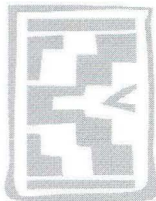

The Kümm isolate of *Ehrlichia ruminantium*: *In vitro* isolation, propagation and characterization

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ABSTRACT

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An effective culture system for *Ehrlichia* (*Cowdria*) *ruminantium* comb. nov. was first established in 1985 and many stocks were subsequently isolated and propagated *in vitro*. A notable exception, however, was the Kümm isolate that resisted all attempts at *in vitro* culture until the successful experiment described here. In one experiment white blood cells were harvested from heparinized blood derived from a sheep infected with the Kümm isolate. The cells were added to DH 82 cells and incubated at 37 °C. The high metabolic activity of the DH 82 cells necessitated that cell growth be retarded by the addition of cycloheximide. Colonies were first detected 19 days after culture initiation and, once the cultures were established, they could be passaged every 3 days. Bovine and sheep endothelial cells were readily infected with culture supernatant obtained from the infected DH 82 cells. In a further experiment another sheep was infected, using a higher dose of the same batch of Kümm stabilate, and we attempted to infect several different cell lines: these were DH 82 cells, bovine aorta (BA 886) cells, sheep brain endothelial (SBE 189) cells and sheep fibroblastoid cells (E_2). Ten days after culture initiation only the E_2 cells had become positive for *E. ruminantium*. Culture supernatant from the first cultured isolate (Kümm-1) was less virulent for mice than that of the second cultured isolate (Kümm-2) which killed all mice. Upon molecular characterization with *E. ruminantium* 16S probes we found that Kümm-1 hybridized with a Senegal 16S genotype probe, whereas Kümm-2 hybridized only with an Omatjenne 16S genotype probe. The original stabilate used to infect the sheep hybridized with both probes. These results clearly indicate that two different stocks had been isolated in culture.

Keywords: Cycloheximide, DH82, *Ehrlichia* (*Cowdria*) *ruminantium*, fibroblastoid cells, 16S genotype, heartwater, *in vitro* cultivation, Kümm isolate

INTRODUCTION

Heartwater is a tick-borne rickettsial disease of ruminants caused by *Ehrlichia (Cowdria) ruminantium* comb. nov. (Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa, & Rurangirwa 2001). The disease is prevalent in sub-Saharan Africa (Uilenberg 1983), and in the Caribbean (Perreau, Morel, Barré & Durand 1980; Birnie, Burridge, Camus & Barré 1984). The *in vitro* culture of *E. ruminantium* was first achieved using bovine umbilical cord endothelial cells as host cells (Bezuidenhout, Paterson & Barnard 1985). Almost all the continuous cultures of *E. ruminantium* which have been achieved have used endothelial cells of various

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animal species and from various anatomical sites (Byrom & Yunker 1990; Byrom, Yunker, Donavan & Smith 1991; Jongejan, Zandbergen, Van de Wiel, De Groot & Uilenberg 1991; Martinez, Sheikboudou, Couraud & Bensaid 1993; Totté, Blankaert, Zilimwabagabo & Wérenne 1993), and several stocks have been isolated, propagated and expanded in vitro using these culture systems. However, the Kümm isolate (Du Plessis & Kümm 1971) has yielded constantly negative culture results (Bezuidenhout, Brett, Erasmus & Rossouw 1988; Bezuidenhout & Brett 1992). Here we report on the first successful attempt at the isolation and *in vitro* propagation of the Kümm isolate of *E. ruminantium* and its subsequent characterization.

MATERIALS AND METHODS

Infective agent

The Kümm isolate of *E. ruminantium* was originally obtained from a naturally infected goat in Rust de Winter, Northern Province, South Africa (Du Plessis 1982). The animal had a disease which was described at the time as heartwater (Du Plessis & Kümm 1971) and a lymph node suspension from the animal was inoculated intravenously into a sheep. The sheep reacted and a saline suspension of its mesenteric lymph nodes was inoculated intraperitoneally (i.p.) into mice, in which it was found to be fatal. Lung and spleen homogenate from infected mice was preserved in liquid nitrogen (Ramisse & Uilenberg 1970) and the isolate has subsequently been passaged more than 100 times in our laboratory, mainly in mice but also in sheep. This isolate has also been used to prepare antigen slides for heartwater serology (Du Plessis 1982). The number of animal passages at which the present experiments were conducted is unknown.

