# THEILERIOSIS IN CATTLE (INFECTION WITH *THEILERIA ANNULATA*, *T. ORIENTALIS* AND *T. PARVA*)

## SUMMARY

*Description and importance of the disease:* Tick-transmitted Theileria parasites of cattle are a major constraint to the improvement of the livestock industry in large parts of Europe, Africa and Asia. Theileria annulata and T. parva, the most economically important species, are responsible for mortality and losses in production. Bovine theileriosis is generally controlled by the use of acaricides to kill ticks, but this method is not sustainable. Acaricides are expensive, they cause environmental damage, and over time ticks develop resistance to them requiring newer acaricides to be developed. More sustainable and reliable methods for the control of theileriosis that deploy a combination of strategic tick control and vaccination are desirable. However, these are yet to be successfully applied on a large scale in endemic areas.

*Identification of the agent:* Diagnosis of a variety of disease syndromes caused by the parasites is principally based on clinical signs, knowledge of disease and vector distribution, and identification of parasites in Giemsa-stained blood and lymph node smears. The presence of multinucleate intra-cytoplasmic and free schizonts, in lymph node biopsy smears, is a characteristic diagnostic feature of acute infections with T. parva and T. annulata. Animals infected with T. parva show enlarged lymph nodes, starting with the parotid lymph node, fever, a gradually increasing respiratory rate, dyspnoea and occasional diarrhoea. Post-mortem lesions observed are pulmonary oedema with froth in the trachea, enlargement of lymph nodes and spleen, haemorrhages in internal organs, abomasal erosions, the presence of parasitised lymphocytes and lympho-proliferative infiltrations in visceral tissues. The gross pathology caused by schizonts of T. annulata resembles that of T. parva, while the piroplasm stages may also be pathogenic, causing anaemia and jaundice. For T. annulata, the first lymph nodes involved are the pre-crural ones as a result of the predilection sites of the vector ticks.

*Molecular-based tests:* In addition, molecular diagnostic tests, particularly those based on the polymerase chain reaction and reverse line blot hybridisation are proving to be powerful tools for detecting theileria parasites in the vertebrate and invertebrate hosts, characterising species and parasite polymorphisms, defining population genetics and generating epidemiological data.

*Serological tests:* The most widely used diagnostic test for Theileria species has been the indirect fluorescent antibody (IFA) test. For the IFA test, both schizont and piroplasm antigens may be prepared on slides or in suspension and preserved by freezing at ≤ −20°C, except in the case of the piroplasm suspension, which is stored at 4°C. Test sera are diluted with phosphate-buffered saline and incubated with the antigen in suspension, and anti-bovine immunoglobulin conjugate is then added. Using the test as described, the fluorescence is specific to the causative agent. The IFA test is sensitive, fairly specific, and usually easy to perform. However, because of the problems of cross-reactivity among some Theileria species, the test has limitations for large-scale surveys in areas where species distribution overlaps. The IFA test for T. parva does not distinguish among the different immunogenic stocks. An indirect enzyme-linked immunosorbent assay for T. parva and T. mutans, based on recombinant parasite-specific antigens, has demonstrated higher sensitivity and specificity.

*Requirements for vaccines:* Reliable vaccines of known efficacy have been developed for T. parva and T. annulata. For T. annulata, the vaccine is prepared from schizont-infected cell lines that have been isolated from cattle and attenuated during in vitro culture. The vaccine must remain frozen until

*shortly before administration. Vaccination against* T. parva *is based on a method of infection and treatment in which cattle are given a subcutaneous dose of tick-derived sporozoites and a simultaneous treatment with a long-acting tetracycline formulation. This treatment results in a mild or inapparent East Coast fever reaction followed by recovery. Recovered animals demonstrate a robust immunity to homologous and, to varying degrees, heterologous challenge, which usually lasts for the lifetime of an animal. In endemic areas characterised by high transmission intensities, immunisation of animals with a stock(s) engendering a broad-spectrum immunity is desirable to cover a range of immunologically distinct* T. parva *strains that exist in the field. Immunised animals may become carriers of the immunising parasite stock. Safety precautions must be taken in the preparation and handling of* T. parva *vaccines to protect the workers and to avoid contamination of the stabilates. Consideration should also be given to the risk of introducing new isolates into an area where they may then become established through a carrier state.*

# A. INTRODUCTION

Theileriae are obligate intracellular protozoan parasites that infect both wild and domestic *Bovidae* throughout much of the world. They are transmitted by ixodid ticks, and have complex life cycles in both vertebrate and invertebrate hosts. There are a number of species of *Theileria* spp. that infect cattle; the two most pathogenic and economically important are *T. parva* and *T. annulata*. *Theileria parva* occurs in 13 countries in sub-Saharan Africa causing east coast fever (ECF), whilst *T. annulata* (tropical/Mediterranean theileriosis) occurs in southern Europe as well as North Africa and Asia. Endemic regions of *T. annulata* and *T. parva* do not overlap. *Theileria annulata* can occur in cattle, yaks, water buffalo and camels and is transmitted by ticks of the genus *Hyalomma*. Tropical theileriosis is more severe in European breeds, with a mortality rate of 40–90%, while the mortality rate in indigenous breeds of cattle from endemic areas can be as low as 3%. In Spain, *T. annulata* infections are mainly restricted to the southern and Mediterranean areas such as Menorca island, where the tick vector (*Hyalomma* sp.) is present. In northern Spain, reports of the presence of *Hyalomma* ticks are sporadic, as are associated *T. annulata* infections. However, tick distribution might change because of changes in climatic conditions. *Theileria orientalis/buffeli* complex is now thought to consist of two species – *T. orientalis*, occurring in the far east, and *T. buffeli* having a global distribution (Gubbels *et al*., 2000; Jeong *et al.,* 2010). Infection is generally subclinical; however, disease can occur in cattle depending on a number of epidemiological factors (including previous exposure to theileriae, stress or health status, and variations in the species pathogenicity, as reported recently in Australia and New Zealand (Gebrekidan *et al.,* 2015; McFadden *et al*., 2011). *Theileria taurotragi* and *T. mutans* generally cause no disease or mild disease, and *T. velifera* is nonpathogenic. These last three parasites are found mainly in Africa, and overlap in their distributions, complicating the epidemiology of theileriosis in cattle.

*Theileria lestoquardi,* also transmitted by *Hyalomma* ticks, is the only species of economic significance infecting small ruminants and it also occurs in north Africa, the Mediterranean basin and Asia. In sheep and goats, the morbidity rate from *T. lestoquardi* can approach 100% with a mortality rate of 46–100% in the most susceptible breeds.

*Theileria uilenbergi* and *T. luwenshuni* are pathogenic ovine piroplasms described in north-western China (People's Rep. of), though *Theileria* parasites with similar sequences have been found in sheep in northern Spain and Turkey, but apparently with a low pathogenicity. *Theileria luwenshuni* has also been detected in sheep in the United Kingdom associated with clinical signs (Phipps *et al*., 2016).

Some *T. parva* stocks produce a carrier state in recovered cattle, and studies using DNA markers for parasite strains have shown that *T. parva* carrier animals are a source of infection that can be transmitted naturally by ticks in the field (Bishop *et al*., 1992; Kariuki *et al*., 1995; Marcotty *et al*., 2002; Maritim *et al*., 1989). The severity of ECF may vary depending on factors such as the virulence of the parasite strain, sporozoite infection rates in ticks and genetic background of infected animals. Indigenous cattle in ECF-endemic areas are often observed to experience mild disease or subclinical infection, while introduced indigenous or exotic cattle usually develop severe disease.

The most practical and widely used method for the control of theileriosis is the chemical control of ticks with acaricides. However, tick control practices are not always fully effective for a number of reasons, including development of acaricide resistance, the high cost of acaricides, poor management of tick control, and illegal cattle movement in many countries. Vaccination using attenuated schizont-infected cell lines has been widely used for *T. annulata*, while for *T. parva* control, infection and treatment using tick-derived sporozoites and tetracycline is being implemented in a number of countries in eastern, central and southern Africa.

Chemotherapeutic agents such as parvaquone, buparvaquone and halofuginone are available to treat *T. parva* and *T. annulata* infections. Treatments with these agents rely on early detection of clinically affected animals and do not completely bring about eradication of theilerial infections, leading to the development of carrier states in their hosts.

The immune response to theileriae parasites is complicated. Cell-mediated immunity is thought to be the most important protective response in *T. parva* and *T. annulata*. In *T. parva,* the principal protective responses are mediated through killing of infected cells by bovine major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes. *Theileria annulata* schizonts inhabit macrophages and B cells. Innate and adaptive immune responses cooperate to protect cattle against *T. annulata* theileriosis. Intracellular parasites are mostly affected by cell-mediated immunity. Infection of leukocytes with *T. annulata* activates the release of cytokines, initiating an immune response and helping to present parasite antigen to CD4$^+$ T cells. These cells produce interferon-γ (IFN-γ), which activates non-infected macrophages to synthesise tumour necrosis factor α (TNF-α) and nitric oxide (NO), which destroy schizont- and piroplasm-infected cells. CD8$^+$ T cells have recently been shown to recognise parasite antigens presented by the MHC and to kill infected leukocytes. B cells produce antibody that along with NO kill extracellular merozoites and intracellular piroplasms. On the other hand overproduction of cytokines, in particular TNF-α, by macrophages generates many of the clinical signs and pathological lesions that characterise *T. annulata* theileriosis and the outcome of the infection depends upon the fine balance between protective and pathological properties of the immune system.

