

## Field trial of immunization with an experimental vaccine against Mediterranean theileriosis in Spain

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**Abstract** — Vaccines against Mediterranean theileriosis have been developed in several countries where this disease is of economic concern. Until recently, tissue culture vaccines were a suitable method for immunizing cattle and they have been widely applied with success. In Spain, Mediterranean theileriosis is an obstacle to the improvement of dairy cattle productivity. No vaccines against this disease have been applied until recently. This report concerns the field trial of an available experimental tissue culture vaccine consisting of attenuated *Theileria annulata* schizont infected cells from an enzootic area of Spain. The vaccinated cattle developed a typical post-vaccination immunological response and were resistant to a field challenge. They showed no clinical signs of theileriosis while 50% of the control cattle showed typical signs of the disease and two of them died (12.5% of control cattle). This vaccine may be useful to protect cattle against Mediterranean theileriosis in enzootic areas of Spain.

**Mediterranean or tropical theileriosis / *Theileria annulata* / tissue culture vaccine / field immunization trial / Spain**

**Résumé** — Essai d'immunisation sur le terrain avec un vaccin expérimental contre la theilériose méditerranéenne en Espagne. Des vaccins contre la theilériose méditerranéenne ont été développés dans plusieurs pays où cette maladie pose un problème économique. Jusqu'à présent, les vaccins produits en cultures de tissus se sont révélés être une méthode d'immunisation des bovins convenable et largement appliquée, avec des résultats très positifs. En Espagne, bien que la theilériose méditerranéenne soit une entrave à l'amélioration de la production des vaches laitières, aucun vaccin n'a encore été utilisé pour lutter contre cette maladie. Nous rapportons ici un essai sur le terrain d'un vaccin expérimental produit en culture de tissus (cellules infectées avec des schizontes de *Theileria annulata* atténués, isolés dans une aire enzootique d'Espagne). Les bovins vaccinés ont développé une réponse

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immunologique classique après vaccination ; de plus, ils se sont montrés résistants à une infection naturelle. Ils n'ont montré aucun signe de theilériose, alors que 50 % des animaux du groupe témoin ont présenté des signes typiques de la maladie, et que deux (12,5 %) d'entre eux sont morts. Ce vaccin devrait donc s'avérer utile pour la protection des bovins contre la theilériose méditerranéenne dans les zones enzootiques d'Espagne.

**theilériose méditerranéenne ou tropicale / *Theileria annulata* / vaccin produit en culture de tissu / essai de terrain / Espagne**

## INTRODUCTION

Mediterranean or tropical theileriosis is a disease caused by the protozoan parasite *Theileria annulata*, which affects cattle within a wide geographic zone, from the Mediterranean littoral of Europe and Africa, through the Near and Middle East, part of the former USSR, to India, and China (Purnell, 1978; Dolan, 1989).

In several countries where tropical theileriosis occurs as an enzootic disease, field immunization has been carried out using local strains. Tissue culture vaccine has been applied in Israel since the sixties (Pipano and Tsur, 1966; Pipano, 1977, 1995). In 1973, researchers from Iran isolated local strains of *T annulata* that made it possible to carry out a vaccination campaign for 14 years (Hashemi-Fesharki, 1988). In the former USSR, vaccinations over periods of several years have been carried out with excellent results (Stepanova and Zablotskii, 1989). Studies in India started by establishing *T annulata* cultures (Gill et al, 1976) and are currently continuing with trials of vaccines against local strains (Singh et al, 1993).

It is considered that any *Theileria annulata*-isolate can be attenuated by in vitro culture, so it should be possible to immunize cattle with local isolates, and thus avoid complications due to a particular strain (Pipano, 1977, 1989).

Until recently, there was no experience of field immunization against Mediterranean theileriosis in Spain, although piroplasmosis

is known in the country since the first third of this century (Salvans- Bonet, 1928). Every year widespread clinical cases of this disease are reported in the existing enzootic areas (Brandau et al, 1989; García-Fernández and Viseras, 1995; García-Fernández et al, 1996).

## MATERIALS AND METHODS

A farm of Friesian dairy cattle with 108 animals was selected for the trial. This farm was located in an enzootic area (province of Cádiz) of Southern Spain and presented a morbidity rate of 25% and a mortality rate of 12.5% per year due to theileriosis.