Host cells

Four different mammalian cell lines were used as potential host cells. The DH 82 cells were from a canine macrophage-monocyte cell line (Wellman, Krakowka, Jacobs & Kociba 1988), the BA 886 cell line was established from a bovine aorta (Yunker, Byrom & Semu 1988), the SBE 189 cell line was from sheep brain endothelium (Brett, Bezuidenhout & De Waal 1992) and the E_2 cells were obtained by cultivation of blood mononuclear cells from a sheep. In order to characterize the E_2 cells we used acetylated low density lipoprotein labelled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL) (Molecular Probes, Eugene, OR, USA). Dil-Ac-LDL has been shown to label endothelial cells metabolically, thus allowing their identification by fluorescence microscopy (Voyta, Via, Butterfield & Zetter 1984). The E_2 cells were labelled, and fluorescence microscopy was carried out, as described in detail by Aherne, Davis & Sordillo (1995). As no fluorescence was observed we believe that the E_2 cells are not of endothelial origin.

Culture medium

Infected and uninfected cell cultures were grown 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. The medium was further supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/mℓ penicillin and 100 µg/mℓ streptomycin.

Infection of host cells

DNA samples from the Merino sheep used in these experiments were initially examined by PCR (Mahan, Waghela, McGuire, Rurangirwa, Wassink & Barbet 1992), and probed with the *E. ruminantium*-specific pCS20 probe (Waghela, Rurangirwa, Mahan, Yunker, Crawford, Barbet, Burridge & McGuire 1991) to ascertain that they were negative for *E. ruminantium*.

A Merino sheep was infected with the Kümm isolate by intravenous injection of 5 mℓ of a blood stabilate prepared in 1994. Body temperature was monitored daily and a blood sample was drawn on day 18 after infection when the body temperature had risen to 41 °C. Blood was collected by venipuncture into sterile Vac-u-test[R] tubes containing heparin (lithium heparin, 14.3 USP $m\ell^{-1}$ 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 solution (PBS). The red blood cells were lysed in 5 mℓ sterile distilled water followed by the addition of an equal volume of 1.8 % (w/v) NaCl solution. The white blood cells were washed twice with PBS solution and then resuspended in complete culture medium containing 1 µg $m\ell^{-1}$ cycloheximide (Ennis & Lubin 1964). The cell suspension was then inoculated into each of two 25-cm² culture flasks containing DH 82 cells and the cultures were incubated at 37 °C. The stock isolated in this experiment is referred to as Kümm-1.

In order to repeat the isolation experiment another Merino sheep was infected with a higher dose (9 mℓ) of the same batch of blood stabilate, using a procedure which was otherwise as described above. In this experiment we attempted to infect all four of the cell lines mentioned earlier, and two flasks of each cell line were initiated using heparinized blood. The stock isolated in this instance is referred to as Kümm-2.

Propagation of cultures

Infected cultures were harvested for subcultivation by scraping the cell monolayer into the medium and the cells were dispersed by pipetting the suspension up and down. The suspension was distributed into two new culture flasks containing uninfected host cells, and 2.5 mℓ of fresh medium were added. After 24 h all the medium was discarded and replaced with 5 mℓ fresh medium.

Infectivity testing

The infectivity of the organisms derived from Kümm-1 and Kümm-2 cultures was tested by injecting culture suspensions, either i.p. or intravenously (i.v.) into mice (0.2 mℓ) or i.v. into sheep (2 mℓ).

Molecular characterization

DNA was extracted from Kümm-1 and Kümm-2 infected cell cultures, as well as from the 1974 stabilate of the Kümm isolate, using either the QIAmp (Qiagen, Germany) tissue kit or the FastDNA® Kit (Bio 101 Inc., California, USA). PCR was performed using primers AB 128 and AB 129 for pCS20 amplification (Mahan *et al.* 1992), and primers 930 and BAA5, which amplify the V1 loop of the 16S rRNA genes of all Rickettsiales species, as previously described (Allsopp, Visser, Du Plessis, Vogel & Allsopp 1997). The pCS20 amplicons were slot-blotted onto nylon membranes (Hybond N+, Amersham International), and probed with the pCS20 probe and the 16S amplicons were slot blotted and probed for five different genotypes of *E. ruminantium* (Ball 3, Senegal, Omatjenne, Mara 87/7 and Welgevonden) using procedures as described by Allsopp, Hattingh, Vogel & Allsopp (1999).

The full-length 16S rDNA genes of Kümm-1 and Kümm-2 were amplified using primers fD1 and rD1 (Weisburg, Barns, Pelletier & Lane 1991) as previously described (Allsopp *et al.* 1997) and cloned into pMOS*Blue* (Amersham International). Clones were sequenced on both strands using T7, M13(–48) and internal primers (Allsopp *et al.* 1997) on an ABI 377 automatic DNA sequencer (BigDye terminator cycle sequencing kit, Perkin Elmer Applied Biosystems) and the data were assembled and analyzed using the Staden package (Bonfield, Smith & Staden 1995).

RESULTS

In vitro cultures

Kümm-1

Nineteen days after culture initiation colonies were found in two culture flasks. Twenty-four days post initiation, cultures were subcultured into BA 886 cells and DH 82 cells. From then on, cultures were subcultured every 3 days for another 14 passages until they were cryopreserved in liquid nitrogen. Treatment of the DH 82 cells with cycloheximide during the culture period was a prerequisite for continuous propagation.