# B. DIAGNOSTIC TECHNIQUES

Diagnosis of acute theileriosis is based on clinical signs, knowledge of disease, and vector distribution as well as examination of Giemsa-stained blood, lymph node and tissue impression smears. *Theileria parva* and *T. annulata* are diagnosed by the detection of schizonts in white blood cells or piroplasms in erythrocytes. The piroplasmic stage follows the schizont stage and, in both *T. parva* and *T. annulata*, it is usually less pathogenic and is thus often found in recovering or less acute cases. It is hoped that a combination of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) will greatly enhance our present capacity to identify infected animals, thus making possible accurate surveys of *Theileria* species. Eventually, the aim would be to develop these technologies for the diagnosis of all the vector-borne diseases.

*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 |
| Agent identification[a] | | | | | | |
| Microscopic examination | – | +++ | – | +++ | – | – |
| PCR | + | ++ | ++ | +++ | + | – |
| Detection of immune response | | | | | | |
| IFAT | + | +++ | ++ | – | +++ | – |
| ELISA | + | + | ++ | – | +++ | – |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.

# 1. Identification of the agent

## 1.1. Microscopic examination

Multinucleate intralymphocytic and extracellular schizonts can be found in Giemsa-stained biopsy smears of lymph nodes, and are a characteristic diagnostic feature of acute infections with *T. parva* and *T. annulata.* Both intracellular and free-lying schizonts may be detected, the latter having been released from parasitised cells during preparation of the smears. Schizonts are transitory in *T. mutans* and the *T. orientalis/buffeli* group, in which the piroplasm stage may be pathogenic. *Theileria taurotragi* schizonts are not readily detected in Giemsa-stained blood smears. A veil to the side of the piroplasm may distinguish *T. velifera.* The schizonts of *T. mutans,* if detected, are distinct from *T. parva,* having larger, flattened, and irregular nuclear particles. The piroplasms (intra-erythrocytic stage) of *T. parva, T. annulata* and *T. mutans* are similar, but those of *T. annulata* and *T. mutans* are generally larger and may be seen to divide. However, for practical purposes schizonts and piroplasms of different theilerias are very difficult to discriminate in Giemsa-stained smears.

The schizont is the pathogenic stage of *T. parva* and *T. annulata.* It initially causes a lymphoproliferative, and later a lymphodestructive disease. The infected animal shows enlargement of the lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and/or diarrhoea. The most common post-mortem lesions are enlarged lymph nodes, a markedly enlarged spleen, pulmonary oedema, froth in the trachea, erosions and ulceration of the abomasum, and enteritis with necrosis of Peyer's patches. Lymphoid tissues become enlarged in the initial stages of the disease, but then atrophy if the animal survives into the chronic stages of the disease. When examined histologically, infiltrations of immature lymphocytes are present in lung, kidney, brain, liver, spleen, and lymph nodes. Schizont-parasitised cells may be found in impression smears from all tissues: lung, spleen, kidney and lymph node smears are particularly useful for demonstrating schizonts. In longer standing cases, foci of lymphocytic infiltrations in kidneys appear as white infarcts. In animals that recover, occasional relapses may occur. A nervous syndrome called 'turning sickness' is sometimes observed in *T. parva*-endemic areas, and is considered to be associated with the presence of intravascular and extravascular aggregations of schizont-infected lymphocytes, causing thrombosis and ischaemic necrosis throughout the brain.

In *T. annulata,* both the schizont and piroplasm stages may be pathogenic. Schizonts are scarce in the peripheral blood of acutely sick animals and their presence in blood smears indicates a poor prognosis. However, schizonts can be easily detected in smears from lymph nodes, spleen and liver tissues obtained by needle biopsy of these organs. The gross pathology caused by schizonts of *T. annulata* resembles that of *T. parva,* while anaemia and jaundice are features of both schizont and piroplasm pathology. Pathogenic strains of *T. mutans* also cause anaemia, as can strains from Japan and Korea referred to as *T. sergenti.*

Piroplasms of most species of *Theileria* may persist for months or years in recovered animals, and may be detected intermittently in subsequent examinations. However, negative results of microscopic examination of blood films do not exclude latent infection. Relapse parasitaemia can be induced with some *Theileria* species by splenectomy. Piroplasms are also seen in impression smears prepared at post-mortem, but the parasites appear shrunken and their cytoplasm is barely visible.

## 1.2. Molecular methods

The early DNA-based molecular methods to detect *Theileria* species were based on Southern blotting using a range of probes, derived from ribosomal RNA gene sequences, to detect all the *Theileria* species that are known to infect cattle (Allsopp *et al.*, 1993; Bishop *et al.*, 1995). DNA probes specific for *T. parva* (Allsopp & Allsopp, 1988; Conrad *et al.*, 1987; Morzaria *et al.,* 1999a) and *T. mutans* (Morzaria *et al.*, 1989) were also developed. The Southern blotting methods have largely been supplanted by species-specific PCR protocols designed to detect *T. annulata* or *T. parva* carrier cattle (Bishop *et al.,* 1992; D'Oliveira *et al.,* 1995; Odongo *et al.,* 2010; Skilton *et al.,* 2002). Several PCR assays have also been developed using specific genes or satellite sequences for characterisation of different isolates/strains/clones of *T. parva* (Geysen *et al.,* 1999; Oura *et al.,* 2003; Patel *et al.,* 2011).

A reverse line blot (RLB) assay based on hybridisation of PCR products to specific oligonucleotide probes immobilised on a membrane for simultaneous detection of different *Theileria* species has been

introduced (Gubbels *et al.,* 1999), and fluorescence resonance energy transfer (FRET)-based real-time assays have been developed for specific diagnosis of *T. parva* (Sibeko *et al.,* 2008).

The primer and probe sequences for several of these assays are presented in Table 2, together with the cycling conditions for the commonly used p104 nested PCR for *T. parva*.

PCR amplification of the p33/34 genes of the *T. orientalis/buffeli* complex followed by restriction enzyme analysis can be used to characterise the various types (Kawazu *et al.,* 1992; Kubota *et al.,* 1995).

**Table 2. PCR primers amd probes for the detection of** **T. annulata** *or* **T. parva**

| Target gene | Primer sequences (5'–3') | Probe sequences | Reference |
|---|---|---|---|
| *T. annulata* | | | |
| 30 kDa protein | GTA-ACC-TTT-AAA-AAC-GT<br>GTT-ACG-AAC-ATG-GGT-TT | n/a | D'Oliviera *et al.,* 1995 |
| *T. parva* | | | |
| p104 | ATT-TAA-GGA-ACC-TGA-CGT-GAC-TGC<br>TAA-GAT-GCC-GAC-TAT-TAA-TGA-CAC-C | n/a | Skilton *et al.,* 2002 |
| p104 (nested) | Primary PCR:<br><br>ATT-TAA-GGA-ACC-TGA-CGT-GAC-TGC<br>TAA-GAT-GCC-GAC-TAT-TAA-TGA-CAC-C<br><br>Secondary PCR:<br><br>GGC-CAA-GGT-CTC-CTT-CAG-ATT-ACG<br>TGG-GTG-TGT-TTC-CTC-GTC-ATC-TGC<br><br>Cycling conditions:<br>Primary:<br><br>• 94°C for 1 minute<br><br>• 40 cycles of 94°C/1 minute, 60°C/1 minute, 72°C/1 minute<br><br>• 72°C for 9 minutes after the last cycle.<br><br>Secondary:<br><br>• 94°C for 1 minute<br><br>• 30 cycles of 94°C/1 minute, 55°C/1 minute, 72°C/1 minute<br><br>• 72°C for 9 minute after the last cycle | n/a | Odongo *et al.,* 2010. |
| 18S RNA (RLB) | GAG-GTA-GTG-ACA-AGA-AAT-AAC-AAT-A<br>TCT-TCG-ATC-CCC-TAA-CTT-TC | TTC-GGG-GTC-TCT-GCA-TGT | Gubbels *et al.,* 1999 |
| 18S RNA (FRET) | CTG-CAT-CGC-TGT-GTC-CCT-T<br>ACC-AAC-AAA-ATA-GAA-CCA-AAG-TC | GGG-TCT-CTG-CAT-GTG-GCT TAT–F<br>LCRed640-TCG-GAC-GGA-G<br>TTC-GCT-PH | Sibeko *et al.,* 2008 |

## 2. Serological tests

### 2.1. The indirect fluorescent antibody test

The indirect fluorescent antibody (IFA) test is the most widely used diagnostic test for *Theileria* spp. The IFA test is robust, easy to perform and provides adequate sensitivity and specificity for use in the field for detection of prior infection with *T. parva* and *T. annulata* under experimental situations and in a defined epidemiological environment where only one theilerial species is present. The IFA test has limitations for large-scale serological surveys due to its reduced specificity in field situations where several *Theileria* species co-exist. There is a need for tests that are more specific, easy to interpret, and robust enough to be used in field conditions.

#### 2.1.1. Preparation of schizont antigen

i) Schizont antigen slides

The antigens used for the IFA test are intracytoplasmic schizonts derived from infected lymphoblastoid cell lines for *T. parva* and from infected macrophage cell lines for *T. annulata.*

Cultures of 200 ml to 1 litre of either *T. parva* or *T. annulata* schizont-infected cells containing $10^6$ cells/ml, of which at least 90% of the cells are infected, are centrifuged at 200 $g$ for 20 minutes at 4°C. The supernatant fluid is removed and the cell pellet is resuspended in 100 ml of cold (4°C) phosphate-buffered saline (PBS), pH 7.2–7.4, and centrifuged as before. This washing procedure is repeated three times, and after the final wash the cell pellet is resuspended in PBS (approximately 20–100 ml) to give a final concentration of $10^7$ cells/ml.

