

## Use of an *in vitro* culture system to detect *Theileria equi* strains from infected equids and/or reservoirs

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### Summary

A horse erythrocyte culture technique, partly modifying that originally developed by Holman, was used to detect the presence of *Theileria equi* strains in 12 horse and 2 mule blood samples. The animals were placed into four groups on the basis of their case history and laboratory test results: the mules and two horses were considered as infected and included in the 'recent infection' group, four horses with a history of past infection were included in the 'past infection' group and four animals subjected to anti-theileria treatment formed the 'treated animals' group. The final group consisted of two horses with an unknown history of infection. Ten *T. equi* strains were isolated and adapted *in vitro* from the fourteen animals tested: nine of these originated from the horse samples and one from mule blood. This is the first time that a *T. equi* strain isolated from a mule has been adapted in erythrocyte cultures. All strains isolated from horses showed growth and *in vitro* adaptation with a parasitaemia peak of over 10%. Following freezing and reculturing, adapted strains showed growth after a quiescence period of eight days. The method proved to be effective for culturing and replicating field *T. equi* strains from horses and mules. The technique was also able to identify carriers of infection which were

negative on microscopic examination, indirect immunofluorescence and complement fixation.

### Keywords

Complement fixation, Horses, Immunofluorescence, *In vitro* culture, Microscopic examination, Mules, *Theileria equi*.

### Introduction

Equine theileriosis is a blood cell infection transmitted by various species of ticks and caused by *Theileria equi* (4), a widespread protozoa infecting up to 90% of the world equid population (18). Transmitted naturally by *Hyalomma*, *Dermacentor* and *Rhipicephalus* ticks (6), in its cycle in vertebrate hosts, *T. equi* undergoes a pre-erythrocytic schizogonic phase in lymphocytes, leading to the formation of macroschizonts and microschantons. The latter contain a large number of merozoites, which lyse the host cells and penetrate the red blood cells (19). The intra-erythrocytic cycle includes the formation of trophozoites, merozoites and dividing forms: trophozoites have a diameter of less than 3  $\mu\text{m}$  and can have various shapes within the erythrocyte, while merozoites, which are generally piriform, can be found in pairs or groups of four in the cell, forming the 'Maltese cross' typical of *T. equi*.

The microscopic and serological tests commonly used for diagnosing theileriosis are not highly

sensitive or specific (21). Microscopic examination is generally positive in the acute phase of disease, but low levels of parasitaemia, which can be found even with acute symptoms, may not be detected (1). In serological diagnosis, the most common methods to detect anti *T. equi* antibodies are complement fixation (CF) and indirect immunofluorescence (IF). The latter is the test most frequently used, as it can reveal antibody concentrations earlier and for longer periods than CF: in fact, it can reveal antibodies from three days after infection up to four years in the absence of reinfection (2).

The ability to produce ad hoc recombinant and monoclonal antigens has enabled the development of highly sensitive and specific immunoenzymatic tests. Competitive enzyme-linked immunosorbent assay (ELISA) is currently the official test for the movement of horses (13). This method can detect antibodies just two days after infection, with high sensitivity and specificity. Recent progress in biotechnology has made the development of fast and simple molecular techniques possible. The use of DNA probes ensures high specificity and sensitivity, detecting blood infections even in animals with minimal parasitaemia (16); sensitivity and specificity can be improved further by using the polymerase chain reaction (PCR) to amplify a given nucleotide sequence (4). These features make PCR an excellent diagnostic technique, although it does require significant financial input (15).

In this study, a continuous *in vitro* technique for culturing *T. equi* was refined and its diagnostic ability evaluated against other routinely used tests.

## Materials and methods

### Animals and laboratory tests

Blood and serum samples taken from the jugular vein of 12 horses and 2 mules were tested for

equine theileriosis. Blood samples with anticoagulant were smeared on slides, fixed and tested for *T. equi* by 10% Giemsa staining (5). The percent parasitaemia for positive samples was then calculated using the conventional method (7). In serum samples, anti-*T. equi* antibodies were detected using CF (8) and IF (11, 12).

### *In vitro* cultures

The blood of all animals was cultured. Samples were washed three times through dilution in phosphate buffer solution (PBS), pH 7.2, and centrifugation at 800 g for 10 min.

