10× first-strand buffer; 3 μl of first-strand methyl nucleotide mixture; 2 μl of linker-primer (1.4 μg/μl); 34.5 μl of DEPC-treated water; 1 μl of RNase Block Ribonuclease Inhibitor (40 U/μl) and 3 μl of 480 ng/μl merozoite poly $(A)^+$ RNA. The reaction was mixed and incubated at room temperature

for 10 min to allow linker-primer annealing to merozoite poly $(A)^+$ RNA. Then, 1.5 μl of StrataScript RT (50 U/μl) was added to initiate the first-strand synthesis reaction. The final volume of the reaction was 50 μl. The reaction was incubated at 42°C for 1 hour to synthesize the first strand cDNA.

For the second strand synthesis, the following components were added on ice in order: 45 μl first-strand synthesis reaction; 20 μl of 10× second-strand buffer; 6 μl of second-strand dNTP mixture; 116 μl of sterile distilled water; 2 μl of RNase H (1.5 U/μl) and 11 μl of DNA polymerase I (9.0 U/μl). The second strand reactions were gently mixed and briefly spun, and then incubated at 16°C for 2.5 hrs. The success of double-stranded cDNA synthesis in samples was confirmed by electrophoresis in agarose gel as shown in Fig. 7.

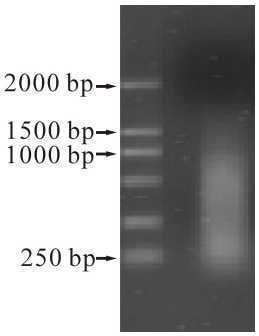


Fig. 7 Electrophoresis of the synthesized cDNA in agarose gel. The size of the cDNA products was ranged in between 250 bp and 2000 bp.

3.1.4.2 Modification of the cDNA termini and size fractionation

During the following process, the uneven termini of the double-stranded cDNA were 'nibbled' back or filled in with cloned *Pfu* DNA polymerase; *Eco*R I adapters were ligated to the blunt ends. After *Xho* I digestion to remove the *Eco*R I adapter at the 3′ end of the cDNA, the double stranded cDNA now possessed an *Eco*R I adapter at the 5' terminus and an *Xho* I adapter at the 3' terminus. The prepared cDNA was ready for orientational ligation into the Uni-ZAP XR vector after fragments fractionation. For blunting the cDNA termini, 23 μl of blunting dNTP mix and 2 μl of cloned *Pfu* DNA polymerase (2.5 U/μl) were added into the double stranded cDNA reaction and incubated at 72°C for 30 min. The cDNA was then extracted by adding 200 μl of phenol-chloroform [1:1 (v/v)]. After centrifugation at 14,000× *g* for 2 minutes, the upper aqueous layer containing the cDNA was collected and re-extracted using only chloroform in the same procedure. The cDNA was finally precipitated by adding 20 μl of 3 M sodium acetate and 400 μl of 100% (v/v) ethanol at -20°C overnight. The cDNA pellet was washed by 500 μl of 70% (v/v) ethanol and air-dried for removal of the additional ethanol. The pellet was resuspended in 7 μl of *Eco*R I adapters by incubation at 4°C for

30 min. Then, 1 μl of the 10× ligase buffer, 1 μl of 10 mM rATP and 1 μl of T4 DNA ligase (4 U/μl) were added and incubated at 8°C overnight.

After *Eco*R I adapter ligation was complete and the ligase had been heat inactivated, the *Eco*R I end was phosphorylated to prevent unspecific digestion by *Xho* I during the following procedure. Briefly, the adapter ends were phosphorylated by adding the following components: 1 μl of 10× ligase buffers, 2 μl of 10 mM rATP, 5 μl of sterile water, 2 μl of T4 polynucleotide kinase (5 U/μl) and the reaction was incubated for 30 min at 37°C followed by heat inactivation for 30 min at 70°C. Then, the reaction was subjected to digesting with *Xho* I by adding 28 μl of *Xho* I buffer supplement and 3 μl of restriction enzyme (40 U/μl), followed by incubation for 1.5 hrs at 37°C. The cDNA was precipitated by adding 5 μl of 10× STE (1 M NaCl, 200 mM Tris-HCl, pH 7.5, 100 mM EDTA) buffer and 125 μl of 100% (v/v) ethanol and resuspended in 14 μl of 1× STE buffer for the fractionation step.

The drip column for fractionation was set up carefully and the Sepharose CL-2B gel filtration medium was loaded following the manufacturer's instructions. Before loading the cDNA sample for fractionation, 10 ml of 1× STE buffer was added to wash the drip column containing the Sepharose CL-2B gel filtration medium. When approximately 50 μl of the STE buffer remained above the surface of the resin, the cDNA sample, previously mixed with 3.5 μl of the column loading dye for tracing the elution process was then loaded onto the column. Three milliliters of 1× STE buffer was added to move the cDNA sample down; the cDNA eluates were collected. Every three drops were collected into one fresh tube; 15 tubes fractionated cDNA sample was collected. These samples were electrophoresed in 1% agarose gel to detect the presence of the cDNA. The cDNA sample with a size of more than 400 bp was observed from tubes 4 to tubes 7, and these samples were combined into one tube for further use. Before ligation of the fractionated cDNA into the vector, it was precipitated and washed following the procedures mentioned above. Finally, the cDNA was resuspended into 3.5 μl of sterile water and measured by a spectrophotometer.

3.1.4.3 Ligation of the cDNA into the Uni-ZAP XR vector

The Uni-ZAP XR vector was double digested with *Eco*R I and *Xho* I so that the cDNA is ready to be orientationally ligated into the vector. The ligation reaction mixture contained 2.5 μl of resuspended cDNA (~100 ng), 0.5 μl of 10× ligase buffer (500 mM Tris-HCl

(pH 7.5), 70 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 µl of 10 mM rATP (pH 7.5), 1.0 µl of the predigested Uni-ZAP XR vector (1 µg) and 0.5 µl of T4 DNA ligase (4 U/µl). The final volume was 5 µl. The ligation reaction was incubated at 4°C for 2 days.

3.1.4.4 Packaging of the ligation reaction product

The packaging was done using the GigaPack®III Gold packaging extract (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. The packaging step was for preparing viable recombinant phages, in which contain the target cDNA samples ligated into the Uni-ZAP XR vector. Packaging was conducted as follows: a tube of the packaging extract (25 µl) was removed from -70°C and thawed quickly by holding the tube between the fingers. Once thawed, 4 µl of the ligation reaction product was added to the tube. (The pre-trial was carried out with 1 µl of the ligation reaction. The results suggested that more ligation reaction should be used for packaging. Thus 4 µl of the ligation reaction product was used). Samples-tubes were stirred and well mixed followed by centrifugation at 14,000× *g* for 1 min and left at room temperature for 2 hrs. The reaction was terminated by adding 0.5 ml of Sodium-Magnesium (SM) buffer (100 mM NaCl, 10 mM $MgSO_4$ plus $7H_2O$, 35 mM Tris-HCl, 0.01% Gelatin, pH 7.5). Twenty microliters of chloroform were added to each tube and the tubes were briefly centrifuged. The supernatant which contained the phages was transferred into a clean 1.5 ml tube, labeled and kept at 4°C until titration.

3.1.4.5 Plating of Bacterial strains

E. coli XL1-Blue MRF' strain, VCS257 strain (this strain was used only for controlling the positive wild-type lambda DNA and the details will not be included in the following sections) and SOLR strain (used for *in vivo* excision only) were provided as a stock in LB medium with 25% glycerol. The frozen cells were revived by scraping off splinters of solid ice with a sterile wire loop and streaking the splinters onto an LB agar plate containing 15 µg/ml tetracycline (Takara, Dalian, China) in case of the *E. coli* XL1-Blue strain, or 50 µg/ml kanamycin in case of the SOLR strain. These were referred to as the primary streak plates. The plates were incubated at 37°C overnight, sealed and stored at 4°C for up to 2 weeks. A working stock plate was prepared by picking a single isolated colony from a primary streak plate and streaking it onto another LB/MgSO4 agar plate containing appropriate

antibiotics. Working stock plates were used as a source of fresh colonies for inoculating liquid cultures and for preparing fresh working stock plates at 2-week intervals.

3.1.4.6 Preparing host bacteria

Liquid cultures of *E. coli* XL1-Blue MRF' for inoculation were prepared as follows: a single colony was picked from the working stock plate to inoculate 10 ml of LB broth containing 10 mM $MgSO_4$ and 0.2% maltose (LB/maltose/$MgSO_4$) without antibiotic in a 50 ml sterile flask. This culture was incubated at 37°C with shaking for 4 hrs until the OD600 reached 0.95. The cells were then centrifuged at 1000× *g* for 10 min. The supernatant was poured off and the pellet was resuspended in a similar volume of 10 mM $MgSO_4$. This was then aliquoted into 200 µl or 600 µl volumes in 5 ml sterile tubes, which received the diluted phage lysates.

3.1.4.7 Titration of the packaging reactions

Four LB agar plates were inverted, partially opened and pre-warmed in a 37°C incubator for an hour. The packaged ligation reactions from section 3.1.4.4 (Phage suspensions) were diluted 10-fold from 1: 10 to 1: 10^4 in SM. Ten microliters of the diluted phage lysates of each dilution was added to 200 µl of the *E. coli* XL1-Blue MRF' and resuspended in 10 mM $MgSO_4$ in different tubes. Phages were allowed to adsorb for 15 min at 37°C. For each sample, 3 ml of melted LB/$MgSO_4$ top agarose (LB medium containing 10 mM $MgSO_4$ and 0.7% w/v agarose), which was cooled to about 48°C, were added, mixed quickly by inverting and immediately poured onto the 90-mm LB agar pre-warmed plates. The plates were quickly swirled to allow the even distribution of the top agarose onto the agar surface. The plates were allowed to solidify for 10 min, then inverted and incubated at 37°C overnight. When 150 mm LB agar plates were used, 600 µl *E. coli* XL1 Blue MRF' cells were inoculated with the appropriately diluted phage lysates, and 7.5 ml top agarose were used. When plaques appeared, the number of plaques was counted and the titer of the phage (plaque forming units per milliliter, pfu/ml) was determined according to the following formula:

$$\text{pfu/ml} = \frac{\text{Number of plaques} \times \text{dilution factor} \times 10^3\ \mu\text{l/ml}}{\text{Number of microliters plated}}$$

The results showed that the yield of the primary library was 8.5× 10^5 plaque forming

units per milliliter (pfu/ml), which was very close to the desired value of 10^6. Titration of the final amplified library showed its efficiency was 6×10^8 pfu/ml, which was also close to the desired value of 10^9.

3.1.4.8 Determining background by blue-white color selection

Blue/white screening of the phage clones in *E. coli* XL1 Blue MRF' was done to estimate the percentage of recombinant clones. The procedure followed was similar to section 3.1.4.6 except that to each 3 ml aliquot of the melted top agarose, 15 µl of 0.5 M IPTG and 50 µl of 250 mg/ml X-gal were added before plating. The ratio of white (recombinant) to blue (non-recombinant) plaques was determined by naked eye. The results showed that more than 70% of the plaques formed in the library were recombinant plaques as shown in Fig. 8, indicating the successful establishment of the merozoite cDNA library.

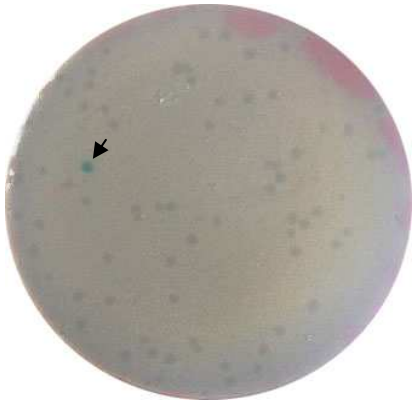


Fig. 8 Screening of the phage clones for recombinant clones. Arrow indicates blue plaques originating from non recombinant clone. White plaques from recombinant clones appear all over the plate as transparent plaques.

3.1.4.9 Amplification, titration and storage of the library

It is usually desirable to amplify libraries prepared in lambda vectors to make a large, stable quantity of a high-titer stock of the library. Thus, five 150 mm LB/$MgSO_4$ pre-warmed agar plates were used to amplify the library. *E. coli* XL1-Blue MRF' cell suspension in 10 mM $MgSO_4$ was aliquoted into 5 aliquots of 600 µl per 10 ml tube. Each tube received 100 µl of the unamplified phage suspension. Phage inoculation and plating were performed as previously described. After overnight incubation, when the plaques were confluent in contact, 10 ml of SM were added to each plate and the plates were stored at 4°C overnight. After which they were incubated for 1 hr on a platform shaker. SM containing the eluted λ-phage lysates was pooled into a sterile beaker. Each plate was washed with 2 ml SM which were added to the pooled lysates. This mixture was mixed and distributed into 50 ml polypropylene sterile screw-cap tubes. Five milliliters of chloroform were added to each tube, which was

followed by vortexing and centrifugation at 3,300× *g* for 10 min. The supernatants were collected into new sterile 50 ml tubes which were placed at 4°C. The titer of the new amplified library was determined by plating 10 µl of the 10^{-4} and 10^{-5} dilutions of the phage lysate/*E. coli* mixture onto 10 mm LB/$MgSO_4$ agar plates as described before, and the number of pfu/ml was determined. The library was divided into 1 ml aliquots, some aliquots were kept at 4°C and the rest was stored at -70°C after adding DMSO to a final concentration of 7%.

3.1.5 Generating subtraction library by *in vivo* mass excision

The Uni-ZAP XR vector is designed to allow simple, efficient *in vivo* excision and recircularization of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert. The ExAssist helper phage and SOLR strain are designed for this purpose, allowing excision of the pBluescript phagemid from the Uni-ZAP XR vector avoiding problems associated with helper phage co-infection. The mass excision procedure was as follows: Separate 50 ml cultures of *E. coli* XL1-Blue MRF´ and SOLR cells in LB broth with supplements were incubated at 30°C overnight. XL1-Blue MRF´ and SOLR cells were gently spun down and the pellets resuspended in 10 mM $MgSO_4$ until the concentration of the cells reached an OD600 of 1.0 (8×10^8 cells/ml). In a 50-ml conical tube, the following components were added: 100 µl of the amplified (lambda bacteriophage) library (approximately 10^7 pfu), 1 ml of XL1-Blue MRF´ cells (approximately 10^8 cells) and 1 µl of ExAssist helper phage (approximately 10^9 pfu). The reaction was incubated at 37°C for 15 min to allow the phage to attachment. Then 20 ml of LB broth with supplements were added. The conical tube was incubated for 2.5 hrs at 37°C with shaking. After incubation, the reaction was heated at 70°C for 20 min to lyse the lambda phage particles and the cells were centrifuged at 1,000× *g* for 10 minutes to remove cell debris and to obtain the supernatant containing the excised phagemids which was transferred into a sterile conical tube. One microliter of the excised phagemids was used to transform 200 µl of the SOLR cells to generate the bacteria colonies. After incubation of the reaction mixture at 37°C for 15 min, separate 50 µl and 100 µl were plated onto LB agarplates containing 100 µg/ml of kanamycin and the plates were incubated overnight at 37°C.

3.1.6 Identifying the cDNA inserts from phagemid subtraction library

3.1.6.1 Isolation of plasmid from overnight bacteria culture

Thirty-six single and separate colonies were picked from the LB plates prepared in section 3.1.5 and inoculated respectively into 36 tubes of 5 ml of LB media containing 100 μg/ml kanamycin then incubated at 37°C overnight. Cells were harvested by centrifugation at 3,300× *g* for 10 min at room temperature. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and a microcentrifuge according to the instructions of the manufacturer; all subsequent centrifugation steps were carried out at 16,000× *g*. Briefly, each of the bacterial pellet was resuspended in 250 μl buffer P1 (RNase A had been added), and then 250 μl buffer P2 were added and mixed thoroughly by inverting the tube 4-6 times. This was followed by adding 350 μl Buffer N3 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 Buffer PE by centrifugation for 1 min. After an additional centrifugation for 1 min to remove residual wash 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, incubation for 1 min and centrifugation for 1 min.

3.1.6.2 PCR identification of the cDNA inserts

The vector specific primers T7 (5'-GTA ATA CGA CTC ACT ATA GGG C-3')/T3 (5'-AAT TAA CCC TCA CTA AAG GG-3') were used, and PCR amplification was performed using an automated DNA thermocycler (PerkinElmer, USA) in a final volume of 30 μl containing 3 μl of 10× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 200 μM of each deoxynucleoside triphosphate (dNTP), 10 μM of each primer, 1 μl of plasmid DNA and 1.5 U of *Taq* DNA polymerase. The cycling conditions of the PCR was 3 min at 94ºC for denaturation followed by 30 cycles with denaturation at 94ºC for 30s, annealing for 40s at 55ºC and extension for 1 min at 72ºC. The final extension step was 7 min at 72ºC.