Using a template or pipette tip, thin layers of the cell suspension are placed on Teflon-coated multispot slides, or on ordinary slides using nail varnish for separation. The smears should give between 50 and 80 intact cells per field view when examined under a ×40 objective lens. The antigens are distributed on to the slides using multichannel or a 100 μl pipette. By dispensing and immediately sucking up the schizont suspension, a monolayer of schizonts remains in each well. This is performed for each enclosure until the volume is exhausted. With this method, approximately 600 good quality slides containing a total of 6000 individual antigen spots can be obtained. The slides are air-dried, fixed in acetone for 10 minutes, individually wrapped in tissue paper and then in groups of five in aluminium foil, and stored in airtight, waterproof plastic containers at either –20°C or –70°C. The antigens keep for at least 1 year at –20°C and longer at –70°C.

ii) Schizont antigen in suspension

First, 500 ml of *T. parva*- or *T. annulata*-infected cells containing $10^6$ cells/ml are centrifuged at 200 $g$ for 10 minutes at 4°C, and the cell pellet obtained is washed twice in 100 ml of cold PBS. The viability of the cells is determined by eosin or trypan blue exclusion (it should be greater than 90%). The cells are resuspended at $10^7$/ml in cold PBS. To this volume, two volumes of a cold fixative solution containing 80% acetone and 0.1% formaldehyde (0.25% formalin) in PBS are added drop by drop while the cell suspension is stirred gently and continuously in an ice bath. The cell suspension is kept at –20°C and allowed to fix for 24 hours. Siphon about 2/3 of the volume off, centrifuge and decant. The fixed cells are then washed three times in cold saline and centrifuged at 200–400 $g$ for 20 minutes at 4°C. After the last wash, the cells are resuspended into 5 ml PBS + 0.2% BSA (bovine serum albumin) at $10^7$/ml. The fixed cells are distributed in aliquots of 0.5 ml. The antigen is stable at 4°C with 0.2% sodium azide as preservative for 2 weeks, and keeps indefinitely at –20°C. This method can also be used to prepare schizont antigen for *T. taurotragi.*

#### 2.1.2. Preparation of piroplasm antigen

The piroplasm stage of *Theileria* spp. cannot be maintained in culture, therefore the piroplasm antigen must be prepared from the blood of infected animals. Due regard should be paid to the principle of 'The Three Rs' as set out in the WOAH *Terrestrial Code,* Chapter 7.8 *Use of animals in research and education.*

i) Piroplasm antigen slides

Experimental infections are induced by infecting cattle subcutaneously with sporozoites, or applying ticks infected with *T. parva*, *T. annulata* or *T. taurotragi*. Infection with *T. annulata* is invariably produced by inoculation of blood drawn from cattle with acute theileriosis. Splenectomy of the recipient cattle prior to the infection considerably increases the piroplasm parasitaemia in red blood cells (RBC). Peak parasitaemias are of short duration and if animals survive the disease the percentage of infected RBC decreases considerably in a few days. Infections with the parasite group referred to as *T. orientalis/buffeli, T. mutans* or *T. velifera* are usually induced by inoculating splenectomised cattle intravenously with blood from a carrier animal, or with a blood stabilate, or by application of infected ticks. When the piroplasm parasitaemia is 10% or higher, 100 ml of the infected blood is collected from the jugular vein in a heparinised or ethylene diamine tetra-acetic acid (EDTA) vacutainer, and gently mixed with 2 litres of PBS. The mixture is centrifuged at 500 *g* for 10 minutes at 4°C; the plasma and buffy coat are removed, the RBC are again resuspended in 2 litres of PBS, and the centrifugation step is repeated. It is important to remove the buffy coat after each wash. This washing procedure is repeated four times. After the final wash, an aliquot of the packed RBC is used to make doubling dilutions in PBS, and a 5 µl drop of each dilution is placed on slides. The dried spots are fixed in methanol and stained with Giemsa's stain, and the concentration of RBC is examined using a light microscope. The dilution that gives a single layer of RBC spread uniformly on the spot is then selected for large-scale preparation of piroplasm antigen slides. Approximately 10,000 antigen slides (100,000 antigen spots) can be prepared from 100 ml of infected blood. The antigen smears are allowed to dry at room temperature before fixing in cold (4°C) acetone for 10 minutes. The fixed smears can be stored as for the schizont antigen slides, and kept for similar periods.

ii) Piroplasm antigen suspension

An alternative method of preparing antigens to that described above is available, and has been tested for *T. parva*. In this procedure, 100 ml of blood are taken from an animal with a high piroplasm parasitaemia and prepared as described previously, and the packed cell volume is adjusted to 5% in PBS.

One volume of the RBC suspension is added to two volumes of the fixative (see Section B.2.1.1.ii above) while stirring. The cells are allowed to fix at –20°C for 24 hours. The fixed cells are then washed three times with PBS and centrifuged at 1000 *g* for 30 minutes. The deposit is resuspended to the original volume of blood with PBS containing 0.2% sodium azide, and distributed in aliquots of 0.5 ml.

The piroplasm antigen is stable at 4°C when preserved with 0.2% sodium azide for a period of at least 3 years.

### 2.1.3. Standardisation of antigen

Schizont or piroplasm antigen suspensions are mixed on a rotor mixer and titrated in PBS by doubling dilution starting from undiluted through to 1/16. The dilution giving a cell distribution of approximately 50–80 schizont-infected cells or 150–200 infected RBC per field view when examined under a ×40 objective lens is recommended for use for that batch of antigen. Using this dilution, test antigen smears are prepared on slides. These antigen smears plus the antigen slides previously frozen (and thawed before use) are tested against a range of dilutions of a panel of known strong, intermediate and weak positive and negative control sera. If the positive control sera titrate to their known titres and the negative control sera give no fluorescence, the antigen is used in the routine IFA test.

Both types of antigen preparations, acetone-fixed smears stored at either –20°C or –70°C, and antigens fixed in suspension and stored at either 4°C or –20°C, are used routinely in many laboratories. The sensitivity of both types of antigen is comparable. In laboratories where adequate low temperature storage facilities and a reliable supply of electricity are available, the antigen slides can be used. However, such antigens can only be transported on dry ice or in liquid nitrogen. Antigens fixed in suspension have the advantage over antigen slides in that the initial method of preparation is simpler and quicker. A large batch of this antigen can be stored in one

container, and aliquots may be taken out as necessary from which fresh smears are prepared for the IFA test. The need for a large storage facility is thereby avoided. The antigens fixed in suspension can also be stored at 4°C and can be safely transported at room temperature without loss of antigenicity.

### 2.1.3.1. Preparation of bovine lymphocyte lysate

A lymphocyte lysate is prepared according to the method described by Goddeeris *et al*. (1982), for use in tests with antigens of *T. parva* in suspension. Briefly, a 3-month-old calf is splenectomised and maintained in a tick-free environment. To exclude the possibility of latent theilerial infections, Giemsa-stained blood smears are examined daily for a period of 4 weeks for parasites. The parasite-free animal is killed and the thymus and all the accessible lymph nodes are removed. These tissues are sliced into small pieces in cold PBS containing 0.45% EDTA as anticoagulant. Cells are teased out of the tissue, separated from the debris by passing through a muslin cloth, and washed three times with PBS/EDTA by centrifugation at 200 $g$ for 20 minutes at 4°C. The washed lymphocytes are resuspended in PBS without EDTA, to give a final concentration of $5 \times 10^7$ cells/ml. The cells are disrupted by sonication in 100-ml aliquots on ice for 5 minutes using the 3/8 probe. The sonicated material is centrifuged at 1000 $g$ for 30 minutes at 4°C, and the supernatant, adjusted to 10 mg protein/ml, is stored at –20°C in 4-ml aliquots.

### 2.1.3.2. Test procedure

i)     With schizont or piroplasm slide antigen

a)     Remove antigen slides from freezer and allow to thaw for 30 minutes at 4°C and then for 30 minutes at room temperature.

b)     Inactivate the sera to be tested for 30 minutes in a water bath at 56°C.

c)     Unpack the slides and label the numbers of the sera tested.

d)     Prepare 1/40 and 1/80 dilutions of sera to be tested. Validated positive and negative sera are included with each test as controls. Further doubling dilutions can be made if end-point antibody titres are desired.

e)     Transfer 25 µl of each serum dilution to a spot of antigen.

f)     Incubate in a humid chamber for 30 minutes at room temperature.

g)     Remove the serum samples from the antigen wells by washing with PBS and rinse by immersing in two consecutive staining jars containing PBS for 10 minutes each time.

h)     Distribute to each well 20 µl of diluted anti-bovine immunoglobulin fluorescein isothiocyanate conjugate at appropriate dilution (generally, dilutions recommended by manufacturers are suitable; however, minor adjustments may be necessary for optimal results). Incorporate Evans blue into the conjugate at a final dilution of 1/10,000 as a counterstain and incubate in a humid chamber for 30 minutes at room temperature.

i)     Repeat step g and mount with a cover-slip in a drop of PBS/glycerol (50% volumes of each).

j)     Read the slides under a fluorescent microscope equipped with epi-Koem illumination (100 W mercury lamp), UV filter block, ×6.3 eyepieces and Phaco FL 40/1.3 oil objective lens.

ii)     With schizont antigen stored in suspension

a)     Thaw frozen antigen at room temperature.

b)     Distribute the antigen suspension on the spots of multispot slides, using multichannel or a 100-µl pipette. By dispensing and immediately sucking up the suspension a monolayer of schizont-infected cells remains on each well.

c)     Allow slides to dry at room temperature or 37°C.

d)     Dilute test and control sera 1/40 in lymphocyte lysate (195 µl lymphocyte lysate + 5 µl serum).

e)     Proceed as described in steps e to j (Section B.2.1.3.2.i).

iii) With piroplasm antigen stored in suspension

a) Resuspend piroplasm antigen (stored at 4°C) by agitation and disperse RBC by passing the suspension through a 25-gauge needle to break the clumps.

b) Dilute the antigen to previously standardised dilutions (see preparation of piroplasm antigen).

c) Allow slides to dry at room temperature or 37°C.

d) Proceed as described in steps d and e (Section B.2.1.3.2.ii).

