THEILERIOSIS IN SHEEP AND GOATS (INFECTION WITH *THEILERIA LESTOGUARDI*, *T. LUWENSHUNI* AND *T. UILENBERGI*)

SUMMARY

Description and importance of the disease: *Theileriosis affects small ruminants in tropical and subtropical regions of Europe, Africa, the Middle East, Far East and Asia. The disease causes clinical signs in domestic ruminants, such as decreased weight and milk production and increased mortality, resulting in significant economic losses. The aetiologic agent is a protozoan transmitted by ixodid ticks, belonging to the* Haemaphysalis, Hyalomma *and* Rhipicephalus *genera. Both internal factors (genetics, breed, lambing and lactation) and external factors (nutrition, other concurrent infections) influence susceptibility to infection. Although sheep and goat breeding is one of the most important economic resources in some regions of the world, parasitic protozoa are not well characterised in these species. Among* Theileria *species affecting small ruminants,* Theileria lestoquardi, T. uilenbergi *and* T. luwenshuni *are considered highly pathogenic, while other* Theileria *species are scarcely or not pathogenic.* Theileria lestoquardi *is the causative agent of malignant ovine theileriosis. The common acute form is characterised by fever, cessation of rumination, swelling of superficial lymph nodes, cardiovascular problems, diarrhoea, jaundice and haemorrhages, and mortality rates of 46–100%. Subacute and chronic forms may also occur, showing intermittent fever, inappetence, anaemia, jaundice and emaciation. Suitable approaches to reducing the risk of theileriosis include the use of chemical repellents and pesticides, habitat management, personal and environment-based preventive and control measures, genetic selection of hosts more resistant to ticks, and vaccines.*

Detection of the agent: *Laboratory diagnosis of acute cases is carried out by the examination of Giemsa-stained smears of peripheral blood or aspirated lymph nodes. However, the technique is not useful for detecting the carrier state nor does it allow species differentiation. The use of molecular biology has provided a useful contribution for* Theileria *species identification and classification in both vertebrate and invertebrate hosts, and for genetic characterisation. The small subunit ribosomal RNA (l8S rRNA) gene is the marker used for* Theileria *spp. characterisation due to its highly conserved sequence interspersed with variable regions differing among species. Several molecular methods targeting different markers have been developed, including polymerase chain reaction (PCR), real-time PCR, reverse line blot hybridisation (RLB), loop-mediated isothermal amplification of DNA (LAMP), and sequencing of the amplified amplicons, allowing not only* Theileria *species identification, but also, in the case of real-time PCR, the simultaneous detection and quantification of pathogens from hosts and tick vectors.*

Serological tests: *Different serological methods have been developed, the most relevant of which are the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay. These methods are easy to perform and assist with diagnosis; however there are problems with cross-reactions and sometimes their sensitivity is low.*

Requirements for vaccines: *A live attenuated cell culture vaccine for* T. lestoquardi (T. hirci) *has been developed through progressive attenuation of schizonts propagated in a lymphoid cell culture. It has been successfully used in the Middle East. Tick vaccines are considered useful integrated tick control methods allowing alternative strategies to reduce acaricide use.*

A. INTRODUCTION

Theileriosis in small ruminants is caused by protozoan parasites of the *Theileria* genus (phylum Apicomplexa, order Piroplasmida), in particular by the species *Theileria lestoquardi* (aetiologic agent of malignant ovine theileriosis [MOT]), *T. ovis* (agent of benign theileriosis), *T. recondita* (causing mild ovine theileriosis), *T. separata*, *T. luwenshuni* (or *Theileria* sp. China 1), *T. uilenbergi* (or *Theileria* sp. China 2), Candidatus *Theileria* sp. and *Theileria* sp. OT3 (Stuen, 2020). Among these species, *T. lestoquardi*, *T. luwenshuni* and *T. uilenbergi* are pathogenic in sheep and goats, *T. ovis*, *T. recondita* and *T. separata* are considered scarcely or not pathogenic (Torina & Caracappa, 2012), even if benign *Theileria* species infections can cause significant production losses in imported, immunocompromised or stressed animals (Stuen, 2020). Experimental infections of sheep and goats with *T. annulata*, the causative agent of tropical theileriosis in cattle, have been reported to induce mild signs with no development of piroplasms. A further indication of the close relationship between *T. annulata* and *T. lestoquardi* is the cross-immunity developed by these two pathogens in sheep (Leemans *et al.,* 1999). *Theileria uilenbergi* and *T. luwenshuni* are pathogenic ovine piroplasms described in north-western China (People's Rep. of). As these pathogens also affect cervids, the disease has been termed cervine theileriosis, and *Theileria* parasites with similar sequences but with a low pathogenicity have been detected in sheep in northern Spain, Turkey and Italy (Torina *et al.,* 2012). Reports of *Theileria luwenshuni* in clinically affected sheep have occurred in the United Kingdom (Phipps *et al.,* 2016). The morbidity rate of *T. uilenbergi* and *T. luwenshuni* infections in sheep and goats varies between 18.8% and 65%, the mortality between 17.8 % and 75.4%.

