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Identification of attenuation markers of a *Theileria lestoquardi* cell line to be used for the development of live vaccine against malignant ovine theileriosis

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1. Introduction

Theileria lestoquardi is a tick-borne protozoan parasite and highly pathogenic for sheep. The disease caused by the pathogen is known as malignant ovine theileriosis (MOT) and is transmitted by *Hyalomma* ticks (Levine, 1973). The disease was first described in Egypt in exported Sudanese sheep and in the meantime has been recorded in south-eastern Europe, North Africa, the Near and Middle East and Southern parts of the former USSR (Dolan, 1989).

Control of the disease can be achieved by chemotherapy, using theilericidal drugs such as buparvaquone (El Hussein et al., 1993), as well as tick control using acaricides (Jongejan and Uilenberg, 2004). However, both methods have shortcomings. Drug treatment is expensive and requires early diagnosis of the disease (El Hussein et al., 1993), whereas acaricide treatment raises environmental concerns and resistance to the chemical is also recorded (Foil et al., 2004). These shortcomings could be overcome by immunoprophylaxis of sheep with attenuated *T. lestoquardi* schizont-infected ovine cells providing the animal with solid immunity (Gill et al., 1978), which has been carried out successfully in Iraq and Iran (Hawa et al., 1981; Hashemi-Fesharki, 1997).

Attenuation is defined as loss of virulence whilst retaining viability and infectivity (Adamson and Hall, 2002). It is possible to attenuate *T. annulata* schizonts, a parasite closely related to *T. lestoquardi* and infective to cattle, by prolonged *in vitro* culture of infected cell lines which can be used as live vaccine (Pipano, 1981; Tait and Hall, 1990). Attenuation of *T. annulata* can be achieved by continuous passage of the original virulent parasite in cell culture for about 60–300 passages over a period of several months to 2 years (Boulter and Hall, 1999) and is usually monitored by examining the clinical and immune reactions of the calves inoculated by these culture cells. This technique has been applied in a number of countries to establish attenuated vaccine against tropical theileriosis (Shkap et al., 2007). However, although successful,

the current strategy for developing attenuated cell line vaccine involves a long and tedious process. Therefore, a better understanding of the properties that determine the virulence of parasitised cell lines could provide markers that would allow more rapid selection of attenuated lines, in order to devise *in vitro* monitoring of attenuation before testing in susceptible animals.

The attenuation phenotype has been extensively studied in *Theileria annulata*. From these studies attenuation has been correlated to a number of processes like loss of induction of host pathogenic effector molecules, particularly metalloproteinases and cytokines by the parasite or alteration in parasite and host gene expression and possible selection of a less virulent parasite subpopulation (Boulter and Hall, 1999). Matrix metalloproteinases have been implicated in the metastatic behavior of schizont infected cells; therefore a reduction in metalloproteinase activity is correlated with attenuation (Adamson et al., 2000; Adamson and Hall, 1996). *T. annulata* infected cells constitutively express mRNA for TNF-alpha and several other proinflammatory cytokines (Brown et al., 1995). The major clinical symptoms of acute tropical theileriosis (anorexia, cachexia, anemia and pyrexia) are characteristic of those induced by TNF-alpha as observed by Bielefeldt Ohman et al. (1989) when cattle were inoculated with recombinant TNF-alpha.

The definition of attenuation at a molecular level could speed production of cell line vaccines and reduce the cost of the dose. Since no work has been reported regarding attenuation mechanisms in *T. lestoquardi*, the following study investigated described potential attenuation markers of *T. annulata* infected cells in a *T. lestoquardi* cell line at different passages. Furthermore, differentially expressed genes in higher passage and lower passage were analyzed using suppression subtractive hybridization in order to identify genes which correlate with subculturing and thus potentially with attenuation.

2. Review of literature

2.1. Genus *Theileria*

Theileria parasites are tick-transmitted, obligatory intracellular parasites from the family Theileriidae in the order Piroplasmida (Levine, 1988). This order belongs to the phylum Apicomplixa, subkingdom Protozoa. This phylum also contains other important parasitic genera such as *Plasmodium*, *Eimeria*, *Toxoplasma*, *Neospora*, *Sarcocystis* and *Cryptosporidium*. The taxonomy of *Theileria* species has been, and still is, the subject of controversy (Uilenberg, 1981).

Subkingdom : Protozoa Goldfuss, 1818

Phylum : Apicomplexa Levine, 1970

Class : Sporozoea Leuckart, 1879

Subclass : Piroplasmia Levine, 1961

Order : Piroplasmida Wenyon, 1926

Family : Theileriidae DuToit, 1918

Genus : *Theileria* Betteneourt, Franca and Borges, 1907

2.2. Life cycle of *Theileria* species

The life cycle is essentially the same for all *Theileria* species and has been extensively documented (Barnett, 1968; Mehlhorn and Schein, 1984, 1993). Development of the parasite takes place within the vertebrate and invertebrate host, with asexual reproduction by schizogony and merogony in the mammalian host followed by sexual reproduction and sporogony in the tick vector.

2.2.1. Life cycle of *Theileria* species in the vertebrate host

Theileria parasites are transmitted to their mammalian host when infected nymph or adult ticks feed on a susceptible host and *Theileria* sporozoites injected with the saliva of the tick rapidly enter lymphoid cells of the host, as has been demonstrated for

T. annulata and *T. parva* by *in vitro* studies (Shaw and Young, 1995). Sporozoites invade different leukocyte sub-types depending upon the *Theileria* species. Sporozoites of *T. parva* can invade and survive in all subpopulations of lymphocytes, although differences in the subsequent rate of replication have been observed between infected T cells and B cells (Morrison et al., 1996). In contrast, *T. annulata* infects bovine MHC class II positive cells of the macrophage/monocyte lineage and to a lesser extent B cells (Spooner et al., 1988; Innes et al., 1989). Inside the leukocyte, they develop into a macroschizont and induce transformation and proliferation of the host cell, leading to a rapid clonal expansion of parasitized cells in the lymphoid tissues (William and Dobbelaere, 1993). Although the parasite and host cell divide in synchrony, schizont DNA synthesis occurs as the host cell enters mitosis and is immediately followed by division when the host cell is in metaphase (Irvin et al., 1982). Merogony occurs within infected lymphocytes (Shaw and Tilney, 1992) and merozoites are released by host cell rupture. The merozoites invade red blood cells (RBCs) and differentiate into piroplasms (Glascodine et al., 1990).

2.2.2. Life cycle of *Theileria* species in the invertebrate host

Ticks become infected after feeding on blood containing infected RBCs. Lysis of infected erythrocytes occur in the gut of the tick and the piroplasms begin to develop to ray bodies from the second to the fourth day of tick feeding (Mehlhorn and Schein, 1984). Ray bodies give rise to uninucleate gametes then syngamy of gametes occurs in the tick gut on about the 6th day of tick feeding and the spherical zygote formed. The zygote invades the gut epithelium and develops eventually into a motile kinete. Fully differentiated kinetes penetrate the gut wall and appear in the haemolymph of engorged nymphs usually 17-20 days after repletion. These kinetes invade and develop in the Type III salivary gland acini (Mehlhorn and Schein, 1993). Inside the salivary gland, the kinetes develop into the sporoblast, which waits locally for coming

blood triggering extensive multiplication known as sporogeny into full grown and infective sporozoites (Mehlhorn and Schein, 1984).

2.3. Malignant ovine theileriosis

Malignant theileriosis of sheep was first described by a team of veterinarians in Egypt in 1914 in Sudanese sheep (Littlewood, 1915). The associated pathogen was described as distinct from *Babesia* in sheep particularly from *Piroplasma ovis* which was well known in Syrian and Egyptian sheep. The smallness of the piroplasm and the exo-erythrocytic schizont led them to describe the pathogen as *Theileria*. The parasite was first described as *Theileria ovis* (du Tiot, 1918) then *T. hirci* (Dschunkovsky and Urodschevich, 1924). The parasite has been named after Lestoquard collated his overview on the subject in 1927 and eventually named as *T. lestoquardi* (Morel and Ulienberg, 1981).

After the first report of the parasite in sheep from Sudan, a similar disease has been reported in Egyptian sheep (Littlewood, 1916). Later the parasite has been reported from sheep and goats in other countries such as Algeria (Lestoquard, 1927), Turkey (Baumann, 1939), Iraq (Khayyat and Gilder, 1947), India (Raghvachari and Reddy, 1959), Serbia (Dschunkovsky and Urodschevich, 1924) and the infection was reported to be common in Iran and Iraq (Hooshmand-Rad, 1974; Hawa et al., 1981). An outbreak of malignant theileriosis in sheep has been reported in Sudan (Tageldin et al., 1992) and recently it was shown to be widely distributed in main sheep grazing areas of the country where 16.2% of sheep surveyed showed reactive antibodies in Indirect fluorescent antibody test (IFAT) (Salih et al., 2003).

Whether all reports on malignant theileriosis of sheep and goats deal with the same parasite is not clear (Brown et al., 1998). Many reports are based on the combination of severe disease with presence of piroplasms and sometimes schizonts. Thus, ovine theileriosis in China was first reported to be caused by *T. lestoquardi* (Luo and Yin,

1997) but later biological and phylogenetic studies revealed that this parasite is divergent from *T*. *lestoquardi* (Schnittger et al., 2000). Further phylogenetic analysis revealed that ovine theileriosis in China is caused by two *Theileria* species (Schnittger et al., 2003; Yin et al., 2004), and these two new species of ovine *Theileria* in China have been named *Theileria luwenshuni* and *T*. *uilenbergi* (Ahmed et al., 2006; Yin et al., 2007). Small ruminant theileriosis in sheep and goats are caused by a variety of *Theileria* species. A minimum of six *Theileria* species infects small ruminants *T*. *separata*, *T*. *ovis* and *T*. *recondita* are nonpathogenic, whereas *T*. *lestoquardi*, *Theileria luwenshuni* and *T*. *uilenbergi* are pathogenic.

2.3.1. Phylogenetic relationships of *T. lestoquardi*

Phylogenetic studies clearly indicated that *T*. *lestoquardi* is more closely related to *T*. *annulata* than to *T*. *parva* or to other sheep and goat *Theileria* and *Babesia* species (Katzer et al., 1998; Schnittger et al., 2003). In a phylogenetic analysis of sheep and goats *Theileria* and *Babesia* parasites, the highest identity values of a pair of 18S rRNA genes belonging to different species were observed between *T*. *annulata* and *T*. *lestoquardi*, with an identity value of 99.7 % (Schnittger et al., 2003). The same results were obtained by Katzer et al. (1998), who used both the rRNA and the major merozoite piroplasm surface antigen (mMPSA) to analyze the relationship between some *Theileria* parasites. Therefore it is considered that both species probably evolved from a common ancestor and this conclusion is supported by the biological data which implies a close relationship, such as the fact that both parasites share the same vector tick.

2.3.2. Transmission

Ticks of the genus *Hyalomma* had been suspected to be responsible for transmission of *T. lestoquardi* (Mazlum, 1970), and their role as a vector was later demonstrated by

transmission of *T. lestoquardi* from stage to stage through *H. a. anatolicum* (Hooshmand-Rad and Hawa, 1973b). This tick was associated with an outbreak of sheep theileriosis in Sudan (Tageldin et al., 1992), and in other reports from Sudan *H. a. anatolicum* ticks were demonstrated to transmit *T. lestoquardi* to sheep (Latif et al., 1994).

H. *a*. *anatolicum* appears to be the only proven vector for *T. lestoquardi* (Uilenberg, 1997) however, other tick species have been suspected to be potential vectors of the parasite such as *Rhipicephalus bursa* (Dschunkovsky and Urodschevich, 1924), *Rhipicephalus* spp. (Sisodia and Gautum, 1983), *H*. *impeltatum* (El-Azazy et al., 2001) and *Rhipicephalus sanguineus* (Razmi et al., 2003).

2.3.3. Clinical signs and pathology

T. lestoquardi is considered to be very pathogenic to sheep, and high morbidity and mortality rates have been reported, even in indigenous stock (Hooshmand-Rad and Hawa, 1973a; Tageldin et al., 1992). Severe malignant theileriosis in goats is very rare excepting the earlier report of Dschunkovsky and Urodschevich (1924). El Hussein et al. (1998) reported enlarged lymph nodes and intermittent parasitaemia in goats experimentally infected with blood from infected sheep while no febrile reaction or hematological changes were associated with the infection.

Several authors have described the symptoms of acute malignant theileriosis in sheep or goats (Lestoquard, 1926; Neitz, 1957; Hooshmand-Rad and Hawa, 1973a; El Hussein et al., 1998) and considered that these were very similar to acute tropical theileriosis in cattle. The symptoms include high fever, listlessness, anorexia, emaciation, diarrhea or constipation, enlarged superficial lymph nodes and pale and icteric mucous membranes. The pathological features of malignant theileriosis of sheep and goats are also very similar to those described for tropical theileriosis of cattle (Neitz, 1957; Hooshmand-Rad and Hawa, 1973a; Tageldin et al., 1992). The main

macroscopical lesions are hyperplasia and oedema of lymphnodes, splenomegaly, a yellowish enlarged liver and the lungs are frequently oedematous. However, typical haemorrhagic ulcers of the abomasum as seen in *T. annulata* infection of cattle were notably absent (Hooshmand-Rad and Hawa, 1973a).

2.3.4. Diagnosis

Diagnosis of *T. lestoquardi* infection is based on the combination of the clinical signs or pathological findings with the demonstration of parasitic stages in blood or organ smears. The provisional diagnosis includes case history, clinical signs, postmortem findings and geographic distribution of disease and vector (OIE, 2000). Generally it is not possible to discriminate *T. lestoquardi* piroplasms from nonpathogenic *Theileria* species that may occur simultaneously within the same ovine host and could confuse accurate diagnosis of *T. lestoquardi*.

Detecting antibodies against *T. lestoquardi* using IFAT was applied in epidemiological surveys (Salih et al., 2003; Taha et al., 2003) but false positive and negative results due to cross-reactions or weak specific immune response are some disadvantages that are commonly observed in this test (Leemans et al., 1997). An enzyme-linked immunosorbent assay (ELISA) has been developed for the serological detection of *T. lestoquardi* using recombinant protein to minimize the chance for cross-reactivity (Bakheit et al., 2006b). This ELISA is based on the newly discovered clone 5 surface protein of *T. lestoquardi* and was applied in field samples collected from northern Sudan (Bakheit et al., 2006a).

Several molecular techniques for detection of ovine *Theileria* species were developed. PCR has been developed using specific primers to amplify the *T. lestoquardi* fragment of the gene coding for a 30-kDa merozoite surface protein from ticks, sheep and goats (Kirvar et al., 1998). The advantage of this PCR is its ability to differentiate between *T. lestoquardi* and *T. annulata* in the *Hyalomma* vector and in sheep and goats

(Leemans et al., 1999 a,b) however, cross reactivity in PCR between other ovine or caprine *Theileria* or *Babesia* species was not tested (Kirvar et al., 1998). This PCR technique cannot be used to detect mixed infections and its sensitivity is poor to detect subclinical infections. In order to overcome these limitations, a reverse line blot (RLB) assay has been developed for detection of *Theileria* and *Babesia* parasites infecting small ruminants (Schnittger et al., 2004). However this method is expensive and requires sophisticated laboratory equipment. Recently, loop-mediated isothermal amplification of DNA (LAMP) has been successfully developed for the detection of some *Theileria* species such as *T*. *annulata*, *T*. *luwenshuni* and *T*. *uilenbergi* (Salih et al., 2008; Liu et al., 2008). This technique is rapid and simple to run, cost effective, sensitive, and specific. Therefore the respective development of LAMP for *T*. *lestoquardi* can be of potential usefulness for application in diagnostics and epidemiological studies.

