

E. Pipano¹ | **Comparison of three methods for
initiating *in vitro* cultures of *Theileria
annulata* schizonts**
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PIPANO (E.), SHKAP (V.), FRANK (M.). Comparaison de trois méthodes de préparation de cultures *in vitro* de schizontes de *Theileria annulata*. *Revue Élev. Méd. vét. Pays trop.*, 1989, 42 (4) : 529-533.

Trois méthodes de préparation de cultures *in vitro* de *Theileria annulata* provenant de bovins infectés ont été comparées : 1) la séparation des cellules du caillot blanc par centrifugation dans une seringue en plastique ; 2) la séparation des lymphocytes par un gradient commercial Ficoll-Paque et 3) la trypsinisation de morceaux de foies prélevés par biopsie. Des cultures positives ont été obtenues dans 26 cas sur 48 pour la méthode numéro 1 (54 p. 100), 28 cas sur 56 pour la méthode numéro 2 (50 p. 100) et 17 cas sur 21 pour la méthode numéro 3 (81 p. 100). Une fois mises en route, les cultures peuvent être repiquées pendant plusieurs années, quelle que soit la technique utilisée. *Mots clés* : Bovin - *Theileria annulata* - Culture *in vitro*.

INTRODUCTION

Theileria annulata is a tick-transmitted protozoan parasite that affects cattle in the Mediterranean area and in a considerable part of Asia. Two main developmental stages of the parasite are known in cattle intralymphocytic schizonts and intraerythrocytic merozoites. *In vitro* multiplication of the schizonts in bovine spleen explants of plasma clots was first described by TSUR (6). Subsequently, schizont-infected monolayer and suspension cultures were obtained with cells from spleen, liver and lymph nodes of *T. annulata*-infected cattle (2, 7, 9), or from peripheral white blood cells of such cattle (3, 4, 8). In no case was the percentage of success in initiating viable cultures reported.

The present report compares the success rate of 3 practical methods for initiating schizont cultures from infected cattle : two using peripheral blood and one using liver biopsy material.

MATERIALS AND METHODS

Source of parasites

Seven 2-10 months old calves were inoculated with *T. annulata*-infected blood. On the first day of fever,

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lymph node and liver biopsies were performed as described by SERGENT *et al.* (5). Smears of biopsy material were stained with Giemsa and examined for schizonts. When schizonts were detected material for cultivation was obtained as described below.

Initiation of cultures

All equipment and reagents in the following procedures should be sterile.

Method 1

Separation of buffy coat cells by centrifugation in a disposable plastic syringe

The jugular vein area of an infected calf was clipped, shaved and disinfected. Using an 18 g x 1.5 inch disposable needle blood was drawn into a 10 ml polypropylene disposable syringe containing 0.2 ml of 0.1 per cent heparin in phosphate buffered saline (PBS). Leaving the needle in place on the syringe, the needle sheath was replaced and the rod of the now extended piston was sheared with scissors at the level of the barrel. The syringe with the sheathed needle in place was centrifuged, needle side up, for 20 min at 2,000 rpm at 4 °C. The syringe now showed a thin layer of buffy coat between an upper plasma layer and a lower red cell layer. With the syringe retained in an upright position, needle side up, the sheath of the needle was removed and the needle carefully bent about 90 ° with a sterile forceps. The piston was now slowly driven upward with the help of the forceps in order to discharge and discard the plasma layer. The next few drops containing the buffy coat were delivered into a separate centrifuge tube containing 5 ml of PBS and the syringe with the red cells was discarded. The buffy coat cell suspension was centrifuged for 10 min at 1,200 rpm and the sedimented white blood cells (WBC), usually accompanied by red blood cells (RBC), were resuspended in 5 ml of culture medium. The WBC were counted in a hemocytometer and viability was determined, using the trypan blue exclusion test. The cell suspension was diluted with cell culture medium to obtain the concentrations shown in table I. Five millilitres of the cell suspension were introduced into 75 ml plastic culture flasks and incubated at 37 °C.