The complete medium used to stabilise and maintain the cultures was prepared according to Holman *et al.* (10), replacing gentamicin with stabilised antibiotic antimycotic solution (Sigma, St Louis, Missouri) and removing AlbuMAX I.

The cell support (uninfected red blood cells) for the cultures was obtained from a donor horse found negative on serological and microscopic testing. The red blood cells were prepared as indicated by Holman *et al.* (10).

Erythrocyte cultures were prepared in 24-well tissue culture microplates and incubated at 37°C in a microaerophilic atmosphere (3% O<sub>2</sub>, 5% CO<sub>2</sub>, 92% N<sub>2</sub>), replacing the culture medium daily with fresh medium preheated at 37°C for 30 min (10).

Parasite growth and red blood cell condition were checked every other day by microscopic examination, for a maximum of 55 days from the start of culture.

Subcultures were performed on the basis of parasitaemia levels and/or degree of red blood cell damage (10). To evaluate the possibility of freezing, storage and conservation, a strain adapted *in vitro* was frozen in liquid nitrogen using 10% polyvinylpyrrolidone (PVP) (Sigma) or dimethyl sulphoxide (DMSO) (Sigma) as cryopreservatives, as described by Palmer *et al.* (14). To test viability, the contents of six vials of infected blood (three

frozen with PVP and three with DMSO) were rapidly thawed after 30 days and washed twice through dilution in PBS and centrifugation (800 g for 10 min). The sediment obtained from the same cryopreserved blood vials was pooled and the two pools that formed were resuspended in PBS and distributed in two wells of the microplate. They were then treated as in the previous phase.

## Results

### Diagnostic tests on animals

Table I shows the results for horse and mule blood samples examined microscopically and serologically for *T. equi* before culture preparation. The animals were divided into four groups on the basis of their case history and laboratory test results: the 'recent infection' group (group 1) included animals with a clear clinical picture, i.e. anaemia, jaundice and haemoglobinuria, associated with a positive microscopic and negative serological examination;

the 'past infection' group (group 2) included clinically healthy animals (negative microscopic and positive serological examination). The third group, 'treated animals' (group 3), consisted of healthy animals previously subjected to anti-theileria treatment, with negative results for both laboratory tests. The final group (group 4) consisted of two subjects with an unknown history of infection: one of these was positive for all diagnostic tests, while the other was negative on microscopic examination and positive for the serological test.

### *In vitro* growth

Growth and adaptation of *T. equi* was evident in 10 of the 14 cultured samples. Three strains were developed from the blood of the horses and one of the two mules in group 1; positive cultures were obtained from two of the group 2 horses, all group 3 horses and the group 4 horse positive for all laboratory tests (Table I).

Before adaptation, all the isolated strains underwent

Table I

Results of parasitological and serological tests for *Theileria equi* in horses and mules

Group	History	Animal ID	Species	Microscopic examination	Serological examination	HC result	Total animals
1	Recent infection	1	Horse	+	-	+	4
		2	Horse	+	-	+	
		3	Mule	+	-	+	
		4	Mule	+	-	-	
2	Past infection	5	Horse	-	+	+	4
		6	Horse	-	+	+	
		7	Horse	-	+	-	
		8	Horse	-	+	-	
3	Treated past infection	9	Horse	-	-	+	4
		10	Horse	-	-	+	
		11	Horse	-	-	+	
		12	Horse	-	-	+	
4	Unreported	13	Horse	+	+	+	2
		14	Horse	-	+	-	

HC horse red blood cell cultures

a period of quiescence of 2-4 weeks. In the erythrocyte cultures from the two horses with recent infection, the first growth was seen after one day and adaptation after 16 and 22 days. The strain isolated from the mule adapted after 20 days (Fig. 1). Strains isolated from group 2 animals showed the first signs of growth ten days after culture and stabilised after 27 and 37 days (Fig. 2). Growth and adaptation of *T. equi* in all erythrocyte cultures from treated animals was seen after 11 to 37 days (Fig. 3). In the horse with no reported history, *T. equi* was detected after 8 days and the strain adapted after 41 days (Fig. 4). Apart from the strain isolated from the mule, all adapted strains had a peak of parasitaemia of over 10% (Figs. 1, 2, 3 and 4).

