



***In vitro* isolation of *Ehrlichia ruminantium* from ovine blood into *Ixodes scapularis* (IDE8) cell cultures**

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ABSTRACT

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Four stocks of *Ehrlichia ruminantium* (Welgevonden, Ball3, Nonile and Blaauwkrans), the causative agent of heartwater in domestic ruminants, were isolated into *Ixodes scapularis* (IDE8) tick cells using the leukocyte fraction of the blood of infected sheep. Organisms of two of the *E. ruminantium* stocks (Welgevonden and Blaauwkrans) propagated in IDE8 cells were also successfully used to infect bovine endothelial cells. All stocks were successfully propagated in IDE8 cells using Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 containing 10% foetal bovine serum (FBS). The technique should be included in any attempt to isolate uncharacterized *E. ruminantium* stocks.

Keywords: *Ehrlichia ruminantium*, heartwater, *in vitro* isolation, tick cell line

INTRODUCTION

Heartwater or cowdriosis is an infectious, non-contagious, tick-borne disease caused by the intracellular rickettsial agent *Ehrlichia ruminantium*. The disease affects cattle, sheep, goats and also some wild ruminants. It is transmitted by ticks of the genus *Amblyomma* and has been reported from almost all African countries south of the Sahara, from the adjacent islands in the Indian and Atlantic Oceans (Uilenberg 1983) and from some Caribbean islands (Perreau, Morel, Barré & Durand 1980; Birnie, Burridge, Camus & Barré 1984). Heartwater is usually an acute disease and may be fatal within hours or days after the onset of clinical signs.

The mammalian cell culture system first described by Bezuidenhout, Paterson & Barnard (1985) is the

method of choice for *in vitro* isolation and propagation of *E. ruminantium*, whereas the successful propagation of *E. ruminantium* in tick cells was only reported recently (Bell-Sakyi, Paxton, Munderloh & Sumption 2000; Bekker, Bell-Sakyi, Paxton, Martinez, Bensaid & Jongejan 2002; Bell-Sakyi 2004). Initiation of infection in tick cell cultures was achieved using elementary bodies derived from mammalian cell cultures (Bell-Sakyi *et al.* 2000) or from other tick cell lines previously infected with mammalian cell culture forms (Bell-Sakyi 2004) but not directly from infected animals. Attempts to infect tick cells with blood from sheep undergoing clinical responses following experimental *E. ruminantium* infection were unsuccessful (Bell-Sakyi 2004).

The present experiments describe the first successful establishment of infection in *Ixodes scapularis* (IDE8) tick cell cultures directly from the blood of four sheep, each infected with a different South African stock of *E. ruminantium*, and the subsequent infection of endothelial cells from infected IDE8 cells.

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MATERIALS AND METHODS

Stocks of *E. ruminantium*

Four stocks of *E. ruminantium* isolated in South Africa were used: the Welgevonden stock was originally isolated by injecting a tick homogenate into a mouse; the tick had been collected on the farm Welgevonden in the Northern Transvaal (Du Plessis 1985); the Ball3 stock was isolated from a non-specified host in the Northern Province (Haig 1952); the Blaauwkrans stock was isolated from an eland near Port Elizabeth in 1996 (Zweygarth & Josemans 2001); and the Nonile stock from a sheep in Kwa-Zulu-Natal (MacKenzie & McHardy 1984).

Culture media

Uninfected and some infected tick cell cultures were propagated in L-15B medium (Munderloh & Kurtti 1989), which was supplemented with 5% heat-inactivated foetal bovine serum (FBS), 10% tryptose phosphate broth (TPB), 0.1% bovine lipoprotein concentrate (ICN, Irvine, CA, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin. The pH was adjusted to 7.2. Infected tick cell cultures were also maintained in Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12, Sigma, St. Louis, MO, USA; D 0547) containing 15 mM HEPES and 1.2 g/l sodium bicarbonate. This medium was further supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin and is referred to as DF-12. DF-12 medium was also used for infected and uninfected endothelial cell cultures.

Cell cultures

The tick cell line IDE8, derived from *I. scapularis* embryos (Munderloh, Liu, Wang, Chen & Kurtti 1994) was used throughout these experiments. IDE8 cultures, infected and uninfected, were maintained at 32°C in complete L-15B medium, unless otherwise stated. IDE8 cell cultures were used at passage levels between 54 and 62. Three fifths of the medium was replaced weekly. Propagation of infected and uninfected bovine aorta endothelial (BA 886) cell cultures was carried out as described previously (Zweygarth, Vogel, Josemans & Horn 1997).