### 2.1.4. Characteristics of the indirect fluorescent test

The incorporation of Evans blue provides a good contrast, enabling good differentiation of non-infected cells from the infected ones under the fluorescent microscope. Mounting the slides in 50% glycerol, at pH 8.0, reduces the rapid fading of fluorescein isothiocyanate and makes photography of the preparation possible. Once prepared, slides are stable and can be read for up to 72 hours after preparation when kept at 4°C in the dark.

The sensitivity of the IFA test depends on the period of time that has elapsed since the onset of infection. Following infection with sporozoites, antibodies to *T. parva* and *T. annulata* are first detected between days 10 and 14 using the schizont antigen. Using the piroplasma antigen, antibodies are first detected between days 15 and 21. Antibodies last for a variable period of time after recovery, depending on such factors as the establishment of a carrier state, chemotherapeutic intervention, and presence or absence of a rechallenge. Following recovery from infection with *T. parva* or *T. annulata*, high levels of antibody are generally detected for 30–60 days. The antibody levels gradually decline and low antibody titres are still detectable 4–6 months after recovery. Later, antibody may become undetectable at a serum dilution of 1/40, but may persist for more than 1 year following a single challenge. In ECF endemic regions, the seroprevalence in cattle population fluctuates considerably depending on the level and regularity of challenge. In an endemic area where a seasonal transmission cycle of ECF occurs, IFA has been shown to lack sensitivity. The overall diagnostic sensitivity of the IFA test has been evaluated as 55% at a cut off titre 1/40 and 28% at cut off 1/160. The specificity of the test for the two cut off points was 86% and 95% respectively (Billiouw *et al.*, 2005).

The IFA test is useful for identifying herds that contain carriers of *T. annulata*, but is not always sufficiently sensitive to detect all infected individuals. Both schizont and merozoite (piroplasm) IFA antigens have failed to detect antibody in some animals despite carrying patent infection with piroplasms (Darghouth *et al.*, 1996).

In *T. mutans* infections induced by sporozoite inoculation, antibodies are first detected between days 10 and 15 after the appearance of piroplasms. Low titres are detectable for at least 12–24 months.

The *T. parva* IFA test is highly sensitive for detection of antibodies in an epidemiological situation where only one species of *Theileria* exists. However, if the test is used to detect antibodies where mixed infections of *Theileria* occur, the specificity of the test needs to be carefully evaluated. For example, *T. annulata* and *T. parva* cross-react, although these cross-reactions are four- to six-fold lower than with the homologous sera. The cross-reactivity between the two species has little practical significanace as the geographical distribution of these two parasites does not overlap. In the IFA test such cross-reactivity does not occur between *T. parva* and *T. mutans* or between *T. annulata* and *T. mutans*. There is a low level of cross-reactivity between *T. parva* and *T. taurotragi*, reducing the specificity of these two tests in many parts of sub-Saharan Africa where their distribution overlaps.

A panel of monoclonal antibodies (MAbs) detecting various epitopes on the polymorphic immunodominant antigen of the *T. parva* schizont stage has been generated. This panel can be used in the IFA test using the schizont-infected lymphoblastoid cells to detect differences between certain stocks of *T. parva* and between *T. parva* and other theilerial species. This test has been deployed as one of the several characterisation tools to differentiate various stocks of *T. parva*, and for quality control during sporozoite stabilate preparation (Bishop *et al.*, 1994).

### 2.2. Enzyme-linked immunosorbent assays

Serological tests based on the ELISA have been developed for the detection of antibodies to *T. annulata* (Gray *et al.*, 1980). Tests used for *T. parva* and *T. mutans* are indirect ELISAs based on parasite-specific antigens, PIM and p32, respectively (Katende *et al.*, 1998; Morzaria *et al.*, 1999a). These ELISAs provide higher (over 95%) sensitivity than the IFA tests. The ELISA reagents are available from the International Livestock Research Institute, Nairobi, Kenya.

# C. REQUIREMENTS FOR VACCINES

## C1. Cell culture live vaccines for *Theileria annulata*

## 1. Background

Vaccination against *T. parva* and *T. annulata* has been attempted since the causal organisms were first recognised early in the last century. However, reliable live vaccines of known potency are a more recent development. The most widely used are attenuated schizont cell culture vaccines against *T. annulata*. The procedures for production and safety testing have been described (Food and Agriculture Organization of the United Nations (FAO), 1984; Hashemi-Fesharki, 1988; Pipano, 1989b), and the vaccine is used in Israel, Iran, Turkey, Spain, India, northern Africa, central Asia and the People's Republic of China.

Despite the fact that vaccination with the cell culture vaccine against *T. annulata* has been available for more than three decades and has shown to be effective under field conditions, the use of this vaccine has been limited. The concern about the introduction of vaccine-derived parasites into the field tick population has led to individual countries developing vaccines from local isolates (Morisson & Mc Keever, 2006). Some attenuated cell lines have lost the ability to differentiate to erythrocytic merozoites (piroplasms) when inoculated to cattle and in one instance, *Hyalomma* nymphs fed on vaccinated cattle did not become infected (Kachani *et al.*, 2004a). However in most cases the loss of differentiation is based on macroscopic examination of blood films from vaccine inoculated cattle. This drawback, the difficulties in standardisation of the antigenic composition of the cultured parasites and the need of a cold chain for distribution of the vaccine to the field are limiting factors in commercialisation of this vaccine (Morisson & Mc Keever, 2006).

## 2. Outline of production and minimum requirements for vaccines

### 2.1. Characteristics of the seed

#### 2.1.1. Biological characteristics of the master seed

Primary cultures of *T. annulata*-infected cells may be established from trypsinised lymph nodes, liver, or spleen taken aseptically from an infected animal after death, or from the buffy coat of heparinised peripheral blood separated on a density gradient (Ficoll Hypaque), or by lymphocytes harvested from lymph node biopsy material using a plastic syringe method (Brown, 1979; FAO, 1984).

Seed cultures are prepared from cryopreserved cell lines that have been isolated from cattle and attenuated as described below. Vaccines should be produced from a seed culture (master seed) that has been passed less than 30 times, because there is some uncertainty about the immunogenic stability of these cultures in long-term passage.

#### 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. As cell culture material is derived from field animals, their cells might be potential sources of contamination of the vaccine with extraneous pathogens. Potential contaminants include bovine leukosis, mycoplasma, bovine viral diarrhoea virus, bovine spongiform encephalopathy (BSE) and other bacteria and viruses.

Cell cross-contamination in cell cultures is a common problem during cell culturing and use. The problem can be solved by increasing the awareness and by introducing regular quality control of cell cross-contamination.

### 2.1.3. Validation as a vaccine strain

Attenuation of *T. annulata* schizonts is achieved by prolonged growth and passage in culture (Pipano, 1989b). The loss of parasite virulence appears to be due to a change in parasite gene expression. Attenuation is assessed by the inoculation of the culture into susceptible calves every 20–30 passages. A sample of culture should be cryopreserved every ten passages in case of accidental loss or contamination. Complete attenuation is achieved when cultures do not cause fever or detectable schizonts and piroplasms in susceptible cattle but could take up to 300 passages. An attenuated culture will reliably infect cattle at $10^5$ cells and induce a serological reaction, and will not produce disease at $10^9$ cells. Cultures may be cryopreserved using either dimethyl sulphoxide (DMSO) or glycerol. Two methods of storing and delivering the vaccine are described below.

## 2.2. Method of manufacture

### 2.2.1. Procedure

The infected cells are cultured initially in Eagle's minimal essential medium (MEM) or Leibovitz L15 medium supplemented with 20% calf serum and containing penicillin (100 units/ml), streptomycin (50 µg/ml), and mycostatin (75 units/ml) in 25-ml plastic screw-cap tissue-culture flasks. An alternative medium is RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin, and is usually used with established cultures. Medium is replenished every 3–4 days. The presence of bright refractile cells free in the medium (on examination using a phase-contrast or inverted microscope) is indicative of infected cell growth. The cultures may establish as a monolayer or in suspension. Passage is effected by decanting the medium, adding 0.025% EDTA (versene) for 15 minutes to monolayer cultures, dispersing the cells, then counting and dispensing according to flask size. Approximately $10^6$ cells are introduced into a 25 cm$^2$ flask, and the same seed rate in 100–200 ml is used in larger flasks. The general culture technique is as described by Brown (1979).