Serologic testing of the herd using immunofluorescent antibody test (IFAT) (Burrige and Kimber, 1972; García-Fernández et al, 1996) was undertaken to assess the extent of the natural challenge present on the farm (Ozkoc and Pipano, 1981). The antigen was the schizonts of *T annulata* from a culture kindly provided by Dr E Pipano (Kimron Veterinary Institute, Beit Dagan, Israel).

### Cattle selection for the vaccination batches

For this trial, 48 animals were placed in four groups including a control group:

- group 1 (control): 16 calves, 10-24 months old, presenting no specific antibodies against *T annulata*;
- group 2: 11 calves, 10-24 months old, presenting no specific antibodies against *T annulata*;
- group 3: five cows, over 3 years old, presenting no specific antibodies against *T annulata*;

— group 4: 16 cows, over 3 years old, presenting specific antibodies against *T. annulata* (titres up to 160).

Groups 3 and 4 were inoculated with a single dose of vaccine ( $1 \times 10^7$  cells). Group 2 was divided in two: sub-group 2a (six calves) was inoculated similarly to groups 3 and 4; and sub-group 2b (five calves) was inoculated with a double number of cells dose ( $2 \times 10^7$  cells) in one shot in order to determine the effectiveness of a single dose.

The 60 remaining animals presented titres higher than 160 against *T. annulata*, and were not inoculated.

The challenge consisted in exposing the animals to natural infection since the farm is situated in an enzootic area (see above).

### Immunization and monitoring of the cattle

The vaccine was an attenuated culture of *T. annulata*-infected bovine lymphoblasts. The cells were isolated from a clinical case of Mediterranean theileriosis and cultured until attenuated (Viseras, 1994; Viseras et al, 1997). The vaccinating cells from 70-80 passage cultures were cultured in RPMI-1640 (Bio-Whittaker, Walkersville, MD, USA) + 20% (v/v) fetal calf serum (Sigma Chemical Co, St Louis, MO, USA) inactivated by heat (56 °C, 30 min) in a monolayer in 75 cm<sup>2</sup> plastic culture flasks (TPP, Trasadingen, Switzerland) in an automatic CO<sub>2</sub> incubator (Forma Scientific, Marietta, OH, USA) at 37 °C in an air/5% CO<sub>2</sub> humid atmosphere. The cells were treated with a detaching solution (EDTANa<sub>3</sub>, 0.025% in phosphate buffer saline (PBS) pH 7.2), collected and their viability determined in an aliquot using Trypan blue exclusion test and haemocytometer. The cells were then centrifuged (400 g, 10 min). The cell pellet was dispersed in culture medium with 8% dimethyl sulfoxide (DMSO) at 4 °C in order to obtain a suitable cell concentration ( $1 \times$  or  $2 \times 10^7$  cells/mL). The suspension was then distributed in 1 mL aliquots into sterile cryotubes and kept at 4 °C for 20 min, and then at -70 °C overnight. The cryotubes were kept in liquid nitrogen until needed.

A bunsen burner, a heat resistant stainless steel tray and a thermometer were used during the actual immunization process.

Before being administered, the frozen vaccine must be resuspended. Therefore, a water bath at 37 °C was prepared and the vials containing the cells were introduced for quick thawing. Once thawed, each vial was diluted with PBS (pH 7.2) to a volume of 5 mL and inoculated subcutaneously within 30 min of thawing. The vaccinations were carried out in February.

