LEIDIANE LIMA DUARTE

Establishment and characterization of embryonic cell lines of *Rhipicephalus* (*Boophilus*) *microplus* (RBME-6) and *Amblyomma sculptum* (ASE-14) (Acari: Ixodida) from Brazil

São Paulo

2021

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Thesis submitted to the Postgraduate Program in Experimental Epidemiology applied to Zoonoses of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

Department:
Preventive Veterinary Medicine and Animal Health

Area:
Experimental Epidemiology Applied to Zoonoses

Advisor:
Prof. Darci Moraes Barros Battesti, Ph.D.

São Paulo

2021

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T. 4116
FMVZ

Duarte, Leidiane Lima
Establishment and characterization of embryonic cell lines of *Rhipicephalus (Boophilus) microplus* (RBME-6) and *Amblyomma sculptum* (ASE-14) (Acari: Ixodida) from Brazil / Leidiane Lima Duarte. – 2021.
95 f. : il.

Título traduzido: Estabelecimento e caracterização de linhagens celulares embrionárias de *Rhipicephalus (Boophilus) microplus* (RBME-6) e *Amblyomma sculptum* (ASE-14) (Acari: Ixodida) do Brasil.

Tese (Doutorado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Medicina Veterinária Preventiva e Saúde Animal, São Paulo, 2021.

Programa de Pós-Graduação: Epidemiologia Experimental Aplicada às Zoonoses.

Área de concentração: Epidemiologia Experimental Aplicada às Zoonoses.

Orientadora: Profa. Dra. Darci Moraes Barros Battesti.

1. Carrapato-do-gado. 2. Carrapato-estrela. 3. Linhagem celular. 4. Cromossomos. 5. Brasil. I. Título.

Ficha catalográfica elaborada pela bibliotecária Camila Molgara Gamba, CRB 7070-8, da FMVZ/USP.

Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

CERTIFICADO

Certificamos que a proposta intitulada "Estabelecimento e caracterização de linhagens de células embrionárias de Rhipicephalus (Boophilus) microplus (RBME-6) e Amblyomma sculptum (ASE-14) (Acari:Ixodida) do Brasil.", protocolada sob o CEUA nº 1732120917 (ID 005688), sob a responsabilidade de **Darci Moraes Barros Battesti** *e equipe; Leidiane Lima Duarte* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 18/10/2018.

We certify that the proposal "Establishment and characterization of embryonic cell lines of Rhipicephalus (Boophilus) microplus (RBME-6) and Amblyomma sculptum (ASE-14) (Acari: Ixodida) do Brazil. ", utilizing 12 Rabbits (12 females), protocol number CEUA 1732120917 (ID 005688), under the responsibility of **Darci Moraes Barros Battesti** *and team; Leidiane Lima Duarte* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/18/2018.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 09/2017 a 12/2018 Área: Epidemiologia Experimental Aplicada As Zoonoses

Origem:	Não aplicável biotério						
Espécie:	Coelhos	sexo:	Fêmeas	idade:	2 a 12 meses	N:	12
Linhagem:	Oryctolagus cuniculus			Peso:	2 a 5 kg		

Local do experimento: As infestações serão realizadas no Biotério de Coelhos do Laboratório de Parasitologia, na sala de experimentação de animais. Cultivos in vitro: Departamento de Patologia Veterinaria-UNESP

São Paulo, 13 de setembro de 2021

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

EVALUATION FORM

Author: LIMA-DUARTE, Leidiane

Title: Establishment and characterization of embryonic cell lines of *Rhipicephalus* (*Boophilus*) *microplus* (RBME-6) and *Amblyomma sculptum* (ASE-14) (Acari: Ixodida) from Brazil.

Thesis submitted to the Postgraduate Program in Experimental Epidemiology applied to Zoonoses of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

Date: _____/_____/_____

Committee Members

Prof. _____

Institution :_____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____Decision:_____

This work is completely dedicated to my family, without whose constant support this thesis paper would not be possible.

ACKNOWLEDGEMENTS

First and foremost, I thank God for supporting me daily. He is the source of my power and inspiration and makes my life more bountiful.

I am extremely grateful to my mother (Antonia) for their love, prayers, caring and sacrifices for educating and preparing me for my future. My sister Sophia, who encourages me to be someone better.

I would like to express my sincere gratitude to my research supervisor, Dr. Darci Moraes Barros Battesti, for giving me the opportunity to do research and providing guidance throughout this research.

I would like to say thanks to my friends and research colleagues, M.Sc. Jaqueline Valéria Camargo, M.Sc. Ana Carolina Castro Santiago, M.Sc. Lívia Perles and Dr. Priscila Ikeda for their constant encouragement.

I express my special thanks Prof. Dr. Marcos Rogério André and Prof. Dr. Rosangela Zacarias Machado, Department of Pathology, Reproduction and One Health, School of Agricultural and Veterinary Sciences, São Paulo State University "Julio de Mesquita Filho" (UNESP), for his genuine support throughout this research work.

To Prof. Dr. Alvimar José da Costa and Dr. Carlos Alberto Perez for providing the ticks. I thank Prof. Dr. Maria Izabel Camargo Mathias, Dr. Luís Adriano Anholeto and M.Sc. Melissa Carolina Pereira, for helping with the morphological characterization of the cells and and improvements in chapters of this thesis.

I thank Prof. Dr. Diogo Cavalcanti Cabral-de-Mello and Ana Beatriz Stein Machado Ferretti for helping with the karyotyping of cell lines and improvements in chapters of this thesis.

I would also like to thank Ricardo Bassini Silva for reviewing the thesis.

I am extending my thanks to the colleagues of Department of Pathology, Reproduction and One Health, School of Agricultural and Veterinary Sciences, São Paulo State University "Julio de Mesquita Filho" (UNESP), for their support during my research work. I also thank all the staff of Department, Arvelino and Mabel.

I thank the staff of the Postgraduate Secretary VPS and Danival Lopes Moreira, for your readiness and kindness.

To my friends: Midian, Izabela, Ana Maria, Ronaldo, Giovanna, you have a special place in my heart.

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico under the Grant Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [CNPq No. 312331/2013-4 and 302420/2017-7]; Fundação de Amparo à Pesquisa do Estado de São Paulo under Grant (FAPESP) [No. 2015/26209-9 and 2020/03190–9]; this study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Last, but not the least, to my caring, loving, and supportive life partner, Marco Antônio de Caires Santana, my deepest gratitude. Your encouragement when the times got rough are much appreciated and duly noted.

“*I was taught that the way of progress was neither swift nor easy*.” - Marie Curi

RESUMO

LIMA-DUARTE, L. Estabelecimento e caracterização de linhagens celulares embrionárias de *Rhipicephalus* (*Boophilus*) *microplus* (RBME-6) e *Amblyomma sculptum* (ASE-14) (Acari: Ixodida) do Brasil. 2021. 95 p. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo, 2021

Os carrapatos são artrópodes de grande importância médica e veterinária, sendo responsáveis pela transmissão de vírus, bactérias, helmintos e protozoários aos seus hospedeiros. Alguns dos patógenos transmitidos por carrapatos são difíceis de cultivar *in vitro* e não se multiplicam em meios artificiais ou outros substratos. As linhagens de células de carrapatos representam uma ferramenta útil para estudar muitos aspectos da pesquisa de carrapatos e patógenos transmitidos por eles. Aqui, estabelecemos e caracterizamos duas linhagens celulares RBME-6 e ASE-14 derivadas de embriões de *Rhipicephalus microplus* e *Amblyomma sculptum*, respectivamente, ambos coletados no Brasil. Culturas primárias de células foram preparadas a partir de ovos embrionados das duas espécies de carrapatos, em meio L-15B, e mantidas à 30 °C. Quando as culturas atingiram uma densidade celular adequada, foram subcultivadas e criopreservadas em várias passagens. As análises citológicas foram feitas usando microscopia de contraste de fase, com células que foram submetidas à citocentrifugação e coradas com Giemsa, enquanto a técnica de coloração com azul de bromofenol foi usada para detectar proteína e a reação com Ácido Periódico de Schiff (PAS) foi usada para detectar polissacarídeos. Não foi detectado o DNA de *Anaplasma* spp., *Anaplasma marginale*, *Babesia/Theileria* spp, *Bartonella* spp., *Coxiella* spp., *Ehrlichia canis*, *Rickettsia* spp. ou *Mycoplasma* spp. nas células por meio de ensaios de PCR. Além disso, realizamos a caracterização cromossômica das linhagens celulares de ambas as espécies de carrapatos e confirmamos a origem dessas linhagens por meio de PCR convencional e sequenciamento de um fragmento do gene mitocondrial 16S rRNA. Em conclusão, duas novas linhagens celulares derivadas dos embriões de carrapatos foram geradas e caracterizadas neste estudo. Essas linhagens celulares poderão ser usadas em estudos futuros sobre diferentes aspectos, como as relações entre carrapatos e patógenos transmitidos por eles, expressão de proteínas em células infectadas e não infectadas e obtenção de antígenos. Além disso, é importante ter um painel maior de linhagens de células de carrapatos, pois elas podem servir como ferramentas eficientes para o avanço de pesquisas em várias áreas da virologia, bacteriologia, biologia e controle desses carrapatos.

Palavras-chave: carrapato-do-gado; carrapato-estrela; linhagem celular; cromossomos; Brasil.

ABSTRACT

LIMA-DUARTE, L Establishment and characterization of embryonic cell lines of *Rhipicephalus* (*Boophilus*) *microplus* (RBME-6) and *Amblyomma sculptum* (ASE-14) (Acari: Ixodida) from Brazil. 2021. 95 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

Ticks are arthropods of great medical and veterinary importance, being responsible for the transmission of viruses, bacteria, helminths and protozoa to their hosts. Some of the tick-borne pathogens are difficult to grow *in vitro,* and do not grow on artificial media or other substrates. Tick cell lines represent a useful tool for studying many aspects of tick and tick-borne pathogen research. Here, we established and characterized two cell lines RBME-6 and ASE-14 derived from embryos of *Rhipicephalus microplus* and *Amblyomma sculptum*, respectively, both from Brazil. Primary tick cell cultures were prepared in L-15B at 30°C.When they reached an adequate density, the cells were subcultured and cryopreserved in several passages. Cytological analysis were performed using phase contrast microscopy and cytocentrifuge smears stained with Giemsa, while periodic acid-Schiff and bromophenol blue staining techniques were used to detect total polysaccharides and the total protein, respectively. *Anaplasma* spp., *Anaplasma marginale*, *Babesia/Theileria* spp., *Coxiella* spp., *Ehrichia canis*, *Mycoplasma* spp. and *Rickettsia* sp. were not detected in the cells through PCR assays. In addition, we performed chromosomal characterization of the tick cell line and confirmed the origin of the cell line through conventional PCR and sequencing of the 16S rRNA gene mitochondrial fragment. In conclusion, two new cell lines derived from embryos of ticks were generated and characterized in this study. These cell lines can be used in future studies on different aspects, such as the relationship between ticks and pathogens transmitted by them, expression of proteins in infected and non-infected cells and obtaining antigens. In addition, it is important to have a larger panel of tick cell lines, as they can serve as efficient tools for advancing research in various areas of virology, bacteriology, biology and control of these ticks.

Keywords: cattle tick; star tick; cell line; chromosome; Brazil.

ABBREVIATIONS

Bp	base pair
CCHFV	Crimean-Congo hemorrhagic fever virus
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
FBS	Fetal Bovine Serum
MEM	Minimum Essential Medium
PCR	Polymerase Chain Reaction
SPG	Sucrose-phosphate-glutamate
Td	Population Doubling Time
rRNA	ribosomal ribonucleic acid
TBEV	Tick-Borne Encephalitis Virus
TPB	Tryptose Phosphate Broth
UUKV	Uukuniemi virus

SUMMARY

1. INTRODUCTION

Ticks are obligate hematophagous ectoparasites that can transmit a variety of pathogens to humans and animals, being the second most common arthropod pathogen vector after the mosquitoes (DANTAS-TORRES *et al*., 2012; MASATANI *et al*., 2017).

The tick-borne pathogens include several species of bacteria belonging to the families Anaplasmataceae and Rickettsiaceae, and protozoa belonging to the genera *Hepatozoon*, *Theileria* and *Babesia* (JONGEJAN *et. al*., 2004; BANETH *et al*., 2014). Ticks also transmit viruses, such as Tick-borne encephalitis virus (TBEV; genus Flavivirus, family Flaviviridae) and Crimean-Congo hemorrhagic fever virus (CCHF) (LANI *et al*., 2014).

Currently, more than 979 valid species are recorded worldwide. They are included in the order Ixodida (Metastigmata), divided in the families – (1) Ixodidae (hard ticks), presenting approximately 758 species; (2) Argasidae (soft ticks), with approximately 221 species; and (3) Nuttalliellidae, represented by a single species, *Nuttalliella namaqua* Bedford, 1931 (GUGLIELMONE *et al*., 2021; MUÑOZ-LEAL *et. al*., 2021).

In Brazil, the tick fauna is composed by 76 species, of these 51 species into the family Ixodidae family and 25 species into the family Argasidae (MARTINS *et al*., 2021; MUÑOZ-LEAL *et al*., 2021). The family Ixodidae is distributed into five genera: *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes* and *Rhipicephalus*. The family Argasidae comprises four genera *Antricola*, *Argas*, *Nothoaspis* and *Ornithodoros* (BARROS-BATTESTI *et al*., 2006; DANTAS-TORRES *et al*., 2019; MUÑOZ-LEAL *et al*., 2020; ONOFRIO *et al*., 2020; MARTINS *et al*., 2021).

One of these genera, *Amblyomma* comprises the most common hard ticks in the Neotropical region, and more than half of species of this genus are endemic to the New World (GUGLIELMONE *et al*., 2010). In addition, some species of this genus are the main vectors of *Rickettsia rickettsii* that cause rickettsiosis, and other bacteria that cause anaplasmosis and ehrlichiosis. (SANGIONI *et al*., 2005; SCHULZE *et al*., 2005; LABRUNA, 2009; WITTER *et al*., 2016).

Amblyomma sculptum Berlese,1888, a member of the *Amblyomma cajennense* complex, has a wide distribution in the Southeastern, Central-Western, and part of Southern Brazil, Northern Argentina, Bolivia and Paraguay (GUGLIELMONE *et al*., 2006; NAVA *et al*., 2014; MARTINS *et al*., 2016). The primary hosts of *A. sculptum* are horses and capybaras (SANGIONI *et al*., 2005; LABRUNA 2009), however this species also feeds on many wild terrestrial and domestic animals (BRITES-NETO *et al*., 2015). This tick species is considered

the main vector of the Brazilian spotted fever (LABRUNA, 2009) and the one most frequently recorded biting humans in Brazil (GUGLIELMONE *et al*., 2006, RAMOS *et al*., 2014).

The genus *Rhipicephalus* comprises 87 species in the world (GUGLIELMONE *et al*., 2021, http://rafaela.inta.gob.ar/nombresgarrapatas/ accessed 07 August 2021), including five species of the subgenus *Rhipicephalus* (*Boophilus*) (Canestrini, 1887) (GUGLIELMONE *et al*., 2010; HORAK *et al*., 2013). In Brazil, this genus is represented by the species *R. sanguineus* s.s. (Latreille, 1806), *R. sanguineus* s.l. and *R. microplus*. It is noteworthy that the species under the name *R. sanguineus* s.l. could be another species (NAVA *et al*., 2018; SANCHES *et al*., 2021). In a recent study, the "tropical lineage" was identified as *Rhipicephalus linnaei* (Audouin, 1826) using material from Australia (ŠLAPETA *et al*. 2021).

Rhipicephalus microplus, known as the cattle tick, has a wide geographic distribution, being found principally in Tropical and Subtropical regions (ESTRADA-PEÑA *et al*. 2006). This species is the most important ectoparasite of cattle and poses as a major economic impact to animal productivity in these regions (JONSSON, 2006; GRISI *et al*., 2014; RODRIGUEZ-VIVAS *et al*., 2018). The economic losses are mainly associated with blood intake, which affects meat and milk production, transmission of infectious agents (e.g., *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale*), inoculation of toxins into hosts, generating several changes and consequences such as food incompetence. In addition, large infestations also reduce the animal's skin quality due to scars generated during feeding (HORN, 1983; FURLONG, 1993; KLAFKE *et al*., 2006).