Amplified PCR products were run on 1% agarose gels 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. Melted agarose was allowed to cool to 55°C before a volume of approximately 40 ml was poured into a beaker. To this volume, 10 µl of 1 mg/ml ethidium bromide (Merck, Darmstadt, Germany) were added, gently swirled and poured into a small gel casting tray (Agagel Mini; Biometra, Goettingen, Germany) fitted with a comb. Samples were loaded at volumes of 6 µl which contained 5 µl PCR product plus 1 µl 6× loading dye (30% (v/v) glycerol, 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol) along with 3 µl LabAid^{TM} GeneRuler DNA ladder (Fermentas, St. Leon-Rot, Germany). Gel electrophoresis was carried out in TBE buffer at 10 V/cm gel width (approximately 70 V) for approximately 1 hr. Visualization and photography of the gels were done using a transluminator equipped with a camera (Biometra, Goettingen, Germany). The PCR identified plasmids containing cDNA inserts were subjected to sequencing (MWG, Ebersberg, Germany).

3.1.7 Sequence analysis and characterization of the clone 2 gene family

Sequences of 12 cDNA library clones were obtained and analyzed by available bioinformatic tools on the Internet; among these clones, Clone 2, Clone 9 and Clone 26 were found to be part of a gene family which was termed Clone 2 gene family in the following. The results of this investigation are presented in publication 1 entitled “Small-scale Expressed Sequence Tag Analysis of *Theileria uilenbergi*: identification of a gene family encoding potential antigenic proteins” attached in the Results section. Since the details of the methods for generating the cDNA library and characterizing the clones was not part of the publication, these were described above and are given in brief as follows:

3.1.7.1 Identification of the Clone 2 gene family orthologous genes from *T. luwenshuni*

Primers K26-F (5’-ATC TCC TCG GCC TGT CTG-3’)/K26-R (5’GGA GGC CCT TGA AGT GGT-3’) were designed based on the Clone 26 sequence (Genbank accession No. DQ187376) and C2UTR-F (5’-CCT TAG GTT ACC AGT TGT G-3’)/C2UTR-R (5’-TAA CAG GGT GGT CCC AGT CAA AC-3’) primers were designed based on the Clone 2 sequence (Genbank accession No. EU016499). PCR reactions and programs were the same as described in section 3.1.6.2., except that the PCR templates were 100 ng of genomic DNA and

the annealing was at 60ºC for 1 min for both primer sets. The PCR products were ligated to 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 µlPCR product, 2 µl distilled water and 5 µl Ligation Master Mix, and then incubated at 16ºC for at least 2 hrs. Two microliters of ligation-reaction mixture were added into a tube of pre-thawed Qiagen EZ Competent Cells, gently mixed and incubated on ice for 5 min, followed by heating in a 42°C heating block for 30s without shaking, then shifted onto ice for 2 min. After incubation, 250 µl room temperature SOC medium were added and 50 µl and 100 µl of transformation mixture were directly plated onto 2 LB agar plates containing ampicillin (100 µg/ml), IPTG (50 µM) and X-gal (80 µg/ml) at 37°C overnight. The white clones were picked and inoculated into LB medium containing ampicillin and cultured at 37°C for 6-8 hrs, after which the bacteria were harvested and subjected to plasmid isolation using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) as described in section 3.1.6.1. The plasmids were sequenced by automated sequencing (MWG, Ebersberg, Germany).

3.1.7.2 Protein expression and purification of Clone 2

PCR primers C26a (5’-GCA CGA GGG TTC AAG GTT -3’)/NC26as (5'- GTG AAC GAG ACC GAA GAC G- 3') were used to amplify a product from the genomic DNA of *T. uilenbergi*. The PCR reaction and cycling conditions were same as that of the C2 primers described above except the annealing temperature was 55ºC. The PCR products were cloned and sequenced as described in section 3.1.7.1. The Clone 2 plasmid and vector pQE 32 were digested by restriction enzymes *Hind* III and *Sp*H I (New England Biolabs, Frankfurt, Germany). The reactions were 17 µl Clone 2 plasmid (120 ng/µl) for restriction or 17 µl pQE 32 vector (60 ng/µl) for vector restriction. Each reaction mixture contained 2 µl 10× NEB buffer II, 0.5 µl *Hind* III (20u/µl) and 0.5µl *SpH* I (20u/µl). The reaction mixture was incubated at 14ºC overnight and then heated at 80ºC for 30 min. The reaction mixture was finally stored at 4ºC until purification.

The reaction mixture was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) as follows: 100 µl of Buffer PB was added to each of 20 µl reaction mixtures, mixed, then applied to the QIAquick column previously placed in a 2 ml collection tube and

centrifuged at 12,000× *g* for 1 min. The QIAquick columns were washed by adding 0.75 ml Buffer PE and centrifuged for 1 min. An additional centrifugation was conducted to remove residual ethanol from Buffer PE and then the purified reaction mixtures were eluted with 30 µl of distilled water. After purification, the restricted plasmid and vector were ligated and used to transform *E. coli* M15. The ligation was done by adding 8.5 µl double digested pQE 32, 8.5 µl double digested Clone 2 plasmid, 1 µl ligase (40 U/µl) and 2 µl ligase buffer (New England Biolabs, Frankfurt, Germany). This incubation was at 16ºC overnight. Ten microliters of ligation mixes were added to 100 µl aliquots of the competent *E. coli* M15 (pREP4) cells previously thawed on ice and gently resuspended. These were kept on ice for 20 min then heat-shocked at 42°C for 90s. After a 5 min incubation step on ice, 500 µl of Psi broth (LB medium, 4mM Mg SO_4, 10 mM KCl) were added, followed by incubation of the cells at 37°C for 60 min with shaking. Aliquots of 50, 100, and 200 µl from each transformation mix were plated on LB-agar plates containing 25 µg/ml kanamycin and 100 µg/ml carbenicillin; the plates were then incubated at 37°C overnight. As controls, competent cells were transformed with 10 ng of the intact undigested pQE 32 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 on a single plate containing antibiotics.

On the second day, 10 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 pQE reverse: 5'-GTTCTGAGGTCATTACTGG-3'), and 2 µl overnight culture as template. PCR conditions were as described (section 3.1.6.2.) 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.

After protein expression, the purification was carried out under denaturing conditions using the Ni-NTA Spin kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two microliters of overnight culture were used to seed 20 ml freshly prepared LB medium containing carbenicillin (100 µg/ml) and kanamycin (25 µg/ml). When the OD600 value reached 0.5 (measured by a spectrophotometer, Eppendorf, Hamburg, Germany), IPTG was added to a final concentration of 0.05 mM. Induction was allowed to continue for 4

hrs, after which the cells were harvested and the pellets were resuspended 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 lysed under rotation at room temperature for 1 h. Lysates were cleared of debris by centrifugation at 10,000× *g* 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 700× *g* 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 pH 4.5).

The concentration of the protein was estimated using the BioRad Micro-DC Assay kit (BioRad, Munich, Germany). BSA (2 mg/ml) was serially diluted to concentrations ranging between 2.0 and 0.2 mg/ml as shown in Table 1. Volumes of 5 µl of the prepared BSA dilutions and water control were pipetted into an ELISA plate in triplicates; the protein samples were diluted 1:1 in water and applied in duplicates, and then 25 µl of reagent A and 200 µl of reagent B were added. The plate was incubated in the dark at room temperature for 30 min then the optical density values were read at 550 nm using an ELISA reader (Asys Hitech, Overath, 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
BSA (µl)	50	45	40	35	30	25	20	15	10	5
ddH_2O (µl)	0	5	10	15	20	25	30	35	40	45

3.1.7.3 Western blot and silver staining

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out to separate proteins according to their molecular size. The 12.5% running gel solution was prepared according to the protocol shown in Table 2. The running gel solution was transferred to the assembled chamber (BioRad, Munich, Germany) using a pipette. Approximately 1.5 cm space was left for the stacking gel. The gels were covered with 70% ethanol and allowed to polymerize for at least 30 min. The 3% stacking gel solution was prepared according to the protocol in Table 2 simultaneously with the running gel. However, the ammonium persulphate solution was added only shortly before transferring the stacking gel solution into the chamber. Alcohol covering the running gel was first discarded by inverting the chamber and the

residual drops were removed using filter paper. After addition of stacking gel onto the running gels in the chamber, combs with the desired number of wells were inserted and the gels were left to polymerize for at least 30 min. The samples were prepared by adding a 4× sample loading buffer (180 mM Tris/HCl, pH 6.8, 40% glycerol (v/v), 4% SDS (w/v), 0.04% bromphenol blue (w/v), 100 mM DTT) in duplicates, in which one was for silver staining and one for western blot and the samples were heated at 98°C for 5 min. The gels were placed into the running chamber which was filled with the running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS). Up to 15 µl of sample (up to 6 µg) was then loaded into the corresponding wells along with a PageRuler™ prestained protein ladder (Fermentas, St. Leon-Rot, Germany). Electrophoresis was carried out in sequence as follows: 50 volts for 5 min, 100 volts for 10 min and 200 volts for 45- 60 min.

Table 2 Composition of the running and the stacking gel

Component	3% stacking gel	12.5% running gel
Distilled autoclaved H_2O	3 ml	4 ml
Acrylamide/bisacrylamide solution	0.5 ml	5 ml
0.5 M Tris-HCl (pH 6.8), 0.4% SDS	1.25 ml	-
1.5 M Tris-HCl (pH 8.8), 0.4% SDS	-	3 ml
TEMED	10 µl	10 µl
Pyronin Y buffer*	10 µl	-
Ammonium persulphate (10% solution)	20 µl	100 µl

* 0.5 M Tris-HCl, 10% Glycerol, 0.4% SDS, 0.01% pyronin Y (Serva, Heidelberg)

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 in 200 ml distilled H_2O for 15 min until they became almost colourless. Distilled H_2O 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 30s in 200 ml H_2O; the H_2O was also frequently changed. The gels were then placed in 50 ml developing solution until it 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.

The western blot was done by blotting the separated proteins samples in an SDS-PAGE gel onto nitrocellulose membranes (0.2 µm, BA 85, Schleicher and Schuell, Dassel, Germany) using a BioRad Transblot-SD semi-dry blotter (BioRad, Munich, Germany). The SDS-PAGE gel was equilibrated in anode buffer II (25 mM Tris, 20% methanol, pH 10.4). Nitrocellulose membranes 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) or 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, and then one piece of anode buffer II wetted blotting paper, equilibrated NC membrane, SDS-PAGE gel and three pieces of cathode buffer wetted blotting papers were placed at the top sequentially. After driving out the air bubbles trapped in between of the blotting papers, the cover of the device was assembled and fastened. Transfer was carried out at 25 V, 120 mA for 60 min for 2 gels or 25 V, 200 mA for 60 min for 4 gels (approximately 2.5 mA/cm^2 of the gel). Immuno-detection of the blotted protein was as described in publication 1 in the results section.

3.2 Practical protocol for loop-mediated isothermal amplification (LAMP) assay

The LAMP method was described in detail in Publication 2 in the Results section. To avoid repetition, only the practical protocol is included as below:

1. Solutions and reagents were prepared as listed in the Table 3, 4, 5 and 6:

Table 3 Preparation of Tris-HCl (1M)

Reagent	Amount
Tris base	12.11 g
ddH_2O, up to	100 ml
Total volume	100 ml

Dissolve in 80 ml of ddH_2O, adjust to pH 8.8 with concentrated HCl.

Table 4 Preparation of 2× LAMP reaction mix without dNTPs as follows

Reagent	Amount for 900ml	Amount for 90ml	Final concentration
1M Tris HCl(pH8.8) stock sol.	40 ml	4ml	40 mM
KCl	1.49 g	0.149 g	20 mM
*$MgSO_4$($MgSO_4.7H_2O$)	*1.93 g (3.94 g)	*0.19 g (0.394 g)	16 mM
$(NH_4)_2SO_4$	2.64 g	0.26 g	20 mM
Tween 20	2 ml	0.2 ml	0.2%

Betaine	187.4 g	18.74 g	1.6 M

*If $MgSO_4 \cdot 7H2O$ is used instead of $MgSO_4$, weights have to be corrected to the values in parenthesis.
The buffer was autoclaved then filtrated.

Table 5 Preparation of 25 mM dNTP stock

Reagent	Volume (Ratio)
100mM dATP	1
100mM dCTP	1
100mM dGTP	1
100mM dTTP	1

Table 6 Preparation of 2× LAMP reaction buffer (working solution)

Reagent	Volume
2× LAMP reaction buffer without dNTPs	900μl
25mM dNTP stock	100μl

Note: Store all solutions at -20°C.

2. Procedures:

1) LAMP reaction was prepared as shown in Table 7.

Table 7 Preparation of LAMP reaction

Reagent	Volume
2× Reaction buffer	12.5 μl
Bst DNA polymerase	1 μl (8units)
Primer mix*	X μl
DNA template	2 μl
H_2O	X μl
Total	25 μl

* Primer Mix contains six primers. Final concentration of FIP and BIP is 40 pmol each, LF and LB is 20 pmol and F3 and B3 is 5 pmol each. LAMP reaction should be prepared on ice.

2) LAMP reactions were incubated in a heating block or water bath at 63°C for 15-30 min, and then heated at 80°C for 5 min to terminate the reaction.

3) The LAMP products were subjected to electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and visualized under ultraviolet light. Alternatively, the reactions were detected by the naked eye by addition of 1.0 μl of tenfold diluted SYBR Green I (Roche Diagnostics, Mannheim, Germany). When the reaction was positive, the color turned green while when negative, the color remained orange.

4 Results

4.1 Publication 1

Annals of the New York Academy of Sciences (2008), 1149: 214-217

Small-scale expressed sequence tag analysis of *Theileria uilenbergi*: identification of a gene family encoding potential antigenic proteins

Zhijie Liu[a,b], Zhisheng Dang[a], Jianxun Luo[a], Hong Yin[a], Jabbar S. Ahmed[b], Ulrike Seitzer[b*]

[a]*State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 730046 Lanzhou, People's Republic of China*

[b]*Division of Veterinary Infection Biology and Immunology, Research Center Borstel, 23845 Borstel, Germany*

Abstract

Recently, *Theileria* sp. (China) has been designated as *T. luwenshuni* [formerly *Theileria* sp. (China 1)] and *T. uilenbergi* [formerly *Theileria* sp. (China 2)]. A cDNA library of *Theileria uilenbergi* merozoites was subjected to random sequencing. Among the obtained sequences were three highly identical cDNA clones, indicating a gene family. Bioinformatic analyses indicated these genes contain signal peptides and encode potential immunogenic proteins. The presence of tandemly arranged and additional variants of these genes was shown. Analysis of one recombinantly expressed clone revealed immunoreactivity for serum from *Theileria*- infected animals. No cross reaction with serum of *T. lestoquardi*-, *Babesia motasi*- or *Anaplasma ovis*- infected animals was observed, indicating a potential antigen for development of serological diagnostic tools.

Key words: *Theileria uilenbergi*; merozoite; cDNA library; sequencing

Introduction

Theileria uilenbergi (formerly *Theileria* sp. (China 2)) is a highly pathogenic protozoan parasite of small ruminants in China.[1] Although diverse approaches have been used, there remains a paucity in knowledge regarding potential antigenic proteins of this parasite.[2-5] This study aimed at generating a cDNA expression library of the pathogenic merozoite stage of *T. uilenbergi* in order to advance possibilities of identifying antigenic proteins as an important prerequisite for both the establishment of serologically based diagnostic tools and vaccine design.

Materials and Methods

T. uilenbergi merozoite cDNA library

T. uilenbergi merozoites were isolated from blood-infected splenectomized sheep as previously described.[4] Analysis of the 18s rRNA gene by PCR confirmed the origin.[6] mRNA (mRNA Purification Kit (Amersham Bioscience)) was purified from total RNA (TRIzol

reagent (Invitrogen)). The ZAP Express cDNA Synthesis Kit and ZAP Express cDNA Gigapack Gold Cloning Kit (Stratagene) were used for cDNA library construction.

Sequence analysis

BLAST searches were performed at the websites of The Institute for Genomic Research (http://tigrblast.tigr.org), Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/t_annulata and http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_bovis), and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The location of signal peptide cleavage sites was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP); antigenic peptides by using MIF (http://bio.dfci.harvard.edu/Tools/antigenic.pl); and prediction of membrane proteins by using TopPred (http://mobyle.pasteur.fr/cgi-bin/ MobylePortal /portal.py?form=toppred).[7-9]

Nucleic acid amplification for characterization of the Clone 2 gene family

The following primers were used for further characterization of the Clone 2 gene family: K26-F (5'-ATC TCC TCG GCC TGT CTG-3')/R (5'GGA GGC CCT TGA AGT GGT-3'); C2UTR-R (5'- TAA CAG GGT GGT CCC AGT CAA AC-3') C2UTR-F (5'- CCT TAG GTT ACC AGT TGT G-3'). PCR amplified products were ligated into pDrive (Qiagen) and selected clones were subjected to sequencing (MWG).