Serum is essential for maintenance of these cultures, and is obtained either from calves up to the age of 6 months, or from commercial sources, and is tested for toxicity through three passages in an established cell line before use.

### 2.2.2. Requirements for ingredients

Before starting to produce vaccine, seed material with known characteristics is required (Pipano, 1997). Three types of seed material are distinguished:

i)    Master seed

Schizont-infected cells from a specific passage that have been selected and permanently stored and from which all other passages are derived. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. As *T. annulata* schizont-infected cells are used for the manufacturing process, the master seed also represents the master cell stock (see Chapter 1.1.8 *Principles of veterinary vaccine production*). To prepare a master seed, schizont-infected cells that have proved to be safe for cattle are propagated to obtain in a single culture passage approximately $5 \times 10^8$ cells. The cells are cryopreserved in about 100 cryotubes each containing $5 \times 10^6$ cells. A viability check of the master seed should be performed once the master seed has been cryopreserved for at least 24 hours by reviving one of the cryotubes.

ii)   Working seed

Schizont-infected cells at a passage level between the master seed and the production seed. To prepare a working seed, the contents of a single cryotube of master seed are transferred to a 10 ml centrifuge tube containing 8 ml complete medium. The tube is centrifuged at 600 *g* for 15 minutes at 4°C and the pellet is transferred into a 75 cm$^2$ culture

flask containing 15–20 ml medium. The medium is replaced the next day, and 4 days later the cells are dispersed and subcultured in larger vessels. After 5–6 subcultivations, a sufficient number of infected cells is available to start the production run.

iii) Production seed

Schizont-infected cells from a specific passage level are used without further propagation for the preparation of a batch of vaccine. The production seed is obtained by propagating large numbers of cells in monolayer or suspension cultures. Monolayer cultures are grown in flasks, 150 cm$^2$ to 175 cm$^2$, which usually provide an average of from $7 \times 10^7$ to $8 \times 10^7$ cells per vessel. About 80 ml of complete medium per flask is required. In a roller bottle culture system, $1.2$–$1.5 \times 10^8$ cells can be obtained in a conventional roller bottle (700 cm$^2$) containing 100–120 ml of medium. To obtain optimal yield of cells, stationary cultures or roller bottles cultures are incubated for 6–7 days with culture media as described previously, see Section C1.2.2.1.

The schizont-infected cells from all vessels are harvested and pooled together and the total number is computed. Alternatively, about 20% of the cells may be seeded again to prepare another batch of vaccine. Several batches of vaccine can be produced using a portion of the production seed as working seed. As prolonged cultivation may generate alteration in the futures of the schizonts, such as immunogenic capacity, after several batches, subsequent vaccine is produced by making a fresh production seed from the master seed.

Schizont-infected cells are mixed with DMSO at a final concentration of 7% or glycerol at a final concentration of 10%, and dispensed in 1.8-ml aliquots into 2-ml plastic vials, each vial containing ten doses of concentrated vaccine. As DMSO immediately penetrates the cell membranes, the time spent in dispensing the vaccine into the vials should be as short as possible. When glycerol is used, an equilibration time of 30–40 minutes is required before freezing the vaccine. There is no consensus on how many schizont-infected cells should constitute one dose of the vaccine. A recommended practical approach is to prepare doses of $10^6$–$10^7$ infected cells in order to counteract variable environmental conditions in the field. However, considerable protection against sporozoite-induced infection has been achieved by vaccination with $10^5$ infected cells (Kachani *et al*., 2004b).

The vaccine is frozen by introducing the vials in an ultracold deep freezer (–70°C) and transferring them 24 hours later to liquid nitrogen containers. Alternatively vials can be introduced in gas phase liquid nitrogen for 3 hours and then immersed in the liquid nitrogen for storage (Pipano, 1989b). Vaccine is transported to the field in liquid nitrogen, and diluted 1/10 in isotonic buffered saline in a screw-cap bottle with a rubber or silicone septum for aseptic withdrawal. For dilution of vaccine frozen with glycerol, isotonic buffered saline should also contain 10% glycerol in order to avoid osmotic damage to the schizonts. The vaccine is administered subcutaneously within 30 minutes of thawing (Pipano, 1977).

The vaccination regimen in Iran up to 1990 was to inoculate two different mild strains 1 month apart. But, in order to reduce cost and save time, a new method was implemented involving only a single dose of a local live attenuated vaccine strain (Hashemi-Fesharki, 1998). A fresh culture vaccine is used in Morocco, usually at a tenfold lower dose ($10^4$ schizont-infected cells) (Kachani *et al*., 2004b). However there are problems with quality control of vaccines with short shelf life.

### 2.2.3. In-process controls

Records of the source and passages of the working seed material should be maintained. Seeds should be free of infective agents such as enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, bovine syncytial virus, Rift Valley fever, etc. Test procedures will depend on availability preferably using DNA analysis.

pH, temperature and coloration of the solutions should be checked during the process and must have been shown to be free from contaminants. Numbers and contamination in growing cell cultures should be checked on a daily basis by examination using an inverted microscope.

### 2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety precautions

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

*Theileria annulata* schizonts are not hazardous for humans or contagious for animals, therefore the main purpose in designing a vaccine production facility is to prevent contamination of the product by extraneous organisms.

a) Freedom from properties causing undue local or systemic reactions

For testing the safety of the master seed, two to four susceptible calves, of the most sensitive stock available, are inoculated with a tenfold greater dose than is recommended for immunisation. This dose should not produce clinical signs beyond a transient rise in temperature. With completely attenuated master seed, no schizonts or piroplasms will be seen in lymph node and liver smears or in blood films. However, different breeds of cattle may show different sensitivities to the vaccine. This should be borne in mind when vaccine from a partially attenuated master seed is to be administered to high-grade cattle stocks.

Following a successful test for safety of a sample, all subsequent batches produced from the same master seed can be released without further testing for safety. However, if parasites are detected in the blood or tissues of vaccinated field animals, or if clinical signs develop following the inoculation of the vaccine, the batch or a parallel batch, from the same master seed, should be retested for safety.

iii) Batch potency

In Israel the schizont vaccines are tested using a documented procedure (Pipano, 1989a) before release.

Usually, the schizont vaccine is produced in small individual batches (3–5 thousand doses), which makes the full testing of each batch impractical for economic reasons. It is recommended therefore that the first batch of vaccine produced from a master seed be tested for safety, efficacy, potency and sterility, while each subsequent batch be tested for sterility and potency only. This recommendation is based on the fact that once the cultured schizonts become attenuated, no reversion to virulence has ever been observed during further cultivation. As far as efficacy is concerned, no obvious alteration of the immunogenic properties has been observed during the limited number (20–30) of passages involved in producing the actual vaccine.

a) Viability of schizont-infected cells

The potency test is conducted by quantitative *in-vitro* methods. Frozen vaccine remains stable during the storage period, even for long periods, but some loss of viability occurs during the freezing and thawing processes. Viability should be tested under conditions as similar as possible to those obtained when the vaccine is used in the field. For this reason, vaccine should be thawed and the diluted suspension of schizont-infected cells should be left at ambient temperature for 60 minutes before performing the viability tests. A simple test for evaluating viability of the infected cells is nigrosin dye exclusion counting (Wathanga *et al.,* 1986). Vaccine that, after being thawed and diluted and left at room temperature for 1 hour, still contains 50% or more live cells can be released for use although in most cases 80–90% of live cells are found.

Viability of the schizonts is also reflected by the plating efficiency of the schizont-infected cells (Wathanga *et al.,* 1986), as only cells containing viable schizonts multiply in culture. For

this purpose, the thawed, diluted vaccine is transferred from the bottle to a centrifuge tube. A sample for counting is taken and the suspension is centrifuged for 15 minutes at 600 *g*. Meanwhile, the total number of cells (live and dead) is determined in order to ascertain that the frozen vaccine had the necessary initial concentration of cells. After centrifugation, the supernatant is discarded and the cells are resuspended to the original volume using complete culture medium. Serial tenfold dilutions of cells in complete medium are performed in sterile 10 ml tubes so that the last two dilutions contain 50, and 5 cells per ml, respectively. Twelve replicates of 200 µl from each of the last two dilutions are introduced into a 96-well culture plate. The plates are incubated at 37°C in a 5% $CO_2$ atmosphere and cultures are checked with an inverted microscope 6 and 9 days after seeding. The number of wells theoretically containing 1 cell each in which growth is observed is counted. Vaccine showing a plating efficiency <2 (cells) are adequate for field use.

### 2.3. Requirements for authorisation/registration/licensing

#### 2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C2.2.1 and C2.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

#### 2.3.2. Safety requirements

i) Target and non-target animal safety

*Theileria annulata* shizonts are not contagious for animals. These vaccines produce no adverse effects in healthy cattle. However, animals with existing infections, particularly viral infections, may not tolerate vaccination well. The administration of a viral vaccine, such as for foot and mouth disease, during the immunisation period (reaction period) is not recommended as the immune response may be compromised (Hashemi-Fesharki, 1988). In Iran, it is not recommended to vaccinate cows that are over 5 months pregnant, although studies in pregnant cattle with the vaccine stocks used in Israel found no effect on pregnancy (Pipano, 1989a). The immunity engendered is long lasting.