1.1. TICK CELL LINES: BACKGROUND, CHARACTERISTICS AND APPLICABILITY

Attempts to grow tick cells date back more than 60 years ago. The first attempt was made by Rehacek (1958), who obtained cells from tissues of *Dermacentor marginatus* (Sulzer, 1776); however, the cultivation only lasted three days. Although, some advances have occurred since the first attempt, the success of tick cell culture *in vitro* was limited to primary cultures capable of surviving up to a maximum of 8 months (PUDNEY *et al*., 1973). Improvements in methodology allowed the establishment of the first cell line obtained from tick tissue of nymphs of the species *Rhipicephalus appendiculatus* Neumann, 1901 (VARMA *et al*., 1975). Currently there are 65 ticks cell lines from different species (Table 1), 15 for the family Ixodidae (*Amblyomma*, *Rhipicephalus*, *Dermacentor*, *Hyalomma*, and *Ixodes*) and three for the family Argasidae (*Argas* and *Ornithodoros*) (BELL-SAKYI *et al*., 2018). Tick cell lines are obtained from cultures derived from eggs, larvae or nymphs. Most tick strains are embryo-derived, as

eggs are an abundant source of rapidly growing undifferentiated cells (BELL-SAKYI *et al*., 2007). Attempts to obtain strains derived from specific organs such as salivary glands, haemocytes, midgut or ovaries have so far not been successful (HOFFMANN *et al*., 1970; FUJISAKI *et al*., 1975; REHACEK, 1976; MOSQUEDA *et al*., 2008; REZENDE *et al*., 2015).

A cell line arises from a primary culture from the first subculture (YUNKER *et al*., 1981). The significant growth of cells from a primary culture can take from eight weeks to three years, and the production of a cell line can take from one to seven years (BELL-SAKYI *et al*., 2018).

Tick cell lines, regardless of species, share some characteristics that differ from insect and vertebrate cell lines (BELL-SAKYI *et al*., 2007; 2018). One of the characteristics of tick cell lines is that the cells are generally not tightly adherent, growing with a combination of incomplete monolayer and suspension cells. Furthermore, do not exhibit contact inhibition and most grow in three dimensions (BELL-SAKYI *et al*., 2007). These cell lines are genotypically and phenotypically heterogeneous, having been derived from multiple individuals (YUNKER, 1987; BELL-SAKYI *et al*., 2007). There are few studies on the characterization of the multiple phenotypes present in the strains (DE ABREU *et al*., 2013, ESTEVES *et al*., 2008; OLIVER *et al*., 2015).

Although, it is known that with the subculture notes is that the diversity of types of cells decreases, and one or two types of cells become dominant (KURTTI & MUNDERLOH, 1982; MUNDERLOH, *et al*., 1994).

Tick cell growth is relatively slow, and this can be measured by population doubling time (PDT). Temperature can interfere with cell growth, and the increase in incubation temperature makes cell multiplication faster, although it should not be much higher than 33°C (YUNKER *et al*., 1981). Most tick cell lines do not require regular subculture and can be maintained as individual cultures with a weekly medium change for many months or years; this characteristic is useful for the attempt of *in vitro* isolation of slow growing microorganisms (BELL-SAKYI *et al*., 2007). The number of subcultures varies between tick cell lines. One culture flask can be expanded to another (LALLINGER *et al*., 2010) or two other flasks (VARMA *et al*., 1975; PUDNEY *et al*., 1979; HOLMAN, 1981), or to three flasks (BAHT & YUNKER, 1977) or to five flasks (MUNDERLOH *et al*., 1994).

Tick species	Cell line	Instar	Reference
Argas reflexus	ARE/LULS41	Eggs	(Bell-Sakyi *et al*.,2018)
Amblyomma americanum	AAE2,12	Eggs	(Kurtti *et al*., 2005) (Singu *et al*., 2006)
Amblyomma variegatum	AVL/CTVM13,17	Larvae	(Bell-Sakyi, 2004) (Bell-Sakyi *et al*., 2000)
Carios capensis	CCE1, CCE2	Eggs	(Kurtti *et al*., 2005)
Dermacentor albipictus	DALBE3	Eggs	(Kurtti *et al*., 2005) (Munderloh *et al*., 1996) (Policastro *et al*., 1997)
Dermacentor andersoni	DAE15, DAE100T		(Kurtti *et al*., 2005) (Simser *et al*., 2001)
Dermacentor nitens	ANE58	Eggs	(Kurtti *et al*., 2005)
Dermacentor variabilis	DVE1	Eggs	(Kurtti *et al*., 2005)
Hyalomma anatolicum	HAE/CTVM8, HAE/CTVM9	Eggs	(Bell-Sakyi,1991)
Hyalomma dromedarii	HDE/PIPA33 HDE/PILS37 HDE/LURF39	Eggs	(Bell-Sakyi *et al*., 2018)
Ixodes ricinus	IRE11 IRE/CTVM18, 19, 20	Eggs	(Lawrie *et al*., 2004) (Bell-Sakyi, 2004) (Simser *et al*., 2002)
Ixodes scapularis	IDE2, 8, 12 ISE6 ,18	Eggs	(Munderloh *et al*., 1994) (Kurtti *et al*.,1996)
Ornithodoros moubata	OME/CTVM21 22,24,27	Eggs/Larvae	(Bell-Sakyi *et al*., 2009)
Rhipicephalus appendiculatus	RAE25 RAE/CTVM1 RA243, 257 RAN/CTVM3 RAE/PIPM38	Eggs Nymph	(Kurtti, *et al*.,1988) (Bell-Sakyi, 2004) (Varma *et al*.,1975) (Bekker *et al*., 2002) (Bell-Sakyi *et al*., 2018)
Rhipicephalus decolaratus	BDE/CTVM14, 16	Eggs	(Bell-Sakyi,2004)
Rhipicephalus evertsi	REE/CTVM31 REN/CTVM32 REN/PIPA34	Eggs Nymph	(Alberdi *et al*., 2012) (Bell-Sakyi *et al*., 2015) (Bell-Sakyi *et al*.,2018)
Rhipicephalus microplus	BmVIII-SCC, BME/CTVM2, BME/CTVM4, BME/CTVM5 BME/CTVM6 BME/CTVM23 BME/CTVM30 BME/PIBB36 RBME-6	Eggs	(Kurtti, *et al*.,1988) (Cossio-Bayugar *et al*., 2002) (Bell-Sakyi,2004) (Holman & Ronald, 1980) (Bell-Sakyi *et al*., 2018) (Lima-Duarte *et al*., 2021)
Rhipicephalus sanguineus s.l.	RSE8 RML-RSE RSE/PILS35	Eggs	(Kurtti, *et al*.,1988) (Yunker *et al*.,1984; 1987) (Koh-Tan *et al*., 2016.)

Table 1. Continuous tick cell lines.

Some changes in the number of chromosomes can occur *in vitro* culture (HOLMAN & RONALD, 1980). In initial stage most tick cell lines are diploid, in which there are male and female chromosomes (PUNDNEY *et al*., 1973; BHAT & YUNKER, 1977; VARMA *et al*., 1975; ESTEVES *et al*., 2008). Cell lines with aneuploid chromosome number have also been reported (MATTILA *et al*., 2007).

Tick cells grow at incubation temperatures between 28°C to 34°C, although some strains also grow at 37°C. Most of the tick cells thrive in acidic conditions (pH 6.5-6.8), similar to the environment of proliferating cells within larvae, nymphs and adults (BELL-SAKYI *et al*., 2007).

The first tick cell cultures were initially maintained in media formulated for mammalian cells. Leibovitz L-15 medium (Leibovitz, 1963) supplemented with TPB (Tryptose Phosphate Broth) and FBS (Fetal Bovine Serum) or their mixture with MEM medium are frequently used to cultivate various tick cultures (KURTTI & MUNDERLOH, 1982). The L-15 medium (LEIBOVITZ, 1963) was modified by the addition of vitamins, trace elements, alpha-ketoglutaric acid, amino acids and glucose (L-15B medium) and has been shown to be an efficient medium for the growth of several tick cell lines (MUNDERLOH & KURTTI, 1989).

The most used supplement in cell culture is fetal bovine serum (FBS), whose concentration varies among tick species, since 5%, 10% and even 20% (KURTTI *et al*., 1982; SAMISH, *et al*., 1985). In addition, another supplement used in culture media is Tryptose Phosphate Broth (TPB) at a concentration of 10%. This supplement provides glucose, which is not present in L-15, as well as disodium phosphate (DSP), which improves the buffering capacity of the medium. It also contains sodium chloride and peptones (KURTTI & MUNDERLOH, 1982). Antibiotics such as penicillin (100 - 1000 units / ml) and streptomycin (0.1-1.0 mg / ml) are also frequently added to the medium (PUDNEY *et al*., 1979; BELL-SAKYI, 2004).

Tick cell line cryopreservation has already been described in detail (LALLINGER *et al*., 2010), however long-term cryopreservation in liquid nitrogen does not guarantee the successful recovery of a cell lineage (BELL-SAKYI, 2007).

As cryopreservation in liquid nitrogen has some limitations (BELL-SAKYI, 1991; MUNDERLOH *et al*., 1994), other short-term storage alternatives under refrigeration conditions can be carried out using temperatures of 4 ºC, 6 ºC and 12 ºC (SAMISH, 1988a; BASTOS *et al*., 2006; MATTILA *et al*., 2007; LALLINGER *et al*., 2010).

Lallinger *et al*. (2010) reported the success of short-term cryopreservation of cells derived from *Rhipicephalus decoloratus* Koch, 1844, *R. microplus* and *Ixodes ricinus* (L., 1758), using dimethyl sulfoxide (DMSO) and sucrose-phosphate-glutamate (SPG) as cryoprotectants. The

cryopreservation process that showed better result has been done mainly using 10% DMSO in the L-15B medium and gradual freezing procedures (BASTOS *et al*., 2006).

Since the first tick cell lines were established, they have been applied extensively to isolate, propagate and study tick-borne viruses and bacteria. They have also been widely used in studies of tick genomics, biology and physiology, innate immunity, tick bite allergy, tick microbiome, anti-tick vaccines and mode of action of acaricides (BELL-SAKYI *et al*., 2007; 2018).

Tick cell cultures provide a simplified *in vitro* vector system that can provide insights into the actual range and potential of possible vector species and the factors that command the ability to transmit a particular pathogen (BELL-SAKYI *et al*., 2018). Some pathogens, such as *R. rickettsii* can exert an inhibitory effect on the apoptosis of tick cells, showing that this pathogen modulates the proteome to ensure cell colonization (MARTINS *et al*., 2020). Propagation of agents as *Ehrlichia*, *Rickettsia* and *Anaplasma* have already been carried out in tick cell lines (MUNDERLOH *et al*., 1996; BLOUIN *et al*., 2002). The bacteria belonging to the genus *Anaplasma* and *Ehrlichia* were the most propagated pathogens in tick cell lines (BELL-SAKYI *et al*., 2018). The cultivation of these pathogens was achieved in cell lines derived from several tick species, predominantly those of *Ixodes scapularis* (ISE6, IDE8) (MUNDERLOHL *et al*., 1994).

Ehrlichia canis has been successfully propagated in *I. scapularis* tick strains (EWING *et al*., 1955; MUNDERLOH *et al*., 1989; MUNDERLOH *et al*., 1998; ZWEYGARTH *et al*. 2014), *I. ricinus* (SINGU *et al*., 2006) and *R. sanguineus* s.l. (FERROLHO *et al*., 2016; BARROS-BATTESTI *et al*., 2018). *Anaplasma phagocytophilum* was successfully cultivated in the IDE8 and ISE6 cell lines (GOODMAN *et al*., 1996; MUDERLOH *et al*., 1996; 1999). The metataxonomic analysis showed that there is a significant diversity of bacterial taxa in females of *R. sanguineus* and in primary embryonic cell cultures of both, the temperate (*R. sanguineus* s.s.) and the tropical (*R. sanguineus* s.l.) lineages. It was showed the presence of *Coxiella* in all the samples, although in different proportions. In addition to *Coxiella* endosymbiont, a putative novel species of *Coxiella* was also observed. These *Coxiella* species present in the two tick lineages and in the tick cell cultures studied, seem to be different and may have co-evolved with these tick lineages, suggesting that the taxon *R. sanguineus* s.l. belongs to a new species that needs to be described (LUZZI *et al*., 2021). So, in this way, the results of metataxonomic analysis confirm the conclusions obtained by Sanches *et al*. (2021). The differential expression of proteins obtained for the species *R. sanguineus* (tropical and temperate lineages), with and without *E. canis* infection, showed important differences in proteomic profiles, which added to genetic, microbiome and biological differences, behavioral

and morphological, reported in previous studies, reinforce the hypothesis of the existence of two different tick species (SANCHES *et al*., 2021), reinforcing also the findings of Nava *et al*. (2018). According to these authors, *R. sanguineus* s.l. is a name applied to ticks that are morphologically close to *R. sanguineus* s.s. but cannot be included under that name (GUGLIELMONE *et al*., 2021).

Ehrlichia ruminantium was propagated in cell lines derived from *I. scapularis*, *I. ricinus*, *Amblyomma variegatum* (Fabricius, 1798), *Rhipicephalus decoloratus* Koch, 1844, *R. microplus* and *R. appendiculatus* (BELL-SAKYI, *et al*., 2000; BELL-SAKYI, 2004).

Anaplasma marginale was first propagated in the lineage derived from *I. scapularis* (IDE8) and remained infective for cattle after several passages in culture (MUNDERLOH *et al*., 1996; BLOUIN, KOCAN 1998). This cell line was then used for the isolation and propagation of several other *A. marginale* isolates (BLOUIN *et al*., 2002; BASTOS *et al*., 2009; BAÊTA *et al*., 2015), and to isolate and propagate *A. phagocytophilum* (WOLDEHIWET *et al*., 2002; SILAGHI *et al*., 2011), also propagated in ISE6.

Other bacteria such as *R. rickettsii*, *R. peacockii* and *Rickettsia buchneri* have also been successfully cultivated in tick cell lines (YUNKER *et al*., 1984; 1987; POLICASTRO *et al*., 1997; KURTTI *et al*., 2005; 2015). In addition, it has already been reported that *Rickettsia raoultii* can infect and propagate in the *R. microplus*-derived BME/CTVM23, *R. sanguineus*-derived RSE/PILS35, and *I. scapularis*-derived IDE8 cell lines (HUSIN *et al*., 2021).

A recent study demonstrated that cell lines from *I. scapularis*, *I. ricinus* and *R. microplus*, can also host multiple *Wolbachia* strains (KHOO *et al*., 2020).

Some studies highlight tick cell lines as research tools, and the advantages that they provide for virologists (BELL-SAKYI *et al*., 2007). Lymphocytic choriomeningitis virus surprisingly also grew in primary cultures of *Hyalomma dromedarii* Koch, 1844 (REHACEK 1964). Tick-borne encephalitis virus (TBEV) was cultivated in tick cells of six genera: *Hyalomma*, *Rhipicephalus*, *Dermacentor*, *Ixodes*, *Amblyomma* and *Ornithodoros* (REHACEK 1964; BHAT, YUNKER, 1979; LAWRIE *et al*., 2004, RŮŽEK *et al*., 2008). As well, it has already been reported the success of the propagation of Crimean-Congo hemorrhagic fever virus (CCHFV) in seven different tick cell lines (BELL-SAKYI *et al*., 2012).

Belova *et al*. (2017) reported that TBEV strain EK-328 of the Siberian subtype effectively replicated and formed persistent infection in ticks and tick cell lines of vector and non-vector species. Mazelier *et al*. (2016), using IRE/CTVM19 and IRE/CTVM20 cell lines derived from *I. ricinus* and the model phlebovirus Uukuniemi virus (UUKV), showed that tick cell-derived

virus particles have specific molecular and structural properties that enhance their infectivity for mammalian cells.

Advances in the identification of host cell defense pathways, and methods for genetic manipulation together with the use of tick cell lines, allowed the elucidation of the complex interactions between arboviruses and vectors at the cellular and molecular levels (NUTTALL, 2009; BELL-SAKYI *et al*., 2012).

Tick cell lines have also been used for the cultivation of the protozoans *Babesia caballi* and *B. bigemina* (KURTTI *et al*., 1983; RIBEIRO *et al*., 2009). *Besnoitia besnoiti* (Apicomplexa: Sarcocystidae) was cultivated in cell lines originating from *R. microplus*, *R. appendiculatus* and *D. variabilis* (SAMISH *et al*., 1987, 1988b).