Protein expression and purification of Clone 2

PCR primers C26a (5'-GCA CGA GGG TTC AAG GTT-3')/NC26as (5'- GTG AAC GAG ACC GAA GAC G- 3') were used to amplify a partial sequence of Clone 2 from genomic DNA of *T. uilenbergi*. The fragment was cloned and subjected to sequencing as described above, then subcloned into the *Hind* III *SpH* I multiple cloning site of the pQE32 expression vector (Qiagen). Recombinant protein expression of the 186-amino acid polypeptide with a deduced molecular weight of 20.2 kD was verified in Western blots, quality was assessed by silver gel staining, and protein concentration was determined using the BioRad Micro-DC Assay kit (Munich, Germany).

Preparation of anitsera

Positive serum of *Theileria* species infected animals was generated by feeding infected *Haemaphysalis qinghaiensis* on experimental sheep. Positive serum of *T. lestoquardi* was from infected sheep in Sudan.[10] Positive serum of *B. motasi* and *A. ovis* were collected from experimentally infected sheep after *Babesia* and *Anaplasma* in erythrocytes were observed.

The negative sheep serum was obtained in Germany.

Detection of serum antibodies to recombinant Clone 2 protein by Western blot

Recombinant protein was subjected to SDS-PAGE and transferred to nitrocellulose membranes. To test its antigenicity, *Theileria* positive serum (1:200) and intact sheep serum (1:200) were applied. To test its specificity, *T. lestoquardi*, *B. motasi* and *A. ovis* positive sera (1:200) were used. Detection of immunoreactivity was assessed by alkaline phospatatase-cojugated Affinipure goat anti-mouse IgG+IgM (H+L) or rabbit anti-sheep IgG (H+L) secondary antibody (Jackson) and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as the substrate.

Results

Twelve clones with different PCR product sizes were sequenced; all contained a complete or incomplete open reading frame (Tab. 1). BLAST and BLASTX searches revealed that 11 expressed sequence tags had a high identity with *T. annulata* or *T. parva* sequences, confirming the *T. uilenbergi* origin. Sequence alignment of three clones (Clone 2 (EU016499), Clone 9 (EU016504) Clone 26 (DQ187376)) showed highly conserved but also variable regions, suggesting a gene family. These proteins were predicted to be apicoplast transmembrane proteins containing a signal peptide cleavage site and antigenic determinants. Sequence analysis of a 1540-bp PCR genomic fragment generated with primers derived from the Clone 26 sequence showed that both 5’ and 3’ termini were highly identical to the Clone 2 gene family (EU488870). These homologous termini were separated by a 136 bp untranslated region, indicating that these Clone 2 gene family members are tandemly arranged. Primers derived from the 3’ untranslated region were designed to amplify the entire Clone 2 or Clone 26 genome sequence. Sequence analysis of three clones showed again three new variants of the Clone 2 gene family (EU488867-EU488869).

Serum from *Theileria*-infected animals showed immunoreactivity to recombinantly expressed Clone 2 protein, whereas serum from *T. lestoquardi*-, *B. motasi*- or *A. ovis*-infected animals showed no reactivity (Fig. 1).

Discussion

The Clone 2 gene family of *T. uilenbergi*, identified by random PCR amplification of an excised merozoite expression cDNA library, is described. Three clones (Clone 2, Clone 9 and

Clone 26) showed high identity with *T. orentalis* ToLocg 1, but not to any of the fully sequenced *T. annulata* or *T. parva* genomes. These three clones were not identical but exhibited a high identity, indicating the possible presence of variant genes representing a gene family. Further evidence for the existence of a gene family arose from PCR experiments specifically designed to amplify the complete genome sequence of Clone 2, which resulted in the detection of further highly identical sequences belonging to this presumed gene family. Furthermore, the presence of tandemly arranged sequences was observed. Analysis of four of these clones (EU016504, EU488867-9) with apparent complete open reading frames showed that not only are there variances in the sequence but also in the length of the predicted proteins.

Bioinformatic analyses indicated that members of the Clone 2 gene family could encode potential immunodominant proteins. Indeed, analysis of recombinantly expressed Clone 2 protein clearly showed specific reactivity with serum of *Theileria* sp. (China) infected animals. Since cross-reactivity with serum of animals infected with other small ruminant piroplasms or *A. ovis* was not present, this protein has the potential to be used for the development of a serological diagnostic tool for monitoring and detecting *Theileria* sp. (China) infection. It remains to be investigated whether members of this gene family may be used to differentiate between the newly defined two species of *Theileria* infecting small ruminants in China, *T. luwenshuni* and *T. uilenbergi*.[11]

Acknowledgements

Supported in part by “863” Project (2006AA10A207), Specific Fund for Sino-Europe Cooperation, MOST, and EU-funded Coordinated Action "Integrated Consortium on Ticks and Tick-borne Diseases" (ICTTD-3), Project number 510561. China. ZL is the recipient of a DAAD scholarship.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Ahmed, J.S. *et al*. 2006. Phylogenetic position of small-ruminant infecting piroplasms. Ann. N. Y. Acad. Sci. 1081: 498-504.

2. Miranda, J. *et al*. 2004. Identification of antigenic proteins of a *Theileria* species pathogenic for small ruminants in China recognized by antisera of infected animals. Ann. N. Y. Acad. Sci. 1026: 161-164.

3. Miranda, J. *et al*. 2006. Identification of Homologous Genes of *Theileria annulata* Proteins in the Genome of *Theileria* sp. (China). Ann. N. Y. Acad. Sci. 1081: 468-470.

4. Miranda, J. *et al*. 2006. Identification and characterization of merozoite antigens of a *Theileria* species highly pathogenic for small ruminants in China. Ann. N. Y. Acad. Sci. 1081: 443-452.

5. Miranda, J. *et al*. 2006. Development of a recombinant indirect ELISA for the diagnosis of *Theileria* sp. (China) infection in small ruminants. Parasitol. Res. 98: 561-567.

6. Li, Y. *et al*. 2007. Experimental transmission of *Theileria* sp. (China 1) infective for small ruminants by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis*. Parasitol. Res. 101: 533-538.

7. Bendtsen, J. D. *et al*. 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340: 783-795.

8. Kolaskar, A. S. & P. C. Tongaonkar. 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 276: 172-174.

9. Claros, M.G. & G. von Heijne. 1994. TopPred II: An improved software for membrane protein structure predictions. CABIOS 10: 685-686.

10. Bakheit , M. A. *et al*. 2006. A new recombinant protein-based ELISA for the diagnosis of malignant theileriosis of sheep and goats. Parasitol. Res. 98: 145-149.

11. Yin, H. *et al*. 2007. Ovine theileriosis in China: a new look at an old story. Parasitol. Res. 101 (S2): 191-195.

Table 1 Blast results of 12 cDNA library clones sequenced at random.

Accession	BLASTX High Score	Identity
EU016498	*Oryza sativa* hypothetical protein OsI_033536	31%
EU016499	*Theileria orientalis* ToLocg 1	42%
EU016500	*Theileria parva* guanine nucleotide binding protein	94%
EU016501	*Theileria parva*, lysyl tRNA synthetase	87%
EU016502	*Theileria parva* RNA helicase	67%
EU016503	*Theileria parva* hypothetical protein TP04_0374	79%
EU016504	*Theileria orientalis* ToLocg 1	47%
EU016505	*Theileria annulata* hypothetical protein TA17355	22%
EU016506	*Theileria annulata* serine/threonine protein phosphatase pp x isozyme	94%
DQ187375	*Theileria annulata* 60S ribosomal protein L5	89%
DQ187376	*Theileria orientalis* ToLocg 1	41%
DQ187377	*Theileria parva* hypothetical protein TP03_0008	20%

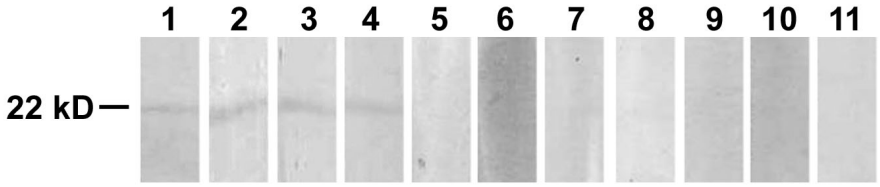


Figure 1 Western blot characterization of recombinant protein Clone 2. Lane 1, anti-His-tag antibody; lanes 2–4, *Theileria* sp. (China)-positive sera; lanes 5 + 6, *T. lestoquardi*-positive sera; lanes 7 + 8, *B. motasi*-positive sera; lanes 9 + 10, *A. ovis*-positive sera; lane 11, negative sheep serum.

4.2 Publication 2

Parasitology Research (2008) 103: 1407-1412

Development of Loop-mediated Isothermal Amplification (LAMP) Assay for Rapid Diagnosis of Ovine Theileriosis in China

Zhijie Liu[1,2], Junlin Hou[1], Mohammed A. Bakheit[3], Diaeldin A. Salih[2], Jianxun Luo[1], Hong Yin[1], Jabbar S. Ahmed[2], Ulrike Seitzer[2*]

[1] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, the People's Republic of China

[2] Division of Veterinary Infection Biology and Immunology, Research Center Borstel, Parkallee 22, D-23845 Borstel, Germany

[3] National Research Center for Protozoan Diseases (NRCPD), Obihiro University for Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

Abstract

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid detection method in which the target DNA can be efficiently amplified with high specificity and sensitivity under isothermal conditions using a set of either four or six specific primers. In this study, we have identified a conserved sequence for *Theileria luwenshuni* (UTRlu8) and for *T. uilenbergi* (UTRu6) suitable for designing a set of six primers for the simultaneous detection by LAMP of these pathogens causing theileriosis in sheep and goats in China. LAMP was performed at 63°C and the amplified DNA was detectable within 15 min. The specificity of the reaction was confirmed through *Eco*RI restriction enzyme digestion analysis and sequencing. The assay was proven sensitive since specific amplification was obtained from 0.1 pg DNA of *T. luwenshuni* or *T. uilenbergi*. The LAMP assay was evaluated by testing 86 field samples in comparison to the reverse line blot method, showing a sensitivity and specificity of 66.0% and 97.4%, respectively. These results indicate that the LAMP assay is rapid and simple to run, cost-effective, sensitive and specific and has potential usefulness for application in diagnostics of and epidemiological studies on *T. luwenshuni* and *T. uilenbergi* infection of small ruminants.

Introduction

Theileria luwenshuni and *T. uilenbergi* are pathogenic *Theileria* species for small ruminants identified in China and transmitted by the three host tick *Haemaphysalis qinghaiensis* (Yin et al. 2002; Li et al. 2007). The infection caused by these parasites represents a major constraint to the development of the sheep and goat industry, mainly in exotic animals, in northwestern China (Luo and Yin 1997; Ahmed et al. 2002). These parasites were previouly referred to as *Theileria* sp. 1 (China) and *Theileria* sp. 2 (China). Recently, based on a molecular phylogenetic study, the names of *T. luwenshuni* and *T. uilenbergi* were designated for *Theileria* sp. 1 (China) and *Theileria* sp. 2 (China), respectively (Yin et al. 2007). *T. luwenshuni* and *T. uilenbergi* are morphologically indistinguishable. They share the same vector tick *H. qinghaiensis* and both are pathogenic for small ruminants.

Generally, diagnosis of theileriosis is based on the microscopic examination of blood smears or lymph node biopsies. This method is useful in detection of acute cases but has limited value in carrier status, where low numbers of erythrocytes remain infected. With

development of molecular biology, some accurate tools became available for the detection of the parasites. PCR assays for the diagnosis of *T. luwenshuni* and *T. uilenbergi* in both transmitting ticks and the ovine host have been developed based on the 18S rRNA gene (Yin et al. 2008; Sun et al. 2008). Also, reverse line blot (RLB) (Schnittger et al. 2004) has been developed for detection and differentiation of these parasites. However, these assays are time consuming, expensive and need a high degree of laboratory experience in molecular biology. Recently a rapid, simple and sensitive technique, loop-mediated isothermal amplification (LAMP), was developed (Notomi et al. 2000). This is a novel strategy for gene amplification which relies on the auto-cycling strand displacement synthesis of DNA by *Bst* DNA polymerase under isothermal conditions. Further improvement of the technique has been achieved by the use of additional loop primers, which increased its efficiency and rapidity (Nagamine et al. 2002a). The LAMP technique allows visual detection of amplified products through the addition of fluorescent dyes such as SYBR Green (Poon et al. 2006) and measurement of turbidity (Mori et al. 2001). LAMP technique has been applied for detection of viral, bacterial and protozoan infections (Seki et al. 2005; Imai et al. 2006; Iseki et al. 2007).

Since *T. luwenshuni* and *T. uilenbergi* both are pathogenic for small ruminants and often occur in mixed infections in the same region, a LAMP method for rapid detection of both parasites simultaneously was developed in the present work based on recently determined gene sequences.

Materials and methods

Parasites and preparation of DNA templates

As previously described (Yin et al. 2004; Yin et al. 2007), *T. luwenshuni* was isolated from Ningxian County in Gansu Province and *T. uilenbergi* was isolated from Longde County in Ningxia Province. Parasite stocks were maintained in liquid nitrogen and parasites were multiplied by inoculating these stocks to experimental sheep under laboratory conditions. DNA was extracted from parasite infected sheep blood using a Genomic DNA Extraction Kit (Qiagen, Hilden, Germany). The species specificity of DNA samples were determined by applying species-specific PCR (Yin et al. 2008). Genomic DNA (gDNA) of *T. lestoquardi*, *T. ovis*, *B. motasi*, *A. ovis*, *T. parva*, *T. annulata* and *T. sergenti* was obtained from the

Division of Veterinary Infection Biology and Immunology of Research Center Borstel except for DNA of *T. sinensis*, *Anaplasma ovis* and DNA from protozoan free sheep, which were prepared in Lanzhou Veterinary Research Institute. Field samples were collected from Gannan region in June 2005 and DNA was isolated using a Genomic DNA Extraction Kit (Qiagen, Hilden, Germany). The amount of isolated DNA was assessed spectrophotometrically and diluted to 100 ng/µl, which served as template for LAMP and RLB.

LAMP primer design

The UTRTu6 gene (Genbank accession: EU488867) is a *T. uilenbergi* specific gene identified in cDNA and genomic DNA by PCR amplification (Liu et al., 2008). The homologous gene in *T. luwenshuni* was amplified using primer C2UTR-R (5'- TAA CAG GGT GGT CCC AGT CAA AC-3')/C2UTR-F (5'- CCT TAG GTT ACC AGT TGT G-3'). PCR amplified product was ligated into the pDrive vector (Qiagen), transformed into *Escherichia coli* cells and selected clones were subjected to sequencing (Eurofins MWG Operon, Ebersberg, Germany). The sequence obtained was submitted to Genbank with accession number EU753118. Alignment analysis using DNAstar software (DNASTAR Inc. Madison, WI, USA) revealed high similarities between these genes. LAMP primers were designed based on these genes to diagnose theileriosis of small ruminants caused by these parasites. In this study, primer F3, B3, FIP and BIP were designed based on UTRTu6 and UTRTlu8 gene using the Primer Explorer V4 program (http://primerexplorer.jp/ elamp4.0.0/index.html), while loop primers (LF and LB) were manually designed. The sequence of each primer and location within the target sequence are shown in Table 1 and Figure 1, respectively. To confirm specificity of LAMP reaction, two *Eco*RI restriction enzyme cleavage sites were added in between F1 complementary and F2 and B1 complementary and B2 (Table 1) for subsequent restriction analysis of LAMP products. The primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Establishment, specificity and sensitivity of LAMP reaction

The reaction was performed in a final volume of 25 µl which contained 12.5 µl 2× LAMP reaction buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$ and 0.2% Tween 20], 125 µM each deoxynucleoside triphosphate, 0.8 M betaine (Sigma-Aldrich Chemie Gmbh, Munich, Germany), 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs, Frankfurt, Germany), 40 pmol each FIP and BIP primers,

20 pmol LF and LB primers, 5 pmol each F3 and B3 primers, and 2 µl of target DNA. The mixture was incubated at 63°C for 30 min using a conventional heating block (Stuart Scientific, Staffordshire, UK) and then heated at 80°C for 5 min to terminate the reaction. The specificity of LAMP primers was examined by testing 100 ng/µl of genomic DNA of *T. luwenshuni, T. uilenbergi, T. lestoquardi, T. ovis, B. motasi, A. ovis, T. parva, T. annulata, T. sinensis,* as well as ovine genomic DNA and water control. To determine the analytical sensitivity of the LAMP assay, 10-fold dilutions were made from 100 ng/µl genomic DNA of *T. luwenshuni* and *T. uilenbergi* which served as templates for the LAMP reaction. The LAMP products were subjected to electrophoresis on a 2% agarose gel containing 0.5 µg/ml ethidium bromide and visualized under ultraviolet (UV) light or stained by SYBR Green.