Theileria species are mainly transmitted by ixodid ticks where sexual life cycle and sporogony of these pathogens occur. When the blood meal takes place, sporozoites are transmitted through the tick's saliva to the vertebrate host where they infect nucleated blood cells and may transform into schizonts. *Theileria* can be grouped into 'schizont-transforming' and 'schizont-non-transforming' species. Transforming *Theileria* species are able to induce uncontrolled proliferation in the infected cells (leukocytes), and schizont-infected cells are often found in the circulating blood. *Theileria* parasites develop within the cytoplasm of host leukocytes where the endosomal cell membrane dissolves, hiding the parasite from antibodies. Following the lysis of the infected leukocytes, the released merozoites infect host erythrocytes (RBCs), developing into piroplasms (Lempereur *et al.,* 2017). *Theileria lestoquardi* causes malignant theileriosis in goats and sheep with indefinite proliferation of schizonts, and appears to transform mainly major histocompatibility complex class II-positive cells. The subsequent switch in cytokine production may induce fever, anaemia, muscle wasting and necrosis. Innate immunity takes part in the initial response against transforming *Theileria* species, with the involvement of natural killer (NK) cells and several plasma proteins, such as the proteins of the complement system. In non-transforming *Theileria* species a further multiplication of the piroplasms (merogony) occurs in the RBCs. Non-transforming *Theileria* species are usually considered benign even if they are able to induce anaemia following the piroplasm stage.

Theileria lestoquardi infects the host's monocytes/macrophages and B cells, and recovered animals are resistant to further infections. Indigenous sheep and goats may acquire immunity at an early age. The main clinical signs of MOT include: generalised enlargement of the superficial lymph nodes, high fever, listlessness, anorexia, emaciation, intermittent diarrhoea or constipation and loss of condition (Leemans *et al.,* 1999). Anaemia due to erythrocyte destruction has been reported in infected sheep as well as a marked fall in white blood cells resulting in leukopaenia, a fall in blood packed cell volume and haemoglobin. Serious cardiovascular problems in sheep have been reported following *T. lestoquardi* infection. Sheep are indeed a very receptive host for *T. lestoquardi*, with infections usually ranging from subacute to acute theileriosis even in indigenous sheep. Goats are more resistant to the infection than sheep despite the natural resistance or tolerance shown by indigenous sheep in *T. lestoquardi* endemic areas. These differences may be related to the parasite epidemiology, the seasonal activity of vector ticks, the severity of host infestation with ticks, behavioral differences between sheep and goats, as well as the small ruminant population in the investigated region (Inci *et al.,* 2010). Analysis of risk factors reported a higher prevalence of *T. lestoquardi* in older sheep, with animals older than 2 years of age showing the highest prevalence of infection. Acute *T. lestoquardi* infection has a morbidity of 30–40% and mortality of 80–100%, with the outcome of the disease influenced by the infection dose (Stuen, 2020). Transplacental transmission has been reported in both sheep and goats.

Fever is usually the first clinical sign of *T. uilenbergi* and *T. luwenshuni* infections, with body temperature reaching 41.0–42.3°C and a continual or intermittent fever persisting for 12–20 days. Other late signs include: inappetence, cessation of rumination, rapid heartbeat, dyspnoea, weakness, listlessness and swelling of the superficial lymph nodes. Marked anaemia and icterus arise in a few days. A single erythrocyte can contain 1–7 piroplasms, which may appear round, oval or pyriform. In sheep, the parasitemia ranges from 3.2% to 3.7%. *Theileria uilenberg* and *T. luwenshuni* merozoites, in addition to schizonts, are involved in the pathogenesis. Some studies reported a higher incidence as well as a higher death rate in young animals than in adults. Incidence and lethality of 28.3% and 75.3%, respectively, have been reported with most cases involving lambs and 1–2 year-old animals.

Theileria species infecting small ruminants are transmitted by ixodid ticks, with different tick genera acting as vectors of the same *Theileria* pathogen in different geographical regions. However, while for some tick species the vector role has been confirmed, for others only pathogen detection in the arthropod has been reported, with no confirmation of the carrier's role. A transstadial mode of transmission has been observed, while no reports of transovarial transmission have been reported. Tick species involved in *T. lestoquardi* transmission are *Hyalomma anatolicum anatolicum, H. detritum, H. impeltatum, H. excavatum, Rhipicephalus turanicus, Rh. sanguineus*. *Theileria ovis* was detected in *Rh. bursa, Rh. sanguineus, Rh. evertsi evertsi, H. impeltatum, H. anatolicum anatolicum*, and *H. marginatum*. *Theileria luwenshuni* and *T. uilenbergi* have been detected in *H. qinghaiensis* and *H. longicornis*. Both field nymphs and adults are able to efficiently transmit the pathogens. *Theileria recondita* is transmitted by *Haemaphysalis punctata*, while *Theileria* sp. OT3 was detected in unfed *Hyalomma detritum* and *Haemaphysalis punctata* (Aktas *et al.,* 2006; Kumar *et al.,* 2020; Li *et al.,* 2009).

A relationship among susceptibility to the infection, internal characteristics (genetics, breed, lactation, parturition) and external determinants (nutrition, concomitant infections) has been reported (Stuen, 2020). Some of the main factors influencing the infection process include host range and number, midgut infection and escape barrier, innate immunity, pathogen characteristics, interplay between tick microbiome and pathogen, cross-immunity interference and abiotic factors. Investigation of molecular drivers affecting vector competence may lead to the identification of novel antigens derived from ticks or pathogens useful for implementing novel control and prevention strategies for tick-borne diseases (de la Fuente *et al.,* 2017)

Chemotherapeutic agents such as parvaquone, buparvaquone and imidocarb have been used for theileriosis treatment. Buparvaquone is effective in clearing *Theileria* parasites from sheep and goats, but it does not completely allow pathogen eradication from the host, leading to the establishment of a carrier status. Acaricides, which reduce tick infestations, have been used to control ovine theileriosis. Vaccines however, remain the most effective approach to the prevention and control of tick borne diseases (TBDs) due to the establishment of tick resistance to acaricides, the short-lasting effect of these chemicals and safety issues. The use of vaccines against ticks as components of integrated tick control methods would reduce the use of acaricides. Among tick vaccines, the ones based on the subolesin antigen act by decreasing both tick infestation and pathogen infection/transmission, thus reducing both the tick population and their vector capacity (Torina *et al.,* 2014). Anti-subolesin antibodies elicited by the vaccine could enter into tick cells and interact with cytosolic subolesin, preventing its translocation to the nucleus and thus its action of regulatory protein. Vaccines based on attenuated schizont-infected cell lines has been widely used for *T. lestoquardi* (Ahmed *et al.,* 2013) and *T. lestoquardi* sporozoite proteins are under evaluation to be included in sub-unit vaccine trials.