Studies have reported the successful growth of the *Borrelia burgdorferi* sensu lato (sl) spirochete bacteria in tick cells (KURTTI *et al*. 1988; 1993). Rezende *et al*. (2008) identified spirochetes of *Borrelia* spp. in primary culture of *R. microplus* cells. Varela *et al*. (2004) isolated *Borrelia lonestari* (strain LS-1) in tick cell line (ISE6).

A cell line of *R. appendiculatus* was used as a substrate for the maintenance of the bovine nematode *Onchocerca lienalis* (Litchfield *et al*., 1991).

Tick cell lines have already been shown to be an effective system for investigating resistance to acaricides and antibiotics. Cossio-Bayugar *et al*. (2002) generated three strains of *R. microplus* resistant to organophosphates, when these strains were exposed to the BmVIII-SCC (HOLMAN, 1980). Furthermore, Blouin *et al*. (2002), when verifying the action mechanism of tetracycline to eliminate *A. marginale*, demonstrated that the action of this antibiotic promotes an interference in the pathogen's capacity to complete its replication cycle in the tick cell cytoplasm.

In a recent study, the *I. ricinus*-derived cell line IRE/CTVM19 was treated with the acaricides amitraz, permethrin or fipronil, and the results of this study supported the proposed application of tick cell lines as *in vitro* models for the study of resistance to these acaricides (MANGIA *et al*., 2018).

Previous studies demonstrated the use of tick cell cultures for the isolation of antigens to be used in diagnostic tests and in the development of vaccines (BLOUIN *et al*., 1998; SALIKI *et al*., 1998; KOCAN *et al*., 2001; DE LA FUENTE *et al*., 2002). The ISE6 and ID8 cell lines, for example, played an essential role in studies of differential gene transcription and outer membrane protein expression, in the search for vaccine candidates against *A. marginale* (BRAYTON *et al*., 2006).

Tick cell lines played an important role involving the genetic manipulation of pathogens. *I. scapularis* cells infected with *R. monacensis* transformants proved to be a useful system for the study of interactions between *Rickettsia* and host cells (BALDRIDGE *et al*., 2005).

Tick cells have also been shown to be a useful tool for studies on phase-specific gene transcription and protein expression. In a study using *I. scapularis* (IDE2) and *D. albipictus* (DALBE3) cells infected with *R. rickettsii* it was shown that protein expression is temperature dependent at 28 °C and 34 °C, this temperature response may be associated with the expression of rickettsial determinants that are pathogenic for mammals (POLICASTRO *et al*., 1997). Singu *et al*. (2005), cultivated *Ehrlichia chaffeensis* in DH82 cells and in tick cell lines and using proteomic approaches, showed that proteins expressed in macrophages are products of genes that differ from those expressed in tick cells.

1.2. CHAPTER PRESENTATION

This thesis is written in two different chapters. Chapter 1, entitled "Establishment and characterization of a cell line (RBME-6) of *Rhipicephalus* (*Boophilus*) *microplus* from Brazil". This chapter is already published as follows: Leidiane Lima Duarte, Jaqueline Valéria Camargo, Ana Carolina Castro-Santiago, Rosangela Zacarias Machado, Marcos Rogério André, Diogo Cavalcanti Cabral-de-Mello, Maria Izabel Camargo-Mathias, Priscila Ikeda, Luís Adriano Anholeto, Melissa Carolina Pereira, Alvimar José da Costa, Darci Moraes Barros-Battesti. Ticks and Tick-Borne Diseases, 12, 101770, 2021. https://doi.org/10.1016/j.ttbdis.2021.101770.

Chapter 2, "Establishment and multiapproach characterization of *Amblyomma sculptum* 2 (Acari: Ixodidae) cell line (ASE-14) from Brazil". This chapter is submitted to Ticks and Tick-Borne Diseases as follows: Leidiane Lima Duarte, Ana Carolina Castro-Santiago, Jaqueline Valéria Camargo, Ana Beatriz Stein Machado Ferretti, Luís Adriano Anholeto, Melissa Carolina Pereira, Priscila Ikeda, Carlos Alberto Perez, Gustavo Seron Sanches, Maria Izabel Camargo Mathias, Diogo Cavalcanti Cabral de Mello, Rosangela Zacarias Machado, Marcos Rogério André, Darci Moraes Barros-Battesti.

1.3. GENERAL OBJECTIVE

- To establish and characterize two new tick cell lines of *Rhipicephalus* (*Boophilus*) *microplus* and *Amblyomma sculptum*.

1.3.1 SPECIFIC OBJECTIVES

- To obtain *R. microplus* and *A. sculptum* embryonic cell cultures and establish new cell lines;

- To characterize the cell lines of *Rhipicephalus* (*Boophilus*) *microplus* and *Amblyomma sculptum*;

- Investigate the presence of *Anaplasma* spp., *Borrelia* spp, *Babesia* spp., *Bartonella* spp., *Coxiella* spp., *Mycoplasma* spp. and *Rickettsia* spp. in these tick cell lines.

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2. CHAPTER 1 - **Establishment and characterization of a cell line (RBME-6) of *Rhipicephalus* (*Boophilus*) *microplus* from Brazil**

Leidiane Lima-Duarte[1], Jaqueline Valéria Camargo[2], Ana Carolina Castro-Santiago[1], Rosangela Zacarias Machado[2], Marcos Rogério André[2], Diogo Cavalcanti Cabral-de-Mello[3], Maria Izabel Camargo-Mathias[3,] Priscila Ikeda[2], Luís Adriano Anholeto[3], Melissa Carolina Pereira[3], Alvimar José da Costa[2], Darci Moraes Barros-Battesti[1,2]

[1]Department of Preventive Veterinary Medicine and Animal Science, School of Veterinary Medicine, University of São Paulo, São Paulo, SP, Brazil.

[2] Department of Pathology, Reproduction and One Health, School of Agricultural and Veterinary Sciences, São Paulo State University "Julio de Mesquita Filho" (UNESP), Jaboticabal, SP, Brazil.

[3]Department of General and Applied Biology, Institute of Biosciences, São Paulo State University "Julio de Mesquita Filho" (UNESP), Rio Claro, SP, Brazil.

Abstract

Tick cell lines have already proved to be a useful tool for obtaining more information about possible vector species and the factors governing their ability to transmit a pathogen. Here, we established and characterized a cell line (RBME-6) derived from embryos of *Rhipicephalus microplus* from Brazil. Primary tick cell cultures were prepared in L-15B medium supplemented with 20% fetal bovine serum and 10% tryptose phosphate broth. The cell monolayers were subcultured when they reached a density of approximately 8 x 10^5 cells/mL (95% viability). Only after the sixth subculture were cells thawed from storage in liquid nitrogen successfully. Cytological analyses were performed using live phase contrast microscopy and cytocentrifuge smears stained with Giemsa, while periodic acid-Schiff and bromophenol blue staining techniques were used to detect total polysaccharides and total protein, respectively. No DNA from *Anaplasma* spp., *Anaplasma marginale*, *Babesia* spp., *Bartonella* spp., *Coxiella* spp., *Ehrlichia* canis, *Rickettsia* spp. or *Mycoplasma* spp. was detected in the cells through PCR assays. In addition, we performed chromosomal characterization of the tick cell line and confirmed the *R. microplus* origin of the cell line through conventional PCR and sequencing of a fragment of the mitochondrial 16S rRNA gene. In conclusion, we established and

characterized a new cell line from a Brazilian population of *R. microplus*, which may form a useful tool for studying several aspects of ticks and tick-borne pathogens.

Keywords: cattle tick; cell line; chromosome; Brazil.

2.1. Introduction

Rhipicephalus (*Boophilus*) *microplus* (Canestrini, 1887), also known popularly as the cattle tick, is an ectoparasite of global economic importance. It is a one-host tick feeding mainly on cattle, although other domestic animals and some wild ungulates can act as secondary hosts (Guglielmone *et al*., 2006). In Brazil, the estimated economic losses caused by this tick are around US$ 3.24 billion annually (Grisi *et al*., 2014). Moreover, the species *R. microplus* is also involved in the transmission of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* (Rosario-Cruz *et al*., 2009). In addition, *R. microplus* has high capacity to develop acaricide resistance, which is seen in many regions of the world (Abbas *et al*., 2014).

Establishment of tick cell lines can provide important information about vector competency and the factors governing pathogen transmission capacity. Furthermore, tick cell lines allow studies on genomics, tick innate immunity, tick microbiomes, development of acaricide resistance and the mode of action of acaricides (Bell-Sakyi *et al*., 2007). Consequently, over recent decades, there have been significant increases in the number of cell lines that have been developed from different tick species in several geographical regions (Bell-Sakyi *et al*., 2018). According to these authors, since the first tick cell lines were established more than 50 years ago, they have become important laboratory tools that enable research relating to the physiology, biology and control of tick-borne pathogens.

Even though *R. microplu*s cell lines have been previously obtained by Pudney *et al*. (1973), Holman and Ronald (1980), Holman (1981), Kurtti *et al*. (1988), Bell-Sakyi (2004), Alberdi *et al*. (2012) and Bell-Sakyi *et al*. (2007; 2018), few studies have addressed the characterization of tick cell lines from *R. microplus* (Esteves *et al*., 2008) and none of them have characterized cell lines derived from ticks in Brazil.

Embryonic tissue cell lines from *R. microplus* have been useful in many studies. For instance, the BME26 cell line (Kurtti *et al*., 1998; Esteves *et al*., 2008), which was derived from *R. microplus* from Mexico, was tested for propagation of *Anaplasma marginale* (Esteves *et al*., 2009). These authors proved the occurrence of the first

infection and propagation of a Brazilian isolate of *A. marginale* in a cell line from *R. microplus*.

Moreover, Koh-Tan *et al*. (2016) indicated that tick cell lines provide an useful experimental tool for acaricide resistance studies and further elucidation of tick genetics. They compared 14 cell lines from resistant and susceptible tick species; identified a new target-site insensitivity gene (βAOR); and demonstrated a higher resistance-detoxification (ABCB10) expression level in a *R. microplus* cell line (BME/CTVM6). In comparison with other *R. microplus* cell lines, the BME/CTVM6 cell line derived from an acaricide-resistant strain showed a different βAOR gene and a higher level of ABCB10 expression.

Indeed, tick cell lines have also been used for their potential as inexpensive ethically correct tools for studying acaricide resistance (Al-Rofaai and Bell-Sakyi, 2020).

Characterization of a cell line should comprise population growth, cell morphology, cytogenetics, absence of contaminants, cell viability before freezing and after thawing and species identification (Freshney, 2005). To characterize specific cell line, several methodologies are used, such as karyotyping, immunological techniques, isoenzymes and molecular analyses (Stulberg *et al*. 1976; Freshney 2005).

Here, we developed and characterized a cell line from *R. microplus* collected in Brazil. The cell line identity was confirmed by means of partial sequencing of the mitochondrial 16S rRNA gene. In addition, the morphology and karyology of this cell line were ascertained, and the absence of microorganisms was confirmed using molecular techniques.

2.2. Material and Methods

Origin of ticks and ethics approval

Engorged female *R. microplus* ticks were kindly sent to the Pathology Department of São Paulo State University (UNESP), by Prof. Alvimar J. Costa, on October 20, 2017. These females had been collected from naturally-infested cattle at the farm belonging to IPESA (Institute for Animal Health Research Ltd.) (20º 31’ 04’’ S; 45º 28’ 28’’ W), in the municipality of Formiga, state of Minas Gerais, Brazil. The procedures were performed in accordance with the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and were approved by the Ethics Committee for

Animal Use (CEUA; no. 06761/19), FCAV/UNESP, Jaboticabal. The tick cell culturing was authorized by the Genetic Asset Management Council (CGEN; no. 010214/2015-1).

Primary tick cell cultures

Cell cultures were obtained from *R. microplus* embryos by following previously described protocols, with some modifications (Pudney *et al*., 1973; Holman and Ronald, 1980). A total of 100 engorged females were disinfected with 70% alcohol for 20 seconds and were immersed in 1% benzalkonium chloride solution (Polyorganic, São Paulo, SP, Brazil) for 15 minutes. They were then washed with 50 mL of sterile distilled water containing antibiotics (penicillin, 50 IU/mL) streptomycin (50 µg/mL), and fungicide (amphotericin B, 0.25 µg/ml) (Vitrocell Embriolife™, Campinas, SP, Brazil) for 5 minutes. The females were then dried on sterile gauze, placed individually in sterile Petri dishes and kept in a BOD incubator (biochemical oxygen demand) at 27 ºC and 80-85% relative humidity to allow oviposition.

Primary cultures were performed using pools of multiple egg batches (around 100 egg batches, in total) from engorged females, 12 days after oviposition began. The eggs were disinfected with 70% ethanol for 5 minutes and were immersed in 1% benzalkonium chloride for 10 minutes. They were then washed with sterile distilled water containing penicillin (100 IU final concentration), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml) for 30 minutes. Lastly, the eggs were washed using 2% hypochlorite for 3 min and sterile distilled water. All the procedures were performed in a class II biosafety cabinet.

The eggs were transferred to tubes containing 500 µL of L-15B medium (Munderloh and Kurtti, 1989) and were crushed using a disposable pellet pestle (VWR Scientific Cat. KT749520-0000). The embryonic tissues were transferred to a centrifuge tube and centrifuged at 300 x *g* for 8 minutes. The pellet was resuspended in 4 mL of complete L-15B medium with 20% FBS and 10% tryptose phosphate broth. Amphotericin B (0.125 µg/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml) were added to the cell suspension. The cultures were incubated at 30 ºC in a 25-cm^2 cell culture flask (Corning™). The culturing medium was fully changed once a week. Cell adhesion was monitored using an inverted microscope (Nikon, Eclipse TS100).

Subcultures

The cell monolayers were subcultured when they reached a density of approximately 10^7 cells/mL. Subcultures were routinely made at a flask ratio of 1:2, i.e., one "parent" flask was subcultured into two "daughter" flasks. The cells were harvested mechanically using a cell scraper (Corning™) and resuspended in fresh complete L-15B medium. After centrifugation for 8 min at 100 × *g*, the supernatant was discarded, and the pellet was resuspended in 8 mL of L-15B medium with 20% fetal bovine serum. Approximately 8 x 10^5 cells/mL were then transferred into two new flasks.

Cryopreservation

From the first subculture, cells were periodically frozen (every 30 days). The tick cells were removed from the flasks by scraping and were then centrifuged at 400 x *g* for 8 minutes. For each 25-cm^2 flask of cells to be frozen, 1 mL of L-15B freezing medium was prepared by adding 20% dimethyl sulfoxide (DMSO) and 1 mL of complete L-15B medium with 25% FBS. First, the cells were resuspended in L-15B medium with 25% FBS and then the freezing medium was added. The cell suspension was placed into cryotubes and was stored at -80 °C (freezer) for 24 hours in a Nalgene™ Cryo 1C freezing container (Thermo Fisher Scientific, cat. no. 5100-001) to achieve a cooling rate of 1 °C/min and was then frozen in liquid nitrogen at -196 ºC.

For thawing, the cryotube was removed from the liquid nitrogen and thawed quickly between the fingers. The cells were then removed from the cryotubes and diluted in 1 mL of fresh L-15B medium. The cells were removed from the freeze-medium by means of centrifugation for 8 min at 400 × *g*; the supernatant was discarded and the pellet was resuspended in 4 mL of L-15B medium containing 20% FBS.

Cell-growth curves

Cells were plated in 12.5 cm^2 cell culture flasks (Corning™) at an initial density of 2 x 10^5 cells per flask in L-15B medium, and incubated at 30 °C. The cells were counted every day over a 20-day period a separate flask was counted on each day (i.e., on a single occasion). During this period, cells were removed by scraping from the flasks and mixed with 0.4% trypan blue (Sigma) at a ratio of 1:1. From this, 20 µL of the mixture were placed on a hemocytometer. The numbers of live cells and dead cells were counted under a microscope. The maximum specific growth rate (lmax) was determined through nonlinear regression curve analysis, and the population doubling time was also evaluated (Munderloh and Kurtti, 1989).

Tick cell identification

Embryonic tick cell samples were scraped from the 25-cm2 flasks and were then centrifuged at 400 x g for 8 minutes. The samples were subjected to a DNA purification protocol using the DNeasy tissue extraction kit (Qiagen, Chatsworth, CA, USA), following the manufacturer's guidelines. The DNA concentration and absorbances rates at 260/280 and 260/230 were measured spectrophotometrically using a Nanodrop ND-1000 device. The cell identity was confirmed using a conventional PCR assay based on mitochondrial 16S rRNA. Primers for amplification of a 460-bp fragment of the 16S rRNA gene were used: forward, 5'-CCG GTC TGA ACT CAG ATC AAG T-3'; reverse, 5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG -3' (Black and Piesman, 1994). The negative control consisted of 5 µl of sterilized ultrapure water and the positive control consisted of 5 µl of extracted *Rhipicephalus sanguineus* DNA.

