A chemically defined medium for the growth of *Cowdria ruminantium*

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ABSTRACT

ZWEYGARTH, E. & JOSEMANS, A.I. 2001. A chemically defined medium for the growth of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, 68:37–40

Chemically defined media, termed SFMC-23 and SFMC-36, were devised for the *in vitro* culture of *Cowdria ruminantium*, the causative agent of heartwater in domestic ruminants. Both media were based on Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12) containing various supplements. Medium SFMC-23 and SFMC-36 supported the long-term growth of the Welgevonden stock of *C. ruminantium* for a total of 55 and 28 passages, respectively, with regular passage intervals of 3 days. Using SFMC-23, split ratios varied from 5–10, depending on which host cell line was used. Other stocks of *C. ruminantium* (Sankat, Blaauwkrantz, Senegal) were successfully propagated for a test period of ten passages.

Keywords: Chemically defined medium, *Cowdria ruminantium*, *in vitro* culture

INTRODUCTION

Heartwater is a tick-borne rickettsial disease of ruminants caused by *Cowdria ruminantium*. The disease is prevalent in sub-Saharan Africa and surrounding islands (Uilenberg 1983), and in the Caribbean (Perreau, Morel, Barré & Durand 1980; Birnie, Burridge, Camus & Barré 1984). The first vector of the disease to be identified was *Amblyomma hebraeum* in South Africa (Lounsbury 1900). Currently, twelve species of *Amblyomma* ticks are known to be capable of transmitting heartwater (Walker & Olwage 1987).

The *in vitro* culture of *C. ruminantium* was first achieved by Bezuidenhout, Paterson & Barnard (1985). They used calf endothelial cells as host cells and Eagle's medium containing 10 % bovine serum. Subsequently, Glasgow minimal essential medium (GMEM) (Bezuidenhout 1987), Leibovitz L-15 (Byrom & Yunker 1990) and Dulbecco's minimal essential medium (DMEM) (Martinez, Sheikboudou, Couraud & Bensaid 1993) supplemented with various amounts of foetal bovine serum and tryptose phosphate broth (TPB) were used for *Cowdria* culture. However, the culture of *C. ruminantium* in the presence of undefined components such as serum and TPB has some serious disadvantages. Batch-to-batch variation of components, lack of standardization of experimental protocols, and the difficulty of identifying specific growth requirements are a few of the factors involved.

Recently, the propagation of *C. ruminantium* in serum-free media supplemented with bovine lipoproteins and transferrin was achieved (Zweygarth, Josemans & Horn 1998). In this report it is shown that *C. ruminantium* can be cultured in a protein-free, chemically defined medium, by replacing bovine lipoproteins by chemically defined lipids and transferrin by protein-free iron complexes.

MATERIALS AND METHODS

Stocks of *C. ruminantium*

Four different stocks of *C. ruminantium* were used, the origins of which are described below. The Welgevonden stock was isolated by injecting a homogenate from a tick collected on the farm Welgevonden (Northern Transvaal, South Africa) into a mouse (Du Plessis 1985). The Senegal stock was isolated in 1981 from cattle in Senegal by subinoculation into sheep (Jongejan, Uilenberg, Franssen, Gueye & Nieuwenhuijs 1988). Both the Senegal and the Welgevonden stock express a conserved immunodominant 32 kDa protein which is currently used for serodiagnosis of the disease (Van Vliet, Jongejan, Van Kleef & Van der Zeijst 1994). The Pokoase stock was isolated in Ghana by Dr Bell-Sakyi, Centre for Tropical Veterinary Medicine, Edinburgh, Scotland. The Blaauwkrantz stock was originally isolated from an eland on the farm Blaauwkrantz (Eastern Cape, South Africa) in 1996 by Dr J.L. du Plessis, formerly of the Onderstepoort Veterinary Institute.

Endothelial cell lines

The E_5 calf endothelial (Bezuidenhout *et al.* 1985) and the bovine aorta BA 886 (Yunker, Byrom & Semu 1988), cell lines were used. Uninfected cells were propagated at 37 °C as monolayers in medium consisting of Dulbecco's modified Eagle's medium (Highveld Biological, Kelvin, South Africa) with 10 % (v/v) heat-inactivated foetal bovine serum (FBS; Delta Bioproducts, Kempton Park, South Africa). The medium was buffered with 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma, St. Louis, MO, USA), and 10 mM sodium bicarbonate, and supplemented with 2 mM L-glutamine, 100 IU/mℓ penicillin and 100 µg/mℓ streptomycin. Before being used for *C. ruminantium* cultivation, each monolayer was rinsed three times with 5 mℓ of phosphate-buffered saline to reduce serum contamination. Endothelial cell lines were used at passage 128–177 (E_5) and 33 to 65 (BA 886).

Media for the propagation of *C. ruminantium*

Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma) containing 15 mM HEPES and 1.2 g/L sodium bicarbonate was used. This was supplemented with 2 mM L-glutamine, 100 IU/mℓ penicillin, 100 µg/mℓ streptomycin, and is referred to as serum-free medium for *Cowdria* no. 36 (SFMC-36). SFMC-23 was prepared by the addition of a mixture containing 10 µℓ of 40 mM ferric chloride hexahydrate (Sigma) in 10 mM HCl and 1 mℓ of 0.5 M glycylglycine (Sigma) to 100 mℓ SFMC-36 supplemented with 0.1 % (v/v) chemically defined lipids (CDL Life Technologies, Paisley, Scotland).