*Eco*RI restriction digestion of the LAMP products and DNA sequencing

The LAMP products were purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany) in order to get rid of the oligo residues. The purified LAMP products were digested by *Eco*RI (New England Biolabs, Frankfurt, Germany) restriction enzyme at 37°C for 1 h. Digested products were analyzed by 2.0% agarose gel electrophoresis as described above and the individual DNA bands were purified from agarose gel using Qiaex Gel Extaction Kit (Qiagen, Hilden, Germany). Then purified DNA bands were cloned into an *Eco*RI-restricted pGEX 4T-1 vector (GE Healthcare Europe GmbH, Munich, Germany), transformed into *Escherichia coli* cells and selected clones were subjected to sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Detection of field samples using LAMP and RLB methods

Eighty six field blood samples of sheep were collected from a theileriosis endemic region in Lintan County of Gannan Region in June 2005. The DNA extraction and preparation were as described above. These DNA samples were subjected to detection by both LAMP method as described above and reverse line blot (RLB) method. RLB was carried out using a previously described protocol (Schnittger et al. 2004). Briefly, 100 ng genomic DNA of each sample was amplified using primer RLB F2 (5'- GAC ACA GGG AGG TAG TGA CAA G-3')/RLB R2 (5'- CTA AGA ATT TCA CCT GTG ACA GT-3') in an automatic DNA thermocycler (PerkinElmer LAS (Germany) GmbH, Rodgau – Jügesheim, Germany) with final volume of 30 µl containing 3 µl of 10× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 200 µM of each deoxynucleoside triphosphate (dNTP), 10 µM of

each primer and 1.5 U of Taq polymerase. The cycling condition of the PCR was 3 min at 94ºC for denaturation followed by 35 cycles with denaturation at 94ºC for 30s, annealing 1 min at 55ºC and extension 1 min at 72ºC. Then the final extension step for 7 min at 72ºC and the samples were kept at 4ºC. The PCR products were subjected to RLB assay following the same protocol described by Schnittger et al. (2004).

Results

Specificity and sensitivity of LAMP

To establish the standard protocol for LAMP method, different reaction temperatures and incubation times were applied. The reaction could successfully take place in temperatures ranging from 60ºC to 65ºC (data not shown), and 63ºC was chosen as the reaction temperature for all following applications. Different incubation times were carried out starting from 15 min to 60 min at 63ºC. LAMP products were detectable after 15 min, and 30 min was chosen as the reaction time. Under these conditions, six LAMP primers specifically produced positive LAMP amplicons of typical ladder patterns from genomic DNA of *T. luwenshuni* and *T. uilenbergi*, while there were no products from DNA of other protozoan parasites, *Anaplasma ovis* as well as naïve sheep DNA and water control (Fig. 2).

The specificity of LAMP products was confirmed by *Eco*RI restriction since *Eco*RI restriction sites were added into FIP and BIP primers, while there was no *Eco*RI restriction site in the target gene sequences. The LAMP products before and after digestion were analyzed in 2% agarose gel electrophoresis (Fig. 3). To further confirm the accuracy of LAMP, a digested fragment of LAMP from *T. uilenbergi* was successfully cloned into pGEX4T-1 vector and subjected to sequencing analysis. The sequence showed that the size of this fragment is 140 bp long, which is a predicted fragment by *Eco*RI digestion. Also, the determined sequence was 100% identical with the sequence of the target gene for LAMP (data not shown).

To evaluate the sensitivity of LAMP, 100 ng/µl *T. luwenshuni* and *T. uilenbergi* DNA samples were 10-fold serially diluted and served as templates for LAMP. Using these serially diluted DNA samples, LAMP could successfully detect parasite DNA down to 0.1 pg dilution (Fig. 4). The amplicons were also confirmed by SYBR Green staining (data not shown).

Evaluation of LAMP by testing field samples in parallel with Reverse Line Blot

A total of 86 field samples were collected from a theileriosis endemic region in Gannan, Gansu Province, China, where *T. luwenshuni* and *T. uilenbergi* are known to be co-existing. DNA extracted from these samples was firstly subjected to RLB (Fig. 5), in which 47 samples were positive. Among these 47 RLB positive samples, 31 were found positive by LAMP, while 1 negative sample of RLB was positive by LAMP (Table 2). Thus, using RLB as the reference method, the sensitivity of LAMP was 66.0% and the specificity was 97.4%.

Discussion

T. luwenshuni and *T. uilenbergi* are the causative pathogens for theileriosis of sheep and goats in northwestern China. Both parasites are similar with respect to the vector, host specificity, morphology, and pathogenicity (Ahmed et al. 2006; Yin et al. 2007), making a simple method for diagnosis and investigation of infections caused by both parasites helpful for local veterinarians. In this study, we report on the successful development of a LAMP method which is highly specific for detecting simultaneously *T. luwenshuni* and *T. uilenbergi* and is simple to perform. The selection of an appropriate gene for development of LAMP is critical. The UTRu6 gene (EU488867) of *T. uilenbergi* (Liu et al., 2008) and its homologous gene UTRlu8 (EU753118) of *T. luwenshuni*, which was cloned and sequenced in this study, were used for LAMP primers design. Highly conserved regions of these homologous genes were selected, and a set of six primers that recognized eight distinct sequences on the target UTRTu6 and URTlu8 genes theoretically unlikely to produce non-specific composition products (Nagamine et al., 2002a) were selected. In fact, only gDNA samples of *T. luwenshuni* and *T. uilenbergi* were amplified under optimized amplification conditions. DNA samples from other protozoan parasites as well as *A. ovis* and the ovine host always tested negative, confirming the high specificity of the assay. Apart from the optimized amplification conditions, different reaction temperatures and durations were also tested, indicating that the temperature can vary from 60ºC to 65ºC, still giving results, and that detectable amounts of LAMP products can be produced within 15 minutes. This efficiency and rapidity may be attributed to the inclusion of loop primers along with the regular LAMP primers (Nagamine et al. 2002a).

Originally, the LAMP inner primers, generally termed FIP and BIP, consisted of F1c, a

TTTT linker and F2 and B1c, a TTTT linker and B2 respectively (Notomi et al. 2000). Recently, restriction sites instead of TTTT linkers have been successfully added to inner primers (Nagamine et al. 2002b; Iseki et al. 2007), allowing enzyme restriction analysis of the amplified DNA products derived from target genes. We artificially inserted a restriction enzyme cleavage site (*Eco*RI site: GAATTC) into the two inner primers UTRFIP and UTRFIB sequences replacing the TTTT linkers. As a consequence, the *Eco*RI enzyme restriction analysis of our LAMP products showed a distinct band pattern. To further confirm the accuracy of the enzyme digestion, one of the digested LAMP fragments of *T. uilenbergi* was successfully cloned and sequenced. The determined sequence showed 100% identity to the UTRTu6 gene of *T. uilenbergi*, confirming the amplification of the target sequence by LAMP and indicating the specificity of this method for detection of *T. luwenshuni* and *T. uilenbergi*.

Regarding the sensitivity of LAMP, it is generally considered that LAMP is highly sensitive compared to PCR methods, being able to detect as few as six DNA copies, 0.001 trypanosomes from 1 ml blood and to detect DNA extracted from 50 *Babesia* infected red blood cells (Notomi et al. 2000; Iseki et al. 2007; Njiru et al. 2008). In our study, the sensitivity of LAMP was tested by subjecting serially 10-fold diluted DNA of *T. luwenshuni* and *T. uilenbergi* to LAMP. The results showed that 0.1 pg DNA can be detected for both parasites, which is 10 times more sensitive than the PCR method for detection of *T. luwenshuni* and as sensitive as PCR for the detection of *T. uilenbergi* (Yin et al. 2008). Determination of sensitivity in relation to parasiteamia using LAMP remains to be completed in the future.

To evaluate the LAMP method, 86 field samples were subjected to LAMP and RLB. RLB is a highly specific and sensitive method for examining the presence of parasite DNA in investigated samples. The sensitivity of RLB demonstrated a detection limit of 10^{-6}% parasitaemia (Schnittger et al. 2004). In this study 47 samples of the 86 were positive by RLB, 31 were positive for LAMP, and additionally, 1 positive sample for LAMP was negative for RLB, resulting in a sensitivity and specificity of 66.0% and 97.4%, respectively, for LAMP. Therefore it appears that the sensitivity of LAMP is poorer than that of RLB, but still gives reliable diagnostic results with a high specificity.

In summary, the LAMP method developed in this study is robust for detection of

infections due to *T. luwenshuni* and *T. uilenbergi* and has a great potential as a test that can be applied easily in theileriosis endemic region in China. Its high specificity suggests this as a very helpful method for local veterinarian to diagnose individual cases of theileriosis and/or make a spot examination.

Acknowledgements

This study was supported in part by “863” Project (2006AA10A207), Specific Fund for Sino-Europe Cooperation, MOST, and EU-funded Coordinated Action "Integrated Consortium on Ticks and Tick-borne Diseases" (ICTTD-3), Project number 510561, National Natural Sciences Foundation of China (№ 30571397), International Cooperation Fund of NSFC, Beijing, China. Zhijie Liu is the recipient of a DAAD scholarship.

References

Ahmed JS, Luo J, Schnittger L, Seitzer U, Jongejan F, Yin H (2006) Phylogenetic position of small-ruminant infecting piroplasms. Ann N Y Acad Sci 1081: 498-504.

Ahmed JS, Yin H, Schnittger L, Jongejan F (2002) Ticks and tick-borne diseases in Asia with special emphasis on China. Parasitol Res 88: S51-S55.

Imai M, Ninomiya A, Minekawa H, Notomi T, Ishizaki T, Tashiro M, Odagiri T (2006) Development of H5-RT-LAMP (loop-mediated isothermal amplification) system for rapid diagnosis of H5 avian influenza virus infection. Vaccine 24: 6679-6682.

Iseki H, Alhassan A, Ohta N, Thekisoe OM, Yokoyama N, Inoue N, Nambota A, Yasuda J, Igarashi I (2007) Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites. J Microbiol Methods 71: 281-287.

Li Y, Luo J, Liu Z, Guan G, Gao J, Ma M, Dang Z, Liu A, Ren Q, Lu B, Liu J, Zhao H, Li J, Liu G, Bai Q, Yin H (2007) Experimental transmission of *Theileria* sp. (China 1) infective for small ruminants by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis*. Parasitol Res 101: 533-538.

Liu Z, Dang Z, Luo J, Yin H, Ahmed JS, Seitzer U (2008) Small-scale expressed sequence tag analysis of *Theileria uilenbergi*: Idenetification of a gene family encoding potential antigenic proteins. Ann N Y Acad Sci 1149: 214-217.

Luo J, Yin H (1997) Theileriosis of sheep and goats in China. Trop Anim Health Prod 29: 8S-10S.

Mori Y, Nagamine K, Tomita N, Notomi T (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation.

Biochem Biophys Res Commun 289: 150-154.

Nagamine K, Hase T, Notomi T (2002a) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16: 223-229.

Nagamine K, Kuzuhara Y, Notomi T (2002b) Isolation of single-stranded DNA from loop-mediated isothermal amplification products. Biochem Biophys Res Commun 290: 1195-1198.

Njiru ZK, Mikosza AS, Matovu E, Enyaru JC, Ouma JO, Kibona SN, Thompson RC, Ndung'u JM (2008) African trypanosomiasis: sensitive and rapid detection of the sub-genus trypanozoon by loop-mediated isothermal amplification (LAMP) of parasite DNA. Int J Parasitol 38: 589-599.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28: E63.

Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W, Tangpukdee N, Yuen KY, Guan Y, Looareesuwan S, Peiris JS (2006) Sensitive and inexpensive molecular test for *falciparum* malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clin Chem 52: 303-306.

Schnittger L, Yin H, Qi B, Gubbels MJ, Beyer D, Niemann S, Jongejan F, Ahmed JS (2004) Simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants by reverse line blotting. Parasitol Res 92: 189-196.

Seki M, Yamashita Y, Torigoe H, Tsuda H, Sato S, Maeno M (2005) Loop-mediated isothermal amplification method targeting the lytA gene for detection of *Streptococcus pneumoniae*. J Clin Microbiol 43: 1581-1586.

Sun C, Liu Z, Gao J, Guan G, Ma M, Luo J, Yin H (2008) Investigations into the natural infection rate of *Haemaphysalis qinghaiensis* with *Piroplasma* using a nested PCR. Exp Appl Acarol 44:107-14.

Yin H, Guan G, Ma M, Luo J, Lu B, Yuan G, Bai Q, Lu C, Yuan Z, Preston P (2002) *Haemaphysalis qinghaiensis* ticks transmit at least two different *Theileria* species: one is infective to yaks, one is infective to sheep. Vet Parasitol 107: 29-35.

Yin H, Liu Z, Guan G, Liu A, Ma M, Ren Q Luo J (2008) Detection and differentiation of *Theileria luwenshuni* and *T. uilenbergi* infection in small ruminants by PCR. Transboundary and Emerging Diseases 55: 233-237.

Yin H, Luo J, Schnittger L, Lu B, Beyer D, Ma M, Guan G, Bai Q, Lu C, Ahmed J (2004) Phylogenetic analysis of *Theileria* species transmitted by *Haemaphysalis qinghaiensis*. Parasitol Res 92: 36-42.

Yin H, Schnittger L, Luo J, Seitzer U, Ahmed JS (2007) Ovine theileriosis in China: a new look at an old story. Parasitol Res 101 Suppl 2: S191-S195.

Figures

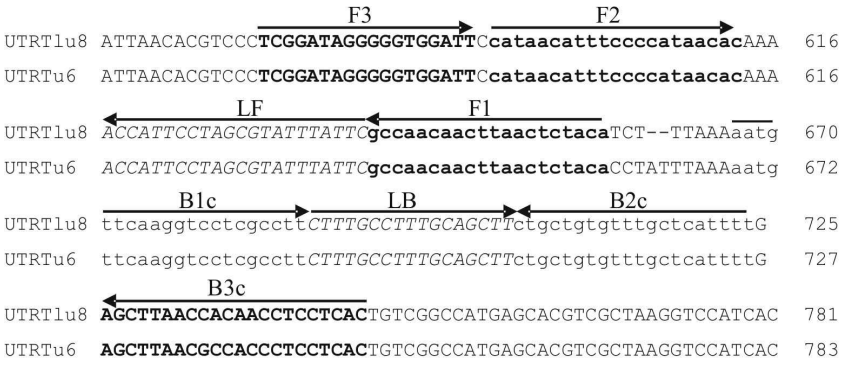


Fig. 1 Schematic diagram showing primers designed for LAMP reaction. The primers were designed based on the homologous genes UTRTu6 (Genbank accession: EU488867) of *T. uilenbergi* and UTRTlu8 (EU753118) of *T. luwenshuni*. F3/B3 binding sites are marked in bold upper case letters, FIP combined by F1c and F2 in bold lower case letters, FIB combined by Bic and B2 in lower case letters and LF/LB in italic upper case letters.

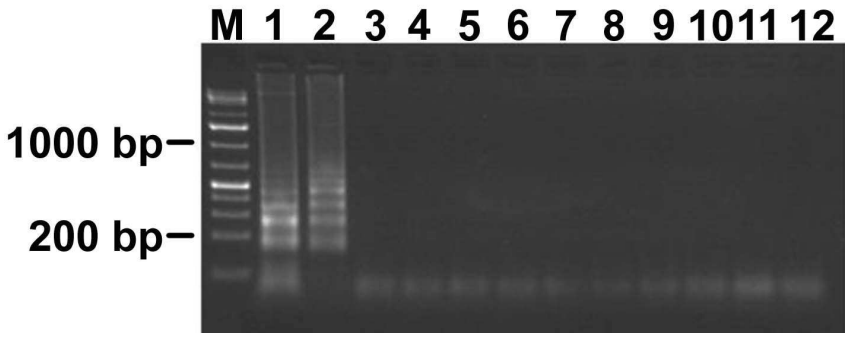


Fig. 2 Specificity of the LAMP method. Genomic DNA of the indicated species was incubated for 30 min at 63°C by using six primers (F3, B3, FIP, BIP, LF and LB) for detection of *Theileria luwenshuni* and *T. uilenbergi*. 1. *T. luwenshuni*, 2. *T. uilenbergi*, 3. *T. lestoquardi*, 4. *T. ovis*, 5. *Babesia motasi*, 6. *Anaplasma ovis*, 7. *T. parva*, 8. *T. annulata*, 9. *T. sergenti*, 10. *T. sinensis*, 11. gDNA of sheep, 12, Water control. Only samples containing *T. luwenshuni* and *T. uilenbergi* genomic DNA resulted in detectable amplification of DNA.