The amplified products were purified using a silica bead DNA gel extraction kit (Thermo Fisher Scientific™, Waltham, Massachusetts, USA) and were sequenced by means of the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using the BigDye™ Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific™, Foster City, California, USA). This was done in a sequencer (ABI PRISM 3700 DNA Analyzer: Applied Biosystems®) at the Center for Biological Resources and Genomic Biology (CREBIO - FCAV - UNESP).

The electropherograms generated through the sequencing were analyzed by observing the quality of the peaks corresponding to each sequenced base. A consensus sequence was built using the Bioedit v. 7.0.5.3 software (Hall, 1999).

This was then analyzed by means of the BLASTn program (Altschul *et al.*, 1990), in order to compare with other sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank).

Molecular analysis on the cell line

The samples that were positive in the cPCR based on the mitochondrial 16S rRNA gene (Black and Piesman, 1994) were subjected to PCR assays to detect the presence of DNA of *Anaplasma marginale*, *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Coxiella* spp., *Ehrlichia canis*, *Mycoplasma* spp. and *Rickettsia* spp. (**Table 1**).

The positive control for *A. phagocytophilum* was kindly supplied by J. Stephen Dumler, Uniformed Services University of the Health Sciences, Bethesda, Maryland,

USA. The positive control of *A. marginale* was obtained from naturally infected buffaloes (Machado *et al*., 2016). *Ehrlichia canis* and *Babesia vogeli* DNA positive controls were obtained from naturally infected dogs sampled in Campo Grande, state of Mato Grosso do Sul (Sousa *et al*., 2013). *Mycoplasma* and *Bartonella* DNA positive controls were obtained from naturally infected cats from the state of Maranhão (Braga *et al*., 2012a, 2012b). *Coxiella burnetii* DNA positive control was provided by Prof. Dr. Renato Arruda Mortara (Universidade Federal de São Paulo, São Paulo, SP, Brazil). *Rickettsia felis* DNA positive control was obtained from cat fleas from Chile (Müller *et al*., 2018). All cPCR runs were performed with nuclease free water (Thermo Scientific) as a negative control.

All the quantitative PCR analyses were performed following the published protocols and in accordance with the MIQE standards ("Minimum Information for Publication of Quantitative real-time PCR Experiments") (Bustin *et al*., 2009). For the conventional/nested PCR protocols, the products were separated by means of electrophoresis on 1% agarose gel, stained with ethidium bromide (Life Technologies™, Carlsbad, California, USA) and imaged under ultraviolet light (ChemiDoc MP imaging system, Bio-Rad™, Hercules, California, USA), using the Image Lab software, version 4.1 as previously described.

Table 1. Oligonucleotide primer sequences and hydrolysis probe sequences for targeted genes used in PCR assays for each agent searched for in samples from *Rhipicephalus microplus* cell line RBME-6.

Agents	PCR type	Target gene	Primer sequence (5'-3')	Reference
***Anaplasma* spp.**	Nested	16S	ge3a:CACATGCAAGTCGAACGGATTATTC ge10r:TTCCGTTAAGAAGGATCTAATCTCC ge9f :AACGGATTATTCTTTATAGCTTGCT ge2: GGCAGTATTAAAAGCAGCTCCAGG	Massung *et al.*,1998
Anaplasma marginale	qPCR	*Msp*1	AM-For:TTGGCAAGGCAGCAGCTT AM-Rev:TTCCGCGAGCATGTGCAT AM-Pb:6FAM TCGGTCTAACATCTCCAGGCTTTCAT-BHQ1	Carelli *et al.*,2007
***Babesia* spp.**	Nested	18S	BTF1:GGCTCATTACAACAGTTATAG BTR1:CCCAAAGACTTTGATTTCTCTC BTF2:CCGTGCTAATTGTAGGGCTAATAC BTR2: GGACTACGACGGTATCTGATCG	Jefferies *et al.*,2007
***Bartonella* spp.**	qPCR	*nuoG*	F-Bart: CAATCTTCT TTTGCTTCACC R-Bart: TCAGGGCTTTATGTGAATAC TexasRed-TTYGTCATTTGAACACG[BHQ2a-Q]	André *et al.*, 2016
***Coxiella* spp.**	Nested	16S	Cox16SF1:CGTAGGAATCTACCTTRTAGWGG Cox16SR2: GCCTACCCGCTTCTGGTACAATT Cox16SF2:TGAGAACTAGCTGTTGGRRAGT Cox16SFR1:ACTYYCCAACAGCTAGTTCTCA	Duron *et al.*,2014
Ehrichia canis	qPCR	*dsb*	Dsb-321:TTGCAAAATGATGTCTGAAGATATGAAACA Dsb-671:GCTGCTCCACCAATAAATGTATCYCCTA Probe:AGCTAGTGCTGCTTGGGCAACTTTGAGTGAA-3 ' e 5'-FAM / BHQ-1,3	Doyle *et al.*,2005
***Mycoplasma* spp.**	Nested	16S	Myco16S-322s:GCCCATATTCCTACGGGAAGCAGCAGT Myco16S-938as:CTCCACCACTTGTTCAGGTCCCCGTC HemMycop16S-322s:GCCCATATTCCTACGGGAAGCAGCAGT HemMycop16S-1420as:GTTTGACGGGCGGTGTGTACAAGACC	Maggi *et al.*,2013
***Rickettsia* spp.**	cPCR	*gltA*	CS-239: GCTCTTCTCATCCTATGGCTATTAT CS-1069: CAGGGTCTTCGTGCATTTCTT	Labruna *et al.*,2004

Cytological analysis

Cytological and histochemical techniques were used to assess the general morphology and distribution patterns of proteins and polysaccharides in cells. The cytological analyses were conducted at the Department of Biology of UNESP, Rio Claro, SP, Brazil.

Round coverslips for microscopy were placed in each well of a 24-well plate. The tick cells were removed from the flask and were inoculated into the wells at a density of 2 x 105 cells per well. The cells were maintained in L-15B medium at 30 ºC for 2 days in a cell culture incubator. In addition to the lid, the 24-well plate was sealed with Parafilm®.

To detect changes such as presence or absence, frequency and distribution of proteins and polysaccharides in the tick cell cultures, the material was prepared as follows:

Periodic acid-Schiff (PAS) technique for polysaccharide detection (Junqueira and Junqueira, 1983). The tick cell cultures were fixed in 4% paraformaldehyde for 15 minutes. Next, 0.4% periodic acid was added to the wells with cells for 5 minutes, and the cells were then washed with distilled water and stained with Schiff's reagent (1%; Merck) for 30 minutes in the dark. The material was then washed twice with water for 3 minutes each time. After drying, the material was clarified with xylol and mounted on slides using Entellan®.

Bromophenol blue (0.1%; Synth®) staining for protein detection (Anholeto *et al*., 2018) - The tick cell cultures were fixed in 4% paraformaldehyde for 15 minutes All wells with cells were stained with bromophenol blue for 30 minutes at room temperature. Afterwards, they were washed with 0.5% acetic acid for 5 minutes and tap water for 15 minutes; then, they were allowed to dry at room temperature, clarified and mounted on slides using Entellan®.

Additionally, to ascertain cell morphology, 100 µl of tick cell suspension were centrifuged onto glass slides (Thermo Scientific Cytospin 4) for 5 minutes at 1000 rpm, fixed in absolute methanol and stained with Giemsa. After staining, the slides were left to dry and examined under a microscope.

Chromosome recovery, slide preparation and fluorescence in situ hybridization (FISH)

Mitotic chromosomes were obtained from cell cultures of *R. microplus* at passages 7 and 29. For this purpose, a protocol previously proposed by Chen *et al*. (1994) with

slight modifications, was used. The cell cultures were used 48 h after medium change. To arrest mitosis, 100 µg/ml of colcemid (GIBCO™) was added to the cell cultures for 6 h. The cells were harvested and lysed in a hypotonic solution of 0.75 mol KCl solution for 30 minutes at 30 °C. Lastly, the material was fixed in modified Carnoy's solution (3:1 absolute ethanol to glacial acetic acid). The chromosome spreads were prepared by spreading 20 µl of fixed material on slides on a hot plate at 40 °C.

For conventional analysis, we stained the slides with 4',6-diamidino-2'-phenylindole (DAPI). The material was mounted on slides using VECTASHIELD (Vector™, Burlingame, CA, USA). FISH was performed as described by Cabral-de-Mello (2015), using the telomeric motif $(TTAGG)_n$. Probes were obtained and labeled with digoxigenin-11-dUTP (Roche™) through non-template PCR, using the complementary primers $(TTAGG)_5$ and $(CCTAA)_5$, as described by Ijdo (1991). These probes were detected using anti-dig-rhodamine (Roche, Mannheim, Germany). The chromosomes were counterstained with DAPI.

These slides were observed under an Olympus BX61 microscope equipped with a fluorescence lamp and appropriate filters. The images were documented using a DP70 cooled digital camera on grayscale. The FISH signals were then pseudo-colored. Images were merged and optimized for brightness and contrast using Adobe Photoshop CS6. To estimate diploid numbers and to check possible variability in chromosome constitutions, we counted 100 metaphase chromosome spreads per passage. For this counting, i.e., diploid number description, we did not consider dot chromosomes. We counted the presence of chromosome fragments (dot chromosomes) independently from the diploid numbers (see results). To compare the mean numbers of chromosome spreads with different diploid numbers and differences in occurrence of chromosome fragments, we used the t-test.

2.3. Results

Primary cultures

In the present study, around 100 batches of eggs from *R. microplus* females were used. Each primary culture was prepared using egg pools weighing 70 mg on average (1–2 cultures per pool). In total, around 10% survived, most of the 84 primary cultures were discarded within 3 months due to cell death or fungal contamination.

Cultures with low numbers of cells were rescued by pooling cells from two flasks, as appropriate. Fragments of tick legs, guts and eggshells were observed, but a large proportion of these non-adherent fragments were eliminated at the time of the first replacement of the medium.

Cell line RBME-6

The cell line is named "RBME-6". RBM represents the abbreviated name of the tick species *R. microplus*; E corresponds to the embryonic cells of *R. microplus*; and the number 6 means that cell cryopreservation success was possible after the 6^{th} passage.

The cells multiplied to form confluent cell layers within three months, reaching a density of approximately 3×10^5 cells/mL. The time interval between starting the primary culture and making the first subculture was six months, when the number of total cells reached 8×10^5 cells/ml (95% live viability). After the first subculture, further subcultures were made at intervals of every 20-30 days. The numbers and diversity of cell types in the line declined, and one or two cell types became dominant. In the present study, rounded cells were dominant after subcultures. Cell morphology, including cell shape and size, was ascertained by looking directly at the cultures under an inverted microscope and by examining cytocentrifuge smears stained with Giemsa (1A, B). Clumps of small, rounded cells were observed, along with cells presenting a fibroblast-like appearance (Fig. 1 A). Giemsa staining revealed cells with rounded nuclei and many cytoplasmic vesicles (Fig. 1 B). The bromophenol blue reaction revealed large amounts of protein in the *R. microplus* cells (Fig. 1 C). Moreover, the use of the periodic acid-Schiff (PAS) histochemical test revealed the presence of polysaccharides homogeneously distributed in those cells (Fig. 1 D).

Analysis on cell growth in the sixth subculture indicated that the population doubling time (Td) during the logarithmic phase of growth was about 62 hours and 56 minutes. The maximum cell density was 2.82×10^6 and the time taken to reach the maximum number of cells was 9 days (Fig. 2).

Only after the sixth subculture were the cells that were thawed from liquid nitrogen storage successfully recovered from the culture medium. Samples of this cell line were deposited at the Tick Cell Biobank, located at the University of Liverpool, in Liverpool, United Kingdom, under the care of Dr. Lesley Bell Sakyi, and at the Tick Cell Biobank South America Outpost, located at the Oswaldo Cruz Foundation (FIOCRUZ),

in Rio de Janeiro, Brazil, under the care of Dr. Flavio Alves Lara. At the time of writing this report, the cultures were at passage level 30.

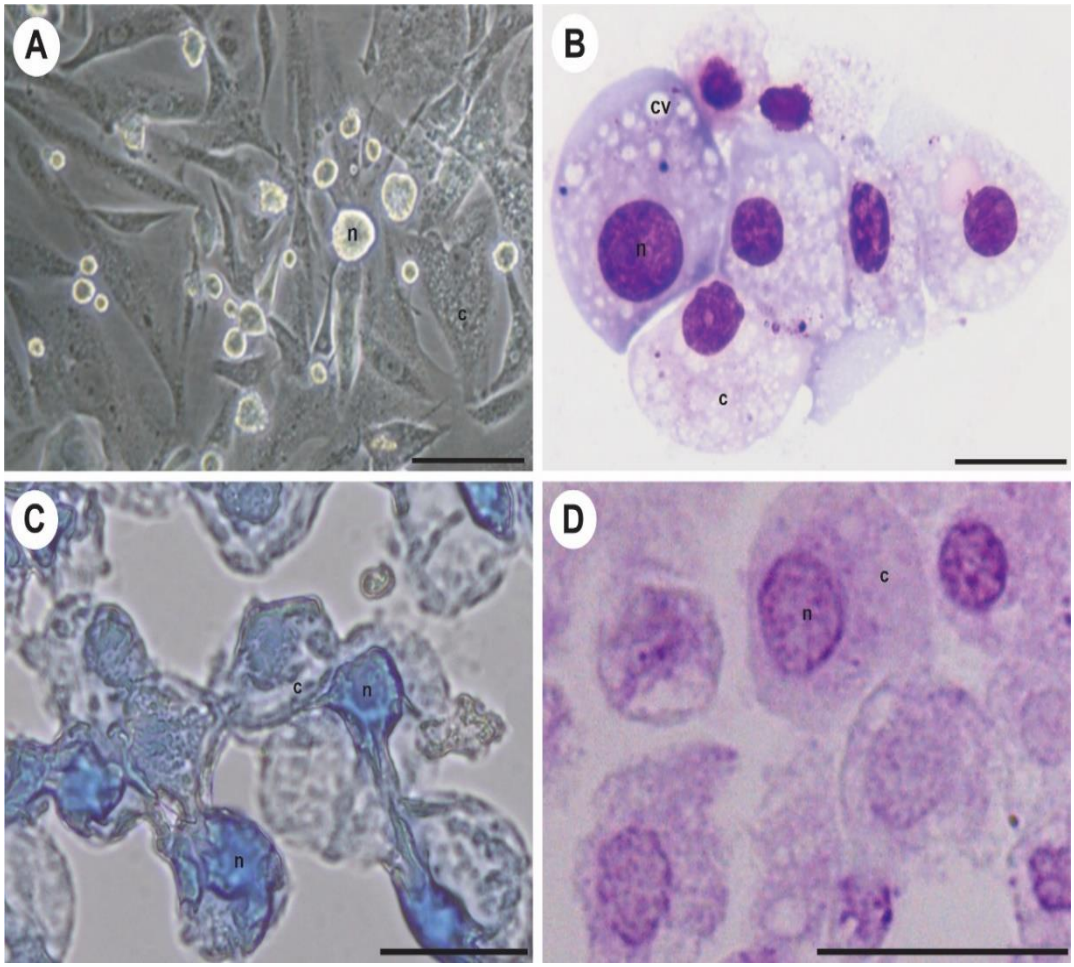

Figure 1. General characterization of cellular morphology of *Rhipicephalus microplus* embryonic cells (RBME-6). A. Phase contrast microscopy of live cells. B. Cytocentrifuge smears stained with Giemsa. C. Cells grown on coverslip and stained with bromophenol blue. D. Cells grown on coverslip and subjected to periodic acid-Schiff reaction. c= cytoplasm; cv= cytoplasmic vesicle; n= nuclei. Bars: A= 10µm; B, C, D= 20µm.