B. DIAGNOSTIC TECHNIQUES

Diagnosis of acute cases, in correlation with clinical data, is based on the examination of smears of peripheral blood or aspirated lymph node stained with Giemsa, which allows the detection of schizonts in white blood cells or piroplasms in erythrocytes.

Table 1. Test methods available for the diagnosis of theileriosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Microscopic examination	–	–	–	+++	–	–
PCR	+	++	+++	+++	+	–
Nested PCR	+	+++	+++	+++	+	–
RLB	+	+++	+++	+++	+	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
IFAT	+	++[b]	++	–	++	++
ELISA	+++	++[b]	++	–	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; RLB = reverse line blot hybridisation;
IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
[a]A combination of agent detection methods applied on the same clinical sample is recommended.
[b]For the purpose of animal movement, serology should not be used alone but always combined with an agent detection test.

The identification of these pathogens by microscopy is difficult, particularly when mixed infections occur. Moreover, expertise in microscopic detection of piroplasm is required for subclinical or chronic infections as parasitaemia is often extremely low and may otherwise be missed. Indirect immunofluorescence and enzyme-linked immunosorbent assays (ELISA) are commonly used serological tests for the detection of specific antibodies as they are easy to perform and useful in diagnosis, but there are problems with cross-reactivity. Techniques based on molecular biology have been developed, such as the polymerase chain reaction (PCR), PCR-RFLP (restriction fragment length polymorphism) methods, nested-PCR, real-time PCR, reverse line blot hybridisation (RLB), loop-mediated isothermal amplification of DNA (LAMP), pan-FRET (fluorescence resonance energy transfer) based assays and the sequencing of several genes, allowing the identification at the species level and the phylogenetic analysis of the pathogens.

1. Detection of the agent

1.1. Microscopic examination

Microscopic examination of thick and thin blood films is the traditional method for identifying the agent. If it is possible to make fresh films from capillary blood, or from venous blood with anticoagulant such as lithium heparin or ethylene diamine tetra-acetic acid (EDTA); samples can be kept at 4°C, until delivery to the laboratory. Macroschizonts and microschizonts cells may be found in impression smears from liver, spleen, lung, kidney, and lymph nodes. Lymph node smears are particularly useful for demonstrating schizonts.

Thin blood smears are air-dried, fixed with methanol for 10–60 seconds, stained with 10% Giemsa for 15–30 minutes, and examined at ×800–1000 magnification under oil immersion. Other stains, such as Romanowsky or Wright's, can also be used.

In erythrocytes, *Theileria* merozoites are predominantly rod shaped, up to 2.0 μm long and 1.0 μm wide. Round, oval, and ringshaped forms also occur. Multiple parasites per erythrocyte are common. In the cytoplasm of lymphocytes, two types of schizonts (Koch's blue bodies) can be found: macroschizonts and microschizonts, both about 8.0 μm, containing up to 8 and 36 small nuclei, respectively (Lempereur *et al.,* 2017). This technique is good for detection of acute infections, but not for detection of carriers, where the parasitaemia is usually very low. Parasite identification and differentiation can be improved by using a fluorescent dye, such as acridine orange, instead of Giemsa. However, negative results from microscopic examination of blood films do not exclude latent infection. Sensitivity may be improved by using 'thick' blood smears, placing a small droplet of blood (~50 μl) on to a clean glass slide and spreading this over a small area using a circular motion with the corner of another slide. This droplet is air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa. RBCs are lysed and parasites concentrated, however, identification of the parasite outside the host cell and in the presence of the many artefacts produced by this method is challenging.

Theileria species can be detected in whole tick salivary glands (SG) using the Feulgen technique. Unfed or 4–5 day engorged ticks are embedded in a small Petri dish in a 1 cm-diameter circle of melted paraffin,

with the tick's dorsal surface facing up. To lift the scutum, an incision is made with a scalpel blade around the margin of the body, starting and ending at the base of the capitulum. This facilitates removal of the gut and exposure of the SG. Trachea fragments are removed, and the whole SG are immersed in physiological saline solution. SG are then fixed for 15–30 minutes in Carnoy's fluid followed by dehydration overnight in absolute ethanol. Using a small brush, samples are washed in a small Petri dish, stained for 2 hours with Feulgen's reagent, washed again, dehydrated, and cleared with xylol. Infected acini that appear as Feulgen-positive bodies (DNA red-purple and cytoplasm green) can be quantified by immersion in xylol or methyl salicylate in a Petri dish or after slide mounting in Canada balm or DPX using a stereomicroscope at magnifications of ×500 or higher (Lempereur *et al.,* 2017).

1.2. Molecular methods

Molecular biology is very useful for the identification and classification of *Theileria* species in vertebrate and invertebrate hosts; these techniques are usually more sensitive than microscopy. Moreover, molecular techniques may allow pathogen identification to the genus or species level or may be used for phylogenetic analysis.