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Method 2

Separation of lymphocytes using Ficoll gradient

A commercial Ficoll-Paque (*) set was employed with a slight modification in the recommended technique (1).

Blood from an infected calf was drawn with a 10 ml plastic syringe containing 0.2 ml of 0.1 per cent heparin in PBS. The blood was ejected into a 50 ml Erlenmeyer flask containing an equal volume of Balanced Salt Solution (BSS) prepared according to the instructions included in the set. Three ml of Ficoll-Paque solution were introduced into each of 4 siliconized tubes and to each tube 4 ml of diluted blood were carefully layered over the contents. The tubes were centrifuged for 30 min at 1,800 rpm (400 g) at room temperature (about 22 °C).

After centrifugation the upper transparent layer of plasma was removed with a pasteur pipette and the next thin, opaque layer containing lymphocytes was transferred with the same pipette to four 15 ml centrifuge tubes each containing 6 ml BSS. The suspensions were centrifuged for 10 min at 1,200 rpm in a refrigerated centrifuge. The pellets were pooled, resuspended in 5 ml culture medium and the WBC were counted as above. After being diluted to the concentrations shown in table I, the suspensions were transferred to 5 ml flasks and incubated at 37 °C.

Method 3

Liver biopsy

The right rib area of the calves was clipped and shaved at a point on the next to last intercostal space, about 10 cm below an imaginary horizontal line traced on the level of the tuber coxae. A shallow incision 0.5 to 1 cm long, was made in the skin. An aspirating hypodermic needle (1.4 mm by 100 mm) was inserted through the incision in the direction of the sternum to a depth of 7-8 cm. Eight to ten puncture movements were made within the abdomen before withdrawing the needle. The collected material was ejected by a syringe into a tube containing 5 ml PBS. The tube was centrifuged for 10 min at 1,200 rpm and the supernatant discarded. Five ml of 0.25 per cent trypsin solution in PBS was added to the sediment and the tube was hand shaken for about 5 min at room temperature. The suspension was allowed to settle for about 1 min and the supernatant was transferred by pipette to a tube containing 1 ml calf serum. The sediment was

subjected to treatment with 5 ml of 0.25 per cent trypsin as before and the entire suspension was added to the first harvest. The suspension was centrifuged for 10 min at 1,200 rpm, the supernatant was discarded and the sediment resuspended in 5 ml culture medium. The number of viable dispersed cells was evaluated and the suspension was transferred into a 75 ml plastic culture flask and incubated at 37 °C.

In all methods described medium was replaced 24 hours after initiation of the cultures and then every 2-3 days.

Culture medium and subcultivation

Eagle's Minimum Essential Medium supplemented with 20 per cent calf serum was used. Penicillin (200 units/ml), streptomycin (100 µg/ml), and mycostatin (75 units/ml), were added to the medium. Primary cultures were maintained up to 3 weeks by changing medium every 2-3 days. When multiplication of cells occurred, the cultures were rinsed with PBS and the cells were detached with 0.025 per cent EDTA (versene) in PBS. Dispersed cells were suspended in culture medium to a final concentration of 2×10^5 cells/ml, dispensed into new culture vessels and incubated at 37 °C. Further passages were carried out in the same way.

Observation of cultures

Living cultures were examined daily on the inverted microscope at 200 x magnification.

For the detection of intracellular schizonts the supernatant fluid of the cultures or cells released by dispersing the culture monolayer with EDTA, were smeared onto slides and stained with Giemsa.

Alternatively, sterile cover-slips introduced into culture flasks were removed after 24 hours of incubation, air dried, fixed with methanol and stained with Giemsa.