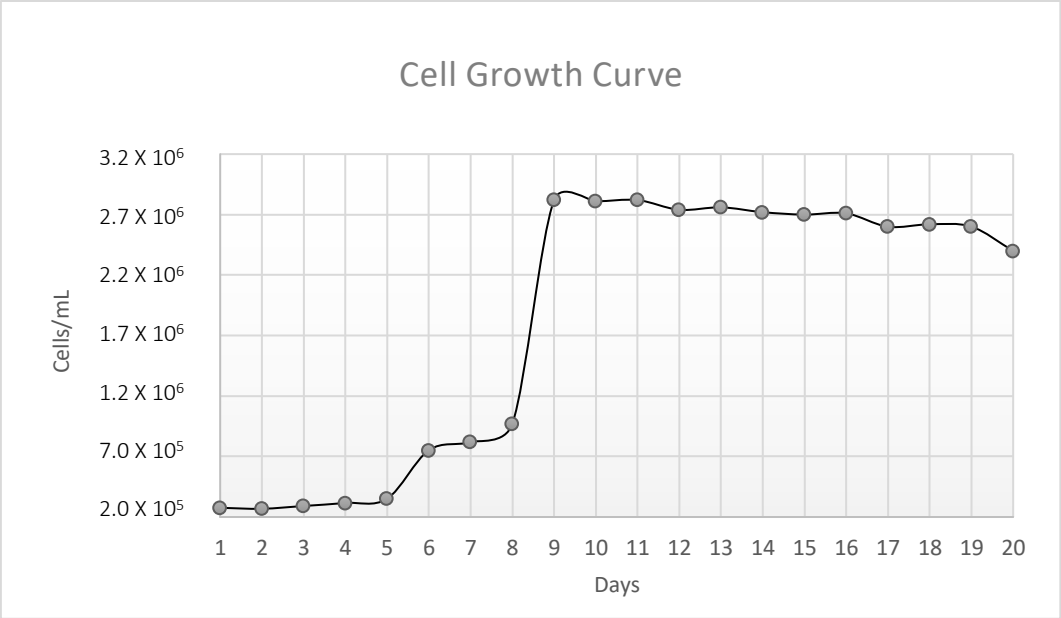

Figure 2. *Rhipicephalus microplus* cell line (RBME-6) growth in the sixth subculture. Tick cells in L-15B medium supplemented with 20% fetal bovine serum. Cells were counted using a hemocytometer

Confirmation of cell line identity and absence of contaminating microorganism

The tick 16S rRNA sequence obtained was submitted to BLASTn analysis to determine its similarity to sequences previously deposited in the GenBank database. The consensus sequence (432 bp), which corresponded to a fragment of the 16S rRNA gene obtained from the RBME-6 cell line (cells from the 4th subculture) showed 100% similarity to *R. microplus*, sequence KY020993 (from Mozambique) and KC170742 (from Brazil) with 99% query coverage and E-value of 0.0, thus confirming that the cells originated from this tick species. The sequence obtained in the present study was deposited in GenBank under accession number MN396583.

To detect microorganism DNA in tick cell cultures, the DNA was extracted from eight cell culture samples (passage 4). The cultures analyzed in duplicate were the primary culture, 4th subculture, 5th subculture and 25th subculture. All the analyzed using the cPCR protocol were positive for the tick 16S rRNA mitochondrial gene. All samples were negative in the PCR assays for *Anaplasma* spp., *Anaplasma marginale*, *Babesia* spp., *Bartonella* spp., *Coxiella* spp., *Ehrlichia canis*, *Mycoplasma* spp. and *Rickettsia* spp.

All the positive control samples were detected in the PCR and there was no amplification in the negative control samples.

Karyotyping

The modal diploid number in both passages analyzed (7 and 29) was 2n = 21, with one X chromosome (Fig. 3A, B). All the chromosomes were acro-telocentric. Slight variations were observed between the two passages, with occurrence of aneuploid metaphase chromosome spreads. For passage 7, the diploid number varied from 20 to 24; and for passage 29, it ranged from 19 to 24 (Fig. 4A). There were no statistical differences between them (p-value = 1). We noticed occurrences of spreads with 2n = 22, harboring two X chromosomes (Fig. 3C); and spreads with 2n = 22, harboring one X chromosome and one extra unidentified autosome (Fig. 3D). No polyploid metaphase chromosome spreads were observed. Lastly, small fragments of chromosomes (dot chromosomes) were noticed in varying numbers (from one to three) in some spreads (Fig. 3E) in both passages. These small fragments of chromosomes were observed in 16 out of 100 metaphase spreads for passage 7 and ten out of 100 metaphase spreads for passage 29 (Fig. 4B). No statistical difference in the number of spreads with dot chromosomes was observed between the passages (p-value = 1).

In both passages, a telomeric probe identified only the actual telomeres (on chromosome termini). The telomeres from the short arm were larger than the telomeres from the long arm, and no differences were noticed between the two passages (Fig. 3F, G). Chromosome fragments were labeled by the telomeric probe (Fig. 3H).

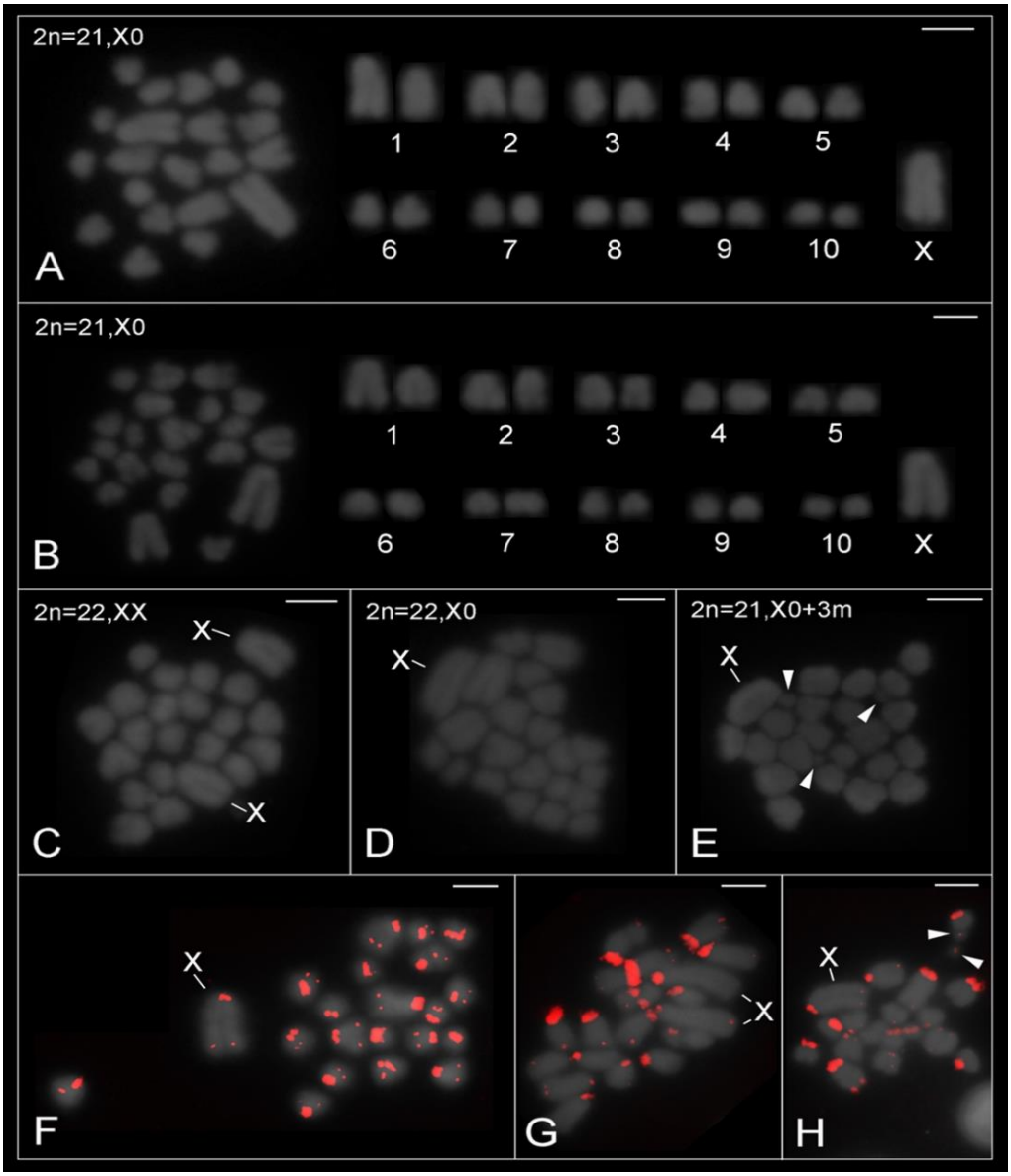

Figure 3. Analysis of chromosomal spreads of *Rhipicephalus microplus* cell line RBME-6. Mitotic chromosome spreads and tentative karyotypes of cell line from passage 7 (A) and passage 29 (B). (C-E) Chromosomal spreads with variations in relation to modal karyotype (2n = 21, X0), (C) 2n = 22, XX, (D) 2n = 22, X0 and (E) 2n = 21, X0+3 small fragments of chromosomes. (F-H) Locations of telomeres in chromosome spreads from passage 7 (F) and passage 21 (G-H). Note the occurrences of telomere foci in each terminal region of chromosomes and their similar size in both passages. In (H), occurrence of the telomere motif in small fragments of chromosome (partial metaphase). is shown. Scale bars=5 μm.

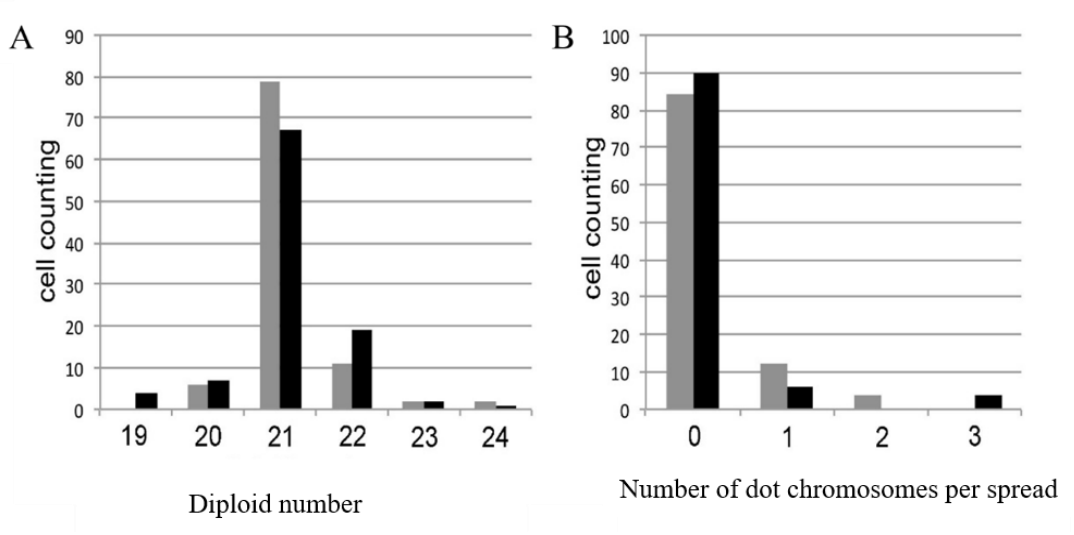

Figure 4. Numbers of chromosomes and chromosome fragments in spreads of *Rhipicephalus microplus* cell line RBME-6. (A) Absolute number of mitotic chromosome spreads with distinct diploid numbers and (B) absolute number of cells with 0-3 small chromosome fragments from passages 7 (gray) and 29 (black). For this estimation we counted 100 cells from each passage. Note the high frequency of chromosome spreads with 2n = 21 and low occurrence of small chromosome fragments.

2.4. Discussion

In this study, we characterized a new cell line from a tick species of considerable veterinary importance worldwide. Even though other tick cell lines from *R. microplus* have already been established (Pudney *et al*., 1973; Holman and Ronald, 1980; Holman, 1981; Kurtti *et al*., 1988; Alberdi *et al*., 2012; Bell-Sakyi, 2004; Bell-Sakyi *et al*., 2018), our study presents the first characterization of a cell line derived from a *R. microplus* population from Brazil.

We started the primary cultures based on protocols described by other authors, with some modifications (Pudney *et al*., 1973; Holman and Ronald, 1980). In some previous studies, primary cell cultures from *R. microplus* tick eggs were incubated at 34 °C and the growth medium consisted of Leibovitz-15, supplemented with 5% to 10% fetal bovine serum (Munderloh and Kurtti, 1989; Esteves *et al*., 2008). In the present study, success was achieved by incubating the primary cultures at 30 °C and using the L-15B

medium with 20% fetal bovine serum. Significant cell growth was observed between three and six months later.

We only succeeded in establishing subcultures six months after starting the primary culture. Subsequently, we made subcultures every four weeks. Previously, *R. microplus* embryonic cell lines had also been subcultured at intervals of three to four weeks (Pudney *et al*., 1973). Given that ixodid ticks have the ability to survive for long periods in the environment between blood meals, some tick cell lines can survive for a long time with occasional subcultures and regular medium changes (Bell-Sakyi *et al*., 2007).

Amplification and sequencing of a partial sequence of the mitochondrial 16S rRNA gene confirmed the identity of the *R. microplus* tick cell line that we established.

In the present study, cell cultures in the sixth subculture, incubated at 30 °C, presented growth with a doubling time of logarithmic growth phase of approximately two days and the overall doubling time of the culture is between 5 and 6 days. In previous established cell lines from *R. microplus*, doubling times of 3.8 days (Holman, 1981), 5.0 days (Cossio-Bayugar *et al*., 2002) and 15 days (Esteves *et al*., 2008) were reported.

Determination of a growth curve is important for evaluation of the specific characteristics of a cell culture. The biology of the cells changes significantly at each phase of the curve (Freshney, 2005). Right after the subculture is started, the cell growth shows a lag phase. The duration of this phase is determined by the time required for a cell to recover from the subculturing, i.e., for it to reconstruct its cytoskeleton and secrete an extracellular matrix that facilitates expansion across the substrate. The cells then enter exponential growth, named the log phase, in which the cell population doubles. When the cell population has become very large and the substrate has practically all been metabolized, the cells enter a stationary phase in which the growth rate drops nearly to zero (Freshney, 2005).

Through understanding the growth cycle of a certain cell line, it becomes possible to predict the most appropriate inoculum density, the expected duration of the experiments and the most appropriate intervals for sampling (Moraes *et al*., 2008).

Tick cells grow relatively slowly in cultures, and this can be measured through the frequency of transfers and the population doubling time. The doubling time for tick cell lines, independent of species, is strongly influenced by medium supplementation, incubation temperature and passage history (e.g. frequency of feeding), and it can range from 5 to 15 days (Munderloh and Kurtti, 1989).

With regard to temperature for maintenance of cell lines, most primary cultures and tick cell lines that have been described were maintained at temperatures ranging from 28 °C to 34 °C. Cell growth varies in response to changes in temperature. In another study on a cell line from *R. microplus*, cells incubated at 28 °C did not survive, whereas those incubated at 32 °C did (Pudney *et al*., 1979). In the present study, the primary cultures and the cell line were maintained at 30 °C.

Regarding the karyotypes, the modal karyotype of 2n = 21 (X0) that was observed in the tick cell line studied here corresponded to the karyotype of male *R. microplus* ticks. This, most parsimoniously, suggested that the cells were derived from male embryos in the initial cultures. Our X chromosome finding was concordant with data from a previous cytogenetic study (Oliver and Bremner, 1968), in which 21 diploid chromosomes and a XX:XO sex determination system in male *R. microplus* ticks were reported. These results are also consistent with the numbers of chromosomes in *R. microplus* in other reports from Mexico (Newton *et al*., 1972; Hilburn *et al*., 1989) and Brazil (Garcia *et al*., 2002).

In a previous study, chromosome preparations of the BmVIII-SCC cell line from *R. microplus* were shown to be predominantly diploid, with 22 chromosomes (female complement). However, in that same study, the cells of the BmVIII-633 cell line from *R. microplus* were considerably more diverse in karyotypes, with diploid predominance of 21 chromosomes (male complement) (Holman, 1981).

Esteves *et al*. (2008) characterized the BME26 cell line of *R. microplus* in Mexico and observed a predominantly male diploid pattern that was similar to the chromosomal pattern obtained in the present study. However, the metaphase chromosomes prepared from the BME26 cell line were from the 20th and 83rd subcultures.

The observation of cells with two X chromosomes indicates the presence of female cells, or the occurrence of aneuploidy for X chromosomes during the cell culture passages. Aneuploidy has also been documented for autosomal chromosomes, along with occurrence of chromosome fragments. These data suggest that although the cell line was chromosomally stable, it would be possible for a certain degree of rearrangement to occur. However, this would not usually compromise the cell culture. The stability of the cell line was emphasized through the observation that stable telomeres with similar sizes were present in both passages. In general, the degree of variability at chromosomal level observed in the present study in relation to the *R. microplus* cell line was less than what had previously been observed in other tick cell lines, in which, for example, large variations in diploid numbers and telomere size were seen (Kotsarenko *et al*., 2020).