2.3.5. Economic impact of malignant ovine theileriosis

Small ruminants have considerable global economic significance. Thus, the assessment of the economic impact of ticks and tick-borne diseases on small ruminants should be one of the priorities that should be addressed (Uilenberg, 1997).

Malignant ovine theileriosis of sheep and goats is a severe disease leading to high mortality rates ranging from 40 to nearly 100% during outbreaks (Friedhoff, 1997). Heavy losses due to malignant theileriosis are recorded even in indigenous animals (Hooshmand-Rad, 1974; Tageldin et al., 1992). In Sudan, the disease was shown to be widely distributed in main sheep grazing areas of the country with a prevalence rate reaching to 23.4% (Salih et al., 2003). It is also known to have a seasonal nature in the northern states of Sudan, and mortalities could reach almost 100% in cases of outbreaks in these areas (Latif et al., 1994; El Ghali and El Hussein, 1995). The disease is widespread in Iraq and causes high morbidity and mortality in local breeds

(Hooshmand-Rad and Hawa, 1975). In Iran it has been reported to cause considerable mortality in lambs less than 9 months old (Hooshmand-Rad, 1977). The disease may be particularly virulent in exotic sheep (Gautam et al., 1975). Accordingly, the disease is expected to be of a high economic importance, especially in countries where export of sheep and sheep products is a major component of their national economy.

2.3.6. Immunity to *Theileria* infection

Cattle that recover from infection with *T. annulata* are solidly protected against subsequent infections with homologous strains and often to heterologous challenge (Preston et al., 1999). It is well established that both antibody-dependent and antibody-independent mechanisms are involved in the protection against theileriosis (Ahmed et al., 2008; Seitzer and Ahmed, 2008). However, T-cells play the crucial role in induction and maintenance of this immunity. Based on the cytokine profile and lytic effect of the T-cells, it seems that both CD4+- and CD8+-T cells are involved in the mediation of the immunity (Preston et al., 1999). Presumably cytotoxic- and helper-T-cells recognize parasite-antigens, which are presented by the infected cells via MHC I and MHC II, respectively. In vitro, it has been shown that both helper- and cytotoxic-T-cells are responding to the infected cells: helper T-cells proliferate and produce interleukin 2 (IL-2) and interferon-gamma (IFN-gamma) (Ahmed et al., 1989); IL-2 is consumed by the cytotoxic T-cells for their clonal expansion and subsequent killing of their target cells in an MHC class I restricted manner. The generation of cytotoxic T-cells is closely related to the control of the infection (Ahmed et al., 1999).

During the course of natural infections with *Theileria*, the host's humoral immune system is stimulated by the various antigens associated with the different stages of the parasite's life cycle (Ahmed and Mehlhorn, 1999). Antibodies reacting with the macroschizont and piroplasm stages of the parasite are usually produced and have been detected by various diagnostic tests (Hooshmand-Rad and Hashemi-Fesharki,

1981; Leemans et al., 1997). There is evidence for a protective humoral response to the sporozoite and probably the merozoite stage of the parasite. *In vitro* studies have shown that immune sera collected from animals recovered from theileriosis are capable of neutralizing sporozoites of both *T*. *annulata* and *T*. *parva* and to prevent infection of monocytes and lymphocytes (Gray and Brown, 1981; Preston and Brown, 1985; Ahmed et al., 1988). Recombinant merozoite surface antigens of *T*. *annulata* have also conferred a degree of protection when the animals were challenged with blood containing infective piroplasms of *T*. *annulata* (d'Oliveira et al., 1997).

2.3.6.1. Immunity to *Theileria lestoquardi*

Little is known about the mechanisms involved in the pathogenesis of the disease and the immune response to *T. lestoquardi* has not been subjected to specific studies. The phenotype of ovine cell lines infected with *T. lestoquardi* or *T. annulata* were studied by cytometric analysis and the results revealed that both parasites had infected the same cell types in sheep as *T. annulata* in cattle, notably monocytes/macrophages and B-cells (Leemans et al., 2001). The antibody responses after inoculation of sheep with *T. lestoquardi* infected cells revealed that a detectable antibody response appeared 15 days after inoculation, a 32-64-fold rise in antibody titres was recorded 1 month after infection, and substantial titres were still observed 90 days after inoculation (Leemans et al., 1997).

There is insufficient knowledge of the relative susceptibility of sheep and goats and of various breeds of each species. The parasite may be particularly virulent in exotic sheep, although it can be a problem in indigenous sheep as well (Hooshmand-Rad and Hawa, 1973a). Goats are less often mentioned; some authors accepted that goats show significant resistance to the disease compared to sheep (Hooshmand-Rad and Hawa, 1973a; Hooshmand-Rad, 1974; Sisodia and Gautam, 1983; Brown et al., 1998; El Hussein et al., 1998).

It is known that animals recovering from *T*. *lestoquardi* infection are immune to super infection and the fact that sheep and goats tend to be indigenous, acquire immunity at an early age (Hooshmand-Rad and Hawa, 1973a). Little is known about the role of cell mediated immunity. Since the clinical course of malignant theileriosis is comparable to tropical theileriosis and the target host cells are very similar in both diseases, it is believed that analogous mechanisms are operating. Thus both CD4+ and CD8+ T cells might be involved.

2.3.7. Control of malignant theileriosis

Some of the theilericidal drugs used for the treatment of *T*. *annulata* and *T*. *parva* infection of cattle are likely to be effective against *T*. *lestoquardi* infection in sheep and goats (Hashemi-Fesharki, 1997; El Hussein et al., 1993).

Following the successful cultivation of *T*. *lestoquardi* schizont infected ovine cells (Hooshmand-Rad and Hawa, 1975) and the discovery that the parasite, like *T*. *annulata*, becomes attenuated but maintains its immunogenicity following prolonged culture *in vitro*, immunization of sheep with such cells has been carried out successfully in Iraq (Hawa et al., 1981). Immunization is widely applied in Iran and has been shown to provide good protection against virulent field isolates (Hooshmand-Rad, 1985; Hashemi-Fesharki, 1997). Although the tissue culture vaccine might be the best method of control for malignant theileriosis of sheep and goats, it remains to be shown whether the immunity induced will be strain-specific (Friedhoff, 1997).

2.4. Vaccination against Theileriosis

2.4.1. Infection and treatment

This method is based on inoculating naive animals with tick stabilate and simultaneous treatment with long acting oxytetracycline (Pipano et al., 1981; Radley, 1981). This method was initially designed for use against *T*. *parva*. A very important point is that it

is not possible to produce an attenuated cell line vaccine against *T. parva* that is equivalent to those used so successfully against *T. annulata*. The principal reason for this is apparently the poor ability of the schizont to transfer from donor host cells to recipient host cells. Indeed, the efficiency of *T. parva* transfer is at least two orders of magnitude lower than that achieved by *T. annulata* (Irvin and Morrison, 1987). There are several drawbacks associated with the use of live parasites. The infection and treatment method uses virulent stocks of parasites for the immunization regime. This method also results in the presence of piroplasms in the immunized animals, which can aid the spread of the disease by ticks to unprotected cattle (Tait and Hall, 1990).

2.4.2. Attenuated cell line vaccine

The most widespread control measure taken against *T. annulata* is the vaccination of animals with an attenuated cell line vaccine and has been widely deployed in North Africa, The Middle East, and South Asia (Boulter and Hall, 1999; Shkap et al., 2007). It is possible to attenuate *T*. *annulata* and *T. lestoquardi* schizonts by prolonged *in vitro* culture of infected cell lines and this phenomenon is exploited to produce the live vaccine that are used to control tropical theileriosis in cattle and malignant theileriosis in sheep (Pipano, 1981; Tait and Hall, 1990; Hawa et al., 1981). Attenuated schizont-infected cell line vaccines protect most breeds of cattle against homologous challenge and usually against heterologous challenge (Pipano, 1981) and have an efficacy approaching 95-100% (Brown, 1990). The duration of immunity varies between 6 months (Ilhan et al., 1998) and lasts as long as 3.5 years (Pipano, 1989). The main practical disadvantage of the cell culture vaccine is the requirement for a cold chain for distribution to remote sites.

2.4.2.1. Attenuation

Attenuation is defined as loss of virulence whilst retaining viability and infectivity (Adamson and Hall, 2002).The poorly understood mechanism of attenuation is likely to be associated with several phenomena that both host and parasite are certainly involved (Hall et al., 1999). Virulence was defined as the propensity of an infectious agent (the protozoan parasite *T*. *annulata*) to cause host pathology and the entities that cause pathology are termed virulence factors (Adamson and Hall, 2002). The virulence factors described to date can be classified as cytokines and matrix metalloproteinases (Adamson and Hall, 2002).

T. annulata-infected cells constitutively express mRNA for TNF-alpha (Tumour necrosis factor alpha) and several other proinflammatory cytokines (Brown et al., 1995). The major clinical symptoms of tropical theileriosis (anorexia, cachexia, anemia and pyrexia) can be induced by experimental administration of TNF-alpha (Bielefeldt Ohman et al., 1989). In a series of elegant experiments, Graham et al. (2001) showed that the levels of proinflammatory cytokine expression of cloned *T. annulata*-infected cell lines could be used as a marker for virulence. Thus, cell lines expressing low levels of proinflammatory cytokines were non-pathogenic whilst their high-expressing counterparts induced pathology and disease. Moreover, high cytokine producing cell lines induced greater non-specific T cell proliferation in the draining lymph nodes, which delayed the onset of a protective parasite-specific response and greatly enhanced the expansion of infected cells, resulting in more severe pathology. The effect of parasite genotype on cytokine expression was investigated by Graham et al. (2001) using PCR-RFLP analysis from which they concluded that differences in cytokine expression and virulence could be parasite-mediated. The cytokines IL-1, IL-6 and TNF-alpha are the most important inducers of an acute phase protein (APP) response which relates to the disease severity (Glass et al., 2003).

Matrix metalloproteinases (MMPs) are a class of enzymes, which degrade components of the extracellular matrix (Stetler-Stevenson et al., 1993). Under normal circumstances these enzymes are under tight regulation and they have several physiological functions including a role in the extravasation of leukocytes (Reponen et al., 1992). However, elevated levels of certain MMPs, including matrix metalloproteinase 9 (MMP9), are associated with tumor cell metastasis (Kohn and Liotta, 1995). The expression of eight host matrix metalloproteinase activities in *Theileria* schizont-infected cells were reported (Hall et al., 1999). The major proteinase activity was shown to be the bovine homologue of the human MMP9 gene (Baylis et al., 1995). The fact that the same enzymes are characteristic of metastatic tumors leads to the obvious suggestion that they contribute to the dissemination of the schizont-infected cells throughout the body of the bovine host (Adamson and Hall, 1997). The evidence in support of this notion was based on the ability of infected cells to invade reconstituted basement membrane as well as spread throughout the bodies of SCID mice (Adamson and Hall, 1996; Somerville et al., 1998).The metalloproteinases were identified as virulence factors of *T. annulata*, as there was loss to absence of the metalloproteinase 9 (MMP9) activity from low (non-attenuated) to high passages (attenuated) of schizont-infected cells (Adamson and Hall, 1997; Adamson et al., 2000). The transcription factor AP-1 binding motif has been identified in the bovine MMP9 gene promoter and was shown by transfection and deletion analysis to be critical in mediating expression of this gene (Adamson et al., 2000). Thus macroschizonts could be stimulating MMP9 expression via a pathway involving AP-1.

Using RFLP analysis on several attenuated lines demonstrated that virulent parasites are mixtures of several genotypes (Darghouth et al., 1996; Sutherland et al., 1996; Somerville et al., 1998). However, upon attenuation, the population complexity decreases but dose not necessarily become clonal. A number of genes have been identified that are either upregulated or downregulated on attenuation using differential

RNA display which was applied to identify alteration to mRNA profiles associated with the attenuation of a *T. annulata* vaccine cell line (Somerville et al., 1998) and the technique also was applied to identify both host and parasite genes that show altered expression during differentiation of *T. annulata* from the machroschizont to the merozoite stage of the life cycle (Oura et al., 2001). Attenuation is likely to be multifactorial, involving selection of an avirulent parasite subpopulation and genotypic alteration in parasite and host gene expression (Hall et al., 1999).

2.4.3. Subunit Vaccine

The practical limitations imposed by using live parasites for vaccination against theileriosis have led to efforts to develop alternative vaccines based on defined parasite antigens.

One approach has been to evaluate a recombinant version of the *T. parva* major surface sporozoite protein (p67), based on the rationale that antibodies against p67 can 'neutralize' sporozoite entry into lymphocytes. A degree of efficacy has been demonstrated by recombinant p67 against field challenge by *T. parva* infected ticks (Musoke et al., 1992; Musoke et al., 2005). A homologue of p67 from *T. annulata* designated SPAG-1 (sporozoite surface antigen 1) shares neutralizing determinants with p67, and the two recombinant proteins are mutually cross-protective on heterologous challenge (Boulter et al., 1999). A homologous sporozoite surface antigen has also been isolated from *T. lestoquardi* and expressed but has yet to be tested as a vaccine (Skilton et al., 2000). Two alleles of merozoite surface antigens of *T. annulata* (Tams) have been used in preliminary immunization experiments specifically designed to test for protection against the intra-erythrocytic stage and have conferred a degree of protection when the animals were challenged with blood containing infective piroplasms of *T. annulata* (d'Oliveira et al., 1997). Recently, immunization of cattle with SPAG-1 or attenuated schizont-infected cells induced limited protection against

homologous or heterologous sporozoites challenge, whereas a combination of recombinant and live vaccine resulted in survival of all vaccinates (Darghouth et al., 2006). These results provide evidence for achieving improved protective immunity by inclusion of sporozoite and schizont antigens in new generation vaccines to be developed. Five of six reported *T. parva* schizont antigens have also been tested for their ability to induce protective immune responses (Graham et al., 2006). In a trial evaluating a cocktail of five antigens, 79% of the vaccinated cattle exhibited antigen-specific CD8+ T cell responses, but only 29% of the vaccinated animals demonstrated cytotoxic T lymphocytes (CTL) activity following *in vitro* restimulation of PBMC with the parasite infected cells (Graham et al., 2006). The extension of this approach to the complete genome of *Theileria* using CTL restricted by additional bovine haplotypes is likely to result in identification of valuable additional vaccine candidates.

3 Materials and methods

3.1. Cell line

A *T*. *lestoquardi* strain was isolated from a sheep showing the typical signs of malignant theileriosis in Atbara, a town in northern Sudan, and maintained in the laboratory as schizont infected cell culture (*T*. *lestoquardi* (Atbara), Bakheit et al., 2006b). PCR experiments confirmed the absence of other contaminating *Theileria* parasites. Cells were cultured in RPMI complete medium (RPMI 1640, 2 mg/ml sodium bicarbonate (Sigma, Deisenhofen, Germany), 2 mM L-glutamine, 100 IU penicillin/ml, 100 µg/ml streptomycin (all from Biochrom, Berlin, Germany) and 20% heat-inactivated foetal calf serum (FCS; PAA, Pasching, Austria) and incubated in a humidified atmosphere with 5% CO_2 at 37℃. This isolate was used for all subsequent investigations. Viable cell count was performed to examine the viability and to estimate the numbers of the cells. The dye exclusion test was used, which is based on the fact that trypan blue only stains dead cells which appear blue under the microscope. Trypan blue stock solution (0.5% w/v) (Biochrom AG, Berlin) was diluted 1:3 in sterile PBS (phosphate buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The cell suspension to be counted was further diluted 1:20 in the dye. Both chambers of a Neubauer slide were filled and the cells in the 4 corner squares of both chambers of the slide were counted. The number of viable cells/ml was calculated using the following equation and the example above: counted cells/number of corner squares counted (8) x 10^4 x dilution factor (20).

