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SUPPLEMENT ARTICLE

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Serum-free in vitro cultivation of *Theileria annulata* and *Theileria parva* schizont-infected lymphocytes

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Abstract

Theileriosis is a tick-borne disease caused by intracellular protozoa of the genus *Theileria*. The most important species in cattle are *Theileria annulata* and *Theileria parva*. Both species transform leucocyte host cells, resulting in their uncontrolled proliferation and immortalization. Vaccination with attenuated *T. annulata*-infected cell lines is currently the only practical means of inducing immunity in cattle. Culture media for *Theileria* spp. typically contain 10%–20% foetal bovine serum (FBS). The use of FBS is associated with several disadvantages, such as batch-to-batch variation, safety and ethical concerns. In this study, the suitability of serum-free media for the cultivation of *Theileria*-transformed cell lines was examined. Three commercial serum-free media (HL-1, ISF-1 and Hybridomed DIF 1000) were evaluated for their ability to support growth of the *T. annulata* A288 cell line. The generation doubling times were recorded for each medium and compared with those obtained with conventional FBS-containing RPMI-1640 medium. ISF-1 gave the shortest generation doubling time, averaging 35.4 ± 2.8 hr, significantly shorter than the 52.2 ± 14.9 hr recorded for the conventional medium ($p = .0011$). ISF-1 was subsequently tested with additional *T. annulata* strains. The doubling time of a Moroccan strain was significantly increased (65.4 ± 15.9 hr) compared with the control (47.7 ± 7.5 hr, $p = .0004$), whereas an Egyptian strain grew significantly faster in ISF-1 medium (43.4 ± 6.5 hr vs. 89.3 ± 24.8 hr, $p = .0001$). The latter strain also showed an improved generation doubling time of 73.7 ± 21.9 hr in an animal origin-free, serum-free, protein-free medium (PFHM II) compared with the control. Out of four South African *T. parva* strains and a *Theileria* strain isolated from roan antelope (*Hippotragus equinus*), only one *T. parva* strain could be propagated in ISF-1 medium. The use of serum-free medium may thus be suitable for some *Theileria* cell cultures and needs to be evaluated on a case-by-case basis. The relevance of *Theileria* cultivation in serum-free media for applications such as vaccine development requires further examination.

KEYWORDS

foetal bovine serum, in vitro cultivation, serum-free, *Theileria annulata*, *Theileria parva*

1 | INTRODUCTION

Theileriosis is a tick-borne disease caused by obligate intracellular protozoa of the genus *Theileria*. The two most important species in cattle are *T. annulata*, which causes tropical or Mediterranean theileriosis, and *T. parva*, which causes East Coast Fever, both having a considerable economic impact on cattle husbandry in endemic countries. *Theileria annulata* occurs from the Mediterranean to the Middle East and parts of Asia where vector ticks such as *Hyalomma scupense* and *Hyalomma anatolicum* are present. *Theileria parva* is found in sub-Saharan Africa, where it is transmitted by *Rhipicephalus appendiculatus* ticks (Dolan, 1989). During tick feeding, sporozoites of *T. annulata* and *T. parva* are transferred with tick saliva into the mammalian host, where they invade lymphocytes and develop to macroschizonts. This results in a transformation of the leucocyte host cells, which subsequently leads to their uncontrolled proliferation and immortalization (Spooner, Innes, Glass, & Brown, 1989). The first in vitro cultures of *T. annulata* macroschizonts (Tchernomoretz, 1945) and *T. parva* macroschizonts (Malmquist, Nyindo, & Brown, 1970) date back more than 50 years. Vaccination with *T. annulata*-infected cell lines attenuated by in vitro propagation remains the only practical means of inducing immunity in cattle. This strategy has been adopted in several countries, whereby there is a tendency for using attenuated cell line vaccines derived from a local parasite isolate (Bilgic et al., 2019; Gharbi et al., 2011; Pipano & Shkap, 2000). The development of similar control strategies against *T. parva* has been constrained by the impractically high amounts of infected T-cells required (Brown, 1981), since the transfer of schizonts from donor culture cells into cells of the recipient animal, a step required for successful induction of immunity, occurs at a lower frequency in *T. parva* compared with *T. annulata* (Dolan, 1989). The propagation of *T. parva* in vitro has nonetheless been useful in pathogenesis studies. Culture media for *Theileria* spp. have remained virtually unchanged over the last decades and commonly contain foetal bovine serum (FBS). Serum supplementation, however, carries the risk of contamination by adventitious agents, particularly viruses and *Mycoplasma*, which could render a culture unusable. Further disadvantages of serum supplementation include batch-to-batch variability, high costs and ethical concerns (van der Valk et al., 2018), making the omission of FBS or newborn calf serum in culture media desirable. Serum-free cultivation techniques have been reported for related apicomplexan parasites such as *Theileria equi*, *Babesia caballi*, *Babesia bovis* and *Babesia bigemina* (Rojas Martinez et al., 2016; Rojas-Martinez et al., 2018; Zweygarth, Just, van Niekerk, & de Waal, 1997; Zweygarth, van Niekerk, & de Waal, 1999).