Traditional cryopreservation using 10% DMSO in the medium, and using gradual freezing procedures, has resulted in resuscitation of tick cells that were frozen in liquid nitrogen (Bastos *et al*., 2006). Besides that, rapid freezing protocols, in which cryotubes are placed immediately in the gas phase of a liquid nitrogen refrigerator at below -120 °C, have also been used to cryopreserve tick cells (Bell-Sakyi, 1991).

Even though a lower concentration of DMSO (7.5%) was used by Holman (1981) for cryopreservation of *R. microplus* cell lines, we successfully recovered frozen cells through using 10% DMSO in the medium and using gradual freezing.

Even though the cell morphology found in the present study was initially heterogeneous, the number and diversity of cell types declined after subculturing. Cell culturing gave rise to clumps of small, rounded cells, along with a few cells of fibroblast-like appearance. Here, most of the cells lost their fibroblast-like appearance and became rounded after subculturing. These results corroborate those of a study in which the BME26 cell line was characterized (Esteves *et al*., 2008). Indeed, occurrence of two or more cell types, which could include fibroblast-like, long, bipolar or epithelial-like muscle cells, had already been observed (Yunker, 1987, Bell-Sakyi, 2007, 2018).

Cell diversity was previously found to decrease through subcultures, to one or two types of dominant cells (Munderloh, *et al*., 1994). Morphological heterogeneity was thought to decline with increasing numbers of subcultures, such that cell lines would end up showing more homogeneous cell populations (Kurtti and Munderloh, 1982). Moreover, the constituents of the media, the pH and the temperature appeared to affect the shape and size of the cells (Pudney *et al*., 1973; Holman and Ronald, 1980).

The present study showed the general pattern of protein and polysaccharide distribution in cells using histochemical techniques (PAS and bromophenol blue, respectively). Besides that, some cytoplasmic vesicles could be observed in the RBME-6 cells. The content of these cytoplasmic vesicles could be mainly lipidic as described by Esteves *et al*. (2008) studying BME26 tick cell line. The same authors, also reported the presence of protein in some cytoplasmic vesicles in BME26 stained with fluorescent metalloporphyrin Pd-mP, indicating a putative cytosolic heme-binding protein. Considering the evidence of a cytosolic heme-binding protein and keeping in mind that heme transport and detoxification systems could be useful targets to new acaricides (Lara *et al*., 2005; Citelli *et al*., 2007; Esteves *et al*., 2008), *R. microplus* cells could be interesting model for drug discovery. In the same study, Esteves *et al*. (2008) also showed that some vesicles could be involved in autophagy and others in endocytosis.

Characterization of cell lines requires a comprehensive strategy involving multiple complementary techniques that identify microbial contaminants. Molecular biology studies have produced a reliable set of tools in this regard (Freedman *et al*., 2015). In the present study, no DNA from *Anaplasma* spp., *Anaplasma marginale*, *Babesia* spp., *Bartonella* spp., *Coxiella* spp., *Ehrlichia canis*, *Rickettsia* sp. or *Mycoplasma* spp. was detected in the cells through PCR assays. These microorganisms are often associated with ticks.

Here, we have established a cell line of *R. microplus* from Brazil. Tick cell lines play an important role as tools for *in vitro* studies to investigate the mechanisms of resistance and tick metabolism. Such studies can lead to development of novel approaches for controlling ticks and tick-borne pathogens (Bell-Sakyi *et al*., 2018). Moreover, studies have shown differences in the susceptibility of *R. microplus* populations to Bm86-based vaccines, which suggests that genetically distinct *R. microplus* strains may exist (Cobon *et al*., 1995; Garcia-Garcia *et al*., 2000).

Over the last few years, several studies have focused on establishing drug-resistant cell lines. The tick cell lines BmVIII-SCC (Holman, 1981) and BME26 (Kurtti *et al*., 1988), derived from acaricide-susceptible ticks from Mexico, were used to obtain acaricide-resistant sub-lines *in vitro* (Cossio-Bayugar *et al*., 2002; Pohl *et al*., 2014). The tick cell lines BME/CTVM2 (Bell-Sakyi, 2004) and BME/CTVM4 (Bell-Sakyi *et al*., 2007) were derived from known acaricide-susceptible ticks from Costa Rica. The cell lines BME/CTVM6 (Bell-Sakyi, 2004) and BME/CTVM5 (Bell-Sakyi *et al*., 2007) were derived from Colombian ticks known to be resistant to organophosphates, organochlorines and amitraz (Koh-Tan *et al*., 2016).

Koh-Tan *et al*. (2016) investigated the expression of genes associated with resistance in *R. microplus* cell lines derived from tick strains that were susceptible or resistant to acaricides, including BmVIII-SCC, BME/CTVM2, BME/CTVM5 and BME/CTVM6. Expression of the βAOR gene, which is associated with amitraz resistance, was detected in all the cell lines, but a novel larger amplicon was detected in BME/CTVM6, which is derived from amitraz-resistant ticks. These results suggest that BME/CTVM6 cells could be a useful model system for studying resistance.

Tick cell lines also provide an *in vitro* system for studying tick-pathogen relationships, through reducing the use of hosts in *A. marginale* research (Bell-Sakyi *et al*., 2007). A Brazilian isolate of *A. marginale* (UFMG) (Ribeiro *et al*., 1997) has been

successfully established in IDE8 cells derived from *Ixodes scapularis* (Bastos *et al*., 2009) and in BME26 cells derived from *R. microplus* ticks (Esteves *et al*., 2009).

Kalil *et al*. (2017) evaluated the immune-related redox metabolism of BME26 cells in response to *A. marginale* infection. According to these authors, the results suggested that this pathogen might manipulate the tick redox metabolism to evade the deleterious effect of the oxidant-based innate immune response. *In vitro* culturing of *A. marginale* in IDE8 cells has already been used in functional studies to discover genes and proteins that are differentially expressed in tick cells in response to infection due to *A. marginale* (De La Fuente *et al*., 2007; Kocan *et al*., 2009). Baêta *et al*. (2014) successfully isolated and propagated two strains of *A. marginale* in IDE8 cells. These two strains were the first *A. marginale* isolates from cattle in the state of Rio de Janeiro to be characterized after propagation in tick cell cultures.

In conclusion, we have established and characterized a new cell line from *R. microplus* embryonic cells that may constitute a useful tool for studying several aspects of tick and tick-borne pathogens. Moreover, the RBME-6 cell line will certainly be useful for future studies relating to the mechanisms involved in resistance of *R. microplus* populations to acaricides.

Authors' contributions

Leidiane Lima-Duarte: Conceptualization, Investigation, Methodology, Writing - original draft. Jaqueline Valéria Camargo: Investigation, Writing - review & editing. Ana Carolina Castro-Santiago: Investigation, Writing - review & editing. Rosangela Zacarias Machado: Formal analysis, Resources, Writing - review & editing. Marcos Rogério André: Formal analysis, Resources, Writing - review & editing. Diogo Cavalcanti Cabral-de-Mello: Investigation, Methodology, Writing - review & editing. Maria Izabel Camargo-Mathias: Investigation, Methodology, Writing - review & editing. Priscila Ikeda: Investigation, Writing - review & editing. Luís Adriano Anholeto: Investigation, Methodology, Writing - review & editing. Melissa Carolina Pereira: Investigation, Methodology, Writing - review & editing. Alvimar José da Costa: Resources. Darci Moraes Barros-Battesti: Conceptualization, Methodology, Supervision, Writing - review & editing

Acknowledgements

This work was supported by the Research Support Foundation of the State of São Paulo (Fundação de Amparo a Pesquisa do Estado de São Paulo, FAPESP) through grants 2007/57749-2, 2015/26209-9 and 2020/03190-9 to DMB-B; and by the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq), through productivity grants 312331/2013-4 to DMB-B and 302420/2017-7 to MRA. It was also partially supported by the Coordination Office for Improvement of Higher-Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES) under finance code 001. We would like to thank Timothy Kurtti and Urlike G. Munderloh, of the Department of Entomology, University of Minnesota, Minneapolis, United States, for training us in Minneapolis/St Paul in 2007, in relation to preparation of tick cell cultures. We would also like to thank Lesley Bell-Sakyi, of the University of Liverpool, Liverpool, United Kingdom, for all her assistance.

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3. CHAPTER 2 - **Establishment and multiapproach characterization of *Amblyomma sculptum* (Acari: Ixodidae) cell line (ASE-14) from Brazil**

Leidiane Lima-Duarte[1], Ana Carolina Castro-Santiago[1], Jaqueline Valéria Camargo[2], Ana Beatriz Stein Machado Ferretti[3], Luís Adriano Anholeto[3], Melissa Carolina Pereira[3], Priscila Ikeda[2], Carlos Alberto Perez[4], Gustavo Seron Sanches[5], Maria Izabel Camargo-Mathias[3], Diogo Cavalcanti Cabral-de-Mello[3], Rosangela Zacarias Machado[2], Marcos Rogério André[2], Darci Moraes Barros-Battesti[1,2]

[1]Department of Preventive Veterinary Medicine and Animal Science, School of Veterinary Medicine, University of São Paulo, São Paulo, SP, Brazil.

[2] Department of Pathology, Reproduction and One Health, School of Agricultural and Veterinary Sciences, São Paulo State University "Julio de Mesquita Filho" (UNESP), Jaboticabal, SP, Brazil.

[3]Department of General and Applied Biology, Institute of Biosciences, São Paulo State University "Julio de Mesquita Filho" (UNESP), Rio Claro, SP, Brazil.

[4]Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, São Paulo, SP, Brazil.

[5]Postgraduate Program on Animal Science, Pontifical Catholic University of Paraná (PUCPR), Curitiba, PR, Brazil.

Abstract

The establishment and characterization of the ASE-14 cell line derived from embryos of *Amblyomma sculptum* is described here. Primary cultures were started in L-15B medium at 30 °C,the medium was changed every week. With 60 days of primary culture, a confluent monolayer was formed and the first subculture was carried out. After the first subculture, the cultures were subculturing every 4 weeks. Cryopreservation of cells was successful only after the 14th subculture. We compared the chromosomes of the ASE-14 cell line with those of parental ticks. Cytogenetic analysis revealed occurrences of variable and increased diploid numbers in the ASE-14 cell line in comparison to adult ticks, probably through polyploidization events, chromosome fusions and translocations, which allowed generation of cells with distinct diploid numbers. Confirmation of the origin of the *A. sculptum* cell line was obtained through conventional PCR and sequencing of a fragment of the mitochondrial 16S rRNA gene. In addition, no DNA from *Anaplasma marginale*, *Anaplasma* spp., *Babesia/Theileria* spp., *Bartonella* spp., *Coxiella* spp., *Ehrlichia canis*, *Mycoplasma* spp. or *Rickettsia* spp. was detected in the cells through

PCR assays. Cytological analyses were performed using live phase contrast microscopy and cytocentrifuge smears stained with Giemsa, while periodic acid-Schiff and bromophenol blue staining techniques were used to detect total polysaccharides and protein, respectively. In conclusion, a new cell line derived from embryos of *A. sculptum* was generated and characterized in this study. This cell line was deposited in the Tick Cell Biobank, located at the University of Liverpool, under the care of Dr. Lesley Bell Sakyi, and in the Brazil Tick Cell Biobank Outpost located at the Oswaldo Cruz Foundation (FIOCRUZ), under the care of Dr. Flavio Alves Lara. s. In addition, it is important to have a larger panel of tick cell lines that can be used as tools for advancing research in various areas of the virology, bacteriology, biology and control of this tick.

Keywords: cell line; chromosome; Brazil.

3.1 Introduction

he ixodid tick, *Amblyomma sculptum* (Berlese, 1888) has been resurrected as a valid species (Nava et al., 2014) within the *Amblyomma cajennense* species complex, which comprises five other species, namely *Amblyomma cajennense* sensu stricto (s.s.) (Fabricius, 1787), *Amblyomma patinoi* Nava, Beati & Labruna, 2014, *Amblyomma mixtum* Koch, 1844, *Amblyomma tonelliae* Nava, Beati & Labruna., 2014, and *Amblyomma interandinum* Beati, Nava & Cáceres, 2014. The geographical distribution of *A. sculptum* includes northern Argentina, Bolivia, Paraguay and Brazil (Beati et al., 2013; Nava et al., 2014).

In Brazil, *A. sculptum* is the most common human-biting tick and it is found mostly in the Cerrado biome and degraded areas of the Atlantic Forest biome, whereas *A. cajennense* s.s. is present within the Amazon biome. Both species occur in areas of transition, corresponding primarily to the states of Maranhão and Tocantins in eastern Brazil, Mato Grosso in central Brazil, and Rondônia in western Brazil (Martins et al., 2016). In these areas, the main hosts for *A. sculptum* and *A. cajennense* s.s. are tapirs and horses, but these ticks also parasitize other animals, such as anteaters, feral pigs and capybaras (Nava et al., 2014; Ramos et al., 2014; Martins et al., 2016; Luz et al., 2019). *Amblyomma sculptum* is the most important tick vector of *Rickettsia rickettsii* in Brazil, which causes the most severe form of spotted fever in the part of the Cerrado located in the states of Minas Gerais and São Paulo. A milder form of spotted fever has been recorded in the central-western region of Brazil, also caused by *Rickettsia rickettsii*

transmitted by *A. sculptum* (Labruna 2009; Bitencourth et al., 2017; Machado et al., 2018).

Attempts to grow tick cells date back more than 60 years ago and several tick cell lines have been established from species of *Amblyomma*, *Dermacentor*, *Ixodes*, *Rhipicephalus* and *Ornithodoros* (Bell-Sakyi, 2018; Lima-Duarte et al., 2021). Currently, there are about 65 cell lines of ticks of different species, including 15 for Ixodidae and three for Argasidae (Bell-Sakyi, 2018; Lima-Duarte et al., 2021). Even though *Amblyomma* cell lines were previously obtained from *Amblyomma variegatum* (Fabricius, 1798) (AVL/CTVM13 and AVL/ CTVM17) (Bell-Sakyi et al., 2000; Bell-Sakyi, 2004) and *Amblyomma americanum* (Linnaeus, 1758) (AAE2 and AAE12) (Kurtti et al., 2005; Singu et al., 2006), only one preliminary report on establishment of an *A. sculptum* cell line (Cirelli-Moraes, 2015) and one on primary culture (Rezende et al., 2012) have been published. Until now, *Amblyomma* tick cell lines had not been characterized concerning cell morphology, growth characteristics or chromosome or cell identity.

In vitro culture systems derived from vector and vertebrate host tissues have an invaluable role in several aspects of tick and tick-borne pathogen research, including host–vector–pathogen relationships, parasite biology and disease control (Bell-Sakyi et al., 2007; 2018).

Here, we describe the process of obtaining and characterizing a new cell line from *A. sculptum* specimens collected in Brazil. In this regard, we performed a comprehensive analysis on some aspects of this cell line, which was named ASE-14, in terms of morphological analysis, karyotyping, presence/absence of contaminants, cell viability before freezing and after thawing, and species identification. The karyotype analysis included diploid numbers and location of structural parts of chromosomes, i.e. telomere and centromere. The cytological and histochemical analyses included general morphology and distribution patterns of proteins and polysaccharides in the cells. We also confirmed the identity of the cell line by means of partial sequencing of the mitochondrial 16S rRNA gene and confirmed the absence of selected microorganisms through molecular techniques.

3.2 Material and Methods

Origin of ticks and ethics approval

Engorged females of *A. sculptum* were collected from capybaras at ESALQ, University of São Paulo (USP), Piracicaba, state of São Paulo, southeastern Brazil (22° 42' south latitude, 47º 38' west), and were sent to the Department of Pathology, Reproduction and One Health of São Paulo State University (UNESP). The tick colonies were maintained in accordance with the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and their maintenance was approved by the Ethics Committee for Animal Use (CEUA; no. 06761/19) of FCAV, UNESP Jaboticabal, São Paulo, Brazil. To maintain colonies, ticks were periodically fed in New Zealand white rabbits. Non parasitic stages werekept under controlled conditions to 27◦C, 80% relative humidity. The tick cell culturing was authorized by the Genetic Asset Management Council (CGEN; no. 010214 / 2015-1) and through SisGen number A33B66E.