In general, cattle should be immunised in the first few months of life, and tick challenge under natural conditions reinforces the immunity. Although antigenically different strains of *T. annulata* have been identified (Pipano, 1977), it is generally considered that there is sufficient cross-protection among strains to provide adequate protection against field challenge as observed in Israel. In the vast infected areas of central Asia, a single stock has proved immunologically effective in 1.5 million cattle (Dolan, 1989; Wathanga *et al*., 1986). However, as described previously, two stocks are used routinely in Iran (Hashemi-Fesharki, 1988).

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Once the cultured schizonts become attenuated, no reversion-to-virulence has ever been observed during further cultivation.

iii) Precautions (hazards)

Different breeds of cattle may show different sensitivities to the vaccine. This should be borne in mind when vaccine from a partially attenuated master seed is to be administered to high-grade cattle stocks.

#### 2.3.3. Efficacy requirements

i) Capacity to protect against naturally transmitted theileriosis

The batch of experimental vaccine used for the safety test can also be used for testing efficacy of the culture-derived anti-theilerial vaccine. Three or four calves are vaccinated with a conventional dose of vaccine and 6 weeks later, the vaccinated calves and the same number of unvaccinated calves are infected with sporozoites of *T. annulata*. Infection can be

induced by live adult ticks issued from *T. annulata*-infected pre-imaginal stages or by inoculation of stabilate prepared from macerated infected ticks (for techniques see Section C2.2) Experience shows that inoculation of stabilate (macerated ticks) generally induces a more severe response than an equivalent number of live, infected ticks allowed to feed on the cattle. However in the long run, the results obtained by challenge with stabilate appear to be more reproducible than those obtained with different batches of live ticks.

There are no internationally agreed standards for the size of a challenge dose used in testing the efficacy of *T. annulata* culture-derived vaccine. Five to ten female and the same number of infected, unfed male *Hyalomma* ticks have been used for infection of cattle. Alternatively, stabilate equivalent to 2–4 macerated ticks inoculated subcutaneously in the neck area will invariably produce acute theileriosis. The responses to the challenge infection of the vaccinated and unvaccinated control calves are monitored using the following parameters: duration and severity of pyrexia, rate of schizont-infected cells in lymph node or liver biopsy smears, rate of piroplasm infected erythrocytes in the blood films, decrease in white and red blood cell counts, and severity of clinical manifestations such us anorexia, depression and recumbency.

The results of the efficacy test depends on factors such us immunological characteristics of the *T. annulata* isolate grown and attenuated in culture, the virulence and dose of the field isolate used for challenge, the species of infected ticks used to produce sporozoites. Research studies (Pipano, 1989b) show that calves vaccinated with schizont vaccine may exhibit an apparently near total protection or show a low level parasitaemia, accompanied by mild fever and insignificant alteration of the remaining parameters from their pre-vaccination values following a potentially lethal homologous challenge. A lesser degree of protection has been exhibited when cattle vaccinated with schizont vaccine were challenged with tick-derived parasites from a geographically remote area. In contrast, in most of the trials the non-vaccinated control calves have exhibited a high level of parasitaemia and pancytopenia accompanied by severe clinical manifestations. In the absence of specific medication, the majority of the control animals have succumbed to the infection (Pipano, 1989b).

Field observations have also been used for evaluation of the efficacy of anti-theilerial vaccines (Pipano, 1989a; Stepanova & Zablotskii, 1989). Susceptible indigenous cattle as well as high-grade exotic breeds were protected against clinical theileriosis and death in pastures on which nonvaccinated cattle succumbed to theileriosis. As completely attenuated schizont vaccine does not yield piroplasms, the presence of this theilerial stage in vaccinated cattle showing no clinical signs is considered to be the result of unapparent tick-induced infection.

The frozen vaccine is viably preserved in large liquid nitrogen refrigerators at production facility and transported to farms in smaller liquid nitrogen containers. Field centres for storage and supply of vaccine can be set up in theileriosis-enzootic areas. The basic equipment required for field application of frozen vaccine includes a wide mouthed jar for preparing a 40°C water bath, a thermometer for measuring the temperature of water, long forceps, face shield and temperature-resistant gloves. Application of the frozen vaccine to field cattle begins by donning the face shield and temperature-resistant gloves. The required numbers of vials are withdrawn with the forceps from the canister of the liquid nitrogen refrigerator. When withdrawing the vials, the canister should be kept as deep as possible in the neck of the refrigerator to avoid quick warming of the remaining vials. Each withdrawn vial should be checked in order to ascertain that liquid nitrogen has not leaked inside. The liquid nitrogen does not alter the vaccine, but may cause the vial to explode when introduced in the water bath. Such a vial should be held at ambient temperature for 1–2 minutes to allow the nitrogen to escape and then processed in the usual way. Leaking of liquid nitrogen into a vial containing frozen vaccine has raised questions to about the sterility of the frozen vaccine. However the system has been used for decades with no significant problem observed. The vaccine is administered subcutaneously within 30 minutes of thawing (Pipano, 1977).

### 2.3.4. Duration of immunity

Controversial results about the length of immunity engendered by vaccination with the cell culture vaccine have been obtained. Periods of from more than 48 months (Stepanova & Zablotskii, 1989) to less than 13 months (Ouelli *et al*., 2004) have been reported.

### 2.3.5. Stability

The frozen vaccine has a practically unlimited shelf life.

## C2. Immunisation of cattle against *Theileria parva* by the infection and treatment method (live vaccine)

## 1. Background

Vaccination against *T. parva* is based on a method of infection and treatment in which an aliquot of viable sporozoites is inoculated subcutaneously, and the animals are simultaneously treated with a formulation of a long-acting tetracycline, the so-called infection and treatment method (ITM) (Radley, 1981). Tetracyclines reduce the severity of the infection, and the resulting mild infection is usually controlled by the host's immune response, so that a carrier state is achieved. There are always risks associated with the use of live parasites for immunisation, however, with appropriate quality control and careful determination of a safe and effective immunising dose, the method can and is being used successfully in the field. Some *T. parva* stocks have been shown to infect cattle reliably without inducing disease, and these can be used without tetracycline treatment. One such stabilate is being applied in the field and offers considerable advantages over potentially lethal stabilate infections and savings in the cost of vaccination. However, different stabilates of these stocks can produce severe disease in cattle, emphasising the importance of a carefully controlled immunising dose.

## 2. Outline of production and minimum requirements for vaccines

### 2.1. Characteristics of the seed

#### 2.1.1. Biological characteristics of the master seed

*Theileria parva* infected ticks can be produced by feeding nymphal *Rhipicephalus appendiculatus* ticks on the ears of an animal undergoing an active ECF infection. After moulting, these ticks, when prefed for 4 days on rabbits will have infective sporozoites in their salivary glands. By grinding these prefed ticks in a specific medium, sporozoites will be released in the supernatants and a stabilate can be produced (FAO, 1984) that can be cryopreserved and when in sufficient quantity be earmarked as a master seed.

If needed, working seed stabilates are prepared by injecting cryopreserved sporozoites from a master seed into experimental cattle and producing a working seed stabilate as described below. Vaccines should be produced from a seed (working seed) that has not undergone further tick passages after its immunological characterisation because this might change after passage,

#### 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

i)     Field collection of ticks

It is important that well characterised laboratory strains of *R. appendiculatus* be used during preparation of immunising stabilates.

If field ticks are collected for experimental purposes, then consideration should be given to the possible hazard to humans from pathogens present in these ticks. The most important pathogen that has been recognised is Crimean–Congo haemorrhagic fever virus, usually associated with ticks of the genus *Hyalomma* and widely prevalent within the geographical distribution of *R. appendiculatus*. Those handling field tick collections should, therefore, be made aware of potential hazards. Ticks of *Hyalomma* species generally should not be removed from hosts; engorged or partially engorged ticks should not be crushed between the fingers. If removed, ticks should be handled with a forceps.

ii)     Tick-handling facilities

The handling of field-collected ticks in the laboratory must be controlled so as to avoid accidental attachment to personnel. Field-collected ticks should be fed on rabbits and cattle in isolation facilities. Animals on which laboratory-infected or field-collected ticks have fed should be destroyed. Following engorgement of field-collected ticks on laboratory animals, aliquots should be homogenised and tested for extraneous human pathogens by inoculation in baby hamster kidney (BHK) and Vero cells. The effects of these inoculations should be studied through three passages. Any unused ticks should be destroyed by chemical means or by incineration.

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. As stabilate material is derived from field animals and rabbits, their cells might be potential sources of contamination of the vaccine with extraneous pathogens. Potential contaminants include bovine leukosis, mycoplasma, bovine viral diarrhoea virus, BSE or other *Theileria* species transmitted by *R. appendiculatus* and alongside other bacteria and viruses.

In the case of different stocks being used on the same premises, problems with labels could be minimised by using appropriate pencils and clear codes. Preparation of different stabiles should be done sequentially to avoid cross-contamination and mislabelling. Regular quality control should be introduced to ensure the right stock(s) are used.

### 2.1.3. Validation as a vaccine strain

A vaccine strain should be identified in cross-immunity trials. These are set up between a vaccine strain and stabilates from *T. parva* field isolates from the area where the protection of the vaccine strain is required. Ideally, five animals and two controls should be used per test, taking into account that these should be set up in two ways. First using the vaccine as a challenge in animals immunised with the local isolates and confirmed by subsequent homologous challenge. Secondly, using the local strain(s) as challenge in animals immunised with the vaccine and confirmed by subsequent homologous challenge. This will give information as to what extent a vaccine strain will give protection. On the other hand, results will indicate if a breakthrough might occur from the local *T. parva* population, present in carrier animals in the region where the vaccine will be deployed. A second test consists in testing if infection with the vaccine strain can be controlled by the intended tetracycline treatment during the vaccination process.