3.2. Sub-culturing

The culture medium was changed by washing the cells in RPMI without additives (L-glutamine, antibiotics and FCS) and centrifuging at 300 x g for 10 min at 4 ℃. This wash step was repeated twice. The cells were resuspended in RPMI complete medium and counted before being sub-cultured at a density of 6 x 10^4 cells/ml in 75 ml flasks

(Sarstedt, Nuernbrecht, Germany) in a final volume of 30 ml complete medium. The sub-culturing was continued every two days till passage 125.

3.3. Cell lysates

The cell lysates were prepared as described by Shkap et al. (2003). Briefly, the schizont infected cells of *T. lestoquardi* (Atbara strain) were collected from culture flasks and washed three times in phosphate buffered saline (PBS, pH 7.2) by centrifugation at 1000 x g for 5 minutes. The pellet of 1 x 10^8 cells was resuspended in an equal volume of twofold concentrated, sterile cold lysis buffer (0.25 % sucrose and 0.5% Nonidet P 40). The cells were left on ice for 30 min and then centrifuged at 13,000 x g at 4 ℃ for 10 min. The supernatant was collected into sterile Eppendorf tubes, transferred on ice and stored at -70 ℃ until used. Cells were collected from passage 5 to passage 125.

3.4. Determination of protein concentration

The concentration of protein was estimated using the BioRad Micro-DC Assay kit (BioRad, Munich, Germany). Bovine serum albumin (BSA) (2 mg/ml) was serially diluted to concentrations ranging between 2.0 and 0.2 mg/ml. The protein samples were diluted 1:1 and 1:2 in water. Volumes of 5 µl of the BSA and the samples were pipetted into an ELISA plate and 25 µl of reagent A and 200 µl of reagent B were added. The plate was incubated at room temperature (20℃) 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.

3.5. Gelatin substrate gel electrophoresis

The protein concentration of samples were adjusted to 2 µg/µl in the sample buffer (2% SDS, 10% glycerol, 0.005% bromophenol blue, 80 mM Tris-HCl, and pH 6.8) and the samples were loaded to the gel without prior boiling. Substrate SDS-page with 0.2% (W/V) gelatin (Sigma porcine type 1) was added to 10 or 8% acrylamide resolving gels according to Shkap et al. (2003). After electrophoresis the gel was rinsed in double distilled water and then immersed in 500 ml of 2.5 %(v/v) Triton X-100 solution on a shaker for 1 hour at room temperature. Then the gel was incubated in incubation buffer (50 mM Tris-HCl and 5 mM $CaCl_2$, pH 8.0) at 37 ℃ overnight. The gel was stained in 0.5% (W/V) coomasie blue R 250 in 45% (v/v) methanol, 45% (v/v) double distilled water and 10 % glacial acetic acid for 24 h and then destained in 45% (v/v) methanol, 45% (v/v) double distilled water and 10% glacial acetic acid until the stacking gel became clear.

To identify the metalloproteinases, specific inhibitors were used. MMP-2 inhibitor 1 was added to the cell lysate at a concentration of 1.7 µM, and MMP-2/MMP-9 inhibitor III (both from Calbiochem, VWR International, Darmstadt, Germany) at a concentration of 10 µM. Densitometric analysis was performed using ImageJ software (Abramoff et al., 2004).

3.6. Isolation of mRNA

Pure mRNA was isolated from *T. lestoquardi* (Atbara). The Oligotex kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. Cell pellets (1×10^7 cells) stored at -70 ℃ were allowed to thaw, then 600 µl of buffer OL2 was added and vortexed for 5-10 seconds. To homogenize the sample, the cell lysate was passed through a sterile plastic syringe fitted with a 20-gauge needle at least 5-10 times then centrifuged at 16,000 x g for 3 min and the supernatant was transferred into a new RNase-free tube. Then 70 µl of Oligotex was added to the sample, mixed thoroughly by

vortexing and placed at room temperature (20 ℃) for 10 min. The Oligotex and mRNA complex was centrifuged at 16,000 x g for 5 min then the pellet was resuspended in 350 µl buffer OW1 by vortexing. The solution was transferred to spin column placed in a 1.5 ml microcentrifuge tube, centrifuged for 1 min at maximum speed (16,000 x g) and the flow-through was discarded. The spin column was placed in a new sterile RNase-free microcentrifuge tube, 350 µl buffer OW2 buffer was added to the column, centrifuged for 1 min at maximum speed and the flow-through was discarded. This step was repeated once using the same microcentrifuge tube. The spin column was transferred to a new RNase-free microcentrifuge tube then 20 µl of hot OEB buffer was added to the column, mixed by pipeting up and down three to four times and centrifuged for 1 min at maximum speed. The mRNA was treated with DNAse 1, Amp Grade (Invitrogen, Karlsruhe, Germany) to remove any contamination with genomic DNA (gDNA). One microgram mRNA was used in a final reaction volume of 10 µl. To the mRNA, 1 µl of 10x DNase 1 reaction buffer (Invitrogen, Karlsruhe, Germany) and 1 µl of DNAse 1, Amp Grade (1 U/ µl) were added and the volume was completed to 10 µl with DEPC-treated water. This mixture was incubated for 15 min at room temperature (20 ℃). To inactivate DNAse 1 µl of 25 mM EDTA solution was added to the reaction mixture, and heated for 10 min at 65 °C. Then the mRNA samples were ready to be used in reverse transcription.

3.7. Synthesis of cDNA

For the synthesis of cDNA from mRNA the Superscript II reverse transcriptase enzyme was used (Invitrogen, Karlsruhe, Germany). One nanogram to 2 µg of mRNA was used in a final reaction volume of 20 µl. To the mRNA, 1 µl oligo (dT) (500 µg/ml) (Invitrogen, Karlsruhe, Germany) and 1 µl 10 mM deoxynucleotide triphosphates (dNTP) mix (10 mM each) were added and the volume was brought to 12 µl with sterile water. This mixture was heated to 65 ℃ for 5 min to allow the oligo (dT) priming and then quickly

chilled on ice. The content of the tube was collected by a brief centrifugation step and 4 µl of (5×) first -strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1 M DTT (dithiothreitol) and 1 µl RNase inhibitor (37 units/µl) (Amersham, Freiburg, Germany) were added. The contents were gently mixed and the tube was incubated at 37 ℃ for 2 min. One microliter (200 units) of Supe rscript was added, mixed by pipetting gently up and down and the tube was incubated for 50 min at 37 ℃. Finally the reaction was inactivated by heating at 70 ℃ for 15 min and the tube was stored at -20 ℃.

3.8. Detection of MMP 9 transcript

The consensus sequence of MMP9 from different species (*Macaca mulatta* (XM_001104871, *Pan troglodytes* (XM_514689), *Oryctolagus cuniculus* (NM_001082209), *Sus scrofa* (NM_001038004), *Canis familiaris* (NM_001003219), *Bos taurus* (NM_174744), *Homo sapiens* (NM_004994)) was used to design primers since the ovine sequence was not available (Table 1). PCR reactions were performed in a final volume of 35 µl which contained 25.5 µl water, 2 µl template (cDNA), 3.5 µl 10x PCR buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 0.1% Tween 20 and 15 mM $MgCl_2$), 0.7 µl dNTPs (final concentration 200 µM each), 1.6 µl each primer (final concentration 450 nM each primer) and 0.15 µl (0.75 units) of Taq DNA polymerase. Thermal cycling was performed using a thermocycler (T3; Biometra, Goettingen, Germany). The following PCR conditions were applied: 35 cycles of 94 ℃ for 30 seconds, 63 ℃ for 1 min and 72 ℃ for 1 min result ing in the amplification of a 502 bp fragment from *T. lestoquardi* cDNA. The fragment was cloned into the pDrive vector (Qiagen, Hilden, Germany) and sequenced (Eurofins MWG operon, Ebersberg, Germany), confirming the detection of MMP9 transcripts.

3.9. SYBR Green quantitative Real Time-PCR (QRT-PCR) for TNF-alpha expression

Complementary DNA (cDNA) from different passages (passage 7, 25, 50, and 100) was amplified using the specific primer for TNF-alpha (Table 1). The transcript of beta 2 microglobulin (β2M) was used as a reference gene for normalization of expression data (Table 1). For amplification, the SYBR Green PCR master mix (Roche, Mannheim, Germany) was used. Amplification reactions were performed in a volume of 10 µl containing 2 µl cDNA as template, 0.8 µl of each PCR primers, 2 µl of the SYBR green master mix and 4.8 µl water PCR grade. Quantitative Real Time-PCR was done in the Light cycler 2.0 (Roche, Mannheim Germany). Amplification conditions were 55 cycles with the following profile: 95 ℃ for 30 s, 60 ℃ for 30 s and 72 ℃ for 1 min, each QRT-PCR experiment was carried out in duplicate. For relative quantification analysis the efficiency calibrated model (Pfaffl, 2001) was used. Relative quantification results were obtained by comparing levels of expression with an internal calibrator consisting of equal amounts of cDNA derived from several different *T*. *lestoquardi* (Atbara) passages (passage 7, 25, 50, 75 and 100).

Table 1: The 5′-3′ sequence of the primers used in the study

Primers	**Forward (5´-3´).**	**Reverse (5´-3´).**	**Reference**
MMP-9	GACGGGCTCCTGGCACACG	TGAAGGGGAAGACGCACAGC	This study
TNF-alpha	CTGGTTCAGACACTCAGGTCCT	GAGGTAAAGCCCGTCATG	(Donaldson et al.,2005)
β2M	TTCTGTCCCACGCTGAGTTCA	CAACCCAAATGAGGCATCT	DQ386890
G3PDH	GGTGGAGTCCACTGGGGTCTTCACT	TGGCAGTGATGGCGTGGACA	This study
Tl_V	GGTGTTCTCGGAATCTACGG	CGAACTCCTGCATCTCCAAC	This study
Tl_ldh	GGTGCTGGAAAATCATTGGA	CGTCCATCGGGTTTGTAATC	This study

3.10. Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. Both mRNA populations are converted into cDNA: we refer to the cDNA that contains specific (differentially expressed) transcripts as tester and the reference cDNA as driver. Differentially expressed genes were enriched following the protocol of the clontech PCR-select ™ cDNA subtraction kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). To construct SSH libraries, mRNA was isolated from passage 4 (low passage) and passage 75 (high passage). Subtractive hybridizations were performed in both directions in order to obtain both downregulated genes (subtraction run in forward direction; transcripts higher expressed in the passage 4) and upregulated genes (subtraction run in reverse direction; transcripts higher expressed in the passage 75). Two different libraries were constructed, for the forward library mRNA from p4 was used as tester and mRNA from p75 was used as the driver. For the reverse library, mRNA from p75 was used as tester and the mRNA from p4 was used as driver. Double-stranded cDNAs synthesized from 2 µg mRNA of passage 4 and passage 75 were subjected to digestion with *Rsa*1 to produce shorter blunt-ended fragments. Digests were used as tester cDNA and driver cDNA (which was used to remove non differentially expressed genes selectively from tester cDNA). The tester cDNA was then subdivided into portions and each was ligated with a different adapter: adaptor 1 or adaptor 2R (Adaptor 1: 5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′; Adaptor 2: 5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3′). No such adaptors were ligated to the driver cDNA. Two hybridizations were then performed. In the first, an excess of driver was added to each sample of tester with a different adaptor. The samples were denatured and then incubated at 68 ℃ for 8 h. The two samples from the first hybridization were mixed together without denaturing, and fresh

denatured driver cDNA was added to the solution and incubated at 68 ℃ for 12 h, enriching it further for differentially expressed sequences. Only the remaining subtracted single-stranded tester cDNAs could reassociate and form double-stranded tester molecules with different ends, which corresponded to the sequences of adaptors 1 and 2R (Fig 1). Dilution buffer (200 µl) (20 mM HEPES pH6.6,20 mM NaCl, 0.2 mM EDTA pH 8.0) was added to the mixture. The entire population of molecules was subjected to two rounds of PCR to amplify the differentially expressed sequences containing both adaptors by exponential amplification of these products. The primary PCR was performed with PCR primer 1 (5′CTAATACGACTCACTATAGGGC3′) against adaptor 1 and adaptor 2R and the amplified products were used as template in the secondary PCR with nested primers (nested PCR primer 1: 5′TCGAGCGGCCGCC CGGGCAGGT3′, nested PCR primer 2R: 5′AGCGTGGTCGCGGCCGAGGT3′).

To evaluate the subtraction efficiency, the relative amount of the constitutively expressed sheep G3PDH was compared in subtracted and un-subtracted PCR samples by PCR. These samples were then diluted ten-fold. PCR was conducted on these samples following the protocol of PCR-select ™ cDNA subtraction kit for 33 cycles using sheep G3PDH primers (Table 1). For each cDNA pool tested, a 5 µl aliquot was removed from the reaction mixture every 5 cycles, starting at the end of cycle 18, and analyzed using agarose gel electrophoresis for the presence of a PCR product.