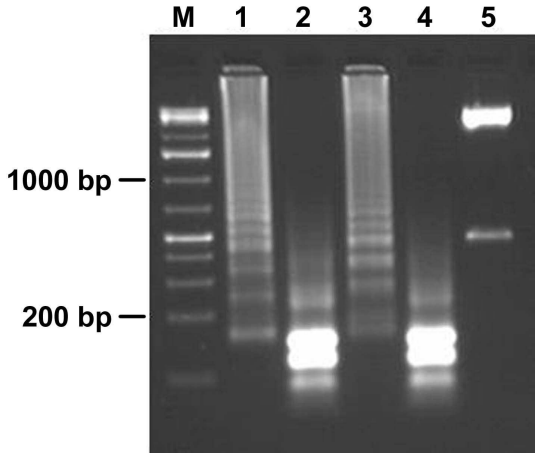


Fig. 3 Restriction digestion of LAMP products from *T. luwenshuni* and *T. uilenbergi*. *Eco*RI restriction sites were introduced into FIP and BIP primers, allowing restriction fragment analysis for the specificity of the LAMP reaction. 1. LAMP products from gDNA of *T. luwenshuni*, 2. *Eco*RI digest of *T. luwenshuni* LAMP, 3. LAMP products from gDNA of *T. uilenbergi*, 4. *Eco*RI digest of *T. uilenbergi* LAMP, 5. *Eco*RI enzyme activity control by digesting reconstructed plasmid DNA in pDrive vector.

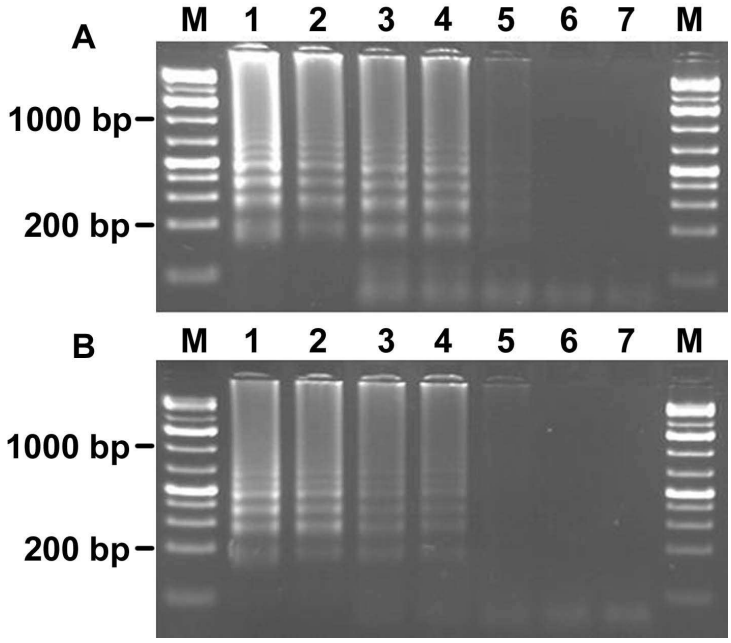


Fig. 4 Sensitivity of the LAMP method. Genomic DNA from *Theileria luwenshuni* and *T. uilenbergi* was diluted 10-fold serially. Lane 1 to 7, 100 pg, 10 pg, 1 pg, 10^{-1} pg, 10^{-2} pg, 10^{-3} pg, 10^{-4} pg. Panel A, LAMP reaction with gDNA of *T. luwenshuni*, Panel B, LAMP reaction with gDNA of *T. uilenbergi*.

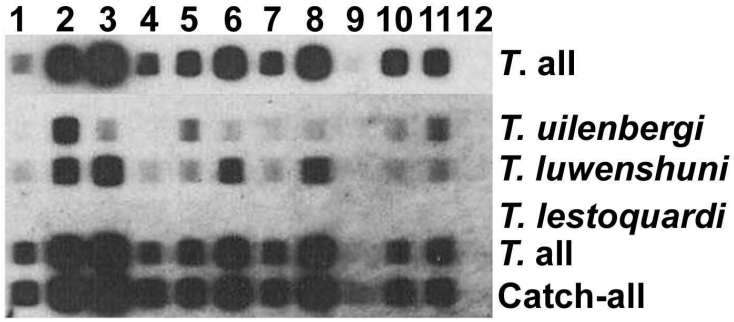


Fig. 5 Detection of field samples using RLB. *T.* all: probe detecting all *Theileria* species; Catch-all: probe detecting all *Theileria* and *Babesia* species. The detection results are listed in Table 2.

Tables

Table 1 Nucleotide sequences of LAMP primers for detection of *T. luwenshuni* and *T. uilenbergi*

Primer	Type	Sequence(5'-3')[a]
URTF3	F3	TCGGATAGGGGGTGGATT
URTB3	B3	TGAGGAGGTTGTGGTTAAGCT
URTFIP	FIP (F1c+F2)	TGTAGAGTTAAGTTGTTGGC*gaattc*CATAACATTTCCCCATAACACA
URTBIP	BIP (B1c+B2)	AATGTTCAAGGTCCTCGCCTT*gaattc*AAAATGAGCAAACACAGCAG
URTLF	LF	GAATAAATACGCTAGGAATGGT
URTLB	LB	CTTTGCCTTTGCAGCTTC

[a] The lower case italic letters in FIP and BIP primer sequences indicate *Eco*RI restriction sites.

Table 2 Evaluation of LAMP method by comparing with RLB method for detection of field samples

Test		RLB		Total
		Positive	Negative	
LAMP	Positive	31	1	32 (37.2%)
	Negative	16	38	54 (62.8%)
	Total	47 (54.7%)	39 (45.3%)	86 (100%)

Sensitivity of the LAMP=66.0%

Specificity of the LAMP=97.4%

5 Discussion

This chapter focuses especially on the discussion of the study design and some complementary points of view and prospects for future work. Further aspects of this doctoral study were elaborately discussed in Chapter 4 (publications).

To date, phylogenetic studies using 18S ribosomal RNA genes have successfully classified *Theileria* species infective to small ruminants in China as two species, namely *T. luwenshuni* and *T. uilenbergi* (Ahmed et al., 2006; Yin et al., 2007). However, little is known about the sequences contained in the genome of these two species. Currently, only a few heterologous genes in other *Theileria* species have been shown to be transcribed in piroplasms of these *Theileria* species (Miranda et al., 2006c). These include TcD, which showed 88% identity to a putative *T. annulata* membrane protein (TaD), TcSP partial cDNA, which showed 94% identity to *T. annulata* surface protein (TaSP), TcSE partial cDNA, which showed 99% identity to a secretory protein of *T. annulata* (TaSE) and Tc Clone-5, which showed 100% identity to *T. lestoquardi* Clone-5 on the genomic level. TcHSP70 was identified as a homologue of *T. lestoquardi* TlHSP70, the latter of which was obtained through immunological screening of a *T. lestoquardi* schizont cDNA library (Miranda et al., 2006a). The identification of the heterologues from other *Theileria* species in *T. uilenbergi* and *T. luwenshuni* is helpful and powerful for finding expressed genes in these species, however, since the heterologous genes usually showed high identity, this could be an obstacle for the development of species specific diagnostic tools, identification of genetic markers for different species and studies on the biological characters of a certain species, especially with respect to the two new *Theileria* species. In fact, a previous study on serological identification of antigens from lysed merozoites showed that the spectrum of merozoite antigens is not identical between *T. uilenbergi* and *T. luwenshuni* (Gao, 2001). Thus, it is of great interest to identify *T. uilenbergi* and/or *T. luwenshuni* specific genes. To meet this demand, the strategy used in this study involved the establishment and screening of a *T. uilenbergi* merozoite cDNA library for the purpose of identifying species specific genes.

Construction of the cDNA library from the merozoite stage was performed considering two aspects. On the one hand, the merozoite material can be obtained relatively easy by isolation and purification from infected blood of experimentally infected animals, whereas it is not possible to obtain other parasite stages such as schizonts, since to date all attempts to

establish schizont infected cell lines have failed (Yin et al., 2007). On the other hand, the piroplasm stage seems to play a pivotal role for the pathogenesis of the disease indicated by the marked anemia in sick animals. Also, considering the close phylogenetic relationship to the *T. buffeli* group (Yin et al., 2003; Yin et al., 2004a) it is likely that these parasites share similar pathogenic features. Thus it is likely that the identification and characterization of unique and/or antigenic genes expressed at the merozoite stage may contribute to the goal of being able to devise control strategies for this disease, including specific diagnostic tests and vaccines.

Pure merozoite material was a prerequisite for construction of a representative expression cDNA library. The more pure the merozoite material, the lesser will be the chance that genes of the ovine host would be included in the cDNA library. For preparation of the merozoites of *T. uilenbergi*, a well known parasite stock was used to infect the experimental sheep. This stock was adequately characterized in previous phylogenetic studies (Schnittger et al., 2003; Yin et al., 2004a) and by PCR (Yin et al., 2008). When the purified erythrocytes were Giemsa-stained and observed under the microscope, no white blood cells were observed, implying complete removal of leukocytes from blood and subsequently, reduced risk of contamination with clones of sheep origin in the library. The hemolysin used to destroy the erythrocytes is known not to damage protozoan cells (Sugimoto et al., 1988, Pearson et al., 1982) and the Percoll gradient centrifugation protocol used in this study has been successfully applied for the purification of the piroplasms from *Babesia* and *Theileria* infected erythrocytes (Shimizu et al., 1988, Ohgitani et al., 1987, Rodriguez et al., 1986, Hauschild et al., 1995) and previously has been used for successful purification of the intact merozoites of *Theileria* sp. (China) (Miranda et al., 2006b). The applied purification protocol has thus greatly reduced the chance of inclusion of host genes in the cDNA library, which was indicated by the fact that all randomly cloned and sequenced inserts of the library were of *Theileria*-origin.

Successful isolation of mRNA from purified merozoites was indicated by agarose gel electrophoresis. Since RNA molecules are exceptionally labile and difficult to amplify in their natural form (Sambrook and Russell, 2001), the cDNA library was constructed from isolated mRNA using ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III Gold cloning kit. The key features of this cDNA library protocol are: (1) use of StrataScript reverse transcriptase,

which is a Moloney murine leukemia virus reverse transcriptase without any RNase H activity, guaranteeing the preparation of the full length and complete cDNA transcripts; (2) directional cloning cDNA transcripts under the *lacZ* promoter of the lambda ZAP vector through enzyme restriction sites ensures the representativeness of the cDNA library. The quality evaluation of the library showed that the yield (6×10^8 pfu/ml) and recombinant ratio (70%) were lower than desired (yields should usually be more than 10^9 pfu/ml) (Sambrook and Russell, 2001). However, the successful identification of a number of *Theileria* genes, especially the Clone 2 gene family and an immunodominant gene TuIP, indicated successful construction and usefulness of the library.

Screening of the library by random PCR amplification of the inserts using vector specific primers led to identification of 12 clones as listed in Table 1 of publication 1. With assistance of published protozoa genomes, particularly the genomes of *T. annulata* (Pain et al., 2005), *T. parva* (Gardner et al., 2005), *Babesia bovis* (Brayton et al., 2007) and bioinformatics tools, three genes encoding hypothetical proteins showing 20-79% identity to either *T. annulata* or *T. parva* genes, three encoding enzymes of *Theileria* parasites, one encoding guanine nucleotide binding protein and one encoding 60S ribosomal protein were found. Additionally, three sequences, Clone 2, Clone 9 and Clone 26 not showing similarity to other parasite genes, composing part of the Clone 2 gene family, have been primarily characterized and discussed in publication 1. Complementarily, the recombinantly expressed Clone 2 protein showed reactivity with *Theileria* positive sera, indicating its potential use as an antigen for the development of serological diagnostic tools or subunit vaccine. PCR amplification of genomic DNA of *T. uilenbergi* using primers designed as described led to the identification of again three new variants of the Clone 2 family (Genbank accession numbers EU488867-9), implying that the gene family is likely variable between isolates since the source of the cDNA library and the genomic DNA was not from the same parasite stock. However, this hypothesis needs to be investigated in more detail. Moreover, a heterologue (EU753118) from *T. luwenshuni* was also obtained as described in publication 2. Interestingly, BLASTn searches through available protozoa genomes including genomes of *T. annulata* and *T. parva* did not identify heterologous genes, the only significant identity was found with the low copy number gene ToLocg 1 (AB159782) of *T. orientalis* (41-47% identity), indicating that the identified Clone 2 gene family is likely specific for *T. uilenbergi* and *T. luwenshuni*. Perhaps this gene

family even has the potential to be used as a genetic marker for differentiation of *Theileria* species closely related to *T. uilenbergi*, or for differentiation of various isolates of *Theileria* species in China. Another interesting feature of this gene family is that it is tandemly arranged. These features are similar to a multicopy locus, *Tpr* of *T. parva*, which consists of tandemly arrayed open reading frames (ORFs) containing several repeated elements (Baylis et al., 1991). However, BLASTp search using amino acid sequences from the Clone 2 family only showed 30-35% identity to *T. parva* hypothetical proteins, but not to the *Tpr* protein family. To further elucidate this gene family, on the one hand more members of the Clone 2 gene family need to be identified and their structure characterized in more detail; on the other hand, it is possible that the genomic syntenic Clone 2 gene family and *Tpr* gene family are highly divergent, since also a striking difference between *Tpr* of *T. parva* and *Tar* of *T. annulata* in their gene arrangement and expression was observed despite the high level of genomic synteny between the two species (Weir et al., 2009). The studies on *Tpr* showed that some of the *Tpr* genes are only transcribed in the piroplasm stage and the domains within the *Tpr* genes are isolate-specific (Gardner et al., 2005), the latter is similar to the findings in this study that the PCR primers designed from the conserved region of one parasite isolate could not amplify an identical product but rather a variant from another, suggesting the divergence between parasite isolates.

Although divergent, a highly conserved region among the Clone 2 gene family was also present. Based on this region, primers were designed for the development of a loop-mediated isothermal amplification method (LAMP) as described in Publication 2. This LAMP method showed many advantages compared to previously established DNA-based detection methods, such as rapidity, efficiency, low price, ease of handling and analysis of results for diagnosis of *T. uilenbergi* and *T. luwenshuni* infections. The LAMP method was designed to detect *T. uilenbergi* and *T. luwenshuni* infection simultaneously in this study, therefore it has a great potential as a test that the local veterinarian can use for diagnosing individual cases of theileriosis and/or making on the spot examination. For studies on individual *T. uilenbergi* or *T. luwenshuni* infection, PCR and RLB methods can still be regarded as helpful. Thus, the LAMP method can be regarded as being able to assist these previous detection methods for alternative investigation of theileriosis in the endemic regions according to different purposes. Although the LAMP method has shown robustness for detection of *Theileria* infections in

China, it still needs more validation with more samples and in comparison with multiple other methods before distribution into field practice.

Besides screening the cDNA library using the PCR approach, the library was screened using serum from an animal infected with *T. uilenbergi*. One positive clone, termed TuIP, was obtained and deposited in Genbank under accession number FJ467922. Partially recombinantly cloned and expressed TuIP has shown excellent reactivity with specific antibodies of *T. uilenbergi* infected animals and has been used to develop an indirect ELISA method in ongoing work.

In summary, a merozoite cDNA library of *T. uilenbergi* was successfully constructed, and its high quality has been proved by identification of genes of interest for development of diagnostics. PCR screening of the library discovered a Clone 2 gene family, and its shared features to *Tpr* gene family of *T. parva* suggests it may play a role in concerted evolution (Bishop et al., 1997). Based on the highly conserved region of this gene family, a set of six primers were designed for successful development of LAMP for detection of *T. uilenbergi* and *T. luwenshuni* infection. Moreover, the serological screening of the library has identified the TuIP antigenic gene, which will be useful for development of serological diagnostic tools and which may be regarded as a candidate for vaccine development in the future.

6 References

Ahmed, J.S., Rehbein, G., Schein, E., 1984. Characterization of *Theileria annulata* infected lymphoblastoid cells. Z. Parasitenkd. 70, 819-821.

Ahmed, J.S., Luo, J., Schnittger, L., Seitzer, U., Jongejan, F., Yin, H., 2006. Phylogenetic position of small-ruminant infecting piroplasms. Ann. N. Y. Acad. Sci. 1081, 498-504.

Aktas, M., Bendele, K.G., Altay, K., Dumanli, N., Tsuji, M., Holman, P.J., 2007. Sequence polymorphism in the ribosomal DNA internal transcribed spacers differs among *Theileria* species. Vet. Parasitol. 147, 221-230.