The strain, frozen using either PVP or DMSO, readapted in culture after 8 days of quiescence and reached 10% to 12% parasitaemia after 12 days (Fig. 5).

## Discussion

Equine piroplasmiasis can occur in hyperacute, acute, subacute and chronic forms. The first two are characterised by a serious, sometimes fatal

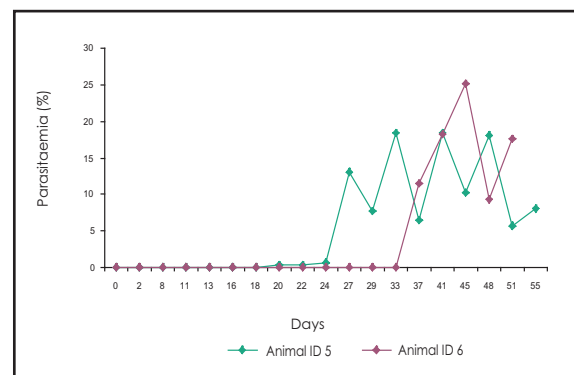


Figure 2  
Growth and adaptation of *Theileria equi* strains isolated in horse red blood cell cultures from the group of animals with a history of past infection

clinical picture, while the subacute and chronic forms are normally insidious and often unapparent. These insidious cases and the consequent formation of permanent reservoirs of infection are responsible for the vast worldwide distribution of the disease. The situation in Italy seems no different to that observed in other countries: the few national surveys conducted over the years have found *T. equi* and/or *Babesia caballi* antibodies in almost a third of the horses tested, almost all of which were attributable to the former – the more serious of the two diseases (3, 17, 20). However, despite

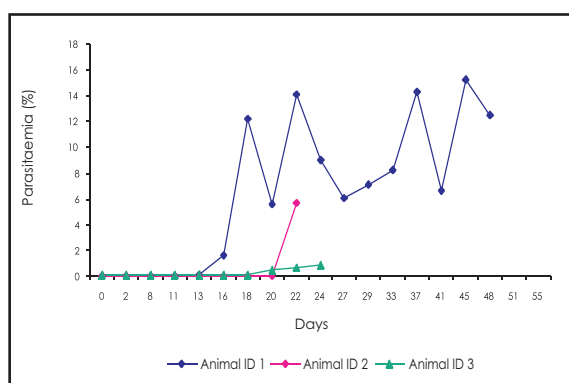


Figure 1  
Growth and adaptation of *Theileria equi* strains isolated in horse red blood cell cultures from the group of animals with recent infection

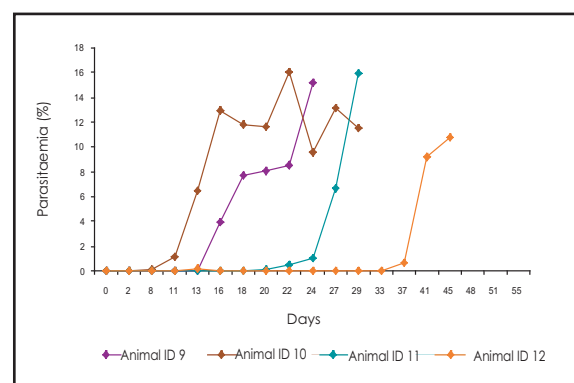


Figure 3  
Growth and adaptation of *Theileria equi* strains isolated in horse red blood cell cultures from the group of animals previously subjected to anti-theileria treatment

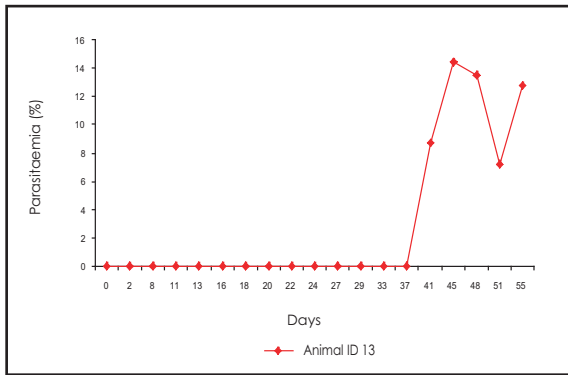


Figure 4  
Growth and adaptation of *Theileria equi* strains isolated in horse red blood cell cultures from the group of animals with an unknown history of infection

such a high percentage of infected animals, a corresponding number of clinical cases has not been encountered. It is therefore logical to suppose that in many areas of Italy, *T. equi* infection is in an endemic phase, which while protecting the horse from clinical signs of equine theileriosis, does not stop the animal from becoming infected and acting as a reservoir. This type of epidemiological situation is accompanied by considerable disadvantages, especially for the breeding of sports horses: many countries still require an import certificate attesting that the