RESULTS

Cultures initiated by methods 1 and 2

The total number of WBC obtained from 10 ml of blood by method 1 and 8 ml of blood by method 2 varied between 10^6 - 10^7 cells and between 3.5×10^6 - 8×10^6 , respectively. Buffy coat cells obtained by method 1 were always contaminated with RBC whereas those obtained by method 2 were practically free of such cells. Following incubation of 3 to 16 days,

(*) Ficoll-Paque, Pharmacia, Uppsala, Sweden.

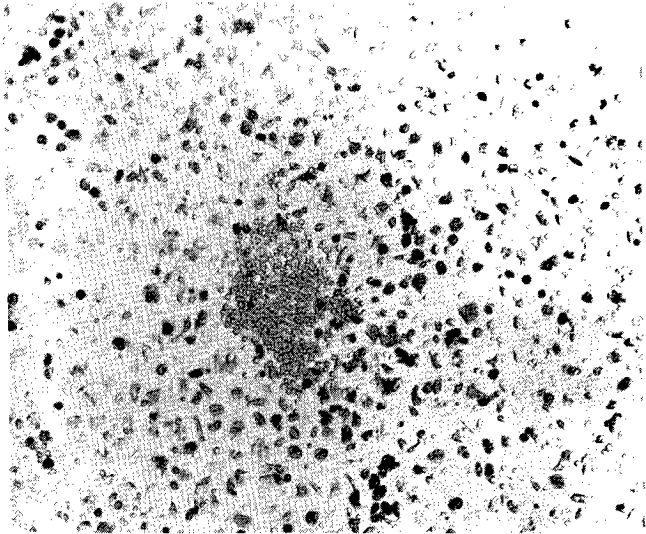


Fig. 1 : Colony of round lymphoblastoid cells in a 12-day old primary culture from buffy coat separated in a plastic syringe (x 100).



Fig. 2 : Same colony as in Fig. 1. Note surrounding degenerated non-infected WBC (arrow) (x 400).

small, refractile, round cells appeared in the cultures as colonies of various size (Fig. 1, 2) or as a diffused growth (Fig. 3). Cells were also observed floating in the supernatant medium. In Giemsa stained preparations, the round cells appeared as lymphoblastoid types containing *T. annulata* schizonts (Fig. 5). A considerable portion of the cells in cultures initiated from buffy coats showed signs of degeneration. During the first medium replacements of the syringe-derived cultures (method 1) erythrocytes were washed away. The degenerated cells seen in cultures started by both methods were also washed away during subcultivation leaving pure cultures of schizont-infected cells. The average percentage of successful cultures obtained from buffy coat cells by method 1 was 54 per cent, versus 50 per cent for cells separated by method 2 (Table I).

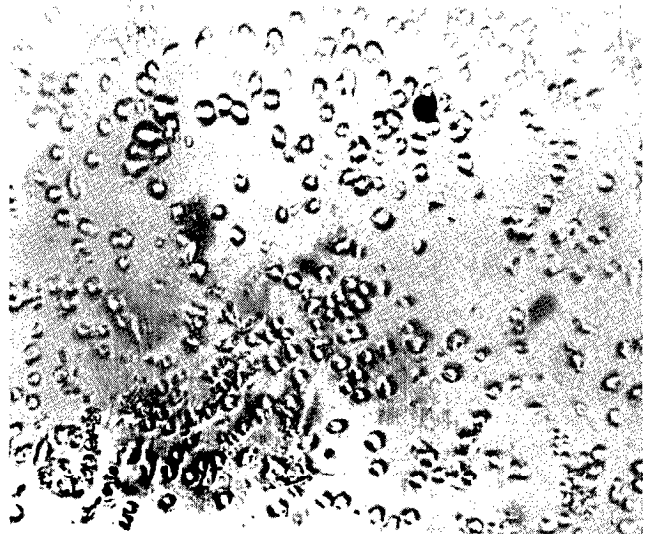


Fig. 3 : Primary culture containing only lymphoid cells derived from blood passed through a Ficoll-Paque gradient (x 300).