Despite advances in molecular-based methodologies for the diagnosis of theileriosis, the use of blood smears is often maintained due to the higher costs associated with molecular techniques. An inexpensive method for extracting *T. orientalis* DNA from bovine blood has been developed to reduce the cost of DNA extraction in molecular assays (Bogema *et al.,* 2015). The method is based on mild hypotonic erythrocyte lysis, followed by centrifugation to remove contaminating PCR inhibitors, and by a detergent-proteinase K treatment (DPK method). 100 µl of blood sample is mixed with 900 µl TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). After centrifugation for 10 seconds at 16,000 g, the supernatant is discarded, the pellet resuspended in 1 ml TE buffer and centrifuged again for 10 seconds at 16,000 g. This step is repeated one additional time. Each pellet is resuspended in 200 µl DPK digest reagent (50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 100 g/ml proteinase K and 0.5% Tween 20) and incubated for 30 minutes at 60°C followed by 10 minutes at 100°C.

Many molecular targets and assays for the detection of ovine and caprine *Theileria* species are the same as those used for cattle *Theileria* agents. The main molecular diagnostic methods specifically developed for detection and identification of small ruminant *Theileria* species are reported below.

The most commonly used marker in the characterisation of *Theileria* spp. is the small subunit ribosomal RNA (18S rRNA) gene (Liu *et al.,* 2010; Sparagano *et al.,* 2006). This gene is highly conserved among the organisms, but it also includes some variable regions differing among species. Primers can therefore be designed in the conserved regions allowing the amplification of gene fragments from all the related species, while species-specific probes can be designed that are complementary to the variable regions to differentiate the species (Mans *et al.,* 2015)

Other genetic markers used in the characterisation and phylogeny of *Theileria* species are: 28S rRNA, 5.8S rRNA and S5 rRNA genes (Mans *et al.,* 2015; Mohammadi *et al.,* 2017), internal transcribed spacers (ITS) (Aktas *et al.,* 2007), micro- and minisatellite markers, and 30 kD merozoite surface protein (Kirvar *et al.,* 1998).

Different techniques have been developed, such as classical PCR (Altay *et al.,* 2008; Kirvar *et al.,* 1998; Mohammadi *et al.,* 2017; Nagore *et al.,* 2004; Yin *et al.,* 2008), multiplex PCR (Zhang *et al.,* 2014), semi-nested PCR (Mohammadi *et al.,* 2017), nested PCR (Altay *et al.,* 2005;), PCR-RFLP (Heidarpour Bami *et al.,* 2009), LAMP (Salih *et al.,* 2012), pan-*Theileria* real-time FRET-PCR (Yang *et al.,* 2014), a multiplex DNA bead-based suspension array (Ros-Garcia *et al.,* 2013) and RLB (Gubbels *et al.,* 1999; Nagore *et al.,* 2004; Schnittger *el al.,* 2004).

Biomolecular methods have partially solved the problems of pathogen detection. *Theileria* species may remain in a latent state in infected animals, manifesting a very low parasitic charge, which is sometimes not enough to stimulate the immune system. Therefore, this latent state cannot be detected even with serological tests unless the animals are in endemic areas and thus subject to continual antigenic stimulation for reinfection by the vector ticks. Identification of carrier animals is very important for animal movement from endemic to free territories.

Another problem for tick-borne diseases is the possibility of co-infections caused by pathogens belonging to the same genus. To overcome this problem, researchers aimed to develop very sensitive and highly specific assays, even if they are sometimes complex and require specialised laboratories. One of these methods is the RLB, based on the hybridisation of PCR products to specific probes immobilised into a nitrocellulose membrane. For detection of *Theileria* spp. DNA, the hypervariable V4 region of the 18S rRNA gene is amplified by PCR (Nagore *et al.,* 2004) using the forward RLB-F (5'-GAC-ACA-GGG-AGG-TAG-TGA-CAA-G-3') and reverse RLB-R (biotin-5'-CTA-AGA-ATT-TCA-CCT-CTG-ACA-GT-3') primers (Georges *et al.,* 2001; Nagore *et al.,* 2004). Some protocol variants include an initial nested PCR to increase the sensitivity of the test (Schnittger *et al.,* 2004). PCR reactions can be performed in a 50 or 100 µl volume, including 1×PCR buffer, 1.5 mM MgCl$_2$, 200 mM each deoxynucleoside triphosphate, 1.125 or 2.5 U of Taq polymerase, respectively, 100 pmol of each primer, and 50–100 ng of purified DNA sample. To minimise nonspecific amplification, a touchdown PCR programme can be used. This protocol includes 3 minutes at 94°C, two cycles of 20 seconds at 94°C, 30 seconds at 67°C, and 30 seconds at 72°C, and then two cycles with conditions identical to the previous cycles but with an annealing temperature of 65°C. During subsequent two cycle sets, the annealing temperature is lowered by 2°C until it reaches 57°C. Then, an additional 40 cycles each consisting of 20 seconds at 94°C, 30 seconds at 57°C, and 20 seconds at 72°C are performed. The PCR ends with an extra incubation for 7 minutes at 72°C.

PCR products are used for hybridisation with specific oligonucleotide probes containing an N-terminal N-(trifluoracetamidohexyl-cyanoethyl,N,N-diisopropyl phosphoramidite [TFA])-C6 amino linker for nitrocellulose membrane binding. Designed *Theileria* spp. oligonucleotides are given in the table below.