The objective of this study was to test commercially available serum-free media (SFM) developed for the propagation of hybridoma and myeloma cells, for their ability to support growth of *Theileria* spp. schizont-infected cell lines in vitro.

2 | MATERIALS AND METHODS

2.1 | *Theileria* spp. schizont-infected cell lines

Four different *T. annulata*-infected cell lines were used. The *T. annulata* strain A288 originated from a parasite stock isolated in Ankara, Turkey (Schein, Buscher, & Friedhoff, 1975). *Theileria annulata* Gharb was isolated in the region of Kenitra, Morocco, in 1980 (Ouhelli et al., 1989), and *T. annulata* strain Assiut was initiated in 2015 from peripheral blood lymphocytes of an ~3-year-old infected cow from El-Ghanayem, Assiut, Egypt (unpublished results). The fourth cell line originated from Bahrain (Uilenberg, Franssen, & Perie, 1986) and was resuscitated in 2017 after 20 years' storage in liquid nitrogen. The experiments with *T. annulata* strain Bahrain started at passage level 12 and with strain Assiut at passage 91. The passage levels of strains A288 and Gharb were not known. Before being adapted to serum-free culture conditions, the *T. annulata* cell lines were propagated in RPMI 1640 medium containing 2 g/L $NaHCO_3$ and 10% (v/v) heat-inactivated foetal bovine serum (FBS). The medium was buffered with 20 mM HEPES (N[2-hydroxyethyl]piperazine-N′-[2ethanesulfonic acid]), further supplemented with 2 mM L-alanyl-L-glutamine, 100 IU/mL penicillin and 100 µg/ml streptomycin. Unless otherwise indicated, all reagents were purchased from Biochrom, Berlin, Germany.

All *T. parva*-infected cell lines originated from South Africa. They were previously established at the Onderstepoort Veterinary Institute (OVI) by feeding ticks collected in a KwaZulu-Natal private game reserve in 2017 at the OVI on calves. Blood from these calves was used to initiate *T. parva* cell cultures 8160, 9596 and 9620. The fourth cell line (KNP II) was isolated by feeding laboratory-reared *Rhipicephalus zambeziensis* ticks on captive buffalo at Skukuza (Kruger National Park, KwaZulu-Natal, South Africa) and subsequently on a bovine at the OVI.

A continuous culture of *Theileria* sp. (roan) schizont-infected cells, originally isolated from a roan antelope (*Hippotragus equinus*) in South Africa (Zweygarth et al., 2009), was also tested. *Theileria parva* cell lines and the *Theileria* sp. (roan) cell line were routinely propagated in HL-1 medium (Lonza Walkersville Inc.) supplemented with 10% FBS, 2 mM L-alanyl-L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. The experiments with the *T. parva* cell lines started at passage level 20, and the *Theileria* sp. (roan) cell line at passage 12.

2.2 | Cultivation in serum-free media

For serum-free propagation, three different commercially available serum-free media were used: HL-1 medium, ISF-1 and Hybridomed DIF 1000. For logistical reasons, the protein-free hybridoma medium PFHM II (Gibco®), an animal origin-free, serum-free, protein-free medium was used for the *T. annulata* Assiut cell line only.

For serum-free propagation of *T. parva* schizont-infected cells and the *Theileria* sp. (roan) cell line, two different serum-free media were used: HL-1 medium and ISF-1 medium.

Weaning the *Theileria* spp. cell lines from serum-containing media was carried out by a weekly stepwise reduction of the FBS content of media initially containing 10% FBS. The serum concentration was reduced from 10% to 5%, 2% and 1%. Thereafter the serum-free media no longer contained any FBS.

All cultures were propagated at 37°C in a 5% CO_2-in-air atmosphere incubator.