## 2.2. Method of manufacture

### 2.2.1. Procedure

Vaccine batches are produced in several institutes in eastern Africa, using different strains, possibly requiring different parameters than the ones described here. For consistency in immunisation in field, it is essential that tick-derived sporozoite stabilates of an immunising stock are prepared from a fully characterised 'working seed stabilate'. The 'working seed stabilate' should be derived directly from the reference 'master seed stabilate', which is available in suitable quantity for future preparation of immunising stabilates. Immunising stabilates can be prepared according to a proposed set of standards (Morzaria *et al.*, 1999b).

### 2.2.2. Requirements for ingredients

Before starting to produce vaccine, seed material with known characteristics is required. Three types of seed material are distinguished:

i)      Master seed

The master seed is a cryopreserved sporozoite stabilate from a specific stock that has been selected and permanently stored and from which all other seeds are derived. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. As *T. parva*-infective sporozoites are used for the manufacturing process, the master seed also represents the master stock (see Chapter 1.1.8). To prepare a master seed, *T. parva*-infected adult *R. appendiculatus* ticks are used that have fed as

nymphs on the ears of an animal going through an active ECF infection. After moulting, these ticks, when prefed during 4 days on rabbits will have infective sporozoites in their salivary glands. These can be quantified by dissecting prefed ticks and determining infection rates in the dissected salivary glands by coloration (Walker *et al.*, 1981). By grinding these prefed ticks in a specific medium, sporozoites will be released in the supernatants and a stabilate can be produced (FAO, 1984) that can be cryopreserved in minimum 100 cryotubes each containing preferably a set of infected acinus equivalent per vial or in case of a new stock an equivalent of 10 infected ticks per vial. A viability check of the master seed should be performed once the master seed has been cryopreserved for at least 24 hours by reviving one of the cryotubes.

ii)   Working seed

The working seed is derived from infective sporozoites at a passage level between the master seed and the production seed. To prepare a working seed, the contents of a single cryotube of master seed are injected into a naive healthy experimental animal to produce an acute ECF infection.

iii)   Production seed

To prepare a vaccine batch, the contents of sufficient cryotubes of working seed is mixed and the appropriate dose is injected into the required number of naive experimental cattle to produce an acute ECF infection.

Infection is established, with the working seed stabilate of *T. parva,* by inoculation of healthy cattle serologically and, ideally, PCR-negative for tick-borne diseases. During the parasitaemic phase of the ensuing disease reaction, clean laboratory-raised nymphs of *R. appendiculatus* are fed on the animals, and the engorged infected ticks are collected. The resultant adult ticks, within 3 weeks to 4 months after moulting, are applied in ear-bags to healthy rabbits. About 600 ticks are applied to each ear and unattached ticks are removed after 24 hours. After 4 days, the ticks are removed and samples (usually 60 ticks) taken to determine infection rates in dissected salivary glands. The remaining ticks are counted into batches of approximately 1000. An estimate of the total number of ticks can be obtained by counting and weighing a given number of ticks and then weighing the total number of ticks. The ticks are washed in a sieve under fast flowing tap water and may be surface disinfected in 1% benzalkonium chloride, or in 70% alcohol, and then rinsed again in distilled water.

The ticks are placed (~1000) in heavy glass specimen jars or plastic beakers, and 50 ml MEM with Hank's or Earle's salts and 3.5% bovine plasma albumin (BPA) is added. The jars are kept on ice, and the ticks are ground using a tissue homogeniser (for instance Silverson LR2) for 2 minutes using a large aperture disintegrating head, and for 3 minutes using a small aperture head (emulsor screen). For smaller batches, an alternative method may consist of grinding the ticks, in batches of 1000 ticks using a mortar and pestle. Ticks are then crushed continuously by teams of two people for 15–30 minutes in a mortar; 30–35 ml cooled MEM/3.5% BSA medium, without glycerine with 50–100 g glass is initially used. The difference (from 50 ml) MEM/BSA without glycerine is used to rinse the mortar and pestle and glass material used in crushing the ticks. Note that most of the crushing is done at the sidewalks of the mortar. Check for good crushing under a stereoscopic microscope and otherwise add glass. ALWAYS KEEP MEDIA AT 4°C.

The ground-up tick material is made up to 50 ml for every 1000 ticks, then centrifuged at 50 $g$ for 5 minutes, and the supernatant is harvested. An equal volume of cold 15% glycerol in MEM/BSA is added dropwise while the tick material is maintained chilled on ice and stirred by a magnetic stirrer. The final volume will contain sporozoites from the equivalent of ten ticks/ml. The number of tick-equivalents/ml can be adjusted if parasite infection rates in a particular tick batch were either very high or very low. The final concentration of glycerol in the sporozoite stabilate is 7.5%.

The bottle containing the ground up tick material (gut) is fitted with a dispenser. Cryotubes of 1 ml are filled with the stabilate (1 ml per vial) while constantly stirring on an ice bath. Aliquots are kept at 4°C. Alternatively, artificial insemination equipment, as used to dispense semen, has been used with pre-labelled plastic straws. This latter system is ideal for large volume stabilates, and colour

coding and labelling provide additional check on the identity of the immunising stabilate. An equilibration time of 30–45 minutes should be allowed

The aliquots are then stored in insulation trays and moved to a –80°C deep-freezer as soon as possible. They are kept there for 24 hours, to allow a gradual cooling down (step-freezing) of the stabilate. On the second day, the aliquots are transferred into liquid nitrogen until use. Alternatively vials can be introduced in gas-phase liquid nitrogen for 3 hours and then immersed in the liquid nitrogen for storage (Pipano, 1989b). Vaccine is transported to the field in liquid nitrogen. Vials are taken out at the place of vaccination, by immersing the liquid nitrogen vials in lukewarm water (38°C) for 30 minutes to allow for good regeneration.It should be administered within 60 minutes after withdrawal from the liquid nitrogen container. Once unfrozen it can be kept alive on ice (+4°C) for another 6 hours (Marcotty *et al.,* 2001; Mbao *et al.,* 2007).The infection and treatment method is usually applied using long-acting tetracycline intramuscularly, and it is recommended that the tetracyline be administered first, in case an animal escapes having received stabilate only. Thereafter the stabilate is inoculated subcutaneoulsy over the parotid lymph node at the base of the ear.

The procedure for the preparation and testing of a multi-valent ITM vaccine (the Muguga cocktail) has been described in detail (Patel *et al.,* 2016). It is important to note that each of the component stabilates is produced before combining the infected and fed ticks immediately before homogenisation. The number of ticks from each component is calculated to produce a final vaccine stabilate containing equal numbers of infected acini from each component.

### 2.2.3. In-process controls

Records of the source and passages of the working seed material should be maintained. Seeds should be free of infective agents like enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, bovine syncytial virus, Rift Valley fever, etc. Test procedures will depend on availability preferably using DNA analysis.

### 2.2.4. Final product batch tests

i)  Sterility

Tests for sterility and freedom from contamination of biological materials for veterinary use may be found in chapter 1.1.9.

ii)  Safety

Both ticks and experimental mammals are potential sources of contamination of stabilates with extraneous pathogens. In both cases, potential contaminants include *Ehrlichia bovis*, bovine *Borrelia* sp., orbiviruses, bunyaviruses, and others. Field-collected ticks should therefore not be used for the preparation of stabilates to be used for immunisation. Well characterised and pathogen-free laboratory colonies of ticks should be used for this purpose. Only healthy cattle and rabbits, free from tick-borne parasites, should be used for tick feeding. Stabilates should be prepared under aseptic conditions. In some circumstances, the use of antibiotics at concentrations appropriate for tissue culture may be indicated. Prepared stabilates should be subjected to routine tests for any viral infections in BHK and Vero cells (as above). Stabilates should be subjected to routine characterisation *in vivo*, which should involve infectivity testing in intact susceptible cattle, sensitivity to tetracyclines and other anti-theilerial drugs, and cross-immunity studies. A characterised 'working seed stabilate' should be prepared to ensure the purity of the *T. parva* stocks in the daughter immunising stabilate.

During stabilate preparation care must also be taken to avoid extraneous contamination of the stock being used with other *T. parva* stocks. Quality assurance procedures must be enforced, for example for the handling of infected ticks, and the rules should be adhered to rigidly. Tick unit facilities should allow for strict separation of infected and uninfected ticks. Tick unit personnel should use separate overalls for each batch of ticks used in stabilate preparation, and the overalls should be sterilised daily. Simultaneous work on many different stocks should be avoided. Stabilate storage systems should incorporate clear

labelling of each stabilate tube or straw also mentioning the preferred number of doses per vial or straw. This will vary according to use in small holder dairy or pastoral herds etc.