TABLE I Relative success of 3 different methods of initiating in vitro cultures of *T. annulata* schizonts.

| N° of cells per ml in initial culture ($\times 10^6$) | Method 1 centrifugation in syringe | | Method 2 Ficoll-Paque gradient | | Method 3 Liver biopsy | |
|---|------------------------------------|---------|--------------------------------|---------|-----------------------|---------|
| | Results | P. cent | Results | P. cent | Results | P. cent |
| 0.2-0.4 | | | | | 11/14 | 79 |
| 0.5 | 11/22* | 50 | 12/23 | 52 | 6/7 | 85 |
| 1.0 | 11/18 | 61 | 13/27 | 48 | | |
| 2.0 | 4/8 | 50 | 3/6 | 50 | | |
| Totals | 26/48 | 54 | 28/56 | 50 | 17/21 | 81 |

* N° of cultures successful/N° of cultures initiated.

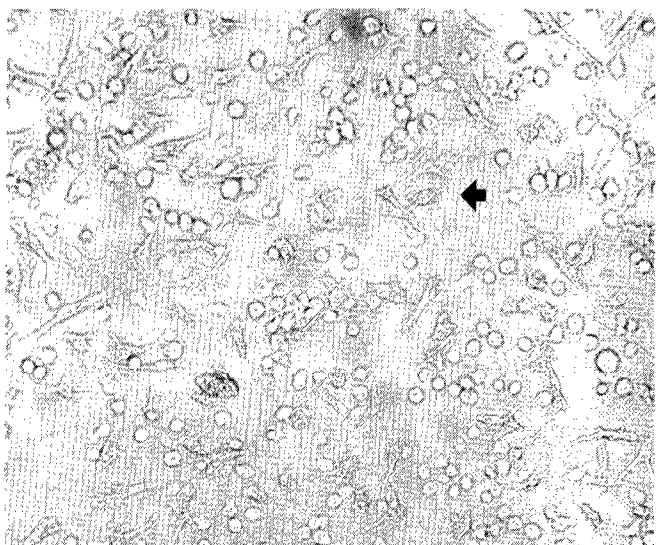


Fig. 4 : Primary culture derived from liver biopsy material. Note fibroblasts among the lymphoid cells (arrow) (x 300).

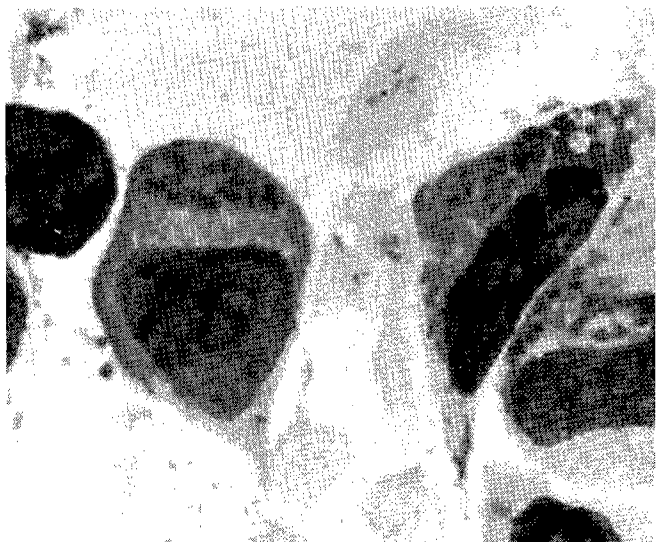


Fig. 5 : Bovine lymphoid cells infected with *T. annulata* schizonts. Giemsa stain (x 2,000).

Cultures initiated by method 3 (liver biopsy material)

Digestion with trypsin did not completely disperse the liver tissue. Although the tissue suspension was allowed to settle before transferring the supernatant into culture vessels small particles of undigested tissues

remained suspended. In the primary cultures, fibroblast cells (Fig. 4), mainly derived from undigested liver particles, usually developed. The small round refractile cells seen in the WBC cultures appeared between the fibroblast cells. Dispersion of the cultures with EDTA and subcultivation into new vessels resulted in a gradual disappearance of the fibroblast cells.