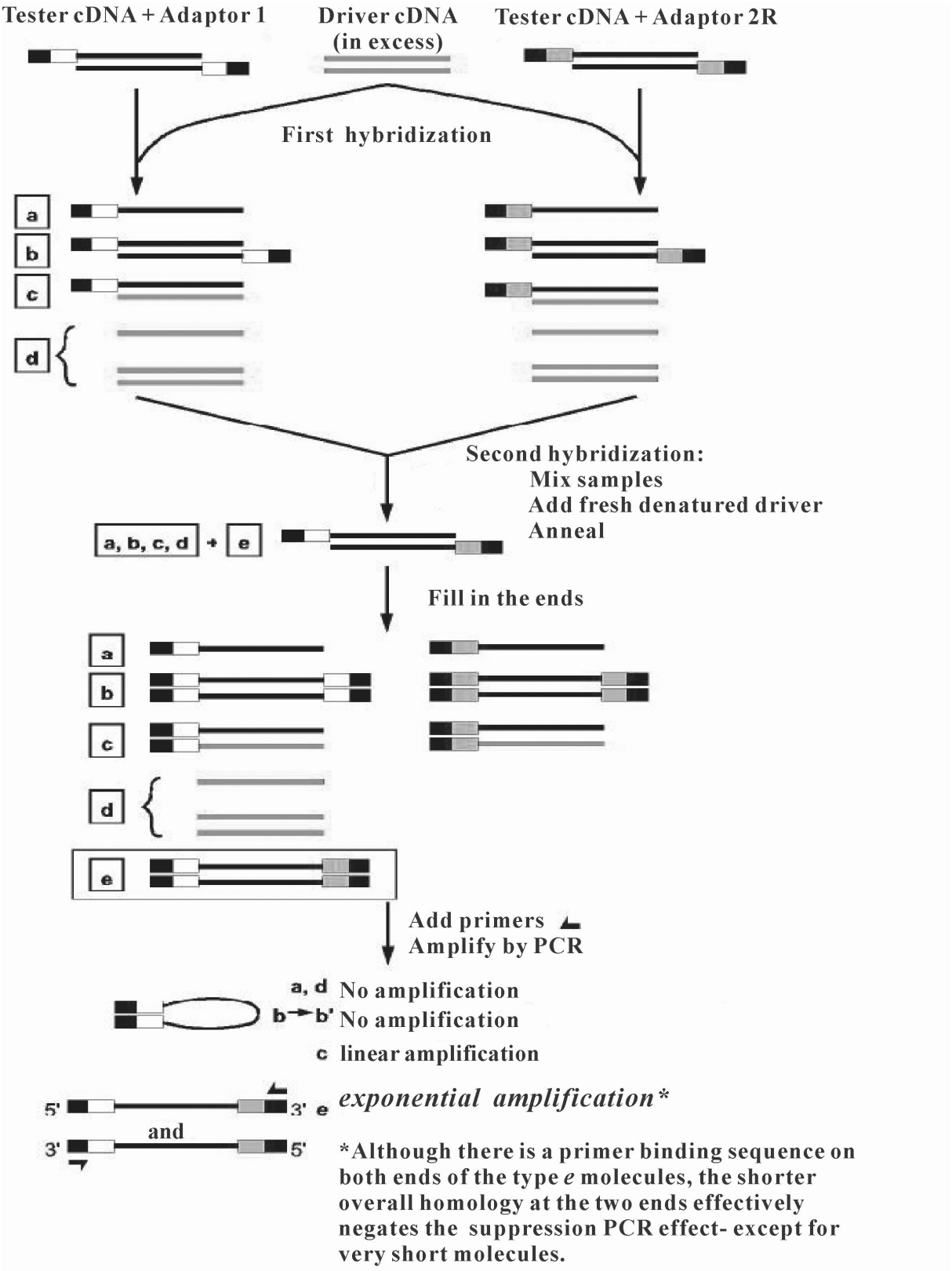

Figure 1. Schematic diagram of PCR-select cDNA subtraction (Figure adopted from clontech PCR-select ™ cDNA subtraction kit manual)

3.11. Construction of the SSH libraries (T/A cloning) and sequencing

The forward and reverse SSH libraries contained many differentially expressed cDNAs. The libraries were constructed by ligating the subtracted cDNAs into the pDrive vector (Qiagen, Hilden, Germany) according to the manufacturer's protocol, a ligation-reaction mixture was prepared in 10 µl: 5 µl of ligation master mix, 1 µl pDrive cloning Vector (50ng/ µl), 2 µl PCR products, 2 µl distilled water. The ligation reaction was incubated at 16° C for 16 h .The ligated product was transformed into *E. coli* competent cells (*E. coli* EZ competent, Qiagen, Hilden, Germany). A volume of 2 µl of ligation-reaction mixture was added per tube containing 50 µl aliquot of the competent cells, mixed gently then incubated on ice for 5 min. Cells were then heated by placing the tubes in a 42° C water bath for 30 s without shakin g. The tubes were then incubated on ice for 2 min then 250 µl of a SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) were added to each tube at room temperature (20 ℃). A volume of 100 µl and 50 µl of the transformed cells were plated onto LB agar plates containing carbenicillin (Roth, Karlsruhe, 100 µg/ml LB agar), 5 µl 10 mM Isopropyl-beta-D-thiogalactopyranoside (IPTG) (Invitrogen, Karlsruhe) and 40 µl 4%5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Roth, Karlsruhe) then the plates were incubated at 37 ℃ for 12 h. White colonies were picked and inoculated into 5 ml of LB broth (Gibco/BRL, Eggenstein) containing 100 µg/ml ampicillin and incubated overnight at 37 ℃. To test for the correct insert, PCR reaction utilizing the M13 forward (5'-TGTAAAACGACGGCCAGT-3') and the M13 reverse (5'-CGAGAAACAGCTATGA CC-3') primers were performed. The cycling program was as follows: 94 ℃ initial denaturation step for 2 min, 30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 3 min depending on the size of the product. The correct product size was determined as the original PCR product size plus 267 bp representing the total distance in the pDrive derived sequence of the M13 primer

annealing sites from the PCR insert. Plasmid DNAs of individual clones were purified using the MiniPrep kit (Qiagen, Hilden, Germany) and sequenced (Eurofins MWG operon, Ebersberg, Germany).

When required an aliquot of the overnight culture was stored in a labelled 1.5 ml screw capped tube using 750 µl of an overnight culture combined with 250 µl glycerol (Sigma, Deisenhofen, Germany). The tube was vortexed and left to equilibrate for 2 h in a refrigerator. It was then snap-frozen in liquid nitrogen and stored at -70 ℃.

3.12. Isolation of plasmid DNA

Plasmid DNA was isolated from overnight culture for sequencing using the QIAprep® Miniprep kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

Bacteria grown overnight in 5-10 ml LB medium (Luria Bertani medium) containing the required antibiotics were pelleted by centrifugation for 10 min at 3300 x g using a Heraeus Bactifuge (Heraeus Instruments, Osterode, Germany). All subsequent steps were performed at 20 ℃ and all subsequent centrifu gation steps were carried out at 15000 x g. Bacterial cells were resuspended in 250 µl buffer P1 containing RNase A (100 µg/ml) and transferred into a 1.5 ml tube. An equal volume of buffer P2 was added and the tube was gently inverted 4–6 times to lyse the cells and denature the plasmid DNA. This was followed by the addition of 350 µl buffer N3 and the tube was inverted immediately but gently 4-6 times to re-nature plasmid DNA and precipitate bacterial genomic DNA and protein. The tube was centrifuged for 10 min during which a compact white pellet was formed. The supernatant was decanted into the QIAprep spin column. The column was centrifuged for 30 s to bind plasmid DNA and the flow-through was discarded. The column was washed using 0.75 ml buffer PB and centrifuged for 30 s. Further two similar washing steps were carried out with the ethanol containing buffer PE. The flow-through was discarded and the column was

centrifuged empty for an additional 1 min to remove residual wash buffer. DNA was eluted into a clean 1.5 ml microcentrifuge by the addition of 50 µl distilled H_2O at 70 ℃ to the center of the column, incubation for 1 min at 20 ℃ and centrifugation for 1 min.

3.13. Sequence analysis and homology comparison

The positive clones were sequenced (Eurofins MWG operon, Ebersberg, Germany). Compilation, editing and assembly of sequences were performed with the EditSeq and SeqMan analysis programme components of Lasergene software package for Windows (DNASTAR, Madison, Wis, USA). BLAST analysis of the cDNA libraries sequences were performed on the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.14. SYBR Green quantitative Real Time -PCR (QRT-PCR)

To confirm the result of SSH the parasite gene was selected to be used in QRT-PCR. Specific pairs of primers for the parasite gene vacuolar H+ ATPase (Tl_V) and parasite housekeeping gene lactate dehydrogenase (Tl_ldh; Accession numbers FJ201249; FJ201250) were designed to target the flanking regions of the intron (Table 1). cDNAs from different passages were used (passages 7, 25, 50, 100). The quantity of cDNA in each sample was normalized to the quantity of the parasite housekeeping gene. Amplification conditions and relative quantification were as described above (3.9.).

3.15. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For detection of Ki-67 antigen expression, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) followed by western blot and detection with specific antibody was performed. The detection of α-tubulin was performed as the loading control. The SDS-PAGE technique used here was similar to the originally described method (Laemmli, 1970). The 5% running gel solution was prepared

according to 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 gel was covered with 70% ethanol and allowed to polymerize for at least 30 min. The 3% stacking gel solution was prepared according to 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 removed by inverting the chamber and draining the residual drops with a filter paper. Combs with the desired number of wells were placed and the gel was left to polymerize for at least 30 min.

Table 2 Composition of the running and the stacking gel

Component	3% stacking gel	5% running gel
Distilled autoclaved H_2O	3 ml	7 ml
Acrylamide/bisacrylamide solution	0.5 ml	2ml
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 (0.5 M Tris-HCl, 10% Glycerol, 0.4% SDS, 0.01% pyronin Y [Serva, Heidelberg])	10 µl	-
Ammonium persulphate (10% solution)	20 µl	100 µl

The samples were prepared by harvesting $1x10^5$ *T. lestoquardi* ovine infected cells by centrifugation (10 min at 400 x g) from passage 7, 25, 50 and 75 and suspended in sample buffer (20% glycine, 0.125 mol/l (molar) Tris-HCI, (pH 6.8), 6% SDS with 10% 2-mercaptoethanol) then samples were heated to 100 ℃ for 5 minutes. The gels were

placed into the running chamber which was filled with the running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS). Depending on the estimation of the protein concentration for each sample the same amount of protein was then loaded into the corresponding wells along with a Precision Plus Protein™ Dual Color Standards (BioRad, Munich, Germany). Electrophoresis was carried out for 50 min as follows: 50 Volts for 5 min, 100 Volts for 10 min and 200 Volts for 35-45 min.

3.16. Semi-dry transfer of protein onto nitrocellulose membranes

Separated proteins were transferred to nitrocellulose membranes (0.2 µm, Schleicher and Schuell, Dassel, Germany) using a BioRad Transblot-SD semi-dry blotter (BioRad, Munich, Germany). After SDS-PAGE, gels were 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. Transfer was carried out at 25 V, 120 mA for 60 min for 2 gels.

3.17. Western blot

Blocking was carried out in PBS containing 3% BSA in PBS at room-temperature for 2 h on a platform shaker, then the membrane was washed 3 times 10 min each in PBS containing 0.1% Tween 20 (PBST). After the last wash in PBST the membrane was probed with the primary antibodies 40 µg/ml MIB-5 (Dako, Glostrup, Denmark) or 1 µg/ml anti-tubulin (Mouse monoclonal antibody) (DMA1, Sigma, Deisenhofen, Germany) in PBS containing 1% BSA in PBS and were incubated overnight. Afterwards, the membrane was washed in PBST as above. Goat anti-mouse alkaline phosphatase (AP)-conjugated Fab fragments (Dako, Glostrup, Denmark) diluted 1:5,000 in 1% BSA in PBS were added. The membranes were incubated then washed

as above. After the last wash in PBST, the membranes were washed once in PBS for 10 min then equilibrated in alkaline phosphatase (AP) buffer (100 mM Tris HCl pH 9.5, 5 mM $MgCl_2$, 100 mM NaCl) for 5 min. Freshly prepared substrate solution was added, which contained 0.33 mg/ml Nitroblue tetrazolium (NBT) and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roth, Karlsruhe, Germany) in AP buffer. Colour development was stopped by rinsing the membranes in a stop solution of 20 mM EDTA. Densitometric analysis was performed using ImageJ software (Abramoff MD, et al., 2004).

3.18. [^{3}H] - Thymidine incorporation assay

T. lestoquardi ovine infected cells from passage 7, 25, 50 and 75 were plated at 10^4 cells/well in a 96–well plate each passage in one raw and one raw of medium was used as a control. The infected cells were cultured in 150 µl RPMI complete medium and incubated in a humidified atmosphere with 5% CO_2 at 37 ℃. After 2 h, ^{3}H-thymidine (0.2 µCi) (ICN Biomedicals, Eschwege, Germany) was added to each well. After 6 h of incubation, cells were collected onto filter paper using a cell harvester. Finally the incorporated radioactivity of the dried filter was counted in a scintillation counter.

4 Results

Influence of subculturing on gene expression in a *Theileria lestoquardi* infected cell line

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Abstract

In this study potential molecular markers for identification of attenuation in a *Theileria lestoquardi* infected cell line to be used in vaccination trials were identified. Two markers associated with attenuation in *T. annulata* vaccine strains were analyzed (metalloproteinase activity and TNF-α mRNA expression). The result showed a decreased activity of MMP 9 and decreased mRNA expression of TNF-α with increasing passage number. Suppression subtractive hybridization was used to identify potential new markers of attenuation. Random screening revealed nine differentially expressed genes, one from the parasite and eight from the host. Quantitative real time-PCR confirmed mRNA expression of the parasite vacuolar H+ATPase to be downregulated at higher passages.

Keywords: Attenuation, cell line vaccine**,** *Theileria lestoquardi*

1 Introduction

Theileria lestoquardi is a tick-borne protozoan parasite and highly pathogenic for sheep. The diseases caused by the pathogen is known as malignant ovine theileriosis (MOT) and is transmitted by *Hyalomma* ticks [1]. The disease was first described in Egypt in exported Sudanese sheep by Mason [2] and has in the meantime been recorded in south-eastern Europe, North Africa, the Near and Middle East and Southern USSR [3]. In the Sudan, the disease was first reported in Khartoum State [4] and then in northern Sudan [5]. Recently it was shown to be widely distributed in main sheep grazing areas of the country where 16.3% of sheep surveyed showed reactive antibodies in IFAT [6]. Case fatality rates may reach up to 100% [7] and hence the disease is of high economic importance.

Control of the disease can be achieved by chemotherapy, using theilericidal drugs such as buparvaquone [8], as well as tick control using acaricides [9]. However, both methods have shortcomings. Drug treatment is expensive and requires early diagnosis of the disease [8], whereas acaricide treatment raises environmental concerns and resistance to the chemical is also recorded [10]. These shortcomings could be overcome by immunoprophylaxis of sheep with attenuated *T. lestoquardi* schizont-infected ovine cells providing the animal with solid immunity [11], which has been carried out successfully in Iraq and Iran [12-14].

Attenuation is defined as loss of virulence whilst retaining viability and infectivity [15]. It is possible to attenuate *T. annulata* schizonts, a parasite closely related to *T. lestoquardi* and infective to cattle, by prolonged *in vitro* culture of infected cell lines which can be used as live vaccine [16, 17]. Attenuation of *T. annulata* can be achieved by continuous passage of the original virulent parasite in cell culture for about 60–300 passages over a period of several months to 2 years [18] and is usually monitored by examining the clinical and immune reactions of the calves inoculated by these culture cells. This technique has been applied in a number of countries to establish attenuated

vaccines against tropical theileriosis [19]. However, although successful, it is an expensive, painstaking and time-consuming process. Therefore, a number of studies have been designed to identify attenuation markers which can be associated with loss of virulence, in order to devise *in vitro* monitoring of attenuation before testing in susceptible animals.

The attenuation phenotype has been extensively studied in *Theileria annulata*. From these studies attenuation has been correlated to a number of alternative processes like loss of virulence factors such as metalloproteinases and cytokines or alteration in parasite and host gene expression and possible selection of a less virulent parasite subpopulation [18]. Matrix metalloproteinases have been implicated in the metastatic behavior of schizont infected cells, therefore a reduction in metalloproteinase activity is correlated with attenuation [20, 21]. *T. annulata* infected cells constitutively express mRNA for TNF-α and several other proinflammatory cytokines [22]. The major clinical symptoms of acute tropical theileriosis (anorexia, cachexia, anemia and pyrexia) are characteristic of those induced by TNF-α as observed by Bielefeldt Ohman et al. [23] when cattle were inoculated with recombinant TNF-α.

Immunization experiments in cattle implied that both cytokine expression and metalloproteinase activity may be regarded as a marker of virulence [24, 25], however there seems to be some differences in the vaccination strains used, since metalloproteinase activity was still found in an Israeli strain [25], and was even high in an Indian strain used for immunization [24].

The definition of attenuation at a molecular level could speed production of cell line vaccines and reduce the cost of the dose. Since no work has been reported regarding attenuation mechanisms in *T. lestoquardi*, the following study investigated described potential attenuation markers of *T. annulata* infected cells in a *T. lestoquardi* cell line at different passages. Furthermore, differentially expressed genes in higher passages and

lower passages were analyzed using suppression subtractive hybridization in order to identify genes which correlate with subculturing and thus potentially with attenuation. These analyses were performed before going into vaccination trials, to prevent adverse effects in experimental animals.