Probe specificity	18S probe sequence (5' → 3')	Reference
Theileria/Babesia catch-all	TAA-TGG-TTA-ATA-GGA-(A/G)C(A/G)-GTT-G	Gubbels *et al.,* 1999
Theileria spp.	TGA-TGG-GAA-TTT-AAA-CC(CT)-CTT-CCA	Nagore *et al.,* 2004
Theileria ovis	TTT-TGC-TCC-TTT-ACG-AGT-CTT-TGC	Nagore *et al.,* 2004
Theileria lestoguardi	ATT-GCT-TGT-GTC-CCT-CCG	Schnittger *et al.,* 2004
Theileria uilenbergi	TGC-ATT-TTC-CGA-GTG-TTA-CT	Schnittger *et al.,* 2004
Theileria uilenbergi	TGC-ATT-TTC-CGA-GTG-TTA-CT	Niu *et al.,* 2009
Theileria luwenshuni	ATC-TTC-TTT-TTG-ATG-AGT-TG	Niu *et al.,* 2009
Theileria luwenshuni	TCG-GAT-GAT-ACT-TGT-ATT-ATC	Schnittger *et al.,* 2004
Theileria annulata	CCT-CTG-GGG-TCT-GTG-CA	Gubbels *et al.,* 1999
Theileria sp. OT1	ATC-TTC-TTT-TTG-ATG-AGT-TGG-TGT	Nagore *et al.,* 2004
Theileria sp. OT3	ATT-TTC-TCT-TTT-TAT-ATG-AGT-TTT	Nagore *et al.,* 2004
Theileria sp. MK	CAT-TGT-TTC-TTC-TCA-TGT-C	Altay *et al.,* 2008

1.2.1. Reverse line blot hybridisation

The RLB protocol has been described (Georges *et al.*, 2001; Gubbels *et al.,* 1999; Niu *et al.,* 2009) and is summarised here. Membrane preparation includes the activation of the nitrocellulose membrane by 10 minutes incubation in 10 ml of 16% (wt/vol) 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDAC) at room temperature. The membrane is then washed in distilled water and placed in a miniblotter. *Theileria*-specific oligonucleotides are diluted to 200–1600 pmol/150 ml in 500 mM NaHCO$_3$ (pH 8.4) and loaded into the miniblotter slots. An incubation step for 1 minute at room temperature is carried out to covalently link the membrane with the amino linker. After aspiration of solutions, the membrane is first inactivated by incubation in 100 ml of a 100 mM

NaOH solution for 10 minutes at room temperature, then washed in 125 ml of 2× SSPE–0.1% sodium dodecyl sulfate (SDS) for 5 minutes at 60°C.

Before hybridisation with PCR products, the membrane is first washed for 5 minutes at 42°C with 125 ml of 2× SSPE–0.1% SDS and placed perpendicular to its previous orientation into the miniblotter. 40 μl of PCR products are diluted with 2× SSPE, 0.1% SDS to a final volume of 150 μl, heated to 95°C for 5 minutes and then cooled on ice. Denatured PCR samples are applied into the slots and incubated for 60 minutes at 42°C, then aspirated and the membrane is washed at 42°C for 10 minutes in 2×·SSPE, 0.1% SDS.

The membrane is subsequently incubated for 30 minutes at 42°C in 10 ml of 1/4000 diluted peroxidase-labelled streptavidin in 2× SSPE–0.5% SDS, and washed twice in 125 ml of 2× SSPE–0.5% SDS for 10 minutes at 42°C with shaking. After two rinses in 125 ml of 2× SSPE at room temperature, the membrane is incubated for 1 minute in 10 ml of ECL detection solution followed by exposure to a chemo-luminescent detection film. The film is then developed in an X-ray film developer and fixed. A black spot in the sample–probe cross in the hyperfilm indicates a positive signal for that pathogen.

After use, all PCR products are stripped from the membrane by two washes in 1% SDS for 30 minutes each time at 80°C. The membrane is rinsed in 20 mM EDTA (pH 8.0) and stored in fresh EDTA solution at 4°C; it can be reused about 20 times (Schnittger *et al.,* 2004).

The RLB finds wide application for its high sensitivity and the ability to identify co-infections. Exceptions may occur when the sensitivity of the assay is affected by the presence of mixed infections. Sensitivity of the RLB is severely affected by the use of universal primers and these are depleted by the predominant species present, thereby suppressing the signal from less abundant templates (Mans *et al.,* 2015).

1.2.2. Polymerase chain reaction

In cases of mixed infections, species-specific PCRs can be useful in investigations of field samples. Some of the most used PCRs for detection of *Theileria* pathogens in small ruminants are reported below.

A PCR and a nested PCR targeting the *ssu rRNA* of *T.ovis* have been developed (Aktas *et al.,* 2006). The first method (Aktas *et al.,* 2006) is based on the amplification of a 520 bp fragment of the *ssu rRNA* gene. Amplification conditions consist in a 50 μl final volume containing: 5 μl of 10× PCR buffer (100 mM Tris–HCl [pH 9], 500 mM KCl, 1% Triton X-100), 250 μM of each of the four deoxynucleotide triphosphates, 2U Taq DNA polymerase, 10 pg of each primer, 5 μl of DNA. The thermal profile includes an initial denaturation at 96°C for 3 minutes, 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes and final extension of 72°C for 10 minutes. The second method (Altay *et al.,* 2005) add a second amplification step using inner primers to the previously described steps, amplifying a 398 bp smaller fragment. The nested PCR is carried out in a total reaction volume of 50 μl containing 5 μl of 10× PCR buffer (100 mM Tris–HCl [pH 9], 500 mM KCl, 1% Triton X-100), 250 μM of each of the four deoxynucleotide triphosphates, 2 U Taq DNA polymerase, and 10 pg of each primer. In the first round of amplification, 5 μl of the DNA suspension is added as template, while for the nested PCR, 5 μl of a 1:20 dilution of the primary product is used. The amplification protocol includes an initial denaturation step at 96°C for 3 minutes, followed by 30 cycles at 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 2 minutes and a final extension of 72°C for 10 minutes.