Alani, A.J., Herbert, I.V., 1988. Pathogenesis of infection with *Theileria recondita* (Wales) isolated from *Haemaphysalis punctata* from north Wales. Vet. Parasitol. 28, 293-301.

Allsopp, B.A., Baylis, H.A., Allsopp, M.T., Cavalier-Smith, T., Bishop, R.P., Carrington, D.M., Sohanpal, B., Spooner, P., 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. Parasitology 107, 157-165.

Bakheit, M.A., Scholzen, T., Ahmed, J.S., Seitzer, U., 2006a. Molecular characterization of a *Theileria lestoquardi* gene encoding for immunogenic protein splice variants. Parasitol. Res. 100, 161-170.

Bakheit, M.A., Seitzer, U., Ahmed, J.S., 2006b. A new recombinant protein-based ELISA for the diagnosis of malignant theileriosis of sheep and goats. Parasitol. Res. 98, 145-149.

Baldwin, C.L., Black, S.J., Brown, W.C., Conrad, P.A., Goddeeris, B.M., Kinuthia, S.W., Lalor, P.A., MacHugh, N.D., Morrison, W.I., Morzaria, S.P., 1988. Bovine T cells, B cells, and null cells are transformed by the protozoan parasite *Theileria parva*. Infect. Immun. 56, 462-467.

Baylis, H.A., Sohal, S.K., Carrington, M., Bishop, R.P., Allsopp, B.A., 1991. An unusual repetitive gene family in *Theileria parva* which is stage-specifically transcribed. Mol. Biochem. Parasitol. 49, 133-142.

Bishop, R., Musoke, A., Morzaria, S., Gardner, M., Nene, V., 2004. *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. Parasitology 129, S271-S283.

Bishop, R., Musoke, A., Morzaria, S., Sohanpal, B., Gobright, E., 1997. Concerted evolution at a multicopy locus in the protozoan parasite *Theileria parva*: extreme divergence of potential protein-coding sequences. Mol. Cell Biol. 17, 1666-1673.

Boulter, N., Hall, R., 1999. Immunity and vaccine development in the bovine theilerioses. Adv. Parasitol. 44, 41-97.

Brayton, K.A., Lau, A.O., Herndon, D.R., Hannick, L., Kappmeyer, L.S., Berens, S.J.,

Bidwell, S.L., Brown, W.C., Crabtree, J., Fadrosh, D., Feldblum, T., Forberger, H.A., Haas, B.J., Howell, J.M., Khouri, H., Koo, H., Mann, D.J., Norimine, J., Paulsen, I.T., Radune, D., Ren, Q., Smith, R.K., Jr., Suarez, C.E., White, O., Wortman, J.R., Knowles, D.P., Jr., McElwain, T.F., Nene, V.M., 2007. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS. Pathog. 3, 1401-1413.

Brown, C.G., Ilhan, T., Kirvar, E., Thomas, M., Wilkie, G., Leemans, I., Hooshmand-Rad, P., 1998. *Theileria lestoquardi* and *T. annulata* in cattle, sheep, and goats. *In vitro* and *in vivo* studies. Ann. N. Y. Acad. Sci. 849, 44-51.

Cavalier-Smith, T., 1993. Kingdom protozoa and its 18 phyla. Microbiol. Rev. 57, 953-994.

Chae, J.S., Allsopp, B.A., Waghela, S.D., Park, J.H., Kakuda, T., Sugimoto, C., Allsopp, M.T., Wagner, G.G., Holman, P.J., 1999. A study of the systematics of *Theileria* spp. based upon small-subunit ribosomal RNA gene sequences. Parasitol. Res. 85, 877-883.

Chansiri, K., Kawazu, S., Kamio, T., Terada, Y., Fujisaki, K., Philippe, H., Sarataphan, N., 1999. Molecular phylogenetic studies on *Theileria* parasites based on small subunit ribosomal RNA gene sequences. Vet. Parasitol. 83, 99-105.

Dchunkowsky, E., Urodschevich, V. (1924). Theileriosis in goats, sheep and cattle with a descrioption of *Theileria hirci* (nov. sp.) from Serbia. Parasitology 16, 107-110.

Dobbelaere, D., Heussler, V., 1999. Transformation of leukocytes by *Theileria parva* and *T. annulata*. Annu. Rev. Microbiol. 53, 1-42.

Dolan, T.T., 1989. Theileriosis: a comprehensive review. Rev. Sci. Tech. Off. Int. Epizoot. 8, 11-36.

Dukes, J.P., King, D.P., Alexandersen, S., 2006. Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Arch. Virol. 151, 1093-1106.

Ellis, J., Hefford, C., Baverstock, P.R., Dalrymple, B.P., Johnson, A.M., 1992. Ribosomal DNA sequence comparison of *Babesia* and *Theileria*. Mol. Biochem. Parasitol. 54, 87-95.

Forsyth, L.M., Minns, F.C., Kirvar, E., Adamson, R.E., Hall, F.R., McOrist, S., Brown, C.G., Preston, P.M., 1999. Tissue damage in cattle infected with *Theileria annulata* accompanied by metastasis of cytokine-producing, schizont-infected mononuclear phagocytes. J. Comp. Pathol. 120, 39-57.

Gajadhar, A.A., Marquardt, W.C., Hall, R., Gunderson, J., Ariztia-Carmona, E.V., Sogin, M.L., 1991. Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata* and *Crypthecodinium cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. Mol. Biochem. Parasitol. 45, 147-154.

Gao, Y.L., 2001. Studies on merozoite antigenic protein analysis of Chinese *Theileria* isolates

and establishment of diagnostic method and chemical treatment of the disease. Dissertation of Master Degree in Inner Mongolia Agricultural University, P1-64.

Gao, Y.L., Yin, H., Luo, J.X., Ouyang, W.Q., Bao, H.M., Guan, G.Q., Zhang, Q.C., Lu, W.S., Ma, M.L., 2002. Development of an enzyme-linked immunosorbent assay for the diagnosis of *Theileria* sp. infection in sheep. Parasitol. Res. 88, S8-10.

Gardner, M.J., Bishop, R., Shah, T., de Villiers, E.P., Carlton, J.M., Hall, N., Ren, Q., Paulsen, I.T., Pain, A., Berriman, M., Wilson, R.J., Sato, S., Ralph, S.A., Mann, D.J., Xiong, Z., Shallom, S.J., Weidman, J., Jiang, L., Lynn, J., Weaver, B., Shoaibi, A., Domingo, A.R., Wasawo, D., Crabtree, J., Wortman, J.R., Haas, B., Angiuoli, S.V., Creasy, T.H., Lu, C., Suh, B., Silva, J.C., Utterback, T.R., Feldblyum, T.V., Pertea, M., Allen, J., Nierman, W.C., Taracha, E.L., Salzberg, S.L., White, O.R., Fitzhugh, H.A., Morzaria, S., Venter, J.C., Fraser, C.M., Nene, V., 2005. Genome sequence of *Theileria parva*, a bovine pathogen that transforms lymphocytes. Science 309, 134-137.

Glass E.J., Innes E.A., Spooner R.L., Brown C.G.D., 1989. Infection of bovine monocyte/macrophage populations with *Theileria annulata* and *Theileria parva*. Vet. Immunol. Immunopathol. 22, 355-368.

Gubbels, M.J., Hong, Y., van der, W.M., Qi, B., Nijman, I.J., Guangyuan, L., Jongejan, F., 2000. Molecular characterisation of the *Theileria buffeli*/*orientalis* group. Int. J. Parasitol. 30, 943-952.

Gubbels, M.J., Yin, H., Bai, Q., Liu, G., Nijman, I.J., Jongejan, F., 2002. The phylogenetic position of the *Theileria buffeli* group in relation to other *Theileria* species. Parasitol. Res. 88, S28-S32.

Guo, S., Yuan, Z., Wu, G., Wang, W., Ma, D., Du, H., 2002. Epidemiology of ovine theileriosis in Ganan region, Gansu Province, China. Parasitol. Res. 88, S36-S37.

Guo, S., Mu, Y., Liu, Z., Ma, D., Yang, S., Ge, G., Fang, B., Ga, D., Ma, M., Luo, J., Yin, H., Seitzer, U., Ahmed, J.S., 2007. Serological investigation of ovine theileriosis by ELISA in Gannan Tibet Region of Gansu Province in China. Parasitol. Res. 101 Suppl 2, S197-S200.

Hashemi-Fesharki, R., 1997. Tick-borne diseases of sheep and goats and their related vectors in Iran. Parassitol. 39, 115-117.

Hauschild, S., Shayan P., Schein E., 1995. Characterization and comparison of merozoite antigens of different *Babesia canis* isolates by serological and immunological investigations. Parasitol. Res. 81, 638–642.

Mehlhorn, H., 2008. Theileriosis. Encyclopedia of parasitiology, 3rd Edition, pp1370-1372.

Hooshmand-Rad, P., Hawa, N.J., 1973. Malignant theileriosis of sheep and goats. Trop. Anim. Health Prod. 5, 97-102.

Hooshmand-Rad, P., Hawa, N.J., 1973. Transmission of *Theileria hirci* in sheep by

Hyalomma anatolicum anatolicum. Trop. Anim. Health Prod. 5, 103.

Hooshmand-Rad, P., Hawa, N.J., 1975. Cultivation of *Theileria hirci* in sheep lymphoid cells. Trop. Anim. Health Prod. 7, 121-122.

Irvin, A.D., 1987. Characterization of species and strains of *Theileria*. Adv. Parasitol. 26, 145-197.

Irvin, A.D., Morrison, W.I., 1987. In immune responses in parasitic infections: immunology, immunopathology and immunoprophylaxis. Vol III Protozoa (Soulsby, E.L., ed.), pp 223-274.

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

Kakuda, T., Shiki, M., Kubota, S., Sugimoto, C., Brown, W.C., Kosum, C., Nopporn, S., Onuma, M., 1998. Phylogeny of benign *Theileria* species from cattle in Thailand, China and the U.S.A. based on the major piroplasm surface protein and small subunit ribosomal RNA genes. Int. J. Parasitol. 28, 1261-1267.

Kawazu, S., Kamio, T., Kakuda, T., Terada, Y., Sugimoto, C., Fujisaki, K., 1999. Phylogenetic relationships of the benign *Theileria* species in cattle and Asian buffalo based on the major piroplasm surface protein (p33/34) gene sequences. Int. J. Parasitol. 29, 613-618.

Kawazu, S., Sato, M., Kamio, T., Yokomizo, Y., Fujisaki, K., Honma, S., Minami, T., 1991. Immunohistological staining for detection of *Theileria sergenti* schizonts. J. Vet. Med. Sci. 53, 743-745.

Kirvar, E., Ilhan, T., Katzer, F., Wilkie, G., Hooshmand-Rad, P., Brown, D., 1998. Detection of *Theileria lestoquardi* (hirci) in ticks, sheep, and goats using the polymerase chain reaction. Ann. N. Y. Acad. Sci. 849, 52-62.

Leemans, I., Hooshmand-Rad, P., Uggla, A., 1997. The indirect fluorescent antibody test based on schizont antigen for study of the sheep parasite *Theileria lestoquardi*. Vet. Parasitol. 69, 9-18.

Levine, N.D., Corliss, J.O., Cox, F.E., Deroux, G., Grain, J., Honigberg, B.M., Leedale, G.F., Loeblich, A.R., III, Lom, J., Lynn, D., Merinfeld, E.G., Page, F.C., Poljansky, G., Sprague, V., Vavra, J., Wallace, F.G., 1980. A newly revised classification of the protozoa. J. Protozool. 27, 37-58.

Levine, N.D., 1988. Progress in taxonomy of the Apicomplexan protozoa. J. Protozool. 35, 518-520.

Li, Y., Luo, J., Liu, Z., Guan, G., Gao, J., Ma, M., Dang, Z., Liu, A., Ren, Q., Lu, B., Liu, J., Zhao, H., Li, J., Liu, G., Bai, Q., Yin, H., 2007. Experimental transmission of *Theileria* sp. (China 1) infective for small ruminants by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis*. Parasitol. Res. 101, 533-538.

Li, Y., Luo, J., Guan, G., Ma, M., Liu, A., Liu, J., Ren, Q., Niu, Q., Lu, B., Gao, J., Liu, Z., Dang, Z., Tian, Z., Zhang, B., He, Z., Bai, Q., Yin, H., 2009. Experimental transmission of *Theileria uilenbergi* infective for small ruminants by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis*. Parasitol. Res. 104, 1227-1231.

Li, Z., Hao, Y., Chen, T., Yu, X., Xi, H., Zuo, Y., Chen, Y., Liu, J., Ding, Y., Meng, Z., 1983. Investigation on ticks and their importance to small ruminants in Dian'an community (in Chinese). Ningxia J. Agric. Sci. Tech. 6, 28–30.

Li, Z., Hao, Y., Chen, T., Yu, X., Xi, H., Zuo, Y., Chen, Y., Liu, J., Ding, Y., Meng, Z., 1985. Investigation on ovine theileriosis in Guyuan District (in Chinese). Chin. J. Vet. Sci. Technol.1 Suppl, 81–85.

Li, Z., Hao, Y., Yu, X., Yan, H., Ding, Y., 1986. Incubation test on *Haemaphysalis qinghaiensis* and *Dermacentor silvarum* and transmission experiment of *Theileria hirci* with them (in Chinese). Chin. J. Vet. Sci. Tech. 7, 36–39.

Li, Z., Niu, Y., 1995. Diagnosis and treatment of *Theileria hirci* of sheep imported from Shandong in Yongshou County, Shaanxi province (in Chinese). Chin. J. Vet. Sci. Technol. 10, 32-33.

Lu, W., Lu, W., Zhang, Q., Yin, H., Dou, H., Luo, J., 1994. Studies on investigations of the tick-borne diseases of sheep and goats in Gansu Province (in Chinese). Symposium of fourth academic meeting of veterinary parasitology association, 41-44.

Luo, J., Yin, H., 1997. Theileriosis of sheep and goats in China. Trop. Anim. Health. Prod. 29, 8S-10S.

Marsh, A.E., Barr, B.C., Sverlow, K., Ho, M., Dubey, J.P., Conrad, P.A., 1995. Sequence analysis and comparison of ribosomal DNA from bovine *Neospora* to similar coccidial parasites. J. Parasitol. 81, 530-535.

Mehlhorn, H., Heydorn, A.O., Schein, E., 1987. Developmental processes in parasitic protozoa. Int. J. Parasitol. 17, 259-267.

Mehlhorn, H., Shein, E., 1984. The piroplasms: life cycle and sexual stages. Adv. Parasitol. 23, 37-103.

Miranda, J., Bakheit, M.A., Liu, Z., Yin, H., Mu, Y., Guo, S., Beyer, D., Oliva, A., Ahmed, J.S., Seitzer, U., 2006a. Development of a recombinant indirect ELISA for the diagnosis of *Theileria* sp. (China) infection in small ruminants. Parasitol. Res. 98, 561-567.

Miranda, J., Nascimento, E., Cruz, H., Hong, Y., Coelho, A., Ahmed, J.S., Oliva, A., 2006b. Identification and characterization of merozoite antigens of a *Theileria* species highly pathogenic for small ruminants in china. Ann. N. Y. Acad. Sci. 1081, 443-452.

Miranda, J.P., Bakheit, M., Schneider, I., Haller, D., Ahmed, J.S., Yin, H., Oliva, A.G., Seitzer, U., 2006c. Identification of homologous genes of *T. annulata* proteins in the genome

of *Theileria* sp. (China). Ann. N. Y. Acad. Sci. 1081, 468-470.

Morel, P.C., Uilenberg, G., 1981. The nomenclature of some *Theileria* species (Sporozoa, Babesioidea) of domestic ruminants (author's transl). Rev. Elev. Med. Vet. Pays Trop. 34, 139-143.

Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004. Identification, genetic diversity and prevalence of *Theileria* and *Babesia* species in a sheep population from Northern Spain. Int. J. Parasitol. 34, 1059-1067.

Neitz, W.O., 1957. Theileriosis, gonderiosis and cytauxzoonosis: a review. Onderstepoort J. Vet. Res. 27, 275.

Niu, Q., Luo, J., Guan, G., Liu, Z., Ma, M., Liu, A., Gao, J., Ren, Q., Li, Y., Qiu, J., Yin, H., 2009a. Differentiation of two ovine *Babesia* based on the ribosomal DNA internal transcribed spacer (ITS) sequences. Exp. Parasitol. 121, 64-68.

Niu, Q., Luo, J., Guan, G., Ma, M., Liu, Z., Liu, A., Dang, Z., Gao, J., Ren, Q., Li, Y., Liu, J., Yin, H., 2009b. Detection and differentiation of ovine *Theileria* and *Babesia* by reverse line blotting in China. Parasitol. Res. 104, 1417-23.