2.3 | Generation doubling time

Cultures were set up in duplicate in 25 cm^2 vented cell culture flasks (Corning, NY, USA) at 2.5 × 10^5 cells/ml. After 3 or 4 days, 50 µl of the cell suspensions was diluted in 450 µl PBS with 0.5% trypan blue and counted in disposable counting chambers (C-Chip; Biochrom). Attached cells were dispersed with 2 ml of 0.025% ethylene diamine tetra-acetic acid (EDTA) solution and added to cell suspension before determining the cell numbers. Only live cells were counted. The schizont-infected cell proliferation was assessed as follows, where ln stands for natural logarithm: number of generations = [ln(final cell number) − ln(initial cell number)]/ln(2) and doubling time = time interval in hours/number of generations. Statistical analysis was performed using GraphPad Prism (https://www.graphpad.com/quickcalcs/ttest1.cfm) using an unpaired *t* test.

3 | RESULTS

The efficiency of the various culture media for the growth of *T. annulata*-infected cells of strain A288 is shown in Table 1. The growth-promoting effect of the serum-free media was similar to serum-containing medium, with ISF-1 being statistically significantly superior to the other media tested. All cultures were successfully propagated for more than 200 days, with the cultures being split every 3–4 days, with a split ratio between 1:2 and 1:4, depending on the density of the cells as subjectively judged using an inverted microscope.

A comparison of the ISF-1 medium with the conventional serum-containing RPMI 1640 medium for two other *T. annulata* strains from Morocco and Egypt showed that the Moroccan *T. annulata* strain Gharb had a statistically shorter generation doubling time in RPMI 1640 than in ISF-1 medium (p = .0004). In contrast, the Egyptian Assiut strain had a significantly shorter generation doubling time when ISF-1 medium was used (p = .0001). The Assiut strain was also cultivated in hybridoma medium PFHM II, a serum-free and protein-free medium, with a generation doubling time similar to that in RPMI 1640 medium. These results are summarized in Table 2.

TABLE 1 Generation doubling time of *Theileria annulata* A288 strain in various culture media

Medium	Duration of serum-free propagation (days)	Generation doubling time (hr) ± SD
RPMI 1640	n.a.	52.2 ± 14.9 (n = 17)[a]
DIF 1000	215	49 ± 7.7 (n = 11)[b]
HL-1	334	57.2 ± 14.2 (n = 11)[c]
ISF-1	220	35.4 ± 2.8 (n = 11)[abc]

[a]Significant difference between indicated values (unpaired t test, p = .0011).
[b]Significant difference between indicated values (unpaired t test, p = .0001).
[c]Significant difference between indicated values (unpaired t test, p = .0001).

TABLE 2 Generation doubling time of *Theileria annulata* Gharb and Assiut strains in various culture media

T. annulata strain	RPMI 1640	ISF-1	PFHM II
Gharb	47.7 ± 7.5 (n = 16)[a]	65.4 ± 15.9 (n = 15)[a]	not done
Assiut	89.3 ± 24.8 (n = 16)[b]	43.4 ± 6.5 (n = 25)[b]	73.7 ± 21.9 (n = 16)

[a]Significant difference between indicated values (unpaired *t* test, p = .0004).
[b]Significant difference between indicated values (unpaired *t* test, p = .0001).

The doubling time of the Bahrain stock of *T. annulata* was not determined by counting, but subculture intervals were used as a semi-objective assessment instead. Within 4 weeks of cultivation, 10 subcultures were carried out using the RPMI1640-based medium whereas when ISF-1 medium was used, 16 subcultures were performed.

In contrast to the *T. annulata* cultures, *T. parva* schizonts were routinely propagated in HL-1 medium supplemented with 10% FBS. Attempts to adapt these strains to HL-1 medium without serum supplementation, however, failed (data not shown). Among the four strains, only strain 8160 could be adapted to ISF-1 culture medium. Considering the generation doubling time of this strain, no significant difference was observed between ISF-1 (62.9 ± 55.1) and HL-1 with serum (63.1 ± 27.3). Attempts to propagate the *Theileria* sp. (roan) in HL-1 or ISF-1 failed.