Quality control checks on the stabilate should determine the similarity to the parent seed stock and also detect any extraneous *T. parva* contamination.

iii) Batch potency

The evaluation of the number of acini infected with *T. parva* in dissected tick salivary glands, before grinding, is a useful indicator of the level of infection but does not take into account the variable loss of viability during stabilate preparation caused by the intensity of grinding and the freeze–thaw processes. Furthermore, the state of maturation of the sporozoites is difficult to estimate by histological examination of the tick salivary glands. Therefore, the infectivity of the stabilate is determined by inoculation of a standard dose of 1.0 ml into susceptible cattle. The contents of 2–4 randomly selected tubes are mixed and then titrated in cattle, and its infectivity and lethality at different dilutions are established for use in immunisation. As the response of cattle to the infection and treatment method is dependent upon their susceptibility to the infection, it is important to titrate stabilates in cattle of the same type as those to be immunised. Titration of vaccine stabilates remains a highly controversial matter. Ideally, a median infectious dose ($ID_{50}$) and median lethal dose ($LD_{50}$) should be determined by titration of the stabilate using a tenfold dilution range (Duchateau *et al.,* 1998; 1999). The $ID_{99+}$ (corresponding to close to 100% infectivity and having minimal lethality) should then be quantified by means of a finer titration, using dilutions around the $LD_{50}$. With respect to compound vaccines, quantification of the vaccine dose is complex as different strains need to be put together, changing the total lethality of the vaccine (Speybroeck *et al.,* 2008). The sensitivity to tetracyclines is also determined, essentially to provide a dose of stabilate that is controlled, preferably by a single dose of long-acting tetracycline administered at the same time as inoculation. The immunising dose should induce a very mild or inapparent infection, and the animal should develop a serological titre and be immune to lethal homologous challenge. Should a single treatment with tetracycline fail to suppress the infection in all cattle, then either a lower dose of the immunising stabilate or two treatments of tetracycline (on days 0 and 4) may be used. A single dose of 30 mg/kg long-acting oxytetracycline has been found to be effective in field immunisations, when used with an appropriate stabilate dilution. An alternative method that has been used involves stabilate infection and treatment with parvaquone at 20 mg/kg on day 8 (depending on the stabilate). This method can be applied where tetracyclines are not reliable, but it requires that the animal be handled more than once. A single treatment with buparvaquone at 2.5 mg/kg at the time of infection has also been shown to be effective with stabilate infections that were not controlled with a single treatment at 20 mg/kg of a long-acting formulation of tetracycline.

Once the procedure that results in a safe and effective immunising dose is established, it must be adhered to strictly in the field, or breakdown of immunisation may occur. It is also important that the stabilate dilution and drug/dose regimen be determined in the most susceptible cattle in which it is likely to be used.

## 2.3. Requirements for authorisation/registration/licensing

### 2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C2.2.2.1 and C2.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

### 2.3.2. Safety requirements

i) Target and non-target animal safety

*Theileria parva* sporozoites are not hazardous to humans, but they are infectious to bovines, and infection of naive animals for production of vaccine batches, as well as the titration experiments for dose quantification should be done in tick-free facilities. Tests for sterility

and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. At a meeting in Malawi in 1988, the following recommendations on safety in the preparation, handling and delivery of *T. parva* infection and treatment vaccines were adopted (Anon, 1989).

ii)   Reversion-to-virulence for attenuated/live vaccines and environmental considerations

The introduction of an immunising stock into an area/country from which it does not originate may result in that parasite, or a component parasite(s) of that stock, becoming established through a carrier state in cattle and transmission by ticks. The long-term effect of the introduction of new (and potentially lethal) parasites on the disease epidemiology should be considered before introduction, and should be monitored carefully following immunisation.

The characterisation of parasites in target populations should be carried out before immunisation, and at intervals following immunisation. At present the characterisation of parasite stocks with reference to vaccination relies primarily on immunisation and cross-challenge experiments in cattle. However a number of methods for characterising parasite stocks *in vitro* have been attempted in laboratories possessing a high degree of expertise. Early studies have shown that parasite stocks that differ in MAb profile may not cross-protect, whereas stocks showing similar profiles give cross-protection (Irvin & Morrison, 1987). However, in more recent experiments using other *T. parva* stocks, this observation has been proven to be wrong. Another method to detect antigenic differences used T cell clones specific for parasitised cell lines, as T cell responses are believed to be important in mediating immunity against *T. parva* and the strain specificity observed in *in-vitro* killing assays reflects the *in-vivo* challenge results. (Irvin & Morrison, 1987; Taracha *et al.,* 1995). Apart from this, there are no other, simpler *in vitro* assays that correlate with protection *in vivo*. Statistically derived disease reaction index, based on parasitological, clinical and haematological measurements, was proposed for characterising levels of infectivity and virulence of different parasite stocks and assessing the impact of control intervention against theileriosis (Rowlands *et al*., 2000; Schetters *et al*., 2010). Recently, DNA typing for characterisation of vaccine stabilates has been used and could be based on multi-locus genotyping using polymorphic antigen genes or satellite markers or a combination of both (Hemmink *et al*., 2016; Patel *et al*., 2011).

iii)   Precautions (hazards)

Care should be taken during the preparation of sporozoite stabilates to avoid aerosol infection of personnel with extraneous pathogens when ticks are being ground. Those grinding ticks should be educated in the potential hazards involved; access to areas where ticks are homogenised should be restricted to specified and informed personnel; personnel should wear protective clothing, including gloves and masks; and tick grinding should be carried out in a microbiological safety cabinet (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

### 2.3.3.  Efficacy requirements

i)   Capacity to protect against naturally transmitted theileriosis

Although successful final product batch testing (see Section C.2.2.4.iii) indicates that a vaccine batch is immunogenic, it is important to note that protection against homologous challenge does not necessarily indicate that the vaccine will protect against all *T. parva* strains encountered in the field, and that the 'needle' challenge used in pen trials does not necessarily reflect a challenge delivered by ticks. This can only be assessed by controlled field trials, which are expensive, complicated and lengthy. The need for and extent of field trials for each vaccine batch is the subject of debate and there is no agreed protocol. The least intensive approach, if veterinary authorities allow, is to closely monitor the initial 'roll-out' of the vaccine after successful final product batch testing as described above, with particular attention being given to the incidence of 'reactors' soon after vaccination and the occurrence of any 'breakthrough' cases following field exposure.

ii) Use in the field

The frozen vaccine is viably preserved in large liquid nitrogen repositories at the production facility and transported to farms in smaller liquid nitrogen containers. Field centres for storage and supply of vaccine can be set up in theileriosis-enzootic areas. The basic equipment required for field application of frozen vaccine includes a wide-mouthed jar for preparing a 38°C water bath, a thermometer for measuring the temperature of water, long forceps, face shield and temperature-resistant gloves. Application of the frozen vaccine to field cattle begins by putting on a face shield and temperature-resistant gloves. The required numbers of vials are withdrawn with the forceps from the canister of the liquid nitrogen refrigerator. When withdrawing the vials, the canister should be kept as deep as possible in the neck of the refrigerator to avoid quick warming of the remaining vials. Each withdrawn vial should be checked to ascertain that liquid nitrogen has not leaked inside. The liquid nitrogen does not alter the vaccine, but may cause the vial to explode when introduced in the water bath. Such a vial should be held at ambient temperature for 1–2 minutes to allow the nitrogen to escape and then processed in the usual way. The reported leaking of liquid nitrogen into a vial containing frozen vaccine has raised questions about the sterility of the frozen vaccine. However the system has been used for decades with no significant problem observed.

### 2.3.4. Breadth of immunity

Unlike *T. annulata*, where considerable cross-protection is observed among different strains in the field, a more complex situation exists for *T. parva*. Two strategies are used to try to overcome this antigenic complexity. A combination of three stocks, which provides a broad spectrum of protection, has been tested in a number of countries. Two large batches of a trivalent stabilate have been prepared by the International Livestock Research Institute (ILRI, Nairobi), the first in 1996 for the FAO and another one in 2008. These stabilates were prepared to the latest proposed standards and have been used safely and effectively in Tanzania. Further batches are being prepared at the African Union Centre for Ticks and Tick-Borne Diseases, Lilongwe, Malawi with increasing demand for the infection and treatment method of immunisation in *T. parva*-endemic areas in sub-Saharan Africa. If an immunising stabilate fails to protect against a 'breakthrough stock', this should be isolated, characterised, tested and considered for use, either alone, or as an addition to the current immunising stabilate. Another strategy is to prepare stabilates of national or local stocks for use within defined areas. This latter strategy is more costly in time and resources, but it avoids, to some extent, the introduction of new stocks into an area. With movement of cattle, there is a risk of the introduction of different stocks into an area, which may breakthrough the immunity provided by the local stock. Therefore the use of local or introduced stocks for immunisation needs to be carefully evaluated (Geysen, 2008; McKeever, 2007).

The infection and treatment method of immunisation is effective provided the appropriate quality assurance measures are enforced. In the longer term, the attendant delivery problems and the risk of induction of carrier states and disease transmission, emphasise the need for the identification of protective antigens for development of subunit vaccines.

### 2.3.5. Duration of immunity

There have been few reports of controlled experiments to determine the duration of immunity induced by infection and treatment, in either the presence or absence of field challenge. However, Burridge *et al.* (1972) established that cattle that had survived an experimental infection (without treatment), and were subsequently maintained in an ECF-free environment, survived a lethal homologous challenge up to 43 months later.

### 2.3.6 Stability

If kept in liquid nitrogen, the frozen vaccine has a practically unlimited shelf life.

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**NB:** There is a WOAH Reference Laboratory for theileriosis (see the WOAH Web site for the most up-to-date list: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the WOAH Reference Laboratory for any further information on
diagnostic tests, reagents and vaccines for theileriosis

**NB:** FIRST ADOPTED IN 1991; MOST RECENT UPDATES ADOPTED IN 2018.