Njiru, Z.K., Mikosza, A.S., Matovu, E., Enyaru, J.C., Ouma, J.O., Kibona, S.N., Thompson, R.C., Ndung'u, J.M., 2008. African trypanosomiasis: sensitive and rapid detection of the sub-genus Trypanozoon by loop-mediated isothermal amplification (LAMP) of parasite DNA. Int. J. Parasitol. 38, 589-599.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Ohgitani, T., Okabe, T., Sasaki, N., 1987. Antigenic properties of *Theileria sergenti* in ELISA serodiagnosis. Jpn. J Vet. Sci. 49, 531-534.

Pain, A., Renauld, H., Berriman, M., Murphy, L., Yeats, C.A., Weir, W., Kerhornou, A., Aslett, M., Bishop, R., Bouchier, C., Cochet, M., Coulson, R.M., Cronin, A., de Villiers, E.P., Fraser, A., Fosker, N., Gardner, M., Goble, A., Griffiths-Jones, S., Harris, D.E., Katzer, F., Larke, N., Lord, A., Maser, P., McKellar, S., Mooney, P., Morton, F., Nene, V., O'Neil, S., Price, C., Quail, M.A., Rabbinowitsch, E., Rawlings, N.D., Rutter, S., Saunders, D., Seeger, K., Shah, T., Squares, R., Squares, S., Tivey, A., Walker, A.R., Woodward, J., Dobbelaere, D.A., Langsley, G., Rajandream, M.A., McKeever, D., Shiels, B., Tait, A., Barrell, B., Hall, N., 2005. Genome of the host-cell transforming parasite *Theileria annulata* compared with *T. parva*. Science 309, 131-133.

Pearson, T.W., Saya, L.E., Howard, S.P., Buckley, J.T., 1982. The use of aerolysin toxin as an aid for visualization of low numbers of African trypanosomes in whole blood. Acta. Trop. 39, 73-77.

Preston, P.M., Brown, C.G., Entrican, G., Richardson, W., Boid, R., 1993. Synthesis of tumour necrosis factor-alpha and interferons by mononuclear cells from *Theileria annulata*-infected cattle. Parasite Immunol. 15, 525-534.

Preston, P.M., Hall, F.R., Glass, E.J., Campbell, J.D., Darghouth, M.A., Ahmed, J.S., Shiels, B.R., Spooner, R.L., Jongejan, F., Brown, C.G., 1999. Innate and adaptive immune responses co-operate to protect cattle against *Theileria annulata*. Parasitol. Today 15, 268-274.

Qiu, Z., Yuan, H., 1982. Investigation on the ‘‘waist stiff disease’’ in Lintao county (in Chinese). Vet. Sci. Technol. 7, 29–31.

Ren, N., Zheng, Z., Xing, D., 1982. Investigation on the piroplasmosis of sheep (in Chinese). Niaoning J. Vet. Sci. Technol. 1, 6–8.

Ren, N., Zheng, Z., Xing, D., 1982. Investigation of theileriosis of sheep (in Chinese). Liaoning J. Vet. Med. Anim. Husb. 1, 6-8.

Rodriguez, S.D., Buening, G.M., Vega, C.A., Carson, C.A., 1986. *Babesia bovis*: purification and concentration of merozoites and infected bovine erythrocytes. Exp. Parasitol. 61, 236-243.

Sambrook, J., Russell, D., 2001. Molecular cloning, A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sato, M., Kamio, T., Kawazu, S., Taniguchi, T., Minami, T., Fujisaki, K., 1993. Histological observations on the schizonts in cattle infected with Japanese *Theileria sergenti*. J. Vet. Med. Sci. 55, 571-574.

Sato, M., Kamio, T., Tanaka, S., Taniguchi, T., Fujisaki, K., 1994. Development of *Theileria sergenti* schizonts in the lymph node of experimentally infected cattle. J. Vet. Med. Sci. 56, 715-722.

Schein, E., 1975. On the life cycle of *Theileria annulata* (Dschunkowsky and Luhs, 1904) in the midgut and hemolymph of *Hyalomma anatolicum excavatum* (Koch, 1844). Z. Parasitenkd. 47, 165-167.

Schnittger, L., Hong, Y., Jianxun, L., Ludwig, W., Shayan, P., Rahbari, S., Voss-Holtmann, A., Ahmed, J.S., 2000a. Phylogenetic analysis by rRNA comparison of the highly pathogenic sheep-infecting parasites *Theileria lestoquardi* and a *Theileria* species identified in China. Ann. N. Y. Acad. Sci. 916, 271-275.

Schnittger, L., Yin, H., Jianxun, L., Ludwig, W., Shayan, P., Rahbari, S., Voss-Holtmann, A., Ahmed, J.S., 2000b. Ribosomal small-subunit RNA gene-sequence analysis of *Theileria lestoquardi* and a *Theileria* species highly pathogenic for small ruminants in China. Parasitol. Res. 86, 352-358.

Schnittger, L., Yin, H., Gubbels, M.J., Beyer, D., Niemann, S., Jongejan, F., Ahmed, J.S., 2003. Phylogeny of sheep and goat *Theileria* and *Babesia* parasites. Parasitol. Res. 91, 398-406.

Schnittger, L., Yin, H., Qi, B., Gubbels, M.J., Beyer, D., Niemann, S., Jongejan, F., Ahmed, J.S., 2004. Simultaneous detection and differentiation of *Theileria* and *Babesia*

parasites infecting small ruminants by reverse line blotting. Parasitol. Res. 92, 189-196.

Seitzer, U., Liu, Z., Yin, H., Beyer, D., Kullmann, B., Miranda, J., Ahmed, J.S., 2008. Immune response of *Theileria* sp.-infected sheep to recombinant *Theileria* proteins. Ann. N. Y. Acad. Sci. 1149, 186-190.

Seki, M., Yamashita, Y., Torigoe, H., Tsuda, H., Sato, S., Maeno, M., 2005. Loop-mediated isothermal amplification method targeting the lytA gene for detection of *Streptococcus pneumoniae*. J. Clin. Microbiol. 43, 1581-1586.

Shaw, M.K., Tilney, L.G., 1992. How individual cells develop from a syncytium: merogony in *Theileria parva* (Apicomplexa). J. Cell Sci. 101, 109-123.

Shaw, M.K., Tilney, L.G., 1995. The entry of *Theileria parva* merozoites into bovine erythrocytes occurs by a process similar to sporozoite invasion of lymphocytes. Parasitology 111, 455-461.

Shaw, M.K., 1997. The same but different: the biology of *Theileria* sporozoite entry into bovine cells. Int. J Parasitol. 27, 457-474.

Shaw, M.K., 2002. *Theileria* development and host cell invasion. In: *Theileria*, Dobbelaere DAE, McKeever DJ (eds) World Class Parasites, Kluwer, Boston, London, UK, pp 1-22.

Shaw, M.K., 2003. Cell invasion by *Theileria* sporozoites. Trends Parasitol. 19, 2-6.

Shimizu, S., Suzuki, K., Nakamura, Y., Kadota, K., Fujisaki, K., Ito, S., Minami, T., 1988. Isolation of *Theileria sergenti* piroplasms from infected erythrocytes and development of an enzyme-linked immunosorbent assay for serodiagnosis of *T. sergenti* infections. Res. Vet. Sci. 45, 206-212.

Spooner, R.L., Innes, E.A., Glass, E.J., Brown, C.G.D., 1989. *Theileria annulata* and *T. parva* infect and transform different bovine mononuclear cells. Immunology 66, 284-288.

Sugimoto, C., Conrad, P.A., Ito, S., Brown, W.C., Grab, D.J., 1988. Isolation of *Theileria parva* schizonts from infected lymphoblastoid cells. Acta. Trop. 45, 203-216.

Sugimoto, C., Sato, M., Kawazu, S., Kamio, T., Fujisaki, K., 1991. Purification of merozoites of *Theileria sergenti* from infected bovine erythrocytes. Parasitol. Res. 77, 129-131.

Sugimoto, C. and Fujisaki, K., 2002. Non-transforming *Theileria* parasites of ruminants. In: *Theileria*, Dobbelaere DAE, McKeever DJ (eds) World Class Parasites, Kluwer, Boston, London, UK, pp93-106.

Uilenberg, G., Andreasen, M.P., 1974. *Haematoxenus separatus* sp. n. (Sporozoa, Theileriidae) a new blood parasite of domestic sheep in Tanzania. Revue d'Elevage et Medicine veterinaire des Pais tropicaux 27, 459-465.

Uilenberg, G., 1981. Theilerial species of domestic livestock. In: Advances in the control of

theileriosis. Irvin, A.D., Cunningham, M.P. and Young, A.S. (Eds). Martinus Nijhoff Publishers, The Hague, pp 4-37.

Uilenberg, G., 1999. Immunization against diseases caused by *Theileria parva*: a review. Trop. Med. Int. Health 4, A12-A20.

van Vorstenbosch, C.J., Uilenberg, G., van Dijk, J.E., 1978. Erythrocytic forms of *Theileria velifera*. Res. Vet. Sci. 24, 214-221.

Wang, F., Li, O., Li, W., Wang, W., Hua, D., Wang, L., Guo, R., Shi, W., Li, Z., 1980. Investigation on the *Theileria hirci* infection in Huangyuan, Menyuan and Jianzha counties (in Chinese). Newsl. Vet. Med. Anim. Husb. (Qinghai) 3: 29–32.

Weir, W., Sunter, J., Chaussepied, M., Skilton, R., Tait, A., de Villiers, E.P., Bishop, R., Shiels, B., Langsley, G., 2009. Highly syntenic and yet divergent: a tale of two Theilerias. Infect. Genet. Evol. 9, 453-461.

Yagi, Y., Ito, N., Kunugiyama, I., 1991. Decrease in erythrocyte survival in *Theileria sergenti*-infected calves determined by non-radioactive chromium labelling method. J. Vet. Med. Sci. 53, 391-394.

Yang, F., Feng, Z., Yu, G., Liu, J., Wei, Z., He, X., 1958. A report on ovine theileriosis in Ganning animal farm station in Ganzi district (in Chinese). Chin. J. Vet. Med. 2,33–37.

Yin, H., Guan, G., Ma, M., Luo, J., Lu, B., Yuan, G., Bai, Q., Lu, C., Yuan, Z., Preston, P., 2002a. *Haemaphysalis qinghaiensis* ticks transmit at least two different *Theileria* species: one is infective to yaks, one is infective to sheep. Vet. Parasitol. 107, 29-35.

Yin, H., Luo, J., Guan, G., Gao, Y., Lu, B., Zhang, Q., Ma, M., Lu, W., Lu, C., Yuan, Z., Guo, S., Wang, B., Du, H., Schnittger, L., Ahmed, J., Jongejan, F., 2002b. Transmission of an unidentified *Theileria* species to small ruminants by *Haemaphysalis qinghaiensis* ticks collected in the field. Parasitol. Res. 88, S25-S27.

Yin, H., Luo, J., Guan, G., Lu, B., Ma, M., Zhang, Q., Lu, W., Lu, C., Ahmed, J., 2002c. Experiments on transmission of an unidentified *Theileria* sp. to small ruminants with *Haemaphysalis qinghaiensis* and *Hyalomma anatolicum anatolicum*. Vet. Parasitol. 108, 21-30.

Yin, H., Liu, G., Luo, J., Guan, G., Ma, M., Ahmed, J., Bai, Q., 2003. Observation on the schizont stage of an unidentified *Theileria* sp. in experimentally infected sheep. Parasitol. Res. 91, 34-39.

Yin, H., Luo, J., Schnittger, L., Lu, B., Beyer, D., Ma, M., Guan, G., Bai, Q., Lu, C., Ahmed, J., 2004. Phylogenetic analysis of *Theileria* species transmitted by *Haemaphysalis qinghaiensis*. Parasitol. Res. 92, 36-42.

Yin, H., Luo, J., 2007. Ticks of small ruminants in China. Parasitol. Res. 101 Suppl 2, S187-S189.

Yin, H., Schnittger, L., Luo, J., Seitzer, U., Ahmed, J.S., 2007. Ovine theileriosis in China: a new look at an old story. Parasitol. Res. 101 Suppl 2, S191-S195.

Yin, H., Liu, Z., Guan, G., Liu, A., Ma, M., Ren, Q., Luo, J., 2008. Detection and differentiation of *Theileria luwenshuni* and *T. uilenbergi* infection in small ruminants by PCR. Transbound. Emerg. Dis. 55, 233-237.

Yu, R., Wei, H., Yu, X., 1982. Diagnosis and treatment of ovine pyroplasmosis in Baijiedian Farm Tunliao County (in Chinese). Chin. J Vet. Sci. Technol. 4, 54–55.

Zhou, B., Cailangdangzhi, Guan, L., La, M., 1981. A primary report of investigations on ovine theileriosis in Banma County of Qinghai Province (in Chinese). Qinghai J. Vet. Husb. 1, 33-37.

Zhu, X., Pu, S., Shi, F., 1990. Report on diagnosis and treatment of ovine theileriosis (in Chinese). Modern Livestock and Poultry 2, 20.

7 Summary

The major aim of this thesis was to identify novel genes of *T. uilenbergi* through establishment and screening of a merozoite cDNA library with the eventual goal to develop diagnostic tools using identified genes for detection of *Theileria* infections. The experiments were initiated by infection of sheep using *T. uilenbergi* stock. When parasiteamia rose, blood was collected and the merozoites were purified. Messenger RNA was isolated from purified merozoites was then utilized to establish a cDNA library. The library was titrated to be 6 x 10^8 pfu/ml and the recombinant clones were estimated to be 70%. Random PCR identification of the library indicated all of the inserts were of parasite origin, indicating the usefulness of the library for the identification of new genes.

Random PCR amplification of inserts of the cDNA library led to the discovery of 12 single clones, among which Clone 2, 9 and 26 exhibited a high degree of identity, especially at the 3' terminus and 3' untranslated region, indicating that they belong to the same gene family. Furthermore, PCR designed to target Clone 2 amplified again four variant genes from genomic DNA of *T. uilenbergi* and one from genomic DNA of *T. luwenshuni*, suggesting this gene family is likely isolate-specific since the DNA samples for PCR were not derived from the same parasite isolate used for library construction. Sequence analysis of another genomic fragment generated with primers targeting the 3' untranslated region of the Clone 26 sequence showed that both 5' and 3' termini were highly identical to the Clone 2 gene family and these homologous termini were separated by a 136 bp sequence fragment highly identical to the 3' untranslated region of the Clone 2 gene family, indicating Clone 2 gene family members are tandemly arranged. Bioinformatic analysis of cDNA sequences of the Clone 2 gene family indicated these genes contain signal peptides and encode potential immunogenic proteins. Analysis of recombinantly expressed Clone 2 revealed immunoreactivity with sera from *Theileria*-infected animals from China. No cross reaction with sera of *T. lestoquardi-, Babesia motasi-* or *Anaplasma ovis-* infected animals was observed, indicating a potential specificity of this gene family. The features of the Clone 2 gene family are similar to the *Tpr* gene family of *T. parva*, which is believed to play a role in concerted evolution.

Based on the highly conserved region of the Clone 2 gene family, a set of six primers were designed for the development of a loop mediated isothermal amplification (LAMP). The established assay allowed the detection of *T. uilenbergi* and *T. luwenshuni* infections

simultaneously and the reaction could be simply accomplished by incubation at 63ºC for 15 min. The specificity of the reaction was confirmed through *Eco*RI restriction enzyme digestion analysis and sequencing. The assay was sensitive as it detected 0.1 pg DNA of *T. luwenshuni* or *T. uilenbergi*. Moreover, the assay was evaluated by testing 86 field samples in comparison to the reverse line blot method, showing a calculated sensitivity and specificity of 66.0% and 97.4%, respectively. These results indicate that the LAMP assay has a potential usefulness for application in diagnostic and epidemiological studies on *T. luwenshuni* and *T. uilenbergi* infection of small ruminants.

In addition, serological screening of the library led to discovery of a positive clone called TuIP, which has been deposited in Genbank under accession number FJ467922. Partially recombinantly cloned and expressed TuIP showed strong reactivity with serum from *T. uilenbergi* infected animals, indicating its potential usefulness for development of novel serological diagnostic tests or serving as a candidate for vaccine development in the future.

8 Zusammenfassung

Das Ziel dieser Arbeit war die Identifikation neuer Gene von *T. uilenbergi* mittels Screening einer Merozoiten cDNA Bibliothek, die für die Entwicklung diagnostischer Verfahren für den Nachweis einer Infektion mit *Theileria uilenbergi* geeignet wären. Zunächst wurde eine *T. uilenbergi* Merozoiten cDNA Bibliothek etabliert. Dazu wurden Schafe experimentell mit *T. uilenbergi* infiziert, um nach erreichen einer hohen Parasitämie, die durch die Untersuchung von Blutproben kontrolliert wurde, Merozoiten aus dem Blut des infizierten Tieres aufzureinigen. Aus den aufgereinigten Merozoiten wurde mRNA (messenger RNA) isoliert, die zur Etablierung der cDNA Bibliothek genutzt wurde. Die Bibliothek wurde auf 6 x 10^8 pfu/ml eingestellt wobei die Menge an rekombinanten Klonen bei schätzungsweise 70 % lag. Zur Konrolle der Qualität der cDNA Bibliothek wurden Stichproben mittels einem PCR-Protokoll analysiert. Es zeigte sich, dass alle untersuchten eingebaute Genfragmente parasitären Ursprungs waren, womit sich die Bibliothek als brauchbar für das Screening nach neuen Genen von *T. uilenbergi* erwies.