Propagation of cultures

Propagation of cultures was carried out as described previously (Zweygarth, Vogel, Josemans & Horn 1997) with minor modifications. Briefly, infected cultures were harvested for subcultivation by scraping off the cell monolayer into the medium using a Pasteur pipette bent at the tip to form a small hook. Cells were dispersed by pipetting the suspension up and down. The cell suspension was centrifuged (800 x *g* for 10 min at room temperature) and the appropriate amount (0.5 mℓ or 1 mℓ) of supernatant was distributed into each of two culture flasks containing the uninfected replacement endothelial cell line. Fresh medium was then added to 2.5 mℓ. The culture flasks were placed onto a rocker platform (three cycles per min) for 30 min, after which a further 2.5 mℓ aliquot of fresh medium was added. After 24 h all the medium was discarded and replaced with 5 mℓ fresh medium. Smears were prepared for microscopic examination by removal of small samples from the monolayer using a sterile 21-gauge needle with a bent tip, in order to determine the presence of *C. ruminantium* in the endothelial cells. The smears were air-dried, methanol-fixed and stained with RapiDiff (Clinical Sciences Diagnostics, Booysens, South Africa).

RESULTS

SFMC-23 and SFMC-36 were successfully used to propagate the *C. ruminantium* (Welgevonden) stock. The growth characteristics obtained with SFMC-23 and SFMC-36 were almost identical with an average passage interval of 3 days. After transfer of the Welgevonden stock into SFMC-36, a 4-day passage interval was initially observed, followed by 3-day

TABLE 1 Cultivation of *C. ruminantium* stocks in medium SFMC-23 or SFMC-36

Stock of *C. ruminantium*	Total number of passages at termination	Number of passages in serum-free medium	Passage intervals (days)	
			Average	Range
Welgevonden	157	55	3.0	3
Welgevonden (SFMC-36)	157	28	3.0	3–4
Blaauwkrantz	25	10	3.3	3–5
Sankat	20	10	4.6	3–12
Senegal	26	10	4.4	3–6

intervals. The split ratio used was five when BA cells were used and ten when E_5 cells were used as host cells. The cultures were terminated after 55 and 28 passages, respectively, due to a *Mycoplasma* contamination of the host cells. The Blaauwkrantz, Sankät and Senegal stocks were propagated in SFMC-23 over an observation period of ten passages with average passage intervals of 3.3, 4.6 and 4.4 days, respectively. A split ratio of two for the latter three stocks was observed throughout the cultivation period. The results are summarized in Table 1.

DISCUSSION

Two protein-free, chemically defined media, referred to as SFMC-23 and SFMC-36, one of which supports the growth of at least four different stocks of *C. ruminantium* are described in this study. The media are, however, only chemically defined to the extent of the purity of culture ingredients themselves. The *in vitro* cultivation of *C. ruminantium* under serum-free culture conditions has been reported previously (Zweygarth *et al.* 1997). Three *C. ruminantium* stocks were propagated in a serum-free medium based on a modified HL-1 medium, a DME/F-12-based medium supplemented with the HL-1 proprietary solution A, consisting of transferrin, testosteron, sodium selenite, ethanolamine, saturated and unsaturated fatty acids, and stabilising proteins. In further experiments, two components, namely bovine transferrin and bovine lipoproteins that have a growth-promoting action on *Cowdria* were identified. These two components were able to replace solution A in the culture medium (Zweygarth *et al.* 1998). A further step towards a completely defined medium was then attempted by replacing the bovine lipoproteins and transferrin with chemically defined compounds. Instead of bovine lipoproteins, a commercially available mixture of chemically defined lipids was used. Transferrin was replaced with a complex prepared from glycylglycine and ferric chloride (Yabe, Kato, Matsuya, Yamane, Iizuka, Takayoshi & Suzuki 1987). This supplemented medium, SFMC-23, supported the propagation of the Welgevonden stock in a way never observed in our laboratory before with respect to regularity of subculture and level of infection. *Cowdria* was propagated for 55 passages at regular 3-day intervals at split ratios, which are rarely achieved by conventional, serum-containing medium. During culture propagation it also became evident that the E_5 cell line allowed a higher split ratio than the BA cell line. The reasons for this are not yet clear. Both cell lines were bovine-derived, although from different anatomical sites, the umbilical cord (E_5) and the aorta (BA). Therefore, careful selection of the host cell line is necessary for optimal *Cowdria* growth.

Surprisingly, SFMC-36, the medium which was supplemented with L-glutamine and antibiotics only, gave similar results to SFMC-23. This conflicted with

previous findings, where supplements of lipids and transferrin had to be added to maintain growth of *C. ruminantium* in culture (Zweygarth *et al.* 1998). Nevertheless, DME/F-12, the basis of the serum-free media, contains ferric nitrate and ferrous sulphate as a source of iron, and linoleic acid as a source of fatty acids. The results presented here demonstrate that supplementation of the *Cowdria* culture medium with components other than L-glutamine and antibiotics is not necessary.

In addition to the Welgevonden stock, three other stocks of *C. ruminantium* were propagated in SFMC-23. However, passage intervals were slightly longer and subculture ratios usually lower than with the Welgevonden stock. This may have been due to the fact that these stocks had far fewer passages in culture than the Welgevonden stock and were therefore less adapted to growth *in vitro*.

Although the experiments are not completely conclusive as to the requirement of iron and/or lipid acids, it has been shown that *Cowdria* can be propagated in a protein-free, completely defined synthetic medium (DMEM/F-12) which is commercially available. The media described here may be of value for experiments in which serum has a negative impact on results. They can certainly contribute to the elucidation of the amino acid requirements of cultured *Cowdria*. Furthermore, these media may contribute to a more economical production of *C. ruminantium* organisms *in vitro*. Whether these media can also be used for culture initiation remains to be evaluated.

ACKNOWLEDGEMENTS

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