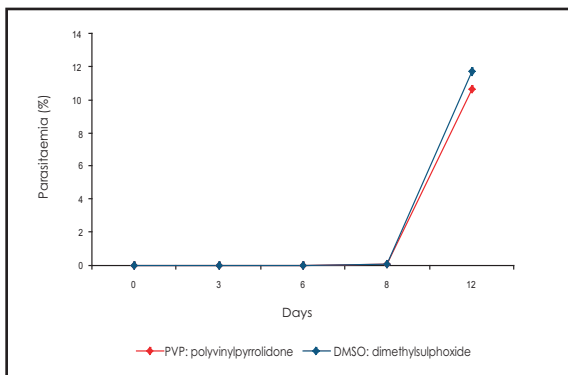


Figure 5  
Growth and adaptation of a *Theileria equi* strain in horse red blood cell cultures after storage in liquid nitrogen

animal was negative on serological testing for piroplasmosis. These infections are thus one of the major obstacles to the movement of horses. In epidemiological scenarios where equine piroplasmosis is mostly due to subclinical infection of *T. equi*, the importance of accurate diagnosis is obvious. While in the acute form, the clinical suspicion is easily confirmed in the laboratory by detection of the parasite in the Giemsa-stained blood smear, in the subacute and/or chronic forms, the absence of diagnostic suspicion is frequently combined with difficult-to-interpret laboratory data, making diagnosis and confirmation difficult if not impossible. Both microscopic and serological examinations are often negative in these forms, especially in the case of treated animals.

One of the objectives of this study was to adapt the technique for adaptation and maintenance of red blood cell cultures developed by Holman *et al.* (10) and modified in regard to some components, and evaluate it in line with the situation in Italy. In easily diagnosable situations, such as recent and/or chronic infections, the erythrocyte cultures used to isolate *T. equi* strains and identify reservoirs of equine infection gave results in conformity with those obtained with traditional laboratory methods. The most significant result was the identification of infected animals that were negative on microscopic and serological testing. In an epidemiological situation such as that in Italy, where insidious forms are those most commonly encountered, the use of erythrocyte cultures in equine theileriosis may thus be of considerable diagnostic importance. In addition, the growth of strains in culture not only demonstrates the presence of the parasite in an animal but can also reveal its viability, which other perhaps more sensitive direct methods such as PCR are unable to do. In contrast with any other diagnostic test, *in vitro* strain isolation is the only method that can be relied upon to identify reservoir animals, thereby



providing essential information to control the spread of infection.

In addition to its peculiar endemic tendency, one of the main causes of the substantial presence of theileriosis reservoir animals is related to the fact that to date there is no treatment capable of sterilising an infected animal. The *in vitro* technique used here to culture horse red blood cells enables isolation of *T. equi*, even from treated animals. This ability, as well as confirming the difficulty in sterilising the animal, suggests its possible use in monitoring the efficacy of a specific treatment.

*Theileria equi* is a protozoa which can also infect donkeys and mules. There is very little information on *T. equi* in these animals, and this seems to be the first time that a *T. equi* strain isolated from a mule has been adapted in an erythrocyte culture. The parasitaemia percentage that was lower than that recorded with strains isolated from horses may have been because the technique did not use red blood cells from mules.

In conclusion, the method used in this study was effective for the *in vitro* culture and replication of wild *T. equi* strains from horses and mules. The detection of parasites in samples found negative to both microscopic and serological tests demonstrates that this method is able to identify carriers of the infection and verify the efficacy of any treatment. It may also be an important diagnostic tool to be used with routine methods. The cultivation of *T. equi* strains *in vitro* also means that diagnostic test reagents can be prepared without the use of laboratory animals (9) and offers the possibility of isolating and studying national strains of *T. equi* in detail, comparing them with those from other countries.

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