Tick cell cultures

Eighty engorged females were surface-sterilized after detachment from capybaras, following previously described protocols, with some modifications (Pudney et al., 1973). The ticks were washed in a solution containing 1% benzalkonium chloride (Polyorganic, São Paulo, SP, Brazil) for 15-20 min. After rinsing twice in sterile distilled water containing antibiotics (penicillin, 50 IU/ml; streptomycin, 50 µg/ml) and fungicide (amphotericin B, 0.25 µg/ml) (Vitrocell Embriolife™, Campinas, SP, Brazil) for 5 min, they were dried on sterilized cotton gauze, put in sterile glass Petri dishes, and incubated at 27 °C and 80-85% relative humidity.

Oviposition started around seven days afterwards and eggs were recovered daily. Eggs that were 12, 16 and 18 days old were used for primary cultures. In a class II biosafety cabinet (BSC), pools of multiple egg batches of the same age were removed using a micro-spatula, to scoop into a 50 ml polypropylene tubecontaining 70% ethanol. After incubation for 5 min at room temperature, the ethanol was discarded and the eggs were immersed in 1% benzalkonium chloride for 10 min. After discarding the benzalkonium chloride, the eggs were washed with sterile distilled water (50 ml of H_2O containing 100 µl of penicillin (10,000 IU/ml) and streptomycin (10 mg/ml) and 50 µl of amphotericin B (1 mg/ml) (Vitrocell Embriolife™) for 30 min. Lastly, the eggs were washed using 2% hypochlorite and sterile distilled water for 3 min.

The eggs were transferred to 2 ml microtubes containing 500 µl of L-15B medium (see below for composition) and were crushed using a disposable pellet pestle (Kimble Chase, Vineland, New Jersey, USA, VWR Scientific Cat. KT749520-0000). The

suspension was transferred to a polypropylene tubeof 50 ml and centrifuged at 300 × g for 8 min. The supernatant was removed and the tissue pellet was resuspended in 4 ml of complete L-15B medium (Munderloh and Kurtti, 1989) with 10% fetal bovine serum (FBS; Vitrocell Embriolife™) and 10% tryptose phosphate broth (Vitrocell Embriolife™) containing 1,000 units of penicillin and 1 mg of streptomycin (Vitrocell Embriolife™) per ml. The cultures were seeded into 25 cm^2 cell culture flasks (Corning Costar ® New York, NY, USA) and incubated at 30 °C. Cultures were monitored weekly, before medium changes, by means of examination under an inverted microscope Nikon, Eclipse TS100 (Nikon Corporation, Tokyo, Japan).

Subculturing was achieved by removing the cells from the flask surface using a cell scraper (Corning™), and these were resuspended in fresh complete L-15B medium. The cell monolayers were subcultured when they reached a density of 10^6 or 10^7 cells/ml. After centrifugation for 8 min at 100 × g, the supernatant was discarded, and the pellet was resuspended in 8 ml of L-15B medium. The cells were split 1:2 into 4 ml L-15B medium per flask.

Cryopreservation

From the first subculture onwards, cultures with cell densities ranging from 2.5 to 3.0 × 10^6 cells/ml were periodically frozen. The tick cells were removed from the flasks by means of scraping and were then centrifuged at 400 × g for 8 min. The supernatant was removed, and cells were resuspended in 1 ml of L-15B medium with 20% FBS. Then, 1 ml of L-15B medium containing 10% [v/v] dimethyl sulfoxide (DMSO) was added to give a final concentration of 5% DMSO and transferred to ice-cold 2 ml cryotubes... Cryotubes were stored at -80 °C using a NALGENE® Frosty™ Cryo (Thermo Fisher Scientific, Waltham, MA, USA) 1 °C freezing container, which resulted in a continuous decrease in temperature at a rate of one degree per minute. After being kept at -80 °C for 24 h, the cryotubes were transferred into a liquid nitrogen tank, where they were stored in the liquid phase for at least 30 d. For resuscitation, the cell suspensions were thawed rapidly by immersing the cryotubes in a water bath at 37 °C. The cryopreserved cells were resuspended in 1 ml of the appropriate L-15B medium and were centrifuged at 400 × g for 8 min. The pellets were resuspended in 4 ml of L-15B medium and transferred to 25 cm^2 flasks.

Cell-growth curves

Once the cells reached 80 to 90% confluence, they were counted in a Neubauer chamber at 1:2 dilution with dye exclusion (Sigma Aldrich, St. Louis, USA).

Cells (at the 24th passage) were plated at a density of 2 x 10^5 cells in 12.5 cm^2 cell culture flasks (Corning™) in L-15B medium with 10% FBS, and were incubated at 30 °C. To determine the numbers of cells, counts were performed in a Neubauer chamber by means of trypan blue dye exclusion. The counts were carried out every 24 h for 20 d (20 flasks seeded and one flask counted on each day), and the medium was not changed. Quantification of doubling time (DT) as a measurement of cell growth for cell line was carried out using counts of total viable cells. The maximum growth rate (μ_{max}) was determined by means of nonlinear regression curve analysis (Munderloh and Kurtti, 1989).

Tick cell identification

DNA was extracted from 10^7 ASE-14 cells (20th subculture, in their exponential phase), using the Qiagen DNA DNeasy® tissue kit (Qiagen, Chatsworth, CA, USA), following the protocol for cultured animal cells. The amounts and the quality of DNA extracted were measured with NanoDrop®1000 and then the DNA was stored at -20 °C until use. The PCR conditions used for amplifying a 460-bp fragment of the 16S rRNA gene of Ixodidae had been described previously, using the following primers: forward, 5'-CCG GTC TGA ACT CAG ATC AAG T-3'; and reverse, 5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3 (Black and Piesman, 1994). The positive control consisted of *A. sculptum* DNA, while the negative control contained sterile ultrapure water instead of sample.

The PCR product was separated by means of electrophoresis on 1% agarose gel and was viewed through ethidium bromide staining. The amplified fragment was purified using the Silica Bead DNA gel extraction kit (Thermo Fisher Scientific,) and was subjected to the dideoxynucleotide chain termination sequencing method, using the BigDye™ Terminator v3·1 cycle sequencing kit (Thermo Fisher Scientific™, Foster City, CA, USA) in the ABI PRISM 3700 DNA Analyzer sequencer (Applied Biosystems®, Foster City, CA, USA), at the Center for Biological Resources and Genomic Biology (CREBIO, FCAV, UNESP).

The electropherograms generated through the sequencing were analyzed to determine the quality of the peaks corresponding to each sequenced base, and a consensus sequence was built using the Bioedit v. 7.0.5.3 software (Hall, 1999). The 16S rRNA gene

sequence obtained from the conventional PCR was subjected to nBLAST analysis (Altschul et al., 1990), to enable comparisons with other sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank).

Molecular investigation of the* ASE-14 *tick cell line for the presence of pathogen DNA

DNA samples for which the 16S rRNA gene was successfully amplified were subjected to PCR assays in order to check for the presence of *Anaplasma* spp., *Anaplasma marginale, Babesia/Theileria* spp., *Coxiella* spp., *Ehrlichia canis*, *Mycoplasma* spp. and *Rickettsia* spp. DNA, following PCR protocols previously described in the literature (Table 1). For the conventional/nested PCR protocols, the products were separated by means of electrophoresis on 1% agarose gel and stained with ethidium bromide (Life Technologies™, Carlsbad, CA, USA), and were imaged under ultraviolet light (ChemiDoc MP imaging system, Bio-Rad™, Hercules, CA, USA), using the Image Lab software, version 4.1. Negative and positive controls were included with each batch of samples analyzed. All PCR runs were performed using nuclease-free water (Thermo Scientific) as a negative control. The positive control for *A. marginale* was obtained from naturally infected buffaloes (Machado et al., 2016). *Mycoplasma* and *Bartonella* DNA-positive controls were obtained from naturally infected cats from the state of Maranhão (Braga et al., 2012a, 2012b). The positive control for *A. phagocytophilum* was kindly supplied by John Stephen Dumler, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA. *Babesia vogeli* and *Ehrlichia canis* DNA positive controls were obtained from naturally infected dogs sampled in Campo Grande, state of Mato Grosso do Sul (Sousa et al., 2013). The *Coxiella burnetii* DNA-positive control was provided by Renato Arruda Mortara (Federal University of São Paulo, São Paulo, SP, Brazil). The *Rickettsia felis* DNA-positive control was obtained from cat fleas in Chile (Müller et al., 2018).

Table 1. Oligonucleotide primer sequences and hydrolysis probe sequences for targeted genes used in PCR assays for each agent searched for in samples from *Amblyomma sculptum* cell line ASE-14.

Cytological analysis

Agents	PCR type	Target gene	Primer sequence (5'-3')	Reference
***Anaplasma* spp.**	Nested	16S rRNA	ge3a:CACATGCAAGTCGAACGGATTATTC ge10r:TTCCGTTAAGAAGGATCTAATCTCC ge9f :AACGGATTATTCTTTATAGCTTGCT ge2: GGCAGTATTAAAAGCAGCTCCAGG	Massung et al.,1998
Anaplasma marginale	qPCR	*msp1β*	AM-For:TTGGCAAGGCAGCAGCTT AM-Rev:TTCCGCGAGCATGTGCAT AM-Pb:6FAM TCGGTCTAACATCTCCAGGCTTTCAT-BHQ1	Carelli et al., 2007
***Babesia/Theileria* spp.**	Nested	18S rRNA	BTF1:GGCTCATTACAACAGTTATAG BTR1:CCCAAAGACTTTGATTTCTCTC BTF2:CCGTGCTAATTGTAGGGCTAATAC BTR2: GGACTACGACGGTATCTGATCG	Jefferies et al., 2007
***Bartonella* spp.**	qPCR	*nuoG*	F-Bart: CAATCTTCT TTTGCTTCACC R-Bart: TCAGGGCTTTATGTGAATAC TexasRed-TTYGTCATTTGAACACG[BHQ2a-Q]	André et al., 2016
***Coxiella* spp.**	Nested	16S rRNA	Cox16SF1:CGTAGGAATCTACCTTRTAGWGG Cox16SR2: GCCTACCCGCTTCTGGTACAATT Cox16SF2:TGAGAACTAGCTGTTGGRRAGT Cox16SFR1:ACTYYCCAACAGCTAGTTCTCA	Duron et al., 2014
Ehrichia canis	qPCR	*dsb*	Dsb-321:TTGCAAAATGATGTCTGAAGATAT GAAACA Dsb-671:GCTGCTCCACCAATAAATGTATCYCCTA Probe:AGCTAGTGCTGCTTGGGCAACTTTGAG TGAA-3 ' e 5'-FAM / BHQ-1,3	Doyle et al., 2005
***Mycoplasma* spp.**	cPCR	16S rRNA	Myco16S-322s:GCCCATATTCCTACGGGA AGCAGCAGT Myco16S-938as:CTCCACCACTTGTTCAGG TCCCCGTC HemMycop16S-322s:GCCCATATTCCTACGGGAAGCAGCAGT HemMycop16S-1420as:GTTTGACGGGCGGTGTGTACAAGACC	Maggi et al., 2013
***Rickettsia* spp.**	cPCR	*gltA*	CS-239: GCTCTTCTCATCCTATGGCTATTAT CS-1069: CAGGGTCTTCGTGCATTTCTT	Labruna et al., 2004

Cytological and histochemical techniques were used to assess the general morphology and distribution patterns of proteins and polysaccharides in cells. The cytological analyses were conducted at the Department of Biology of UNESP, Rio Claro, SP, Brazil.

To detect changes such as presence or absence, frequency and distribution of proteins and polysaccharides in the tick cell cultures, the material was prepared in accordance with previously described protocols (Lima-Duarte et al., 2021).

The tick cells were removed from the flask and were inoculated into a 24-well plate containing round coverslips for microscope, at a density of 2 x 10^5 cells (per well). In addition to the lid, the 24-well plate was sealed with Parafilm®. The cells were then maintained in L-15B medium at 30 ºC for 2 days, in a cell culture incubator.

Obtaining of metaphase chromosome spreads

We obtained chromosome spreads from *A. sculptum* adult males and cell cultures at passage 42. The adult males were obtained from University of São Paulo (USP), Piracicaba, state of São Paulo, southeastern Brazil, and were sent to the Department of Pathology, Reproduction and One Health of São Paulo State University (UNESP). The adult males were obtained from University of São Paulo (USP), Piracicaba, state of São Paulo, southeastern Brazil, and were sent to the Department of Pathology, Reproduction and One Health of São Paulo State University (UNESP).

New Zealand white rabbits (*Oryctolagus cuniculus*) were used as the host. Ticks were placed in feeding chambers, fixed with synthetic glue (Brascoplast®, Brascola Ltda., Brazil) on the dorsal region of the animal. All procedures were approved by the Institutional Animal Care and Use Committee (CEUA; no. 06761/19 and 1732120917). After feeding, we dissected the testes of these male adults under a stereomicroscope and fixed them in modified Carnoy's solution (absolute ethanol: glacial acetic acid, 3:1). For each slide, one pair of testes was macerated in a drop of 60% glacial acetic acid. The material was then spread and the slides were dried on a hot plate at 45 °C. The chromosomes were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich; 2 µg/ml) and the slides were mounted using VECTASHIELD (Vector™, Burlingame, CA, USA). Mitotic metaphase chromosome spreads from cell culture were obtained from the cell culture following the protocol proposed by Chen et al. (1994), with modifications described by Kotsarenko et al. (2020), including the incubation with colcemid (10 µg/mL) during 18h. The slides obtained from the cell cultures were used for molecular cytogenetic analysis (see Section 2.9).

Chromosome counting and molecular cytogenetic analysis With the aim of facilitating chromosome counting and recognition of putatively rearranged chromosomes on mitotic metaphase chromosome spreads, we performed fluorescence *in situ* hybridization (FISH) using probes that identify the centromeric and terminal regions of the chromosomes. In this way, we obtained telomeric probes through non-template PCR,

as described by Ijdo et al. (1991), using the complementary primers $(TTAGG)_5$ and $(CCTAA)_5$, which were labeled with biotin-14-dATP (Invitrogen Life Technologies, Paisley, UK). The probe used for centromere recognition was obtained using the whole genome of the cell culture. The genomic DNA (gDNA) was extracted by means of the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA), in accordance with the manufacturer's recommendations. About 500 ng of gDNA was labeled through nick-translation with digoxigenin-11-dUTP (Roche, Mannheim, Germany).

FISH was performed as described by Cabral-de-Mello and Marec (2021) and the probes were detected using anti-digoxigenin-rhodamine (Roche) for the digoxigenin-labeled probe or using streptavidin Alexa-Fluor-488 conjugate (Invitrogen) for the biotin-labeled probe. The chromosomes were counterstained with DAPI and the slides were mounted on slides using VECTASHIELD (Vector™). The results were observed using an Olympus BX61 microscope equipped with a fluorescence lamp and appropriate filters, and photomicrographs were recorded using a DP70 cooled digital camera. The images were merged and optimized for brightness and contrast using Adobe Photoshop CS2 software.

We counted 100 good-quality metaphase chromosome spreads in order to estimate diploid number variation, occurrence of putative rearranged chromosomes and presence of microchromosome or chromosome fragments.

3.3 Results

Primary culture and establishment of ASE-14 cell line

Among the ages of the batches of *A. sculptum* eggs (12, 16 and 18 d after oviposition), the ideal age for preparation of primary cultures was found to be 16 d after the beginning of oviposition, which allowed us to obtain the largest number of viable cells for adhesion. In the present study, approximately 80 batches of *A. sculptum* female eggs were used and the cultures were prepared using egg pools weighing 60 mg on average.

Furthermore, adhesion of cells to the flasks was observed for L-15B medium supplemented with 10% FCS. After 60 d of culturing, formation of a confluent cell monolayer was observed, reaching a density of approximately 4 x 10^5 cells/ml, thus enabling the first subculture.

Freezing was started from the first subculture onwards, and frozen cells were kept in liquid nitrogen for 3 months before being resuscitated. After thawing, within two weeks of culturing under standard conditions, the samples reached levels of viability similar to those of the unfrozen culture, showing slow but steady cell growth. Only cultures frozen in the 14th subculture were recovered after thawing.

The cell line was named ASE-14, in which “AS” represents the abbreviated name of the tick species; "E" means embryonic cells; and the number "14" means that cell cryopreservation was achieved from the 14th subculture. Cell line ASE-14 in the 14th subculture was deposited at the Tick Cell Biobank located in the University of Liverpool, under the care of Dr. Lesley Bell Sakyi, as well as in the Brazil Tick Cell Biobank Outpost located at the Oswaldo Cruz Foundation (FIOCRUZ), under the care of Dr. Flavio Alves Lara. At the time of writing this report, the cultures were at passage level 45.