For detection of *T. lestoquardi*, a specific PCR has been developed (Kirvar *et al.,* 1998), amplifying a fragment of 785 bp of the 30 kDa merozoite surface gene (*Tlms*). The amplification is carried out in a final volume of 50 μl containing: 5 μl of 10× PCR buffer (2.5 mM MgCl$_2$, 20 mM Tris-HCl pH 8.55, 16 mM [NH4]$_2$SO$_4$, 150 μg/ml bovine serum albumin), dNTP mixture 0.2 mM each, primers 0.5 μM each, 0.025 U/μl Taq polymerase and 5 μl DNA. The amplification protocol includes an initial denaturation at 94°C for 3 minutes followed by 40 cycles at 94°C denaturation, 65°C annealing and 72°C extension and a final extension step of 72°C for 5 minutes. A semi-nested PCR has been also developed for detection of *T. lestoquardi* (Mohammadi *et al.,* 2017), amplifying a 235 bp fragment of the *SSU-rRNA* gene of *T. lestoquardi*. The semi-nested PCR is carried out in

50 µl total reaction volume containing 5 µl of 10× PCR buffer, 2 mM MgCl₂, 250 µM of each of the four deoxynucleotide triphosphate, 1.25 U Taq DNA, 50 pmol of each primer, and 10 ng of first PCR product. Cycling condition for the semi-nested includes an initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 94°C for 45 seconds, 55°C for 90 seconds and 72°C for 45 seconds and a final additional extension step for 5 minutes in 72°C.

Specific PCRs for detection of *T. luwenshuni* and *T. uilenbergi* (Yin *et al.,* 2008) have been developed, based on the amplification of a 389 and a 388 bp 18S rRNA gene fragment, respectively. Two primer pairs have been designed and PCR amplification was performed in a final volume of 30 µl containing 3 µl of 10× PCR buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm MgCl₂, 0.01% gelatin), 200µm of each deoxynucleoside triphosphate, 10 µm of each primer, 100 ng of genomic DNA and 1.5 U of Taq polymerase. The cycling conditions of the PCR were 3 minutes at 94°C for denaturation followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing 1 minute at 57°C for *T. luwenshuni* specific primers or 1 minute at 55°C for *T. uilenbergi* specific primers and extension for 1 minute at 72°C. The final extension step was 7 minutes at 72°C.

A specific PCR was also developed for *Theileria* sp. MK targeting a 757 bp fragment of the *18S ssu rRNA* gene (Altay *et al.,* 2008). The PCR was performed in a total reaction volume of 50 µl containing 5 µl of 10× PCR buffer (750 mM Tris–HCl [pH 8.8], 200 mM [NH₄]2SO₄, 0.1% Tween 20), 2 mM MgCl₂, 250 µM of each of the four deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase, and 37.5 pmol of each primer and 5 µl of template DNA. The reaction was repeated for 35 cycles under the following conditions: 1 minute at 94°C, 1 minute at 57°C and 1 minute at 72°C. Interestingly, this PCR resulted as sensitive as the RLB as both tests were able to detect one infected cell out of 10^7 sheep erythrocytes.

Target gene	Primer sequences	Amplicon size (bp)	Amplification conditions	Amplification protocol	Reference
Theileria ovis					
ssu rRNA	TSsr 170F: TCG-AGA-CCT-TCG-GGT TSsr 670R: TCC-GGA-CAT-TGT-AAA-ACA-AA	520	50 µl containing: 5 µl of 10× PCR buffer (100 mM Tris–HCl [pH 9], 500 mM KCl, 1% Triton X-100), 250 mM of each dNTP, 2U Taq DNA polymerase , 10 pg of each primer, 5 µl of DNA	• Denaturation at 96°C for 3 minutes • 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes • Final extension of 72°C for 10 minutes	Aktas *et al.,* 2006
SSU rRNA	Primary PCR TSsr 170F: TCG-AGA-CCT-TCG-GGT TSsr 670R: TCC-GGA-CAT-TGT-AAA-ACA-AA Nested PCR TSsr 250FN: CGC-GTC-TTC-GGA-TG TSsr 630RN: AAA-GAC-TCG-TAA-AGG-AGC-AA	520 398	50 µl containing 5 µl of 10× PCR buffer 5100 mM Tris–HCl [pH 9], 500 mM KCl, 1% Triton X-100), 250 µM of each dNTPs, 2 U Taq DNA polymerase, and 10 pg of each primer and: • For primary PCR: 5 µl of the DNA • For nested PCR: 5 µl of a 1/20 dilution of the primary product	• Denaturation at 96°C for 3 minutes • 30 cycles at 94°C for 30 seconds, 54°C for 30 seconds and 72 °C for 2 minutes • Final extension of 72 °C for 10 minutes	Altay *et al.,* 2005
Theileria lestoquardi					
30 kDa mero-zoite surface gene (Tlms)	TlestF: GTG-CCG-CAA-GTG-AGT-CA TlestR: GGA-CTG-ATG-AGA-AGA-CGA-TGA-G	785	50 µl containing: 5 µl of 10× PCR buffer (2.5 mM MgCl₂, 20 mM Tris-HCl, pH 8.55, 16 mM [NH4]₂SO₄, 150 µg/ml bovine serum albumin), dNTP mixture 0.2 mM each, primers 0.5 µM each, 0.025 U/µl Taq polymerase and 5 µl DNA	• Denaturation at 94°C for 3 minutes • 40 cycles at 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 2 minutes • Final extension of 72°C for 5 minutes	Kirvar *et al.,* 1998