Sheep-derived infective culture inoculum

Each of the four *E. ruminantium* stocks—Ball3, Blaauwkrans, Nonile or Welgevonden—was used to infect a Merino sheep by intravenous injection of a 5 ml blood stablate. The body temperature of

each sheep was monitored daily and a blood sample was drawn when it had risen to more than 41.5°C, unless stated otherwise. Blood was collected by venipuncture into sterile Vac-u-test® tubes containing heparin (lithium heparin, 14.3 USP per ml blood) as anticoagulant and put on ice. The cooled blood was centrifuged (800 x g; 10 min; 4°C) and the buffy coat was collected and washed with cold phosphate-buffered saline (PBS). The buffy coat was again collected, and the red blood cells were lysed for approximately 30 s in 20 ml sterile distilled water followed by the addition of 5 ml of a 5 x concentrated physiological NaCl solution (4.5% w/v). The leukocytes were centrifuged and the resulting cell pellet was re-suspended in 5 ml of DF-12 or complete L-15B medium. The leukocyte suspensions were then inoculated into 25 cm² culture flasks containing IDE8 cells. The cultures were incubated at 32°C.

Attempts to infect tick cell cultures with the Welgevonden stock of *E. ruminantium* were also carried out according to the method described by Byrom, Yunker, Donovan & Smith (1991) with minor modifications. Briefly, heparinized blood (lithium heparin, 14.3 USP per ml blood) was centrifuged (800 x g; 10 min; room temperature) and 3 ml of plasma were inoculated into each of two 25 cm² culture flasks containing a layer of IDE8 cells. The cultures were put on a rocking platform for 3 h at three cycles per min at 25°C, after which the plasma was decanted and the cell monolayer was rinsed three times with 5 ml PBS. Finally DF-12 medium was added.

In vitro infection of bovine endothelial cells by *E. ruminantium* organisms derived from IDE8 cell cultures

IDE8 cells infected with the Welgevonden and Blaauwkrans stocks of *E. ruminantium* were used to demonstrate infectivity of IDE8-derived organisms for BA 886 cells. Aliquots of 2.5 ml of infected IDE8 cell suspension were distributed into culture flasks containing BA 886 cells. Fresh DF-12 medium was added to give a final volume of 5 ml. The cultures were then incubated at 37°C and the medium was replaced every 3 days. IDE8 and BA 886 cultures were monitored for infection by microscopic examinations. Small samples from the cell layer were removed and smears were prepared. Cytospin smears were made from cultures where some of the cells were in suspension. Smears were allowed to dry before being fixed with methanol and stained with eosin-methylene blue.

Molecular characterization

Extraction and amplification of DNA

DNA was extracted from the four stocks of *E. ruminantium* with the QIAamp DNA kit extraction kit (Qiagen). The primers for the pCS20 PCR diagnostic test (Van Heerden, Steyn, Allsopp, Zwegarth, Josemans & Allsopp 2004) specific for *E. ruminantium* was used to sequence of *E. ruminantium* in the blood and in tick cell cultures. Briefly, the PCRs were performed with 2 µl of genomic DNA extracted from cell culture as template in a 50 µl reaction with 0.5 mM of each of the primers HH1F and HH2R, 2.5 mM dNTP; 25 mM MgCl₂, 10 x PCR reaction buffer and 0.5U of TaKaRa EX Taq enzyme (Takara Shuzo Co., Ltd Japan). PCR conditions were: incubation of 25 s at 94 °C, 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 45 s, elongation at 72 °C for 30 s, final elongation at 72 °C for 10 min and hold at 4 °C (Gene Amp PCR System 9700, Applied Biosystems). Each set of PCRs included a positive control containing 1 µl purified genomic DNA of *E. ruminantium* (Welgevonden) and a negative control containing 5 µl distilled water. Ten microliter of each sample was separated on a 1% agarose gel with molecular mass marker PHIX74.

Sequencing

The PCR amplicons from samples showing bands of the expected size were purified with a High Pure PCR product purification kit (Roche) and sequenced using an ABI Prism 3100 Automatic DNA sequencer (BigDye terminator cyclor sequencing kit, Perkin Elmer Applied Biosystems) with primers HH1F, HH2R (Van Heerden *et al.* 2004), and the data was assembled in gap 4 (Staden, Beal & Bonfield 2000) and analysed using ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997).

RESULTS

Sheep-derived infective culture inoculum

Leukocytes isolated from the blood of infected sheep were used as infective inoculum. All four South African *E. ruminantium* stocks were established successfully in IDE8 cell cultures by this method. The Welgevonden stock was detected in stained smears 9 days after initiation, when leukocytes harvested 14 days after infection were used. In contrast, all attempts to initiate the Welgevonden stock in the "conventional" way (Byrom *et al.* 1991), i.e. by incubating plasma from the infected animal together with host

cells (IDE8), failed (data not shown). During prolonged incubation of the latter IDE8 cultures some cytotoxic effects induced by the ovine plasma became manifest as the pH of the medium failed to fall in the same way as it did in untreated controls. DF-12 medium was used for these experiments.