Kümm-2

All flasks containing DH 82, BA 886 and SBE 189 cells remained negative throughout the observation period (30 days). One of the two flasks containing E_2 cells became positive 10 days after culture initiation. This culture had been initiated with whole blood. This flask was incubated for 3 h before the blood was removed and replaced with medium. Regular subculture intervals could not be achieved because of the slow growth of the organisms and their low infection rate for the host cells. Kümm-2 cultures were passaged ten times with an average splitting interval of 10.5 days (range 8–21 days). Once the number of infected cells increased, attempts were made to infect DH 82, BA 886 and SBE 189 cells. The SBE 189 cells became infected and were subcultured after 15 days but failed to grow subsequently. Neither the DH 82 nor the BA 886 cells became infected by the Kümm-2 stock. The major differences observed between Kümm-1 and Kümm-2 are summarized in Table 1.

Animal infectivity

Culture supernatant harvested from Kümm-1 cultures was injected i.p. and i.v. into mice. None of the mice died nor did they develop signs of disease. However, in a second experiment, culture material was injected into a group of 20 mice, 13 of which died. A Merino sheep which was injected with 2 mℓ of culture supernatant became sick and its body temperature rose to 42 °C 6 days after

TABLE 1 Differences observed between *Ehrlichia* stocks Kümm-1 and Kümm-2

	Stock Kümm-1	Stock Kümm-2
Growth in BA886 or DH82 cells	+	–
Growth in E_2 cells	+	+
Regular subculture intervals	+	–
Virulence in mice	+/–	+
16S genotype	Senegal	Omatjenne

infection. The sheep was cured after being treated with oxytetracycline.

Culture supernatant obtained from Kümm-2 cultures was highly virulent for mice, all of which died 9–11 days after infection regardless of whether the inoculum was injected i.p. or i.v. Seven days after injecting a Merino sheep with 2 mℓ of culture supernatant its body temperature rose to 42 °C and was elevated for 3 days. The sheep was treated on the third day of elevated body temperature with oxytetracycline.

Molecular characterization

The pCS20 probe hybridized with amplicons of Kümm-1 and Kümm-2, indicating that both stocks were *E. ruminantium*. The 16S V1 loop PCR amplification and probing results were as follows. The *E. ruminantium* (Omatjenne) probe hybridized with amplicons from the 1974 Kümm stabilate and the Kümm-2 culture material, but not with the Kümm-1 culture material. The *E. ruminantium* (Senegal) probe gave hybridization signals with amplicons from the 1974 Kümm stabilate and the Kümm-1 culture material, but not with the Kümm-2 culture material (Table 1). The 16S rDNA gene sequence of Kümm-1 was identical to that of the Senegal stock of *E. ruminantium* (Senegal, Genbank accession #U03775) while that of Kümm-2 was identical to that of the Omatjenne isolate of *E. ruminantium* (Omatjenne, Genbank accession #U03776).

DISCUSSION

In vitro cultivation allowed the discrimination and separation of two different organisms, here referred to as Kümm-1 and Kümm-2, within the stock known as the Kümm isolate of *E. ruminantium*. Many previous attempts to isolate this organism using the conventional method, by inoculation of blood onto a layer of endothelial cells, have failed. It was previously reported that the Kümm stock infects mouse peritoneal macrophages (Du Plessis 1982) so a range of monocyte cell lines was used in initial experiments which are not described here. The DH 82 cell line was the most promising candidate and all subsequent experiments were carried out using this cell line.

In the first experiment described here colonies were first detected 19 days after culture initiation. Shortly thereafter the cultures could be subcultured and, surprisingly, bovine endothelial cells (BA 886) were easily infected. These Kümm-1 organisms proliferated in a manner typical of other *E. ruminantium* stocks, with a period from infection to subculture within 3 days. The reason why the Kümm-1 stock has not been previously isolated in attempts using BA 886 cells remains unclear, although a similar phenomenon has been observed using tick cell cultures for *E. ruminantium* isolation (Dr Lesley Bell-Sakyi, personal communication 2001). In that work, *E. ruminantium* organisms grew if they were derived from infected endothelial cell cultures, but not if they were derived from the blood of an infected animal.

The purpose of the second series of experiments was to prove that the Kümm stock only infects DH 82 cells and all the other cell lines which were available in our laboratory were used as controls. Interestingly, only one culture of E_2 cells became infected, with a few colonies being detected. This culture, Kümm-2, showed different behaviour to that of the first isolation. Kümm-2 could be propagated only at irregular intervals and cell infection rates remained rather low. BA 886 and DH 82 cells could not be infected at all, SBE 189 cells were infected, but infection was lost on subculturing. In contrast, Kümm-1 was subcultured regularly at 3-day intervals and infection rates were high.