Target gene	Primer sequences	Amplicon size (bp)	Amplification conditions	Amplification protocol	Reference
SSU-rRNA	• Primary PCR Theil-F: CAC-AGG-GAG-GTA-GTG-ACA-AG Bab-R AAG-AAT-TTC-ACC-TCT-GAC-AG • Emi-nested: ATT-GCT-TGT-GTC-CCT-CCG	426-430 235	Emi-nested : 50 µl containing 1× PCR buffer, 2 mM MgCl$_2$, 250 µM of each dNTPs, 1.25 U Taq DNA polymerase, 50 pmol of each primer, and 10 ng of amplified PCR DNA	Emi-nested: • Denaturation at 95°C for 5 minutes • 35 cycles at 94°C for 45 sec, 55°C for 90 seconds and 72°C for 45 seconds. • Final extension for 5 minutes at 72°C.	Mohammadi *et al.,* 2017
Theileria luwenshuni					
18S rRNA gene	Tluw310s: GGT-AGG-GTA-TTG-GCC-TAC-TGA 374as: TCA-TCC-GGA-TAA-TAC-AAG-T	389	30 µl containing: 3 µl of 10× PCR buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm MgCl$_2$, 0.01% gelatin), 200 µM of dNTPs, 10 µM of each primer, 100 ng of genomic DNA and 1.5 U of Taq polymerase	• Denaturation at 94°C for 3 minutes • 35 cycles at 94°C for 30 seconds, 57°C for 1 minute and 72°C for 1 minute • Final extension of 72°C for 7 minutes	Yin *et al.,* 2008
Theileria uilenbergi					
18S rRNA gene	Tuil310s: GGT-AGG-GTA-TTG-GCC-TAC-CGG 689as: ACA-CTC-GGA-AAA-TGC-AAG-CA	388	30 µl containing: 3 µl of 10× PCR buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm MgCl$_2$, 0.01% gelatin), 200 µM of dNTPs, 10 µM of each primer, 100 ng of genomic DNA and 1.5 U of Taq polymerase	• Denaturation at 94°C for 3 minutes • 35 cycles at 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute • Final extension of 72°C for 7 minutes	Yin *et al.,* 2008
Theileria sp. MK					
18S ssu rRNA gene	Tmk-F: CAT-TGT-TTC-TTC-TCA-TGT-C 990: TTG-CCT-TAA-ACT-TCC-TTG	757	50 µl containing: 1× PCR buffer, 2 mM MgCl$_2$, 250 µM of each dNTPs, 1.25 U Taq DNA polymerase, 37.5 pmol of each primer and 5 µl of DNA	• Denaturation at 94°C for 3 minutes • 35 cycles at 94°C for 1 minute, at 57°C at 1 minute and at 72°C 1 minute • Final extension of 72°C for 10 minutes	Altay *et al.,* 2008

dNTPs: deoxynucleotide triphosphate

2. Serological tests

Detecting antibodies against *Theileria* using serological tests is a useful technique in epidemiological surveys. False positive and negative results due to cross-reactions or weak specific-immune responses are some of the main disadvantages of these tests.

Antibodies first appear at 15 days post-infection (d.p.i) with *T. lestoquardi* in sheep, shortly after the detection of schizonts; an increase in antibody titre of at least four times is observed when compared with samples collected before inoculation. Peak antibody levels, ranging from 1/640 to 1/1280, are reached between 30 and 40 d.p.i., indicating a 32- to 64-fold increase in antibody titres. Serum antibodies are still detectable at 90 d.p.i.

2.1. The indirect fluorescent antibody test

The indirect fluorescent antibody test (IFAT) based on *T. lestoquardi* schizont antigen has been described, but the test has several disadvantages as the subjective operator-dependent interpretation of results and the cross-reactivity of the test against other *Theileria* species. IFAT assay for *T. lestoquardi* showed significant cross-reactivity with *T. annulata* and *T. parva* anti-sera and vice versa. As schizont antigens are mainly used, such cross-reactivity is attributed to shared antigens between *T. lestoquardi* and other tick-borne pathogens.

The protocols for the preparation of schizont antigen and piroplasm antigen as well as the IFAT procedure are provided in Chapter 3.4.13 *Theileriosis in cattle* (*infection with* Theileria annulata, T. orientalis *and* T. parva), Section B.2.1. As samples from small ruminants are used, in step h) of the procedure it is necessary to use anti-sheep or anti-goat immunoglobulin preparations conjugated to fluorescein isothiocyanate. Schizont antigen are intracytoplasmic schizonts derived from *T. lestoquardi*-infected lymphocyte (mostly B cells) cell lines; piroplasm antigen of transforming *Theileria* are prepared from the blood of infected animals or, in the case of *T. uilenbergi* (non-transforming *Theileria*), from long-term culture of erythrocytic stages (Miranda *et al.,* 2006b).

2.1.1. Preparation of *Theileria uilenbergi* long-term *in-vitro* cultures

Theileria uilenbergi long-term *in vitro* cultures are based on the microaerophilous stationary phase method.

i)   A blood sample heparinised from *T. uilenbergi*-infected sheep, with a percentage of parasitised erythrocytes (PPE) of 6% and a haemocrit of 30%, and a blood sample from uninfected sheep (to obtain uninfected RBCs) are washed three times with mVYM (modified Vega and Martinez solution) by centrifugation at 850 g for 10 minutes at 4°C, the supernatant is discarded and the packed RBCs are used to initiate cultures.

ii)   Infected and uninfected RBCs are then prepared in basic culture medium consisting of commercial HL-1 medium supplemented with 20% inactivated lamb serum. Instead of serum, 1% lipid-rich bovine serum albumin supplemented with either 1% NEAA (non-essential amino acids solution) or chemically defined lipids (CDL) can be used. The basic medium is supplemented with 25 mM HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), 2 mM L-glutamine, 24 mM sodium bicarbonate, gentamicin (50 µg/ml), amphotericin B (2.5 µg/ml), 1 mM L-cysteine, 0.02 mM bathocuproinedisulfonic acid disodium salt and 200 µM hypoxanthine.

iii) The parasited and normal RBC suspensions are mixed for a PPE of 3% and a haemocrit of 10%, 0.2 ml of final volume of suspensions are dispensed in duplicate into 96-well culture plates and incubated under O_2 reduced atmosphere (gas mixture of 2% O_2, 5% CO_2 and 93% N_2) in a humidified atmosphere at 37°C.

iv)   Change medium daily removing 0.14 ml of medium from the culture well and replacing with an equal volume of fresh medium.

v)   For subcultures, remove 0.15 ml of medium from the culture well every 4–5 days and replace with 0.15 ml of a suspension of uninfected RBCs in fresh medium at a final concentration of 1/30. Gently resuspend cultures in 1:8 ratio by trasfering 0.025 ml to new wells and bring to a final volume of 0.2 ml by addition of a suspension of 10% RBCs in fresh colture medium.