Observations on Giemsa stained coverslip preparations showed that schizonts occurred only in the lymphoid cells, never in the fibroblasts. Although fewer initial cells were obtained from the liver biopsy material than from peripheral blood, 81 per cent of 21 cultures seeded with the former cells produced viable cultures of *Theileria*-infected cells. Two cell lines, one originating from liver material and the other from buffy coat have been passaged 400 times during about 4 years and show no signs of loss of vitality.

DISCUSSION

Each of the 3 methods used here for initiating cultures of *T. annulata* has its specific technical advantages and disadvantages. The relatively high rate of success in all of them, 50-81 per cent, means that the choice of one method over another can be made primarily on the basis of personal preference and available facilities. In method 1 (buffy coat separation in the syringe) the primary cultures contain erythrocytes, mononuclear and polynuclear cells. However the non-infected cells degenerate and are washed out in the first few transfers leaving only the infected cells in a successful culture. In method 2 (Ficoll-Paque gradient) the primary cultures contain only lymphocytes, of which only the infected cells become attached and remain behind during the initial period of medium replacement.

Method 3 (trypsinized liver biopsy) yielded the highest success rate. This may be related to the fact that primary cultures contain living non-infected fibroblast cells as well as infected lymphoid cells. Some investigators (3) have used non-susceptible feeder layer cells in primary isolation procedures in order to promote growth of infected cells. Although it is obvious from the work reported here that feeder layers are not indispensable for obtaining successful cultures, their use may improve the likelihood of success with primary cultures.

Initiation of primary cultures with more than 0.5×10^6 WBC per ml did not increase the percentage of successful cultures. This fact and the formation of small colonies in the initial stage of the cultivation

indicate that not all of the infected cells introduced in the flask multiply and transform to infected cell lines.

It appears that, once established, the infected cells may have an essentially unlimited potential for multiplication, since no signs of deterioration have been detected in 2 cell lines maintained during a 4-year period of cultivation.

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Three methods for initiating *in vitro* cultures of *Theileria annulata* from infected cattle were compared : 1) separation of buffy coat cells by centrifugation in a plastic syringe ; 2) separation of lymphocytes by a commercial Ficoll-Paque gradient and 3) trypsinization of liver biopsy material. Successful cultures were obtained in 26 out of 48 trials (54 p. 100) by method 1, in 28 out of 56 trials (50 p. 100) by method 2 and 17 out of 21 trials (81 p. 100) by method 3. Once established, cultures, initiated by any method can be subcultivated for years. *Key words* : Cattle - *Theileria annulata* - *In vitro* culture.

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PIPANO (E.), SHKAP (V.), FRANK (M.). Comparación de tres métodos de preparación de cultivos *in vitro* de esquizontes de *Theileria annulata*. *Revue Élev. Méd. vét. Pays trop.*, 1989, 42 (4) : 529-533.

Se compararon tres métodos de preparación de cultivos *in vitro* de *Theileria annulata* proviniendo de bovinos infectados : 1) la separación de las células del cuajarón blanco por centrifugación en una jeringuilla de plástico ; 2) la separación de los linfocitos por un gradiente comercial Ficoll-Paque y 3) la tripsinización de pedazos de hígados tomados por biopsia. Se obtuvieron cultivos positivos en 26 casos de 48 por el método n°1 (54 p. 100), 28 casos de 56 por el método n°2 (50 p. 100) y 17 casos de 21 por el método n°3 (81 p. 100). Se puede trasplantar los cultivos en cuanto establecidos durante varios años, cualquiera que sea la técnica utilizada. *Palabras claves* : Bovino - *Theileria annulata* - Cultivo *in vitro*.

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