2 Material and Methods

2.1 Cell line

A *T. lestoquardi* strain was isolated from a sheep showing the typical signs of malignant theileriosis in Atbara town in northern Sudan and maintained in the laboratory as schizont infected cell culture (*T. lestoquardi* (Atbara)) [26]. PCR experiments confirmed the absence of contaminating *Theileria* parasites. Cells were cultured in RPMI complete medium (RPMI 1640, 2 mg/ml sodium bicarbonate (Sigma, Deisenhofen, Germany), 2 mM L-glutamine, 100 IU penicillin/ml, 100 µg/ml streptomycin (all from Biochrom, Berlin, Germany) and 20% heat-inactivated foetal calf serum (FCS; PAA, Pasching, Austria) and incubated in a humidified atmosphere with 5% CO2 37℃. This isolate was used for all subseque nt investigations.

2.2 Gelatin substrate gel electrophoresis

The cell lysates were prepared as described by Shkap et al. [25]. Briefly, the schizont infected cells of *T. lestoquardi* (Atbara) were collected from culture flasks and washed three times in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) by centrifugation at 1000 xg for 5 min. The pellet was resuspended in an equal volume of twofold concentrated, sterile cold lysis buffer (0.25 % sucrose and 0.5% Nonidet P 40). The cells were left on ice for 30 min and then centrifuged at 13,000 xg at 4℃ for 10 min. The sup ernatant was collected into sterile Eppendorf tubes, transferred on ice and stored at -70℃ until use. Cells were collected from passage 5 to 125. Protein concentration was measured using the BioRad Mirco-DC Assay kit (BioRad, Munich, Germany).

For gelatin substrate gel electrophoresis samples were adjusted to 2 µg/µl in the sample buffer (2% SDS, 10% glycerol, 0.005% bromophenol blue, 80 mM Tris-HCl, pH 6.8) and were loaded on the gel (20 µg/lane) without prior boiling. Substrate SDS-PAGE with 0.2% (w/v) gelatin (porcine type 1, Sigma, Deisenhofen, Germany) was added to 10 or 8% acrylamide resolving gel according to Shkap et al. [25]. After electrophoresis the gel was rinsed in double distilled water and immersed in 500 ml of 2.5 % (v/v) Triton X-100 solution on a shaker for 1 hour at room temperature. Then the gel was transferred to incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.0) at 37°C overnight. The gel was stained in 0.5% (w/v) Coomasie Blue R 250 in 45% (v/v) methanol, 45% (v/v) double distilled water and 10 % glacial acetic acid for 24 h and then destained in 45% (v/v) methanol, 45% (v/v) double distilled water and 10 % glacial acetic acid until the stacking gel became clear.

To identify the metalloproteinases, specific inhibitors were used. MMP-2 inhibitor 1 was added to the cell lysate at a concentration of 1.7 µM, and MMP-2/MMP-9 inhibitor III (both from Calbiochem, VWR International, Darmstadt, Germany) at a concentration of 10 µM. Densitometric analysis was performed using ImageJ software [27].

2.3 RNA extraction and Reverse Transcriptase-PCR

Pure mRNA was isolated from *T. lestoquardi* (Atbara) at different passages. The oligotex kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. The mRNA was treated by DNAse 1, Amp Grade (Invitrogen, Karlsruhe, Germany) for 15 min at room temperature to remove any contamination of genomic DNA (gDNA). First strand complimentary DNA (cDNA) was reverse transcribed using oligo (dT) and Superscript II (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

2.4 Detection of MMP 9 transcripts

The consensus sequence of MMP9 from different species (Macaca mulatta (XM_001104871, Pan troglodytes (XM_514689), Oryctolagus cuniculus (NM_001082209), Sus scrofa (NM_001038004), Canis familiaris (NM_001003219), Bos taurus (NM_174744), Homo sapiens (NM_004994)) was used to design primers since the ovine sequence was not available (Table 1). The following PCR conditions were applied: 35 cycles of 94℃ for 30 seconds, 63° C for 1 min and 72℃ for 1 min resulting in the amplification of a 502-bp fragment from *T. lestoquardi* cDNA. The fragment was cloned into pDrive vector (Qiagen, Hilden, Germany) and sequenced by MWG (Eurofins MWG operon, Ebersberg, Germany), confirming the detection of MMP9 transcripts.

2.5 SYBR Green quantitative Real Time -PCR (QRT-PCR)

Complementary DNA (cDNA) from different passages (passage 7, 25, 50, and 100) was amplified using the specific primer for TNF-α (Table 1). The transcript of beta 2 microglobulin (β2M) was used as a reference gene for normalization of expression data (Table 1). For amplification, the SYBR Green PCR master mix (Roche, Mannheim, Germany) was used. Quantitative Real Time-PCR was done in a final volume of 10 µl in the Light cycler 2.0 (Roche, Mannheim Germany). Amplification conditions were 55 cycles with the following profile: 95 ℃ for 30 s, 60℃ for 30 s and 72℃ for 1min, each QRT-PCR experiment was carried out in duplicate. For relative quantification analysis the efficiency calibrated model [28] was used. Relative quantification results were obtained by comparing levels of expression with an internal calibrator consisting of equal amounts of cDNA derived from several different *T. lestoquardi* (Atbara) passages (passage 7, 25, 50, 75 and 100).

To confirm the result of SSH the parasite gene was selected to be used in QRT-PCR. Specific pairs of primers for the parasite gene vacuolar H+ ATPase (Tl_V) and parasite

housekeeping gene lactate dehydrogenase (Tl_ldh; Accession numbers FJ201249; FJ201250) were designed to target the flanking regions of the intron (Table 1). cDNAs from different passages were used (passages 7, 25, 50, 100). The quantity of cDNA in each sample was normalized to the quantity of the housekeeping gene. Amplification conditions and relative quantification were as described above.

2.6 Suppression subtractive hybridization

Two libraries were constructed from passage 4 (low passage) and passage 75 (high passage)**.** For the two passages cDNA synthesis as well as subtractive suppression hybridization (SSH) including steps of adaptor ligation, subtractive hybridization, and selective amplification were performed following the protocol of the Clontech PCR-select™ cDNA subtraction kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye France).

The efficiency of the SSH procedure was detected by comparing the abundance of ovine G3PDH (primers see Table 1) in the subtracted and unsubtracted PCR samples as recommended in the clontech PCR-select™ cDNA subtraction kit (BD Biosciences). These samples were then diluted ten-fold. PCR was conducted on these samples following the protocol of PCR-select™ cDNA subtraction kit for 33cycles. For each cDNA pool tested, a 5 µl aliquot was removed from the reaction mixture every 5 cycles, starting at the end of cycle 18.

2.7 Cloning, sequencing, and sequence analysis and homology search

For each SSH generated library, the amplification product was cloned into the pDrive vector (Qiagen, Hilden, Germany), resulting in a range of cloned cDNA fragments. Plasmids DNA from randomly selected individual colonies were purified using MiniPrep kit (Qiagen) and sequenced (Eurofins MWG operon, Ebersberg, Germany).

Compilation, editing and assembly of sequences were performed with the EditSeq and SeqMan analysis programme components of Lasergene software package for

Windows (DNASTAR, Madison, Wis, USA). BLAST analysis of the cDNA libraries sequences were performed on the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1: The 5′-3′ sequence of the primers used in the study.

Primers	Forward (5´-3´).	Reverse (5´-3´).	Reference
MMP-9	GACGGGCTCCTGGCACACG	TGAAGGGGAAGACGCACAGC	This study
TNF-α	CTGGTTCAGACACTCAGGTCCT	GAGGTAAAGCCCGTCATG	[55]
β2M	TTCTGTCCCACGCTGAGTTCA	CAACCCAAATGAGGCATCT	Accession number DQ386890
G3PDH	GGTGGAGTCCACTGGGGTCTT CACT	TGGCAGTGATGGCGTGGACA	This study
Tl_V	GGTGTTCTCGGAATCTACGG	CGAACTCCTGCATCTCCAAC	This study
Tl_ldh	GGTGCTGGAAAATCATTGGA	CGTCCATCGGGTTTGTAATC	This study

2.8 SDS-Page and Western blot

For detection of Ki-67 antigen expression , sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blot and detection with specific antibodies was performed. Briefly, $1x10^5$ cells per slot in sample buffer (20% glycine, 0.125 mol/l (molar) Tris-HCl, (pH 6.8), 6% SDS with 10% 2-mercaptoethanol) were heated to 100℃ for 5 minutes and then applied to a 5% acr ylamide slab gel system with a 3.5% stacking gel. After transferal onto a nitrocellulose membrane (0.2µm, Schleicher and Schuell, Dassel, Germany), membranes were blocked with 3% BSA in PBS for 2h before probing with the primary antibodies (40 µg/ml MIB-5 (Dako, Glostrup, Denmark) or 1µg/ml anti-tubulin (DMA1, Sigma, Deisenhofen, Germany)). Detection was performed using alkaline phosphatase (AP) conjugated goat anti-mouse antibody

(Dako, Glostrup, Denmark) followed by incubation with NBT/BCIP (BioRad Labratories, Munich, Germany). Densitometric analysis was performed using ImageJ software [27].

2.9 Proliferation assay

Cell proliferation was assessed by pulsing the cells with ^{3}H-thymidine. Briefly, 10^{4} cells/well were cultured in 150 µl RPMI complete medium in 96-well culture plates. After 2h, the cells were pulsed with 0,2 µCi ^{3}H-thymidine (ICN Biomedicals, Eschwege, Germany) for 6h, and incorporation was measured in a scintillation counter.

3 Results

3.1 Metalloproteinase activity

The metalloproteinase (MMP) activity in the *T. lestoquardi* (Atbara) cell line appeared in two bands of approximately 94 and 62 kDa, corresponding to the expected molecular weights of MMP 9 and MMP2 respectively (Fig.1). The metalloproteinase activity gradually decreased as the passage number increased (Fig. 1), particularly regarding the 94 kDa band. The gelatinase activity observed at 62 kDa also appeared to be regulated, being increased at low passage (passage 5), more intense in passage 15 and 30, and then being decreased again in high passages (passage 83) (Fig. 1). To identify the metalloproteinase the cell lysates were treated with specific inhibitors for MMP9&2 and MMP2. Only the 62 kDa band was diminished using the MMP2 inhibitor (Fig. 2). Activity previously observed at 94 kD was decreased using MMP9&2 inhibitor, the MMP2 inhibitor had no effect on this band (Fig. 2). These results indicated that the two bands of approximately 94 and 62 kDa corresponded to MMP9 and MMP2, respectively. The expression of MMP9 was confirmed on the mRNA level by reverse transcriptase-PCR (Fig. 3) and sequencing (GenBank accession No. FJ185130).

Figure 1 Gelatin substrate SDS-PAGE (8%) showing gelatinase activity in lysates of schizont infected cells of *T. lestoquardi* (Atbara) in different passages (p). U937 is a human leukemic monocyte lymphoma cell line used as a positive control.

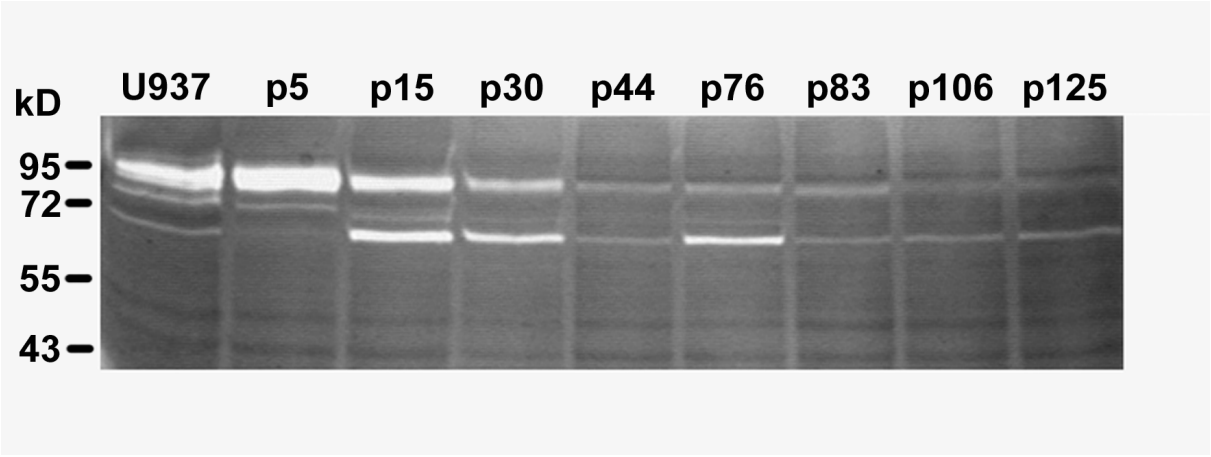


Figure 2 Gelatin substrate SDS-PAGE (8%) showing the reduction in MMP activity when using specific inhibitors for MMP2 and MMP 9&2. For experiments lysates of schizont infected cells of *T. lestoquardi* (Atbara) at passage 5 (MMP2&9inhibitor) and at passage 15 (MMP2 inhibitor) were used. Lane 1: passage 15 without inhibitor; lane 2: passage 15 with MMP2 inhibitor; lane 3: passage 5; lane 4: passage 5 with MMP2&9 inhibitor; lane 5: passage 5 with MMP2 inhibitor. Densitometric evaluation of the gels is shown below the depiction of the gels.