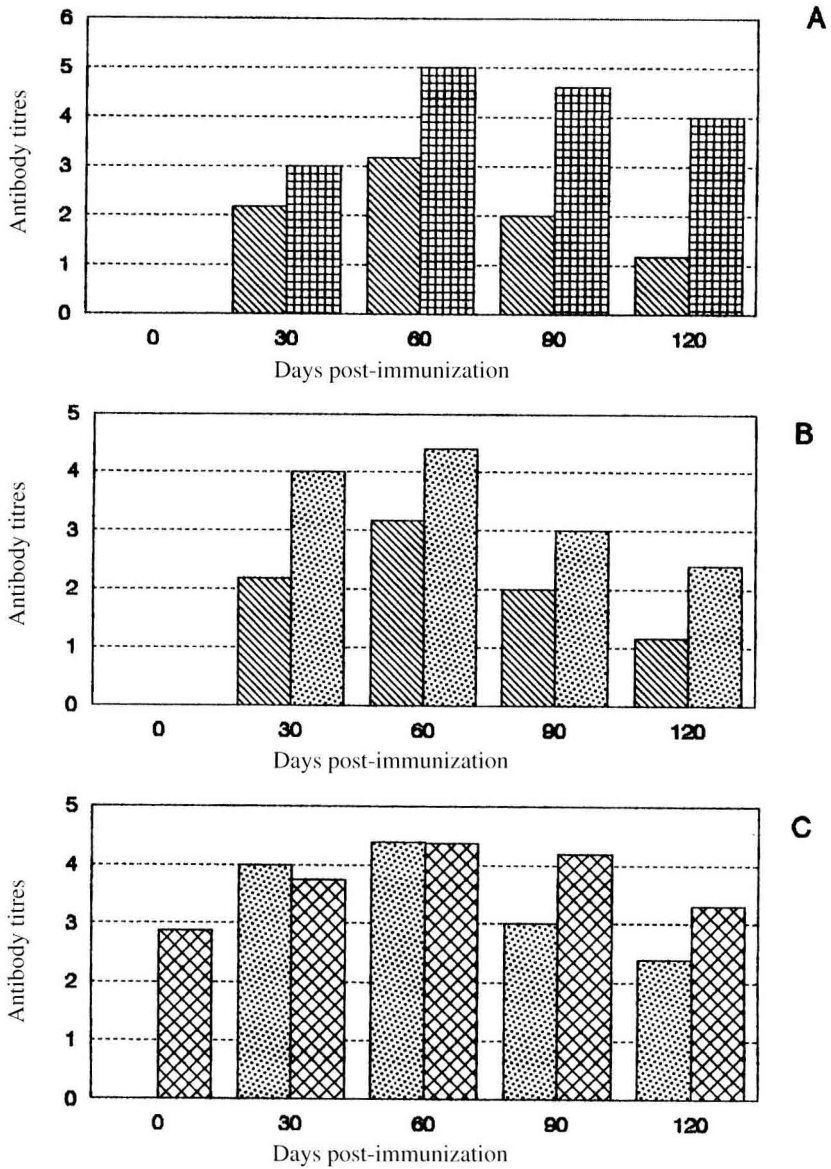
The immunization process was monitored by controlling the antibody response to *T. annulata* following the suggestions of several authors (Pipano et al, 1969; Pipano, 1971; Subramanian et al, 1989). Blood samples were taken from the animals on the day of vaccination (day 0), and on days 30, 60, 90 and 120. The samples were processed for IFAT using the antigen mentioned above. The herd was monitored for two years following the vaccination. It consisted in looking out for clinical signs (fever, lymph nodes enlargement, jaundice, anaemia). When these signs were observed, samples of peripheral blood were collected to prepare thin smears. In fatal cases, post-mortem samples of blood, lymph nodes, liver, kidney, and spleen were taken, when considered necessary. Diagnosis of clinical cases of Mediterranean theileriosis was confirmed when the parasites were seen in the Giemsa stained blood and tissue smears examined under the light microscope. All events such as clinical cases and deaths were recorded.





Following this trial, the control group and any newly introduced cattle were vaccinated in April to control the illness in the farm.

### RESULTS

Once all the antibody titre data were collected (over a 4-month period post-immunization) and all the clinical and fatal cases were recorded (the typical delay before the appearance of clinical cases of the disease in this area, from June to September mainly, according to our experience), the effectiveness of the immunization was assessed.

To test the effect of the number of cells per dose, two groups of cattle, sub-groups 2a and 2b, were inoculated with vaccine at  $1 \times 10^7$  and  $2 \times 10^7$  cells per animal respectively. The evolution of the antibody titres was similar in both groups although the titres were clearly higher in sub-group 2b, as



**Fig 1.** Evolution of specific antibody titres against *Theileria annulata* throughout the post-immunization period. A) Comparing sub-groups 2a and 2b immunized with single and double doses of vaccine, respectively. B) Comparing sub-group 2a (10-24 months old) and group 3 (3 years old) immunized with single doses of vaccine. C) Comparing groups 3 (negative by IFAT to *T. annulata* with titres up to 160) and 4 (positive by IFAT to *T. annulata* with titres up to 160) immunized with single doses of vaccine. Presence of specific antibodies against *T. annulata* by IFAT: titre 40 (1), titre 80 (2), titre 160 (3), titre 320 (4), titre 640 (5), and titre 1280 (6). Symbols: sub-group 2a , sub-group 2b , group 3 , and group 4 .

shown in fig 1A, especially from 60 days post-immunization.