2.2. Enzyme-linked immunosorbent assay

The ELISA is easy to perform, reproducible and it allows for high throughput assays. Using clones of the cDNA library followed by bioinformatics analysis, several recombinant proteins were studied to develop indirect ELISA.

Several indirect ELISAs for the detection of *T. lestoquardi* antibodies have been developed that are suitable for epidemiological studies and large-scale studies because of the objectivity of the results and the ability to process a large number of samples every day; no commercial kits are available. The ELISAs are based on crude merozoite antigen (Gao *et al.,* 2002), or partially expressed *T. lestoquardi*

recombinant heat shock protein 70 (rTlHSP 70) (Miranda *et al.,* 2006a)., or *T. lestoquardi* recombinant clone-5 schizont surface antigen (Bakheit *et al.,* 2006).

The crude antigen (merozoite lysate)-based ELISA is highly sensitive for detection of antibodies against both *T. uilenbergi* and *T. luwenshuni* (Gao *et al.,* 2002). However, it showed a cross-reaction with antibodies against *Babesia ovis* infection and has the inherent problem of obtaining large amounts of antigen based on time-intensive and expensive and sophisticated procedures. In contrast, large batches can be prepared using recombinant protein-based ELISAs making them amenable for standardisation and increasing specificity.

Recombinant protein-based ELISAs have been established for detection of small ruminant theileriosis in China. The antigens used were heat shock protein 70 from the merozoite of *Theileria* sp. China (TcHSP70) (Miranda *et al.,* 2006a), *T. uilenbergi* immunodominant protein (TuIP) (Liu *et al.,* 2010), *T. luwenshuni* recombinant surface protein (rTlSP) and clone-9 from a merozoite cDNA library of *T. uilenbergi* (Abdo et al., 2010).

The indirect ELISA based on recombinant *T. uilenbergi* immunodominant protein (TuIP) has been validated; it showed a high sensitivity and was able to detect early and persistent infection with *T. uilenbergi*. It was also the most extensively characterised regarding the absence of cross-reaction with related pathogens, and was only found to show cross-reactivity with antibodies against the closely related pathogen *T. luwenshuni* (Liu *et al.,* 2010). *Theileria uilenbergi* and *T. luwenshuni* antibodies can be detected using an ELISA based on crude antigen (merozoite lysate).

An indirect ELISA using the recombinant immunogenic pyroplasmic proteins partially expressed by the clone-9 gene for the detection of circulating antibodies in the sera of sheep infected with *T. uilenbergi*; for this test, no cross-reactivity was observed in the serum of animals infected with *T. lestoquardi*.

Theileria luwenshuni recombinant surface protein (rTlSP)-based ELISA demonstrates reactivity with *T. luwenshuni, T. uilenbergi, T. ovis, T. annulata, T. orientalis*, and *T. sinensis* positive sera, but no cross-reactivity was found in sera from *T. lestoquardi*-infected animals. This test could be potentially used for the detection of antibodies against *Theileria* in bovine and ovine sera, but it is currently validated only for cattle.

The recombinant *T. uilenbergi* immunodominant protein (rTuIP) was also used as antigen for a colloidal gold-based immunochromatographic strip (ICS) for the detection of *T. uilenbergi* or *T. luwenshuni* infections (Lu *et al.,* 2015).

C. REQUIREMENTS FOR VACCINES

No commecial vaccines are available for this disease.

For *T. lestoquardi,* an attenuated live vaccine based on inoculation of schizont-infected leukocytes has been used for the control of malignant theileriosis and successfully applied in some countries.

Lambs immunised with 3×10^6 attenuated schizonts obtained at the 63rd passage showed a mild febrile reaction and parasitological reactions. In another trial, sheep immunised with different doses of attenuated *T. lestoquardi*-infected cells at the 105th passage showed a mild reaction with fever lasting for 1–5 days and parasitaemia of <0.2% (Ahmed *et al*., 2013).

Several studies were also conducted to identify antigens for inclusion in a subunit vaccine against *T. lestoquardi* infection in sheep and goats. Sporozoite lestoquardi antigen 1(SLAG-1), p67:SPAG-1 homologue of *T. lestoquardi* and a 73-kDa protein could be identified as possible candidates showing a potential as sporozoite-neutralisation.

Some approaches have been implemented for reducing the risk of tick-borne diseases (TBDs). Vaccines using tick-derived antigens were not intended to prevent tick infestations, but rather to reduce tick populations and the prevalence of tick-borne pathogens (TBPs) by influencing the feeding, fertility, and growth of ticks feeding on immunised animals and ingesting antigen-specific antibodies that interact with and affect protein function. These vaccines should be developed with tick vector species, TBPs, and TBD-affected hosts in mind, and they can be used alone or in conjunction with other control measures. Tick-derived antigens with various functions can be used alone

or in combination with pathogen-derived antigens to provide vaccine efficacy in reducing tick infestations, TBP infection and multiplication in the tick, TBP transmission, tick attachment and feeding time, or host TBP infection (de la Fuente *et al.,* 2017).

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NB: There are WOAH Reference Laboratories for theileriosis and ovine theileriosis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratory for any further information on
diagnostic tests, reagents and vaccines for theileriosis

NB: FIRST ADOPTED IN 2022.