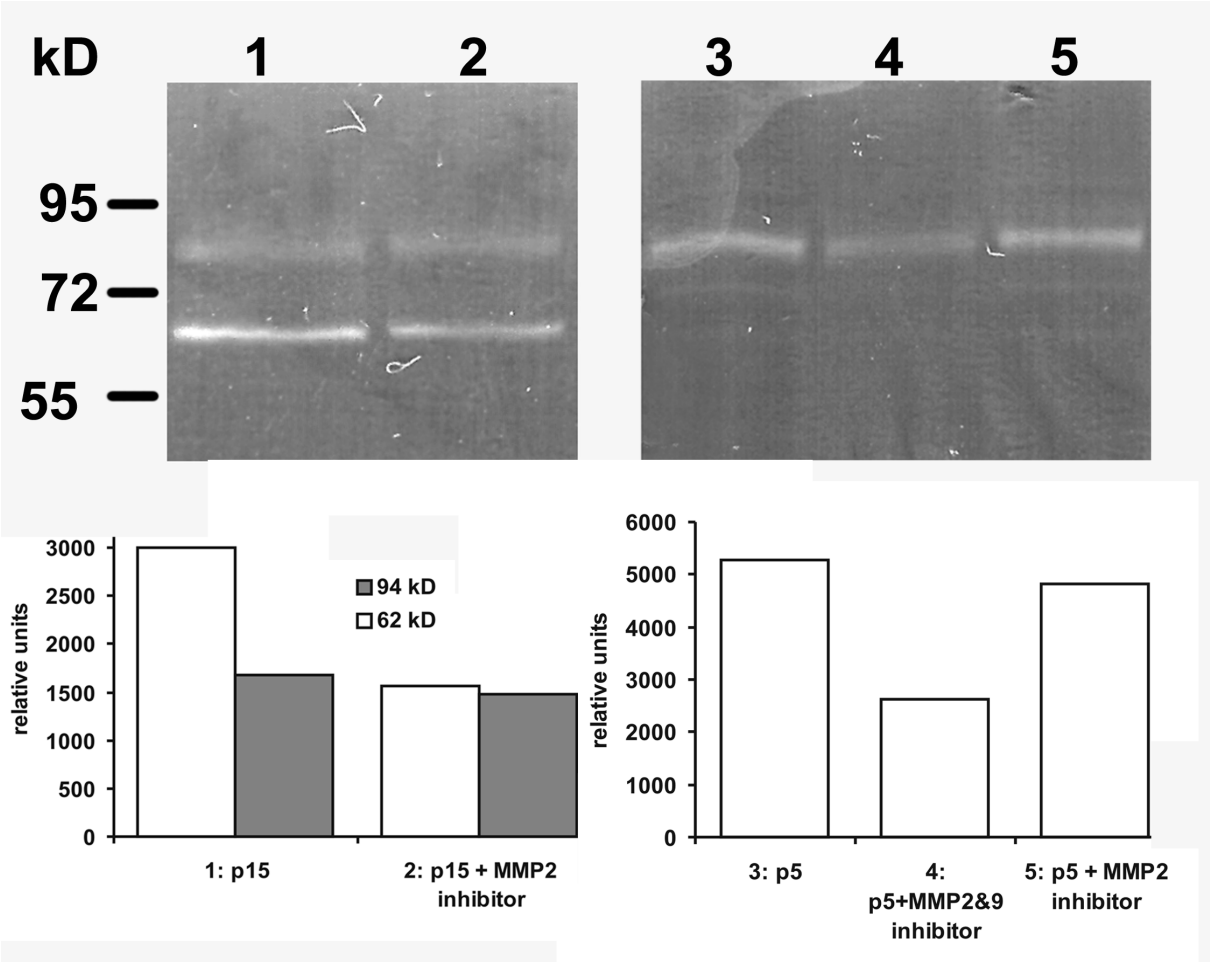
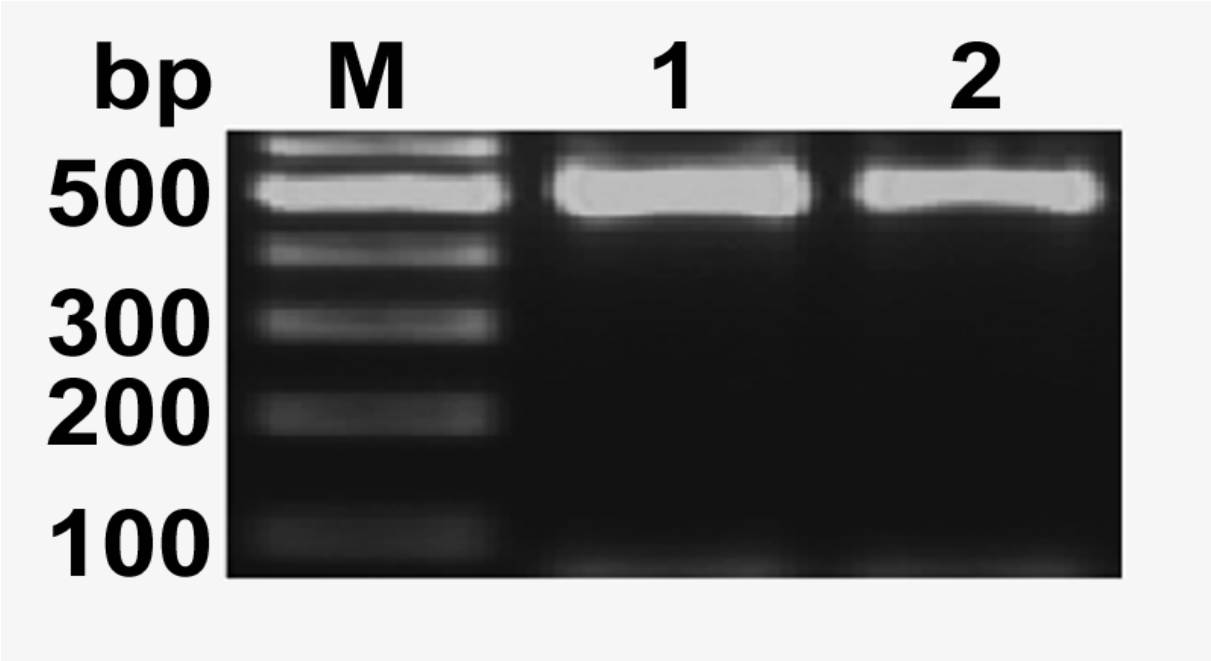


Figure 3 MMP9 mRNA transcripts in *T. lestoquardi* (Atbara) cell culture (1). The *T. annulata* infected cell line 288 (2) was used as a control. M, DNA molecular weight marker.



3.2 Quantitative Real Time-PCR for TNF- α mRNA expression

Quantification analysis of TNF-α mRNA transcripts in different passages of the *T*. *lestoquardi* (Atbara) cell line showed a higher abundance of TNF-α mRNA in passage 7 (Fig. 4). The results showed a decrease in expression of TNF-α mRNA when the passage number increased. TNF-α mRNA had a 3 fold higher expression level in passage 7 compared with passage 50 and 7 fold higher expression level compared with passage 100 (Fig. 4). Therefore the results indicate down regulation of TNF-α mRNA expression through the passaging of the *T. lestoquardi*-infected cell *in vitro* cultures.

3.3 Suppression subtractive hybridization

SSH was used to make two libraries; forward and reverse subtraction of *T. lestoquardi* (Atbara) low passage (passage 4) and high passage (passage 75). The subtraction efficiency was analyzed by comparing the abundance of G3PDH in the subtracted and unsubtracted cDNA pools. Because G3PDH is a house keeping gene, it should be subtracted out during the SSH procedure. In the subtracted sample, the G3PDH product was observed about 15 cycles later than in the unsubtracted sample. Primary random screening of the two libraries revealed 9 differentially expressed transcripts, whereby one gene from the parasite and eight genes of host origin were identified. Transcripts of retinoblastoma binding protein 7, Enolase 1 (ENO 1), Ki-67 antigen and H2A histone from the host and vacuolar H+ATPase from the parasite were more plentiful in passage 4 (Table 2). RAB14, a member of the RAS oncogene family, glucose transporter type 3, creatine kinase B, and cytochrome C Oxidase transcripts from the host were more abundant in passage 75.

Figure 4 Relative expression level of TNF-α transcripts in the *T. lestoquardi* (Atbara) cell line. The results are expressed as fold change in normalized mRNA levels relative to the calibrator.

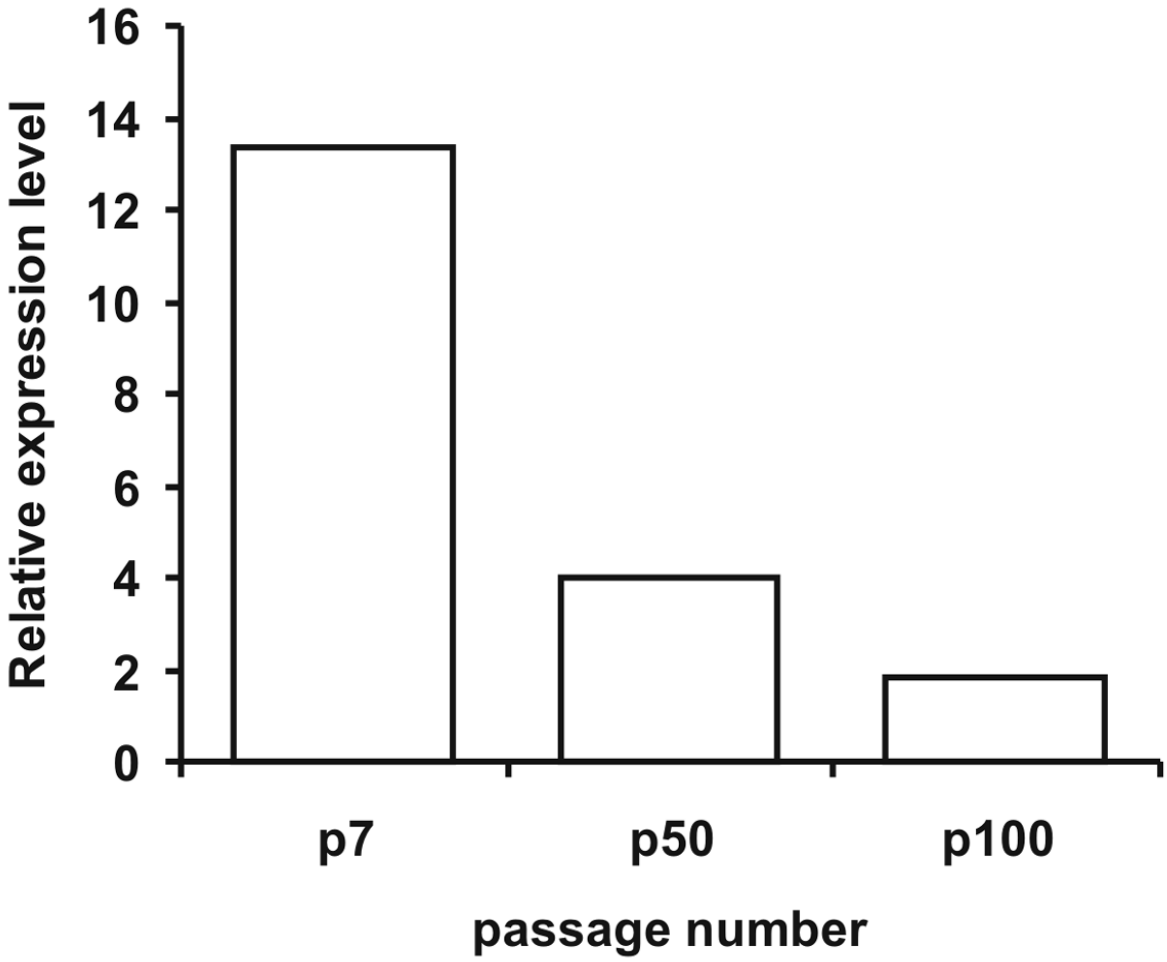


Table. 2: List of genes obtained from two libraries of suppression subtractive hybridization (SSH) with cell cultures of *T. lestoquardi* (Atbara)Passage 4 (Tl p4) and passage 75 (Tl p75).

Gene	Origin	Tl p4	Tl p75	Accession
Vacuolar H+ATPase	parasite	+		FJ201240
Retinoblastoma binding protein 7	Host	+		FJ201247
Enolase 1 (ENO 1)	Host	+		FJ201248
Antigen identified by monoclonal antibody Ki-67	Host	+		FJ201246
H2A histone family	Host	+		FJ201244
RAB14, member RAS oncogene family	Host		+	FJ201245
Glucose transporter type 3	Host		+	FJ201243
Creatine kinase B (CKB)	Host		+	FJ201242
Cytochrome C Oxidase subunit 4	Host		+	FJ201241

+ = higher levels of mRNA transcripts

3.4 Quantitative Real Time-PCR for parasite vacuolar H+ATPAse mRNA expression

The result of QRT-PCR for the parasite vacuolar H+ATPase mRNA expression levels confirmed the result of the SSH. The expression of this parasite gene was decreased around 7 fold in passage 25 compared with passage 7 and the expression remained at a low level in passages 50 and 100 (Fig 5).

3.5 Protein expression of the Ki-67 antigen and proliferation rate

As the mRNA for the proliferation associated Ki-67 antigen appeared downregulated in later passages, the expression of this protein was investigated in Western blot experiments. As depicted in Fig. 6, the amount of expressed Ki-67 395 kD antigen clearly decreased with increasing passage number. Moreover, assessment of thymidine incorporation as a measure for the proliferation rate clearly showed that with increasing passage number, the proliferation rate of the *Theileria*-infected cells decreases (Fig. 7).

Figure 5 Relative expression level of *T. lestoquardi* vacuolar H+ATPase transcripts in different passages of the *T.lestoquardi* infected cell line. The results are expressed as fold change in normalized mRNA levels relative to the calibrator.

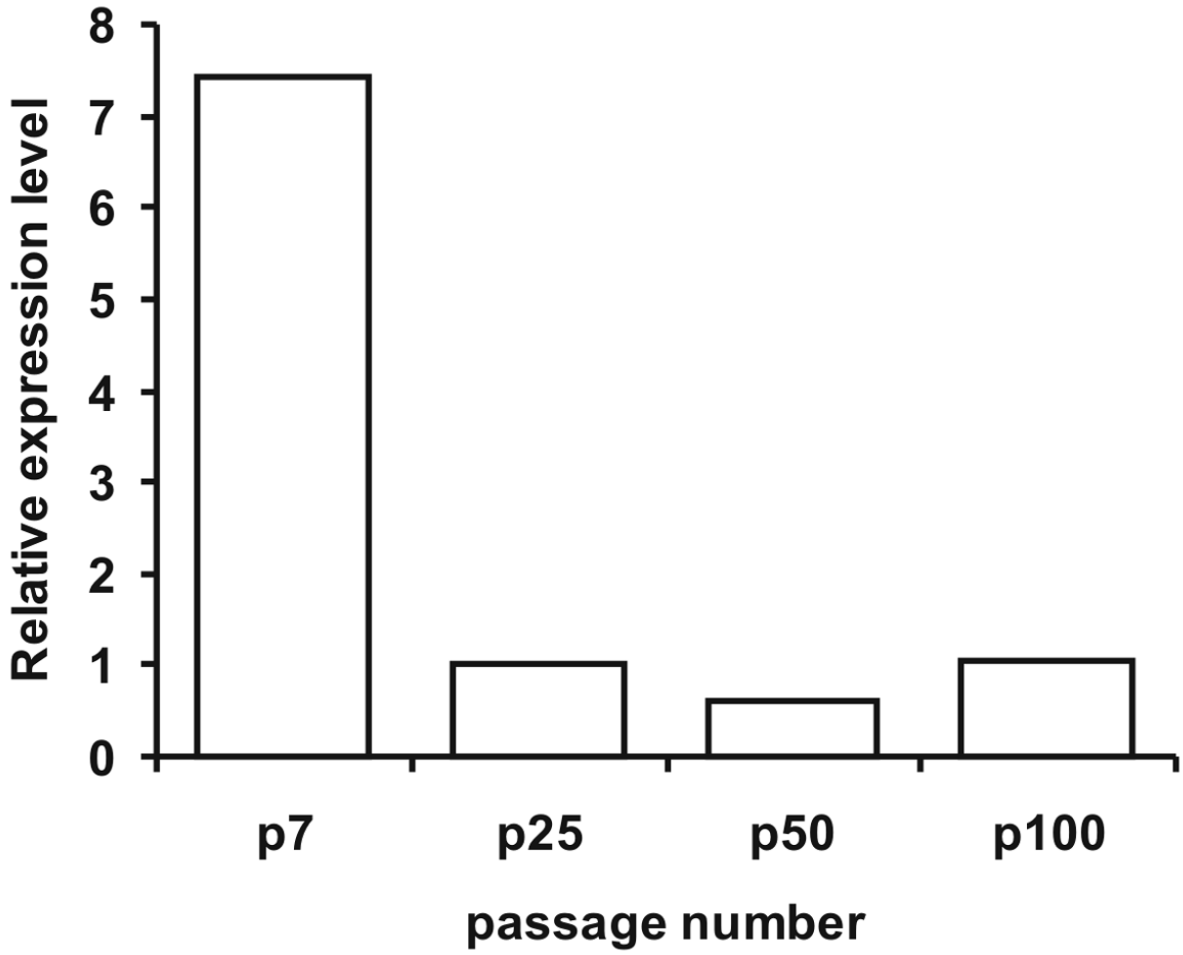


Figure 6 Protein expression of the proliferation associated Ki-67 antigen. A) Western blot for detection of Ki-67 antigen expression using the MIB-5 antibody. Lane 1) Hela cells; 2) *T. lestoquardi* infected cell line passage 7; 3) *T. lestoquardi* infected cell line passage 25; 4) *T. lestoquardi* infected cell line passage 50; 5) *T. lestoquardi* infected cell line passage 75. The 345 kD and 395 kD protein bands of the Ki-67 antigen are expressed by the Hela cell line (1), whereas only the 395 kD band is expressed in the *T. lestoquardi* infected cell line. The lower panel depicts detection of alpha-tubulin as the loading control. B) Semi-quantitative densitometric evaluation of the Western blot in A.