Taking into account the age of the cattle, two groups of different ages (sub-group 2a, 10-24 months old, and group 3, 3 years old or over) were immunized with the same vaccine doses,  $1 \times 10^7$  cells per animal. In this case, the evolution of the specific antibody titres was also similar, although the response was higher for group 3 than for sub-group 2a, as shown in fig 1B.

Fig 1C shows the evolution of the specific antibody titres of cattle with (group 4) or without (group 3) specific antibodies against *T annulata* on day 0 of the vaccination. The specific humoral response presented a maximum at 60 days post-vaccination in both groups, but this response decreased more rapidly in animals from group 3.

The clinical cases in the herd were recorded to assess the actual effectiveness of the vaccine. No clinical case was reported among the cattle selected for the vaccination groups, nor among the other animals who had been eliminated because of their high antibody titres. Eight clinical cases (50%) were found in the control group and two of them (12.5%) had fatal consequences.

We recorded the events on the farm over the next two years and there were no clinical cases of theileriosis among the vaccinated cattle. In the second year, however, the farmer introduced new unvaccinated cattle, and several clinical cases occurred among them.

Afterwards, the immunization of the susceptible cattle was carried out, which led to the control of theileriosis on the farm, as there were no further clinical cases of this disease within the subsequent 6 months (including the June-September period).

## DISCUSSION

A farm with high levels of morbidity and mortality related to Mediterranean theile-

riosis was selected for this study. It was considered to be particularly suitable to assess the effectiveness of the vaccine as a natural challenge from ticks was certain to occur.

As many of the animals would have been exposed to theileriosis throughout their lives in this enzootic area and might have been already naturally immunized, it was necessary to carry out a serological evaluation of the herd first.

After vaccination, the animals were monitored to check for potential clinical cases. The specific antibody titre evolution was monitored as well as recommended by several authors to follow the immunization process (Pipano et al, 1969; Pipano, 1971; Subramanian et al, 1989).

In addition, Pipano (1989) suggested that the production of specific antibodies is useful to assess the success of the vaccination, since the inoculation of dead schizonts does not induce a significant level of antibodies. None of the vaccination groups had specific antibodies against *T annulata*, at the beginning of the study except group 4. All the animals developed a humoral response with maximum titres approximately 2 months post-vaccination (fig 1). Other authors have obtained similar results (Pipano and Cahana, 1968; Pipano et al, 1969; Pipano, 1970; Ozkoc and Pipano, 1981). Sub-group 2b, immunized with doubled dose ( $2 \times 10^7$  parasitized cells) of vaccine, developed a higher response than sub-group 2a (fig 1A), but the effectiveness of the vaccine was apparently the same, in protecting the cattle against Mediterranean theileriosis.

The intensity of the specific humoral response of cattle of sub-group 2a was lower than in group 3 (fig 1B), probably because these animals were younger (10-24 months old). In this sense, Reddy et al (1994) have also found that the animals aged between 2 and 3 years showed a significantly higher antibody response compared to animals under 2 years old.

When the specific post-vaccination humoral response of groups 3 and 4 was compared, the observed intensity was different after the second month post-vaccination (fig 1C). The higher intensity of this response for the previously positive animals could be due to a secondary response against *T annulata* (group 4) in contrast to a possible primary response of the animals in group 3.

This vaccine has been previously tested with cattle inducing a successful protection against an experimental challenge of virulent schizonts (Viseras, 1994). Bearing in mind that the vaccinated cattle have been protected (clinical cases occurred only within the non-vaccinated susceptible control group) for, at least, 2 years against the disease, the duration of protection induced by the vaccine appears sufficiently lasting to allow safe introduction of vaccinated cattle into enzootic areas and provides for the development of a long-term protection after natural exposure, since, according to Pipano (1979), the immunity produced by the vaccine against *T annulata* will actually be reinforced by exposure to infected ticks in the field. Therefore, the vaccine may be useful for vaccination of cattle in enzootic zones, assuming it is used before natural exposure of animals to *T annulata* in these areas occurs.

We consider that this is a potential vaccine that provides good protection against Mediterranean theileriosis, at least in the area of Spain where this immunization trial took place.

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