The E_2 cell line, obtained by cultivation of blood mononuclear cells from a sheep, did not accumulate Dil-Ac-LDL, from which it was assumed that they were not of endothelial origin. It is generally accepted that *E. ruminantium* infects only endothelial cells, neutrophils and monocytes, and although there are scattered reports of *E. ruminantium* being found in other cell types, their accuracy has been questioned (Uilenberg 1983), and no real cultural evidence has been presented. Da Graça (1966) reported finding *E. ruminantium* in fibroblasts of the interstitial spaces and alveolar septa, while Ilemobade (1976) described the presence of *E. ruminan-*

tium in the epithelium of a renal tubule of one animal. If E_2 cells are in fact fibroblastoid cells then this is, to the best of our knowledge, the first report of *E. ruminantium* cultures initiated in mammalian cells other than endothelial cells, apart from the short-term cultures of *E. ruminantium* in neutrophils (Logan, Whyard, Quintero & Mebus 1987). Yunker *et al.* (1988) have reported that a cell line changed from an endothelioid cell morphology, susceptible to *E. ruminantium* infection, to a fibroblastic morphology, therefore losing its susceptibility to infection. Furthermore, they claimed that E5 cells, which were of fibroblastic morphology and negative for Factor VIII, were susceptible to infection. In our hands E5 cells, although of questionable morphology, stained positively with Dil-Ac-LDL (data not shown). Bezuidenhout (1987) found that Vero cells and lamb foetal kidney cells did not support the growth of the Ball 3 isolate of *E. ruminantium*, and that mouse L-cells did not become infected with the Welgevonden isolate. These conflicting data suggest that further experiments need to be carried out to investigate the infectivity of *E. ruminantium* to fibroblastoid cells.

The original Kümm isolate was the first isolate of *E. ruminantium* described to be pathogenic for mice by i.p. injection. Mice infected with spleen and liver suspension via the i.p. route consistently died from 10–14 days after injection. Sheep inoculated with these organisms developed severe febrile reactions and some of the animals died (Du Plessis & Kümm 1971). In contrast, in our first experiment described here, mice injected with Kümm-1 did not develop any clinical signs of disease, nor did they die. However, when the experiment was repeated 65 % of the infected mice succumbed to the infection. Despite these conflicting results Kümm-1 was consistently less virulent for mice than Kümm-2, which was highly virulent in this host, even after i.p. injection. The Kümm-2 stock, therefore, bears the closest resemblance to the description of the original Kümm isolate (Du Plessis & Kümm 1971). The fact that the Kümm stabilate (original) and the Kümm-2 stock cause more than 90 % mortality in mice when injected i.p. is a characteristic not shared by other isolates (MacKenzie & McHardy 1987).

The striking differences in growth patterns, host cell repertoire and subculture intervals clearly indicated that two different organisms were isolated. The molecular characterization further confirmed that the Kümm-1 and Kümm-2 stocks were genetically distinct organisms, with the Kümm-1 stock being of the Senegal 16S genotype while the Kümm-2 stock was of the Omatjenne 16S genotype. It is interesting to note that the Kümm-1 stock's 16S gene sequence is an exact match with the original Senegal stock isolated in 1988 by Jongejan, Uilenberg & Franssen. The Kümm-2 stock was of the Omatjenne *E. ruminantium* 16S genotype, and this isolate was originally made by infecting a mouse with homogenate prepared from a single *Hyalomma truncatum* tick collected in a heartwater- and *Amblyomma*-free area of Namibia (Du Plessis 1990). Previous attempts to molecularly characterize the Kümm isolate gave conflicting results. Examination of a long-stored Kümm blood stabilate revealed the presence of Omatjenne type V1 loop sequences and also of sequences of the *E. ruminantium* Crystal Springs 16S genotype (Allsopp *et al.* 1997). Subsequently, ticks experimentally infected with the Kümm isolate were positive only with the Omatjenne 16S probe (Allsopp *et al.* 1999).

The presence of the two different genotypes poses the question as to whether they were already present in the original isolate of 1971. As described above, the characteristics of Kümm-2 appear to be typical of the original description, so it is possible that the Kümm-1 component was introduced during passages, which were carried out over many years. This is not impossible, given the difficulty of detecting *E. ruminantium*-carrier animals in the pre-PCR era (Andrew & Norval 1989; Camus 1992). The question could be resolved by examining the original stabilate of the Kümm isolate, but unfortunately, the earliest stabilate still available stored in liquid nitrogen originates from 1974. The presence of both genotypes was demonstrated in this material, so we can be sure that, if inadvertent contamination occurred, it was at an early stage in the stock's history. Unfortunately the question as to whether both genotypes were present in the original isolate in 1971 cannot now be answered.

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