Infection with the Blaauwkrans and Ball3 stocks of *E. ruminantium* was detected in stained smears in IDE8 cell cultures after 21–29 and 18–25 days, respectively. Both stocks were initiated using DF-12 medium. Leukocytes contained morula-like inclusions, presumably *E. ruminantium*. These were only demonstrated with the Blaauwkrans stock before culture initiation. The results of successful culture initiations are shown in Table 1.

Attempts to infect IDE8 cell cultures using the Nonile stock were carried out on two successive occasions. The first attempt was carried out on Day 13 post infection of the donor sheep when its body temperature was 41.3 °C. A Giemsa-stained cytocentrifuge smear prepared from the leukocyte inoculum revealed that more than 99% of the cells were mononuclear. Cultures initiated on this occasion remained negative throughout an observation period of 60 days. In contrast, the initiation experiment carried out the next day revealed a switch to a granulocyte cell distribution pattern, with mononuclear cells being a minor contaminating population only. Thirteen days after initiation, infected tick cells were detected in Giemsa-stained cytocentrifuge smears prepared from culture supernatant. Complete L-15B medium was used for the initiation, but was replaced by DF-12 medium 39 days after culture initiation due to unsatisfactory growth of the *E. ruminantium* organisms. The first subculture was carried out 100 days after culture initiation. These results are summarized in Table 2.

***In vitro* infection of BA 886 cells by *E. ruminantium* organisms derived from IDE8 tick cell cultures**

IDE8 cultures infected with the Welgevonden stock for 149 days and the Blaauwkrans stock for 165 days were used successfully to infect BA 886 cell cultures. When the Welgevonden stock was used, both BA 886 cultures were positive 3 days after inoculation as determined by stained smears, whereas the Blaauwkrans stock was detected in the two infected cultures on Days 9 and 15, respectively. BA 886 cultures infected with both stocks were then subcultured after a further 15 days. The results are summarized in Table 3.

TABLE 1 Infection of IDE8 cell cultures using leukocytes isolated from sheep infected with three *E. ruminantium* stocks

<i>E. ruminantium</i> stock	Number of flasks	Time to detection of <i>E. ruminantium</i> in IDE8 cultures (days)	Time to first subculture (days)
Ball3	2	18, 25	63
Blaauwkrans	3	21, 21, 29	60
Welgevonden	2	9, 9	37

TABLE 2 Infection of IDE8 cell cultures using leukocytes isolated from sheep infected with the Nonile stock of *E. ruminantium*

Inoculum	Number of flasks	Time to first detection of <i>E. ruminantium</i> in IDE8 cultures (days)	Time to first subculture (days)
Mainly mononuclear cells	3	— ⁽¹⁾	— ⁽²⁾
Mainly granulocytes	3	13 ⁽³⁾	100

⁽¹⁾ Remained negative for 60 days

⁽²⁾ Not applicable

⁽³⁾ Detected in a cytocentrifuge smear of pooled samples from the three culture flasks

TABLE 3 *In vitro* infection of BA 886 cells by *E. ruminantium* organisms derived from IDE8 tick cell cultures

<i>E. ruminantium</i> stock	Time in IDE8 culture (days)	Number of flasks	Time to detection of <i>E. ruminantium</i> in BA 886 cultures (days)	Time to first subculture (days)
Welgevonden	149	2	3, 3	15
Blaauwkrans	165	3	9, 15, 15	15

Molecular characterization

Sequence analysis using the pCS20 specific primers confirmed that the *E. ruminantium* stocks recovered from the tick cell cultures were the same as those which were injected into the donor sheep, showing that the organisms used to infect the sheep were also of the stocks isolated in the respective cultures.

DISCUSSION

The first continuous propagation of *E. ruminantium* in a tick cell line was achieved by Bell-Sakyi *et al.* (2000), who cultivated the Gardel stock in the *I. scapularis*-derived cell line IDE8 (Munderloh *et al.* 1994). Elementary bodies derived from bovine endothelial cell cultures of several stocks of *E. ruminantium* were used to establish continuous, infected tick cell cultures (Bell-Sakyi 2004). However, attempts to infect three different tick cell lines, AVL/CTVM13, IDE8 and RAN/CTVM3, with blood from sheep undergoing clinical responses following experimental *E. ruminantium* infection were unsuccessful (Bell-Sakyi 2004). Similar unsuccessful results were obtained by us when we attempted to infect IDE8 cultures using heparinized plasma from infected sheep.