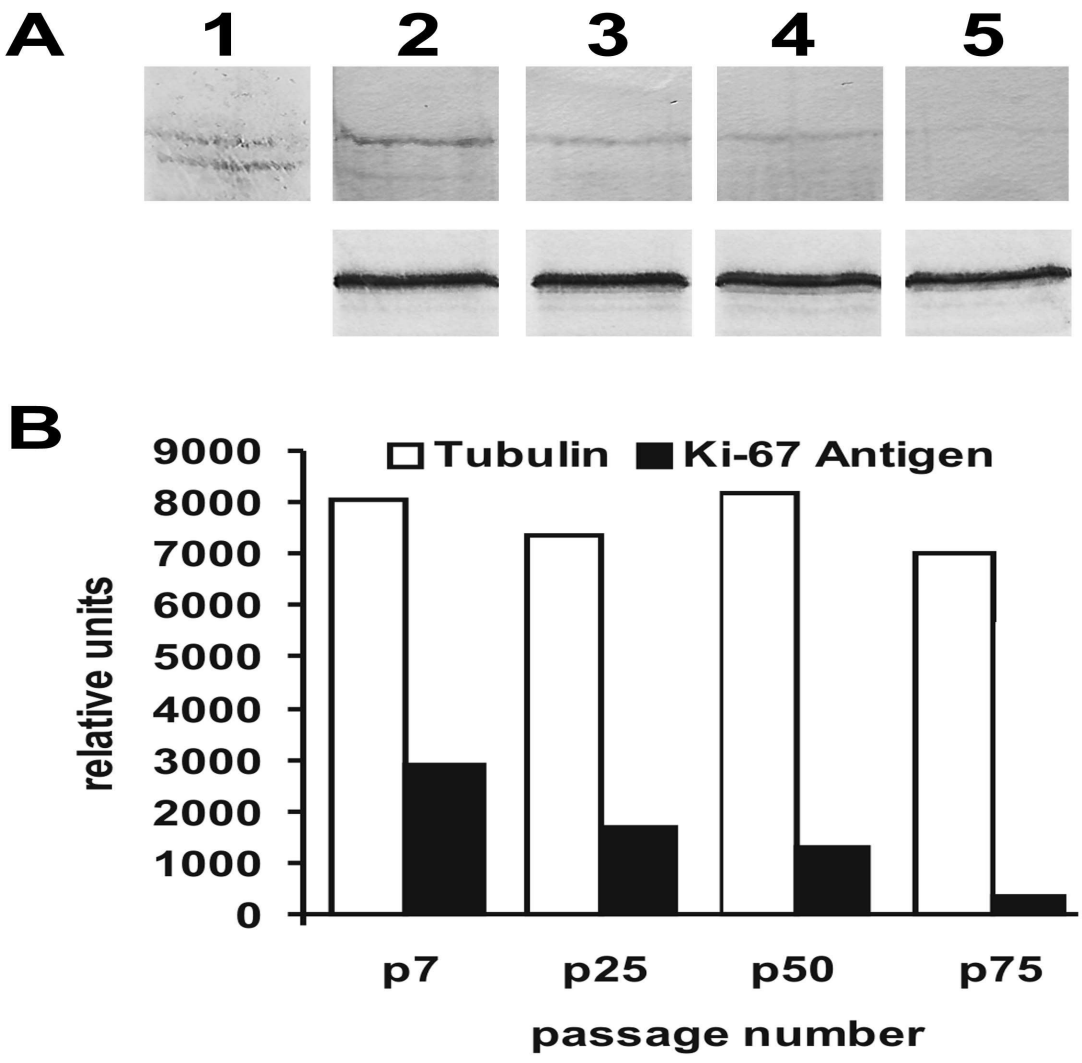
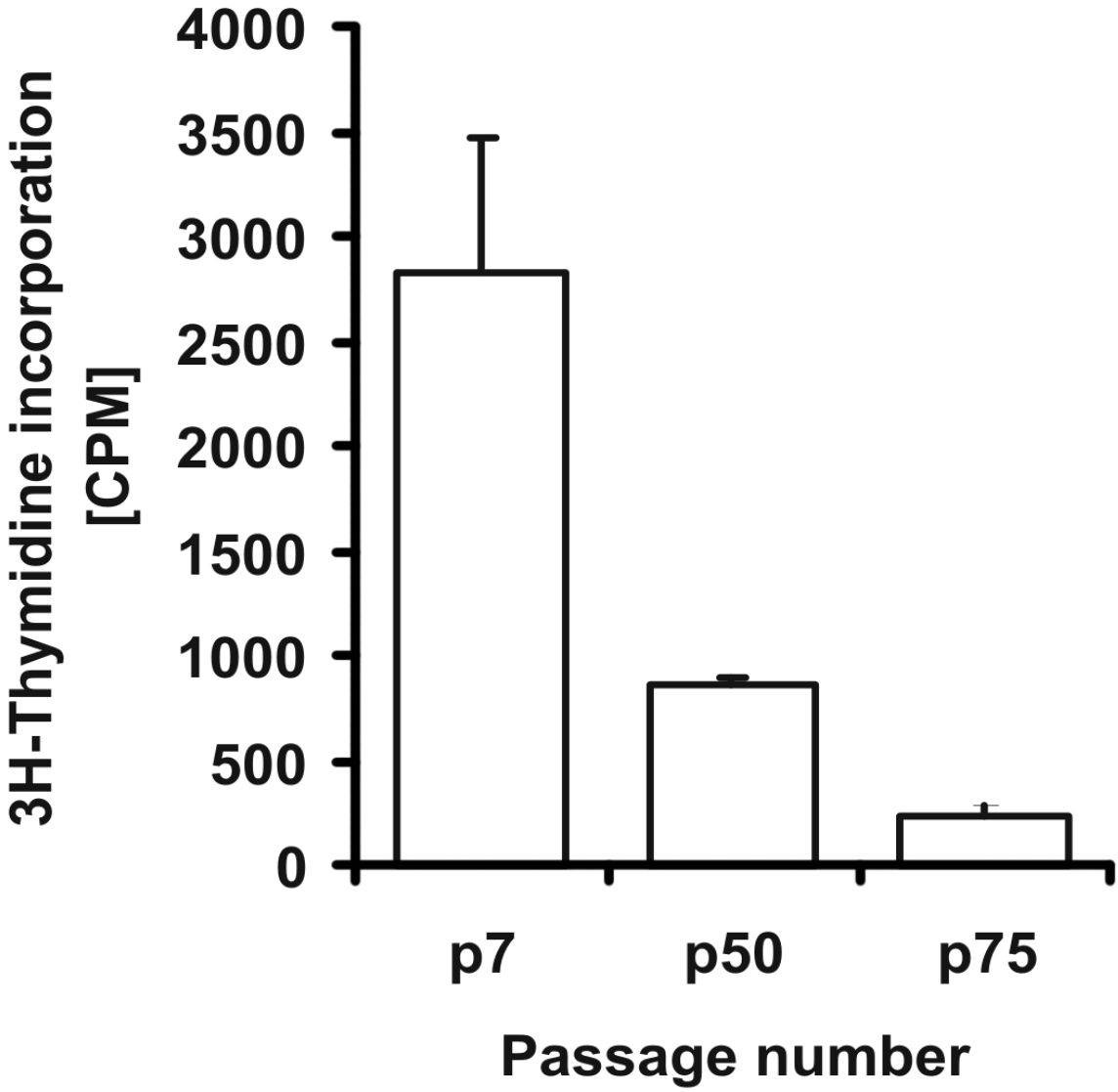


Figure 7 Thymidine incorporation of *Theileria lestoquardi* infected cells at passage 7, 50 and 75.



4 Discussion

This study was aimed at identifying the potential markers for attenuation at the molecular level in a *Theileria lestoquardi* cell line which was previously established to generate a live vaccine for immunprophylaxis against malignant theileriosis. This approach was chosen before commencing vaccination trials. Firstly to keep adverse effects of immunization on experimental animals to a minimum, and secondly, to be able to carry out vaccination trials using passage numbers the effects of which may be set in correlation to potential attenuation markers.

Regarding the investigation of known attenuation markers for *T. annulata* cell lines, this study provides details about the MMP activity in the *T. lestoquardi* (Atbara) cell line. Thus, the expression of MMP9 and MMP2 in this cell line was confirmed by using specific inhibitors and specific detection of MMP9 transcripts. The gradual reduction in the activity of MMP9 with increasing passage number was observed. These results are in agreement with previously made observations in the related parasite *T. annulata* [18, 25, 29], in which the attenuation of *T. annulata*–infected cell lines was associated with the reduction of MMP activity. Since a loss down to the absence of MMP9 activity from low passage (non-attenuated) to high passage (attenuated) in schizont infected cells was observed [20, 21], MMP9 is considered a virulence factor of *T. annulata*-infected cells. However, so far immunization trials could not entirely confirm the use of metalloproteinase activity as a marker for attenuation [24, 25], although for an Uzbek vaccine strain this was confirmed [25].

Analysis of the mRNA expression of TNF-α in different passages revealed down regulation of this cytokine from the low passage compared with high passages. The levels of pro-inflammatory cytokine mRNA in infected cells are correlated to the pathology produced by inoculation in susceptible cattle [24]. The cytokines IL -1, IL-6 and TNF-α are the most important inducers of an acute phase protein (APP) response

which relates to the disease severity [30]. Furthermore, several symptoms of tropical theileriosis can be induced by experimental administration of TNF-α [23]. Taken together, these findings suggest an important role of pro-inflammatory cytokines generally and particularly TNF-α in the pathology of theileriosis. The significant reduction in the clinical signs of theileriosis after immunization with live vaccine at a high passage (attenuated) compared with low passage (non-attenuated) is well documented [25, 31]. Therefore the down regulation of TNF-α through the passaging of the parasite might suggest TNF-α as a virulence factor.

Fundamentally the attenuated phenotype must be due to an alteration in parasite and host gene expression since the attenuated macroschizont transfers from the donor to the host leucocytes. As an approach to identify parasite or host transcripts involved in the attenuation phenotypes the suppression subtractive hybridization technique was applied on the *T. lestoquardi* (Atbara) cell line. Suppression subtractive hybridization technique is powerful and efficient for generating cDNAs highly enriched for differentially expressed genes of both high and low abundance [32]. The related technique mRNA differential display has been applied to identify alteration in mRNA profiles associated with the attenuation of a *T. annulata* vaccine cell line [31] and the technique was also applied to identify both host and parasite genes that show altered expression during differentiation of *T. annulata* from machroschizont to the merozoite stage of the life cycle [33]. In this study, suppression subtractive hybridization technique revealed some interesting genes that appear to be differentially expressed between high and low passage.

The retinoblastoma binding protein-7 (RBBP7) is potent suppressor of cell growth in transformed cell lines and inhibits tumorigenesis in nude mice [34]. While RBBP7 may act to control cell cycle progression through its association with retinoblastoma (Rb), its ability to cause growth inhibition in Rb-deficient cells suggests that it is also likely to

function in an Rb-independent manner [35]. The regulation of RBBP7 protein could be contributed to the transformation induced by *Theileria* which is entirely reversible. Therefore it is most likely that the parasite somehow regulates the gene associated with Rb without permanent change in retinoblastoma (Rb). As RBBP7 is a component of multiple chromatin remodeling complexes [36], our finding may suggest involvement of epigenetics in the transformation induced by *Theileria* parasite.

Enolase is the glycolytic enzyme that catalyzes the formation of phosphoenolpyruvate from 2-phosphoglycerate, the second of the two high energy molecules that generate ATP in glycolsis [37]. Enolase-α (ENO 1) was found to exist on the cell surface functioning as one of the plasminogen receptors [38], implying that it may play a role in cancer invasion and metastasis. Furthermore, by using an alternative translation start codon, ENO 1 transcripts can be translated into MBP1 (the Myc promoter-binding protein-1), which is localized to the nucleus and binds to the c-myc p2 promoter [39]. Upregulation of ENO 1 has been reported in several tumorigenic or metastatic cell lines [40, 41]. The upregulation of enalose-α in low passage of *Theileria*-infected cells could be contributed to invasion and metastasis and on the other hand may ensure the survival of the parasite in their host leukocyte since the activation of c-Myc was involved in mediating survival of *Theileria*–transformed cells [42].

The Ki-67 antigen is a protein which is an excellent marker for determining the so-called growth fraction of a given cell population [43]. The expression of Ki 67 antigen was detected in *Theileria*-infected cells and was related to the presence of the parasite within the cytoplasm of the host cell [44]. In this study, the up regulation of the Ki-67 antigen mRNA in the low passage compared with the high passage may indicate the difference in the proliferation rate of the parasite-infected cells between the two passages. The corresponding downregulation of Ki-67 protein expression in higher passages and the lower proliferation capacity of the high passages corroborated this

finding. A lower proliferation rate of infected cells may contribute to attenuation by allowing the host more time to induce protective responses against the schizont stage.

Vacuolar ATPase (V-ATPases), a family of ATP-dependent proton pumps, is responsible for acidification of intracellular compartments in eukaryotic cells [45, 46]. Acidification of intracellular compartments mediated by V-ATPase is essential for a variety of cellular functions, including receptor recycling, intracellular protein degradation, and regulation of membrane flow and trafficking [47-49]. They also play an important role in the entry into animal cells of many enveloped viruses [50]. The Vacuolar H+- ATPases is expressed in the plasma membrane and cytoplasmic organelles and can contribute to the metastasis potential of many tumor cells [51, 52]. The knocking down of the 16 kDa subunit of Vacuolar H+- ATPases in human hepatocellular carcinoma cell line resulted in inhibition of growth and metastasis in nude mice [53]. These findings strongly support a key role of vacuolar H+- ATPases in metastasis. The metastatic behavior of *Theileria*-infected cells was reported in the natural bovine host and immune–deficient mice [54]. Taken together these observations with the finding that a vacuolar H+- ATPase of parasit origin is expressed in a *Theileria lestoquardi* cell line could suggested that the vacuolar H+- ATPase plays an important role in the metastatic phenotype of the infected cells. Moreover, the down regulation of *Theileria* vacuolar H+- ATPases could be contributing to attenuation.

In conclusion, the metalloproteinase enzymes (9 and 2) and TNF-α could be potential molecular markers for identification of attenuation in the *Theileria lestoquardi* (Atbara) cell line. Also the down regulated parasite gene, vacuolar H+- ATPase could be considered as a molecular marker for attenuation. Immunization trials in sheep with different passages are required to provide *in vivo* evidence to support these findings.

Acknowledgments

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5 Discussion

Theileriosis, caused by *Theileria* parasites, is among the major tick-borne diseases, and economic losses are attributed in particular to those caused by the leukoproliferative *Theileria* like *T*. *parva*, *T*. *annulata* and *T*. *lestoquardi*. It is possible to attenuate *T. annulata* schizonts, a parasite closely related to *T. lestoquardi* and infective to cattle, by prolonged *in vitro* culture of infected cell lines which can be used as live vaccine (Pipano, 1981; Tait and Hall, 1990). This technique has been applied in a number of countries to establish attenuated vaccine against tropical theileriosis (Shkap et al., 2007).This vaccine is effective because the schizont establishes itself in the recipient cells, an essential requirement if it is to engender protective immunity.