In einem ersten Verfahren führten PCR Amplifikationen der Genfragmente und anschließende Sequenzierung zu der Entdeckung von 12 einzelnen Klonen. Klone 2, 9 und 26 wiesen einen hohen Grad an Identität auf, besonders am 3'-Terminus und der 3'-UTR (‘untranslated region’), was darauf hindeutete, dass diese drei Klone zu einer Genfamilie gehören. Eine auf der Sequenz von Klon 2 basierende PCR führte zur Amplifikation von vier weiteren spezifischen Fragmenten genomischer DNA von *T. uilenbergi* und einem Fragment aus genomischer DNA von *T. luwenshuni*. Dieser Befund ließ vermuten, dass diese Genfamilie Isolat-spezifisch ist, da die für die PCR verwendeten DNA-Proben nicht von den Parasiten-Isolaten stammten, welche für die cDNA-Bibliothek Generierung verwendet wurden. Sequenzanalysen eines weiteren genomischen Fragments, das mit Primern basierend auf der Sequenz der 3´UTR von Klon 26 generiert wurde, zeigten, dass sowohl der 5´ als auch der 3´ Terminus im Vergleich zur Klon 2 Genfamilie eine hohe Identität aufwies. Diese homologen Termini waren durch ein Sequenzfragment von 136 bp getrennt, das im Vergleich zu der 3´UTR der Klon 2 Familie hoch identisch war. Aus diesen Beobachtungen wurde geschlossen, dass die Mitglieder der Klon 2 Genfamilie hintereinander angeordnet sind. Bioinformatische Analysen von cDNA Sequenzen der Klon 2 Genfamilie zeigten, dass diese Gene Signalpeptidsequenzen beinhalten und potentielle immunogene Proteine kodieren.

Analysen des rekombinant exprimierten Klon 2 ließen eine Immunreaktivität mit Seren von *Theileria*-infizierten Tieren aus China erkennen. Diese Immunreaktivität war spezifisch, da keine Kreuzreaktivitäten mit Seren von *T. lestoquardi-*, *Babesia motasi-* oder *Anaplasma ovis*-infizierten Tieren beobachtet werden konnten. Die Eigenschaften der Klon 2 Genfamilie sind der *Tpr* Genfamilie von *T. parva* sehr ähnlich, von der angenommen wird, dass sie eine Rolle in der Evolution gespielt hat.

Basierend auf der hoch konservierten Region der Klon 2 Genfamilie wurde ein Satz von sechs Primern für die Entwicklung einer „Loop mediated isothermal Amplification“ (LAMP) konstruiert. Die etablierte Methode erlaubte den gleichzeitigen Nachweis einer Infektion mit *T. uilenbergi* und *T. luwenshuni*. Die Reaktion konnte in einem einzigen Inkubationsschritt bei 63°C für 15 min durchgeführt werden. Die Spezifität der Reaktion wurde durch *Eco*RI Restriktions-Enzym-Verdauungs-Analyse und Sequenzierung ermittelt. Die LAMP war mit der Detektion von 0,1 pg DNA von *T. luwenshuni* oder *T. uilenbergi* in der Sensitivität mit PCR vergleichbar. Weiterhin ist die Methode durch den Vergleich von 86 Feldproben mit einem PCR-basierendem Nachweisverfahren, dem 'Reverse Line Blot', evaluiert worden. Der LAMP Test wies dabei eine Sensitivität und Spezifität von 66,0% bzw. 97,4% auf. Diese Ergebnisse deuten darauf hin, dass die LAMP einen potentiellen Nutzen für den Einsatz in diagnostischen und epidemiologischen Studien von Infektionen mit *T. luwenshuni* und *T. uilenbergi* bei kleinen Wiederkäuern hat.

In einem zweiten Verfahren führte ein serologisches Screening der Bibliothek zur Identifikation des positiven Klons TuIP, welcher in der Genbank unter der Zugangsnummer FJ467922 zu finden ist. Dieses Gen wurde partiell geklont und rekombinant exprimiert. Dieses TuIP-Peptid wies eine starke Reaktivität mit Antikörpern von *T. uilenbergi* auf, und ist damit für die Entwicklung eines neuen serologischen Tests geeignet. Weitere Untersuchungen könnten auch klären, ob TuIP für die Entwicklung eines Impfstoffes dienen könnte.

9 Abbreviations

AP	Alkaline phosphatase
BCIP	5- Bromo-4-chloro-3-indolyl phosphate
BLAST	The Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complimentary Desoxyribonucleic acid
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxynuclease
dNTP	Deoxynucleotide triphosphates
DTT	Dithiotreitol
ECF	East Coast fever
EDTA	Ethylene diamine tetracetic acid
ELISA	Enzyme linked immmunosorbent assay
HSP	Heat shock protein
IFAT	Indirect fluorescent antibody test
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITS	Internal transcribed spacers
kDa	Kilo Dalton
LAMP	Loop-mediated isothermal amplification
LB medium	Luria Bertani medium (broth)
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MPSP	Major piroplasm surface protein
MW	Molecular weight
NC	Nitrocellulose
NBT	Nitroblue tetrazolium

Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pfu	Plaque forming units
RLB	Reverse line blot
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SM	Phage suspension medium
TBD	Tick borne-diseases
TBE	Tris/Boric acide/EDTA
TE	Elution buffer
TBS	Tris buffered saline
TNF	Tumour necrosis factors
Tris	3,3’,5,5’-Tetramethylbenzidine
UTR	Untranslated region
UV	Ultra violet
WB	Western-blot
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

10 Publications

1. Li, Y., Luo, J., Guan, G., Ma, M., Liu, A., Liu, J., Ren, Q., Niu, Q., Lu, B., Gao, J., **Liu, Z.**, Dang, Z., Tian, Z., Zhang, B., He, Z., Bai, Q., Yin, H., 2009. Experimental transmission of *Theileria uilenbergi* infective for small ruminants by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis*. Parasitol. Res. 104, 1227-1231.

2. Niu, Q., Luo, J., Guan, G., **Liu, Z.**, Ma, M., Liu, A., Gao, J., Ren, Q., Li, Y., Qiu, J., Yin, H., 2009a. Differentiation of two ovine *Babesia* based on the ribosomal DNA internal transcribed spacer (ITS) sequences. Exp. Parasitol. 121, 64-68.

3. Niu, Q., Luo, J., Guan, G., Ma, M., **Liu, Z.**, Liu, A., Dang, Z., Gao, J., Ren, Q., Li, Y., Liu, J., Yin, H., 2009b. Detection and differentiation of ovine *Theileria* and *Babesia* by reverse line blotting in China. Parasitol. Res. 104, 1417-23.

4. Gao, J., Luo, J., Fan, R., Schulte-Spechtel, U.C., Fingerle, V., Guan, G., Zhao, H., Li, Y., Ren, Q., Ma, M., **Liu, Z.**, Liu, A., Dang, Z., Sugimoto, C., Yin, H., 2009. Characterization of a concealed antigen Hq05 from the hard tick *Haemaphysalis qinghaiensis* and its effect as a vaccine against tick infestation in sheep. Vaccine 27, 483-490.

5. **Liu, Z.**, Hou, J., Guan, G., Luo, J., Yin, H., 2008. Development and application of loop-mediated isothermal amplification (Review in Chinese). Research and Exploration in Laboratory 27, 22-27.

6. **Liu**, **Z.,** Dang, Z., Luo, J., Yin, H., Ahmed, J. S., Seitzer U., 2008. Small-scale expressed sequence tag analysis of *Theileria uilenbergi*: Identification of a gene family encoding potential antigenic proteins. Ann. N.Y. Acad. Sci. 1149, 214–217.

7. Liu, Z., Hou, J., Bakheit, M.A., Salih, D.A., Luo, J., Yin, H., Ahmed, J.S., Seitzer, U, 2008. Development of loop-mediated isothermal amplification (LAMP) assay for rapid diagnosis of ovine theileriosis in China. Parasitol Res. 103, 1407-1412.

8. Seitzer, U., **Liu**, **Z.,** Yin, H., Beyer, D., Kullmann, B., Miranda, J., Ahmed, J.S., 2008. Immune response of *Theileria* sp.-infected sheep to recombinant *Theileria* proteins. Ann. N.Y. Acad. Sci. 1149, 186–190.

9. Salih, D.A., **Liu, Z.**, Bakheit, M.A., Ali, A.M., El Hussein, A.M., Unger, H., Viljoen, G., Seitzer, U., Ahmed, J.S., 2008. Development and evaluation of a loop-mediated isothermal amplification method for diagnosis of tropical theileriosis. Transbound. Emerg. Dis. 55, 238-243.

10. Guan, G., Chauvin, A., Luo, J., Inoue, N., Moreau, E., **Liu, Z.**, Gao, J., Thekisoe, O.M., Ma, M., Liu, A., Dang, Z., Liu, J., Ren, Q., Jin, Y., Sugimoto, C., Yin, H., 2008. The development and evaluation of a loop-mediated isothermal amplification (LAMP) method for detection of *Babesia* spp. infective to sheep and goats in China. Exp. Parasitol. 120, 39-44.

11. Li, Y., Luo, J., **Liu, Z.**, Guan, G., Gao, J., Ma, M., Dang, Z., Liu, A., Ren, Q., Lu, B., Liu, J., Zhao, H., Li, J., Liu, G., Bai, Q., Yin, H., 2007. Experimental transmission of *Theileria* sp. (China 1) infective for small ruminants by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis*. Parasitol. Res. 101, 533-538.

12. Liu, A.H., Yin, H., Guan, G.Q., Schnittger, L., **Liu, Z.J.**, Ma, M.L., Dang, Z.S., Liu, J.L., Ren, Q.Y., Bai, Q., Ahmed, J.S., Luo, J.X., 2007. At least two genetically distinct large *Babesia* species infective to sheep and goats in China. Vet. Parasitol. 147, 246-251.

13. Gao, J., Luo, J., Fan, R., Fingerle, V., Guan, G., **Liu, Z.**, Li, Y., Zhao, H., Ma, M., Liu, J., Liu, A., Ren, Q., Dang, Z., Sugimoto, C., Yin, H., 2008. Cloning and characterization of a cDNA clone encoding calreticulin from *Haemaphysalis qinghaiensis* (Acari: Ixodidae). Parasitol. Res. 102, 737-746.

14. Miranda, J., Bakheit, M.A., **Liu, Z.**, Yin, H., Mu, Y., Guo, S., Beyer, D., Oliva, A., Ahmed, J.S., Seitzer, U., 2006. Development of a recombinant indirect ELISA for the diagnosis of *Theileria* sp. (China) infection in small ruminants. Parasitol Res. 98, 561-7.

15. Luo, J., Yin, H., **Liu, Z.**, Yang, D., Guan, G., Liu, A., Ma, M., Dang, S., Lu, B., Sun, C., Bai, Q., Lu, W., Chen, P., 2005. Molecular phylogenetic studies on an unnamed bovine *Babesia* sp. based on small subunit ribosomal RNA gene sequences. Vet Parasitol. 133, 1-6.

16. Luo, J., Yin, H., Liu, G., Guan, G., **Liu, Z**., Liu, A., Dang, Z., Ma, M., Lu, B., Sun, C., Bai, Q., Lu, W., Chen, P., 2005. Studies on Comparison of 18S rRNA Gene Sequences of *Babesia* Species Infective to Cattle (in Chinese). Xu Mu Shou Yi Xue Bao, 36, 906-911.

17. Gao, J., Guan, Q., Ma, M., **Liu, Z**., Dang, Z., Liu, A., Li W., Ren, Q., Luo, J., Yin, H., 2005. Construction of cDNA expression library of *Haemaphysalis qinghaiensis* (in Chinese). Xu Mu Shou Yi Xue Bao, 36,1187～1191.

18. **Liu, Z.**, Luo, J., Bai, Q., Ma, M., Guan, G., Yin, H., 2005. Amplification of 16S rRNA genes of *Anaplasma* species in China for phylogenetic analysis. Vet Microbiol. 107, 145-8.

19. Guan, G., Luo, J., Ma, M., Yang, D., Wang, Y., Gao, J., Sun, H., **Liu, Z.**, Liu, A., Dang, Z., Boulard, C., Yin, H., 2005. Sero-epidemiological surveillance of hypodermosis in yaks and cattle in north China by ELISA. Vet Parasitol. 129, 133-7.

20. Yang, D., Luo, J., Tang, L., Guan, G., Ma, M., **Liu, Z.**, Liu, A., Dang, Z., Wang, Y., Yin, H., OU YANG, W., 2005. Cloning and construction of recombinant expression vector of Hypodermin C (in Chinese). Chin. J Vet. Sci. Technol. 33, 3-6.

21. Li, W., Luo, J., Yin, H., Gao, J., Guan, G., **Liu, Z**., Dang, Z., Ma, M., Liu, A., Ren, Q., 2005. Construction of cDNA expression library from larvae of *Boophilus microplus* (in Chinese). Chin. J Vet. Sci. Technol. 35, 994-996.

22. Li, W., Luo, J., Liu, L., Dang, Z., Gao, J., Guan, G., **Liu, Z**., Liu, A., Ma, M., Ren, Q., Yin, H., 2005. Cloning of Bm86 gene of *Boophilus microplus* and construction of recombinant expression vector (in Chinese). Chin. J Vet. Sci. Technol. 35, 124-128.

23. Ma, M., Guan, G., Lu, B., Liu, A., **Liu, Z.**, Chang, Z., Li, F., Chang, F., Luo, J., Lu, W., Zhang, Q., Yuan, G., Yin, H., Boulard, C., 2003. Efficacy of different dosages of ivermectin injectable against the *Hypoderma* spp. in yaks. Vet Parasitol. 117, 147-51.

24. Luo, J., Yin, H., Guan, G., Yang, D., Liu, A., Ma, M., **Liu, Z.**, Dang, Z., Liu, G., Bai, Q., Lu, W., Chen, P., 2003. Experimental transmission of an unnamed bovine *Babesia* by *Hyalomma* spp., *Haemaphysalis longicornis* and *Boophilus microplus*. Vet Parasitol. 116, 115-24.

11 Acknowledgements

I am heartily thankful to my supervisor, Prof. Dr. Jabbar Sabir Ahmed, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject and I am also grateful to Prof. Dr. Kurt Pfister for his kind evaluation of my thesis and offering me an opportunity to obtain my doctorate title in Veterinary Faculty of Ludwig Maximillians University, in Munich.

I wish to express my sincere gratitude and deep thanks to Prof. Dr. Ulrike Seitzer for her valuable suggestions, patient supervision and critical correction of the thesis. I would like to thank Prof. Rosevelt L. Party from the School of Biological Science in the University of Nebraska and his wife Ann B. Party for reading and correction of my thesis.

This thesis would not have been possible without the nice team work of members of the Division of Veterinary Infection Biology and Immunology in Research Centre Borstel, particularly the technical assistance from Ms. Birgit Kullmann and Doreen Beyer; helps and valuable discussions from Drs. Mohammed Bakheit and Silke Gelber, and Ph D. students Diaeldin Salih, Awadia Ali, Heike Mueller, Jassim Abdo, Stefanie Renneker and Monika Mackiewicz. Hereby, I would like to express my sincere thanks and greetings to them.

I would like to also show my gratitude to Mrs Gudrun Lehwark-Yvetot for her unyielding help in many aspects of daily life, and to all Borstel friends for creating happy atmosphere of life, which I will never forget.

Sincere thanks and gratitude are also due to my scholarship sponsor, the Germany Academic Exchange Service (DAAD, Deutcher Akademischer Austauschdienst), and particularly to Mrs. Cordula Behrsing for her help.

In China, I am greatly indebted to Prof. Dr. Yin Hong and Prof. Dr. Luo Jianxun for not only their kind permission, guidance, but also their encouragement and endless supporting for me to study in Germany for years. Special thanks to all my Chinese colleagues and students in Parasitology Department of Lanzhou Veterinary Research Institute, CAAS, China, for assistance to prepare experimental materials and samples collection.

I owe my deepest gratitude to my parents, parents-in-law, wife and son for their understanding, supporting, encouragements and forgiveness, particularly to my parents-in-law for looking after my son. This work is dedicated to them.

Lastly, I offer my regards and blessings to all of those who supported me in any respect.