4 | DISCUSSION

The primary objective of this study was to test three commercially available serum-free media for their ability to support the growth of *T. annulata* strain A288 in vitro: HL-1, ISF-1 and Hybridomed DIF 1000. These media were recommended by the respective manufacturers for the cultivation of hybridomas, which share with the *Theileria*-infected cell lines the characteristic of being immortal. All media tested supported the growth of *T. annulata* strain A288. The performance between them was similar, with the exception of ISF-1, since cultivation in this medium resulted in significantly shorter generation doubling times. A wide range of generation doubling times was found, in particular for RPMI-1640 and HL-1 (Table 1), which

might be due to the fact that the cell lines used were not clonal or synchronised. Further flow cytometry studies could be performed to elucidate which stages of the cell cycle are mainly affected by changes in the media composition. Due to the superior performance of ISF-1, subsequent experiments using additional *T. annulata* strains, *T. parva* and *Theileria* sp. (roan) were performed using this medium.

FBS accounts for a high percentage of the overall cost of the culture medium. The relative costs of the different media, with ISF-1 set at 100, were 91 for DIF 1000, 345 for HL-1, 270 for PFHM II and 285 for RPMI 1640 with 10% FBS. So in the experiments presented here, ISF-1-based culture medium was one of the more economical media and fell within the same cost range as DIF 1000, which was considerably cheaper than HL-1, PFHM II or RPMI 1640 with 10% FBS. Although ISF-1 was slightly more expensive than DIF 1000, the additional costs could be offset by the shorter generation doubling time as far as *T. annulata* strain A288 was concerned. Similar results were observed with the Assiut and Bahrain strains which seemed to perform better in ISF-1 than in the FBS-containing medium. In contrast, the Moroccan Gharb strain performed better in RPMI 1640-based medium. The question of why the Moroccan strain grew less well in ISF-1 remains unanswered. One of the reasons could be that the FBS batch used had better growth-promoting abilities for the Gharb strain and/or its host cell than for the others. FBS batches tend to vary in composition and as a result in quality, which may manifest itself in their growth-promoting effects.

Furthermore, we demonstrated that PFHM II supported the propagation of the Assiut strain of *T. annulata*. PFHM II is totally chemically defined medium that is entirely free of animal-derived components, minimizing the risk of poorly-defined contaminants. Immunisation of cattle using a culture-derived vaccine makes it imperative that no viruses are distributed through a contaminated vaccine. Currently, *T. annulata* vaccines are derived from cultures using FBS or other animal serum supplementation thus posing a potential danger. The easiest way to prevent this would be the use of a medium free of undefined animal-derived products.

It is interesting to note that although *T. parva* schizont-infected cells were routinely propagated in HL-1 medium supplemented with 10% FBS, none of these strains could be adapted to HL-1 medium without serum supplementation. Although HL-1 is optimized for the propagation of hybridomas, it seems that serum contains substances which are required by *T. parva*, but are absent in HL-1 medium. As far as the propagation of *T. parva* in ISF-1 is concerned, the results were rather disappointing and only one *T. parva* strain could be propagated, whereas all *T. annulata* strains grew in ISF-1 medium.

Our results showed that not all serum-free culture media support the different *Theileria* strains/species equally well. The reasons for this observation remain unclear, but it is interesting to note that the media used were specifically developed for the optimization for hybridoma cells, which belong to the B cell lineage. *Theileria parva* sporozoites infect B and all subsets of T lymphocytes in vitro, whereas *T. annulata* was found to infect monocytes and B lymphocytes, but not T lymphocytes (Baldwin et al., 1988; Spooner et al., 1989). Furthermore, *T. parva* cell lines obtained by infection of PBM with sporozoites in vitro exhibited T-cell markers (Emery, MacHugh, & Morrison, 1988). Therefore, it may well be that bovine cells infected with *T. annulata* generally respond better to media developed to support B cell growth than *T. parva* schizont-infected cells.

This study should be seen as a first step towards the optimization of serum-free *Theileria* cultivation. Further optimization of serum-free media would be useful, but is partially hampered by the fact that the exact composition of commercial serum-free media is not known to the user.

In conclusion, it was shown that *T. annulata* strains could be propagated in serum-free media optimized for hybridoma growth. The advantages of this are clear, as the serum-free cultivation of *Theileria* spp. macroschizonts is cheaper, safer and more ethical than FBS-containing alternatives. Nevertheless, the propagation in serum-free media of additional *T. annulata* strains and other *Theileria* spp. requires further investigation. It would in particular be important to examine whether attenuated *T. annulata* strains used for vaccination remain immunogenic when produced under serum-free culture conditions.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of the work described in this manuscript.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as human or animal subjects were not involved in this study.

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