The current strategy for developing attenuated cell line vaccine involves a long and tedious process, involving *in vitro* passage and testing in animals which need to be duplicated in each country. A better understanding of the properties that determine the virulence of parasitised cell lines could provide markers that would allow more rapid selection of attenuated lines. The definition of attenuation at a molecular level could speed production of cell line vaccine and reduce the cost of the dose.

The attenuation phenotype has been extensively studied in *Theileria annulata*. *Theileria annulata* is particularly virulent since, in addition to immortalizing the host cell, a range of host effector molecules, particularly proinflammatory cytokines and matrix metalloproteinases are induced. The cytokines have a range of effects, whilst the MMPs are responsible for numerous pathological outcomes. Adamson and Hall (2002) have proposed that virulence is caused by having a panoply of parasite-induced, interacting host cell virulence factors above a threshold. The antithesis of virulence, attenuation, is believed to be due to a reduction in the genotypic complexity of the parasite population through selection in *in vitro* culture, reducing the range of induced virulence factors below the threshold. An important point to stress is that the loss of parasite virulence must be due to a change in parasite gene expression. This assertion

is based on the knowledge that the schizonts in the donor vaccine cells are transferred to the leukocytes of the recipient bovine host. Therefore mRNA differential display has been applied to identify alteration in mRNA profiles associated with the attenuation of a *T. annulata* vaccine cell line (Somerville et al., 1998) and the technique was also applied to identify both host and parasite genes that show altered expression during differentiation of *T. annulata* from macroschizont to the merozoite stage of the life cycle (Oura et al., 2001). Since no work has been reported regarding attenuation mechanisms in *T. lestoquardi*, this study investigated potential attenuation markers of *T. lestoquardi* cell line at different passages. Furthermore, differentially expressed genes in higher passage and lower passage were analyzed using suppression subtractive hybridization in order to identify genes which correlate with subculturing and thus potentially with attenuation. These analyses were performed before going into vaccination trials, to prevent adverse effects in experimental animals.

Matrix metalloproteinases (MMPs) are a class of enzymes, which degrade components of the extracellular matrix (Stetler-Stevenson et al., 1993). Under normal circumstances these enzymes are under tight regulation and they have several physiological functions including a role in the extravasation of leukocytes (Reponen et al., 1992). However, elevated levels of certain MMPs, including MMP9, are associated with tumor cell metastasis (Kohn and Liotta, 1995). The expression of eight host matrix metalloproteinases activities in *Theileria* schizont-infected cells were reported (Hall et al., 1999). This study provides details about the MMP activity in a *T. lestoquardi* cell line. The expression of MMP9 and MMP2 in this cell line was confirmed by using specific inhibitors. The gradual reduction in the activity of MMP9 with increasing passage number was observed. These results are in agreement with previous observations in the related parasite *T. annulata* attenuated cell lines (Baylis et al., 1992; Adamson et al., 2000; Shkap et al., 2003). In these studies the attenuation of *T. annulata* is associated with the reduction of MMP activity. There was a loss down to

absence of the metalloproteinase 9 (MMP9) activities from low passage (non-attenuated) to high passage (attenuated) of schizont infected cells (Adamson and Hall, 1997; Adamson et al., 2000). Therefore the matrix metalloproteinases were considered as a virulence factor.

Following the expression of TNF-alpha in different passages revealed down regulation of this cytokine from the low passage comparing with the high passage. Graham et al. (2001) showed that the levels of proinflammatory cytokines expression of cloned *T*. *annulata*-infected cell lines could be used as a marker for virulence. Thus, cell lines expressing low levels of proinflammatory cytokines were non-pathogenic whilst their high-expressing counterparts induced pathology and disease. Moreover, high cytokines producing cell lines induced greater non-specific T-cell proliferation in the draining lymph nodes, which delayed the onset of a protective parasite-specific response and greatly enhanced the expansion of infected cells, and resulted in more severe pathology. The cytokines IL -1, IL-6 and TNF-alpha are the most important inducers of an acute phase protein (APP) response which relates to the disease severity (Glass et al., 2003). Furthermore, several symptoms of tropical theileriosis can be induced by experimental administration of TNF-alpha (Bielefeldet Ohman et al., 1989). Taken together, all of this evidence suggests an important role of pro-inflammatory cytokines generally and particularly TNF-alpha in the pathology of theileriosis. The significant reduction in the clinical signs of theileriosis at the high passage (attenuated) comparing with the low passage (non-attenuated) is well documented (Somerville et al., 1998; Shkap et al., 2003). Therefore our results of down regulation of TNF-alpha through the passaging of the parasite could be accepted and could suggest TNF-alpha as a virulence factor also in *T. lestoquardi* infected cells.

As an approach to identify parasite or host transcripts involved in the attenuation phenotypes suppression subtractive hybridization technique on the *T. lestoquardi* cell line was applied. Suppression subtractive hybridization technique is powerful and

efficient for generating cDNAs highly enriched for differentially expressed genes of both high and low abundance (Diatchenko et al., 1996). In this study, suppression subtractive hybridization technique revealed some interesting genes that appear to be differentially expressed between high and low passage.

Interestingly, suppression subtractive hybridization technique revealed vacuolar H+-ATPase of parasite origin is downregulated. Upregulation or overexpression of vacuolar H+- ATPases is frequently observed in several types of solid tumours and causative acidic microenvironment offers an advantage in tumour progression and metastatic behaviour, therefore this gene contributes to the metastasis potential of many tumour cells (Sennoune et al., 2004; Martin-Zaguilan et al., 1993). Moreover It has been shown that inhibition of vacuolar H+- ATPases by knockdown of the subunit ATP6L of vacuolar H+- ATPases using small interfering RNA (siRNA) significantly decreased the growth and metastasis of human hepatocellular carcinoma cell line in nude mice (Lu et al., 2005). Since the metastatic behaviour of *Theileria*-infected cells was reported in the natural bovine host and immune-deficient mice (Fell et al., 1990) these findings support a possible key role of parasite vacuolar H+- ATPases in the metastasis induced by *Theileria* parasite. Therefore the down regulation of *Theileria* vacoular H+- ATPases could be contributing to the attenuation. Further work is required to establish a direct/indirect link between the expressions of MMPs with this parasite gene. Recently it was reported that vacuolar H+- ATPases inhibitors induce the dysfunction of anti-apoptotic Bc1-2/Bcl-xL and this finding demonstrates the potential use of vacoular H+- ATPases inhibitors as anticancer drugs for Bc1-2/Bcl-xL over expressing malignancies (Sasazawa et al., 2009).

Theileria parasites induce transformation and proliferation of infected cells as evident by the staining for Ki-67 in infected cells in the lymph node draining the tick attachment site of a cow infected with *T.annulata* (Campbell et al., 1995). The expression of Ki-67 was detected in *Theileria*-infected cells and was related to the presence of the parasite

within the cytoplasm of the host cell (Shayan et al., 1999). Suppression subtractive hybridization technique in this study showed the down regulation of the Ki-67 antigen mRNA in the high passage compared with the low passage. This result may indicate the difference in the capacity of the proliferation rate of the parasite-infected cells between the two passages. It was reported that the parasite infected cells induce surrounding T-cells to proliferate (Campbell et al., 1995) and the expansion of the T-cell may be particularly important to the pathogenesis in tropical theileriosis (Brown et al., 1995). The proliferation of both infected cells and T-cells are responsible for the draining lymph node enlargement which is a feature of *T. annulata* infection (Campbell et al., 1995). Taken together with results of down regulation of Ki-67 protein expression in higher passages which mean lower proliferation capacity of the high passages may contribute to attenuation by inducing moderate pathogenesis allowing the host more time to induce protective responses against the schizont stage.

The results of suppression subtractive hybridization technique also showed a regulation of some interesting genes such as the retinoblastoma binding protein-7 (RBBP7) and Enolase-α (ENO 1). The regulation of RBBP7 protein could be involved in the transformation induced by *Theileria* which is strictly dependent on the presence of the parasite and is reversible by treatment. The regulation of RBBP7 may suggest involvement of epigenetics as a mechanism of transformation induced by *Theileria* parasite since RBBP7 is a component of chromatin remodeling complexes (Yang et al., 2002). The up regulation of Enalose-α in low passage of *Theileria*-infected cells could be contributed to invasion and metastasis. In addition to that, Enolase-α may be ensuring the survival of the parasite in the host lymphocyte by using an alternative transcript translated into MBP1 (the Myc promoter-binding protein-1) and it has been shown that the activation of c-Myc was involved in survival of *Theileria*-transformed lymphocytes (Dessauge et al., 2005).

6. Summary

Theileria lestoquardi is a tick-borne protozoan parasite and highly pathogenic for sheep. The disease caused by the pathogen is known as malignant ovine theileriosis (MOT) and is transmitted by *Hyalomma* ticks. Control of the disease can be achieved by immunization of sheep with attenuated *T. lestoquardi* schizont-infected ovine cells that provides the animal with solid immunity. The approach of using the attenuated vaccine against malignant ovine theileriosis has been carried out successfully in Iraq and Iran. Better characterization of attenuated cell lines could result in the identification of markers that would allow more rapid selection of attenuated vaccine and reduce the cost of vaccine production.

Since no work has been reported regarding attenuation mechanisms in *T. lestoquardi*, the following study investigated potential attenuation markers of *T. annulata* infected cells in a *T. lestoquardi* cell line at different passages. Two markers associated with attenuation in *T. annulata* vaccine strains were analyzed, matrix metalloproteinase activity and TNF-alpha mRNA expression. Furthermore, differentially expressed genes in higher passage and lower passage were analyzed using suppression subtractive hybridization in order to identify genes whose expression correlates with subculturing and thus potentially with attenuation.

The expression of matrix metalloproteinase-9 (MMP9) and matrix metalloproteinase-2 (MMP2) in the investigated cell line was confirmed by using specific inhibitors. The results showed gradual reduction in the activity of matrix metalloproteinase-9 (MMP9) with increasing passage number. Following the mRNA expression of TNF-alpha in different passages revealed down regulation of this cytokine from the low passage compared with high passage. Analysis of randomly selected clones in the suppression subtractive hybridization libraries identified nine differentially expressed genes, one from the parasite and eight from the host. Transcripts of retinoblastoma binding protein 7, Enolase-α (ENO 1), Ki-67 antigen and H2A histone from the host and

vacuolar H+ATPase from the parasite were more plentiful in low passage culture. RAB14, a member of the RAS oncogene family, glucose transporter type 3, creatine kinase B, and cytochrome C oxidase transcripts from the host were more abundant in high passage culture. Quantitative real time-PCR confirmed mRNA expression of the parasite vacuolar H+ATPase to be downregulated at higher passages. The expression of the Ki-67 protein was clearly decreased with increasing passage number in western blot using specific antibody. Moreover, assessment of thymidine incorporation as a measure for the proliferation rate clearly showed that with increasing passage number, the proliferation rate of the *T. lestoquardi* infected cells decreases.

This study revealed that the matrix metalloproteinase enzymes (9 and 2) and TNF-alpha could be potential molecular markers for identification of attenuation in the *Theileria lestoquardi* (Atbara) cell line. Also the down regulated parasite gene, vacuolar H+- ATPase could be considered as a molecular marker for attenuation. Immunization trials in sheep with different passages are required to provide *in vivo* evidence to support these findings.

7. Zusammenfassung

Theileria lestoquardi, ein durch Zecken übertragenes Protozoon, ist ein für Schafe hochpathogener Parasit. Die Krankheit, die durch diesen Erreger ausgelöst wird, ist als maligne Schaf-Theileriose (malignant ovine theileriosis, MOT) bekannt und wird durch *Hyalomma*-Zecken übertragen. Sie kann durch Immunisierung der Schafe mit attenuierten *T. lestoquardi*-Schizonten-infizierten Schafzellen verhindert werden, wobei das Tier mit einer robusten Immunität ausgestattet wird. Diese Art der Vakzinierung gegen MOT wurde erfolgreich im Irak und Iran durchgeführt. Eine bessere Charakterisierung der Attenuierung könnte helfen, Marker zu identifizieren, die eine schnellere Auswahl einer attenuierten Vakzine und Reduktion der Kosten ihrer Herstellung ermöglichen.

Da bisher keine Arbeiten über den Attenuierungsmechanismus von *T. lestoquardi* publiziert wurden, stand die Untersuchung potentieller Attenuierungsmarker *T. annulata*-infizierter Zellen in *T. lestoquardi*-infizierten Zellen – bei unterschiedlichen Passagen – am Anfang der vorliegenden Studie. Zwei dieser mit *T. annulata* assoziierten Marker, Matrix-Metalloproteinase-Aktivität und TNF-α-mRNA-Expression, wurden analysiert. Des Weiteren wurden differenziell exprimierte Gene bei niedriger oder hoher Passagenzahl durch „Suppression subtractive hybridization“ ermittelt, um solche Gene zu identifizieren, deren Expression sich im Laufe der Subkultivierung verändert und so potentiell mit der Attenuierung korreliert.

Die Expression von Matrix-Metalloproteinase 2 (MMP2) und Matrix-Metalloproteinase 9 (MMP9) wurde in den untersuchten Zelllinien durch Verwendung spezifischer Inhibitoren bestätigt. Die Ergebnisse zeigen eine allmähliche Reduktion der MMP9-Aktivität mit ansteigender Passagendauer. Die Untersuchung der TNF-α-mRNA-Expression in Zellen, die unterschiedlich lange passagiert wurden, zeigt eine Herunterregulierung dieses Zytokins in Abhängigkeit der Kultivierungsdauer. Die Analyse zufällig aus der „Suppression subtractive hybridization“-Bibliothek

ausgewählter Klone ergab neun differenziell exprimierte Gene, von denen eines vom Parasiten stammte und acht von der Wirtszelle. Transkripte des Retinoblastoma-bindenden Proteins 7, Enolase-α (ENO1), Ki-67-Antigen und Histon H2A der Wirtszelle sowie vakuoläre H^+-ATPase des Parasiten waren in Zellen niedriger Passagenzahl höher exprimiert. RAB14, ein Mitglied der Ras-Onkogen-Familie, Glukose-Transporter Typ 3, Kreatinkinase B und Cytochrom-C-Oxidase der Wirtszelle wurden bei höherer Passagezahl verstärkt exprimiert. Durch quantitative Real-time-PCR konnte die Herunterregulierung der vakuolären H^+-ATPase des Parasiten bei höherer Passagenzahl bestätigt werden. Die mit fortdauernder Passage deutlich abnehmende Expression des Ki-67-Proteins konnte im Western Blot unter Verwendung spezifischer Antikörper nachgewiesen werden. Darüber hinaus konnte durch die Bestimmung des Thymidin-Einbaus eine abnehmende Proliferationsrate der *T. lestoquardi* infizierten Zellen höherer Passagenzahl ermittelt werden.

Im Rahmen dieser Arbeit konnte gezeigt werden, dass Matrix-Metalloproteinasen (MMP2 und MMP9) als mögliche molekulare Marker zur Identifizierung der Attenuierung in der *Theileria lestoquardi*-Zelllinie (Atbara) dienen können. Auch die vakuoläre H^+-ATPase des Parasiten besitzt Potential als ein solches Markerprotein. Immunisierungsversuche in Schafen mit Zellen unterschiedlicher Passierungszahl sind nun notwendig, um die gezeigten Ergebnisse *in vivo* zu bestätigen.

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