However, infective organisms are not only found free-floating in the blood or plasma of an infected animal but also in circulating leukocytes, in which the organisms are able to proliferate. Logan, Whyard, Quintero & Mebus (1987) observed *E. ruminantium* colonies in up to 35% of neutrophils maintained *in vitro* for between 18 h and 5 days. Furthermore, leukocytes from infected animals were able to transmit *E. ruminantium* to naive animals (Ilemobade & Blotkamp 1978). The experiments in the present study show that leukocytes may also be used to initiate *in vitro* cultures. In fact, IDE8 tick cell cultures can be infected with *E. ruminantium* directly from the blood of infected sheep, provided leukocytes are used as the inoculum. Cultures of all four stocks used in the experiments—Ball3, Blaauwkrans, Nonile and Welgevonden—were successfully initiated in IDE8 cells using this technique. Similar approaches were carried out with *Ehrlichia canis* (Ewing, Munderloh, Blouin, Kocan, Kurti 1995) and with the other former *Ehrlichia* species: *Ehrlichia equi* (Munderloh, Madigan, Dumler, Goodman, Hayes, Barlough, Nelson & Kurti 1996), the human granulocytic ehrlichiosis (HGE) agent (Munderloh, Jauron, Fingerle, Leitritz, Hayes, Hautman, Nelson, Huberty, Kurti, Ahlstrand, Greig, Mellencamp & Goodman 1999), and *Ehrlichia phagocytophila* (Woldehiwet, Horrocks, Scaifer,

Ross, Munderloh, Brown, Edwards & Hart 2002) (all now reclassified as *Anaplasma phagocytophilum* [Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa & Rurangirwa 2001]) which have been successfully isolated from infected blood into *I. scapularis* cell cultures using leukocytes as infective inoculum. Besides neutrophils, *in vitro* cultured macrophages also revealed the presence of inclusion bodies of *E. ruminantium* (Sahu 1986). Interestingly, infection of IDE8 cells with the Nonile stock using a leukocyte fraction containing almost exclusively mononuclear cells failed, although monocytes/macrophages have been described as containing the organism. In contrast, the Nonile stock could be established in IDE8 cells using granulocytes from the same infected sheep.

Ehrlichia ruminantium is regarded as an obligatory parasite of endothelial cells (Martinez, Sheikboudou, Couraud & Bensaid 1993) but it has been shown recently that not all *E. ruminantium* isolates can be initiated in endothelial cells. Different types of host cell were required to isolate these organisms which have previously resisted all attempts at isolation in the conventional way (Zweygarth, Josemans, Van Strijp, Van Heerden, Allsopp & Allsopp 2002). Consequently, isolating *E. ruminantium* using only endothelial cells limits the probability of success. Here we show that IDE8 cells can be used as an alternative to conventional methods. Tick cell cultures may thus increase the chances of isolating organisms which do not generally grow in endothelial cells as the primary culture.

Both the Welgevonden and Blaauwkrans stocks in IDE8 cells, which were used to inoculate BA 886 cells, not only infected them but also gave rise to continuous infected mammalian cell culture lines. Similar results were reported by Bell-Sakyi *et al.* (2000). However, their IDE8 cultures were not consistently infective for bovine pulmonary artery endothelial cells. The fact that endothelial cells can be infected with IDE8-derived organisms makes the system very valuable, especially when cultures cannot be directly initiated in endothelial cells. Infected IDE8 cells can thus be used as a stable source of inoculum to identify suitable mammalian cell lines that support growth of the organisms. It has been reported recently that the Kümme isolate, which resisted all attempts at conventional *in vitro* culture, was isolated in culture in non-endothelial cells (Zweygarth *et al.* 2002).

Unlike others (Bell-Sakyi *et al.* 2000; Bekker *et al.* 2002; Bell-Sakyi 2004), who only used a L-15B-based

medium for the propagation of *E. ruminantium* in IDE8 cultures, we also used DF-12 medium, which was originally devised for the propagation of *E. ruminantium* in mammalian cell cultures at 37°C (Zweygarth *et al.* 1997). DF-12 medium was a suitable alternative for the propagation of *E. ruminantium* in IDE8 cells and it also supported the growth of uninfected IDE8 cells over a three-month period with several subcultures (data not shown).

In conclusion, it has been shown that the technique of isolating *E. ruminantium* from infected animals using IDE8 tick cell cultures is a valuable method which should be included in any attempt to isolate uncharacterized *E. ruminantium* stocks.

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