Cell morphology, including cell shape and size, was ascertained by looking directly at the cultures under an inverted microscope and by examining cytocentrifuged smears stained with Giemsa (Fig. 1 A-E).

The cultures showed two cell types: fibroblast-like cells with granularity in their cytoplasm; and rounded cells (Fig. 1 B-D). Clumps of small, rounded cells were observed at the 24th passage (Fig. 1C). Nuclei were strongly stained by the bromophenol blue reaction (Fig. 1F) and by the PAS reaction (Fig. 1G). Histochemical techniques showed the homogeneous distribution of proteins and polysaccharides in the cells. The cytoplasm showed cytoplasmic projections and inclusions for which the composition was not identified through the techniques applied. Fibroblast-like cells predominated in the ASE-14 line (Figure 1 A-D).

The cell growth parameters of *A. sculptum* are shown in Figure 2. The population doubling time (Td) during the logarithmic phase of growth was about 67 h. The maximum cell density was 4.25 x 10^6 and the time taken to reach the maximum number of cells was 11 d.

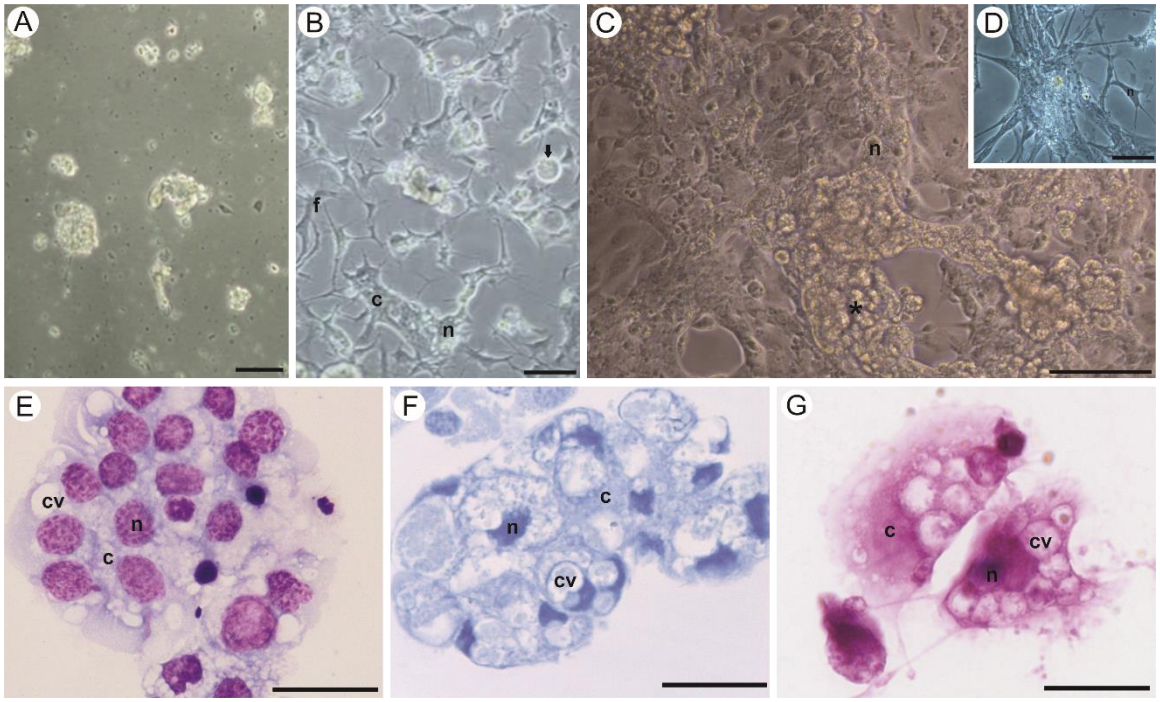

Figure 1. General characterization of cell morphology of *Amblyomma sculptum* embryonic cells (ASE-14). A. Primary cultures of *Amblyomma sculptum* on day 16 after start. B. *Amblyomma sculptum* cell line ASE-14 at passage 15, 60 days after start. C. Cell line ASE-14 at passage 24. D. Detail of spindle-shaped cell type. E. Cytocentrifuged tick cell smears stained with Giemsa. F. Cells grown on coverslip and stained with bromophenol blue. G. Cells grown on coverslip and subjected to periodic acid-Schiff. A-D = Phase contrast microscopy on live cells. E-G = Bright-field microscopy. c = cytoplasm, cv = cytoplasmic vesicles, f = spindle-shaped cells, n = nuclei, * = clumps of cells, arrow = rounded cells. Bars A-E = 10 µm, F-G = 20 µm.

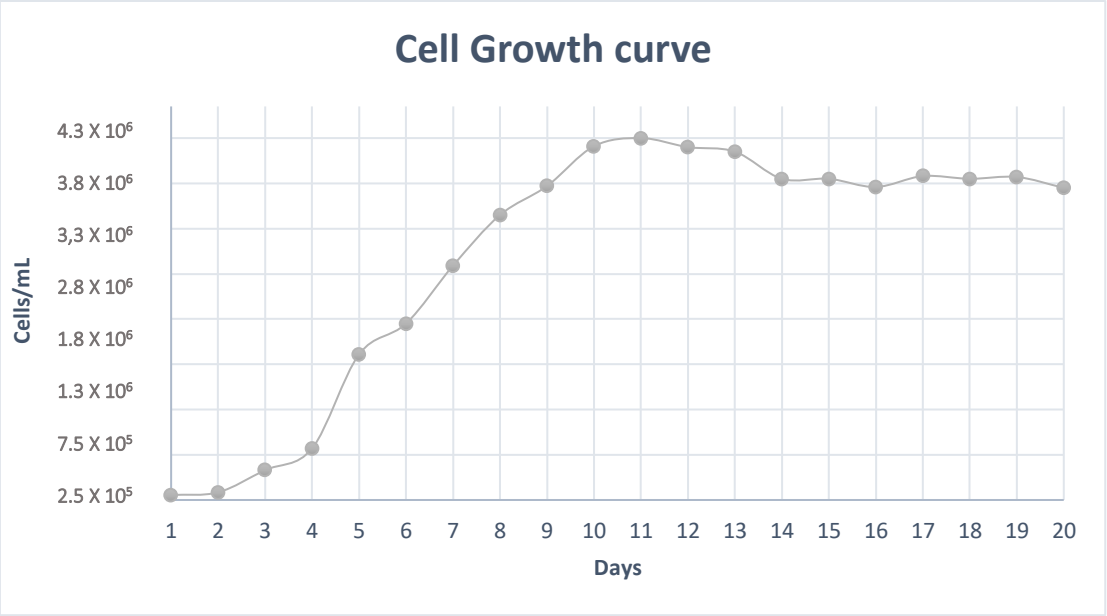

Figure 2. *Amblyomma sculptum* cell line (ASE-14) growth in the 30th subculture. Tick cells were maintained in L-15B medium supplemented with 10% fetal bovine serum. Cells were counted using a hemocytometer.

Confirmation of cell line species identity

he 16S rRNA sequence obtained of ASE-14 cell line was subjected to BLASTn analysis to determine the similarity with sequences previously deposited in the GenBank database. The consensus sequence (432 bp), which corresponded to a fragment of the 16S rRNA gene obtained from the ASE-14 cell line (cells from the 20th subculture) showed 100% identity to *A. sculptum*, sequences MF353127 and KY172626 from Brazil, with 93% query coverage and E-value of 0.0, thus confirming that the cells originated from this tick species. The sequence obtained in the present study was deposited in GenBank under accession number MZ913260.

Molecular analysis on the cell line obtained

The cultures analyzed for detection of microorganism DNA were the following: the primary culture and cultures from the 23rd, 24th, 41st and 42nd subcultures.

The samples analyzed were positive in the PCR protocol for the tick's 16S rRNA mitochondrial gene. All samples were negative in the PCR assays for *Anaplasma* spp., *Anaplasma marginale*, *Babesia/Theileria* spp., *Bartonella* spp., *Coxiella* spp., *Ehrlichia*

canis, *Mycoplasma* spp. and *Rickettsia* spp. Positive control samples were detected by the PCR and there was no amplification in the negative control samples.

Karyotyping

The karyotype from the adult males of *A. sculptum* was 2n=21, X0 with occurrence of exclusively acrocentricchromosomes. The chromosomes were similar in size, and from pair 1 to 10 only slightly decreasing size was noticed. The X chromosome is the largest element of the karyotype (Figure 3a), as in other species of Amblyoma (Gunn and Hilburn 1995). In metaphase I, we confirmed the diploid number of the species through observation of 10 bivalents, corresponding to 20 autosomes, along with one large univalent, the X chromosome (Figure 3b). As expected, 11 or 10 chromosomes were noticed in metaphase II, corresponding to chromosome spreads with the X chromosome (Figure 3c) and without it (Figure 3d), respectively.

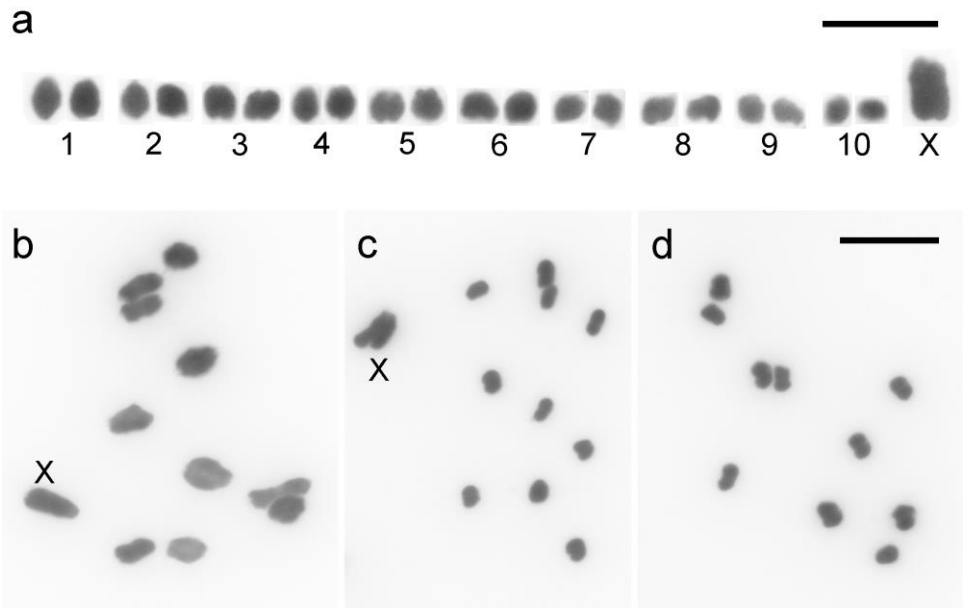

Figure 3. Chromosomal analysis from adult testes of *Amblyomma sculptum* (a-e) and from cell culture (f). (a) A tentative karyotype from spermatogonial mitotic metaphase with 21 chromosomes, 2n=21, X0 organized based in the decreasing of chromosome size. (b) Metaphase I with 10 autosomal bivalents and one univalent, the X chromosome. (c, d) Metaphase II harboring the X chromosome (c) and with no X chromosome (d). (e) Metaphase II harboring X chromosome probed for telomeric probe (green) and gDNA

probe (red). (f) mitotic metaphase spread from cell culture of passage 42 of *Amblyomma sculptum* harboring 34 chromosomes, i.e. the most common diploid number for the cell culture. Scale bars = 5 μm.

Our FISH mapping of the telomeric motif $(TTAGG)_n$ and gDNA in cell cultures helped us to establish chromosomal landmarks that facilitated counting of chromosome numbers on mitotic spreads and also analysis of chromosome structure and documentation of putative chromosome rearrangements. From the cell culture of passage 42, our analysis on 100 mitotic metaphase spreads revealed variation of diploid number from 27 to 108 chromosomes. The modal number was 34 (Figure 3f), which occurred in 26% of the metaphase mitotic spreads, and 32, 33 and 36 chromosomes were also quite common. Moreover, we noticed occurrence of microchromosome or chromosome fragments on 27 mitotic metaphase spreads, with a range from one to seven. Most commonly, one microchromosome or chromosome fragment occurred (13 spreads), followed by two (seven spreads) and three (four spreads). Mitotic spreads with diploid numbers from 32 to 34 presented microchromosome or chromosome fragments more frequently (Figure 4).

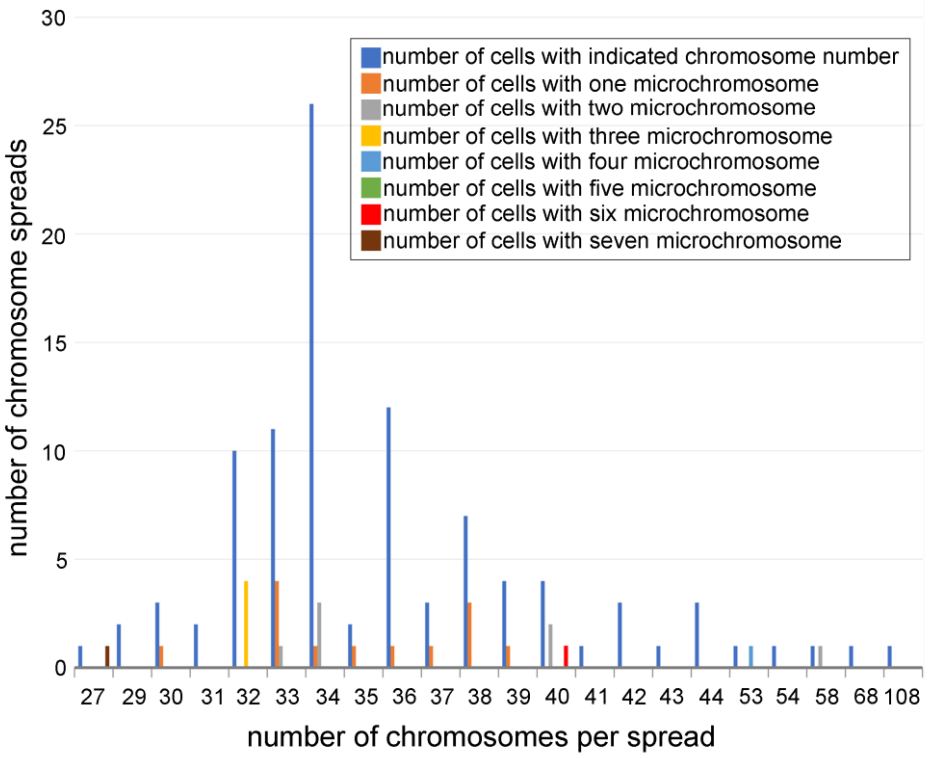

Figure 4. Variability of numbers of chromosomes and occurrence of microchromosome or chromosome fragments in metaphase mitotic spreads from cell culture on passage 42 of *Amblyomma sculptum*. A total of 100 metaphase mitotic spreads were counted. Note the higher number of chromosome spreads with 34 chromosomes, which was thus the modal number. The microchromosome or chromosome fragments were more common in metaphase mitotic spreads with numbers from 32 to 34.

The mapping of the gDNA probe revealed strong signals in the pericentromeric regions of all chromosomes, which is putatively the region most enriched with repetitive DNA, thus allowing higher degrees of probe hybridization and clear signal visualization. In all cases, only one mark for the gDNA probe was observed per chromosome, thus suggesting the presence of only one centromere per chromosome. This pattern was similar for cells from adults and from culture (Figure 3e). Through mapping of $(TTAGG)_n$, we observed only regular telomeres (located on terminal regions of the chromosomes) on cells from adult males (Figure 3e). But for cell culture we observed not only the usual telomeres in the terminal region of chromosomes, but also evidenced signals in interstitial areas of multiple chromosomes, i.e. interstitial telomeric sites (ITS). Acrocentricchromosomes as well as observed in *A. sculptum* male adults were more common (Figure 5a). We noticed that the centromeres and telomeres were maintained

independently of the number of chromosomes in the mitotic spreads, given that probe signals were observed in all cells. The ITS occurred in centromeric regions or in the chromosome arms (Figure 5b). Concerning the microchromosome or chromosome fragments, we noticed three patterns for the chromosomal markers studied: i) presence of centromere and telomere; ii) exclusive presence of telomeric repeat; and iii) exclusive presence of centromere (Figure 5c, d). These chromosomes were slightly variable in size (Figure 5c).

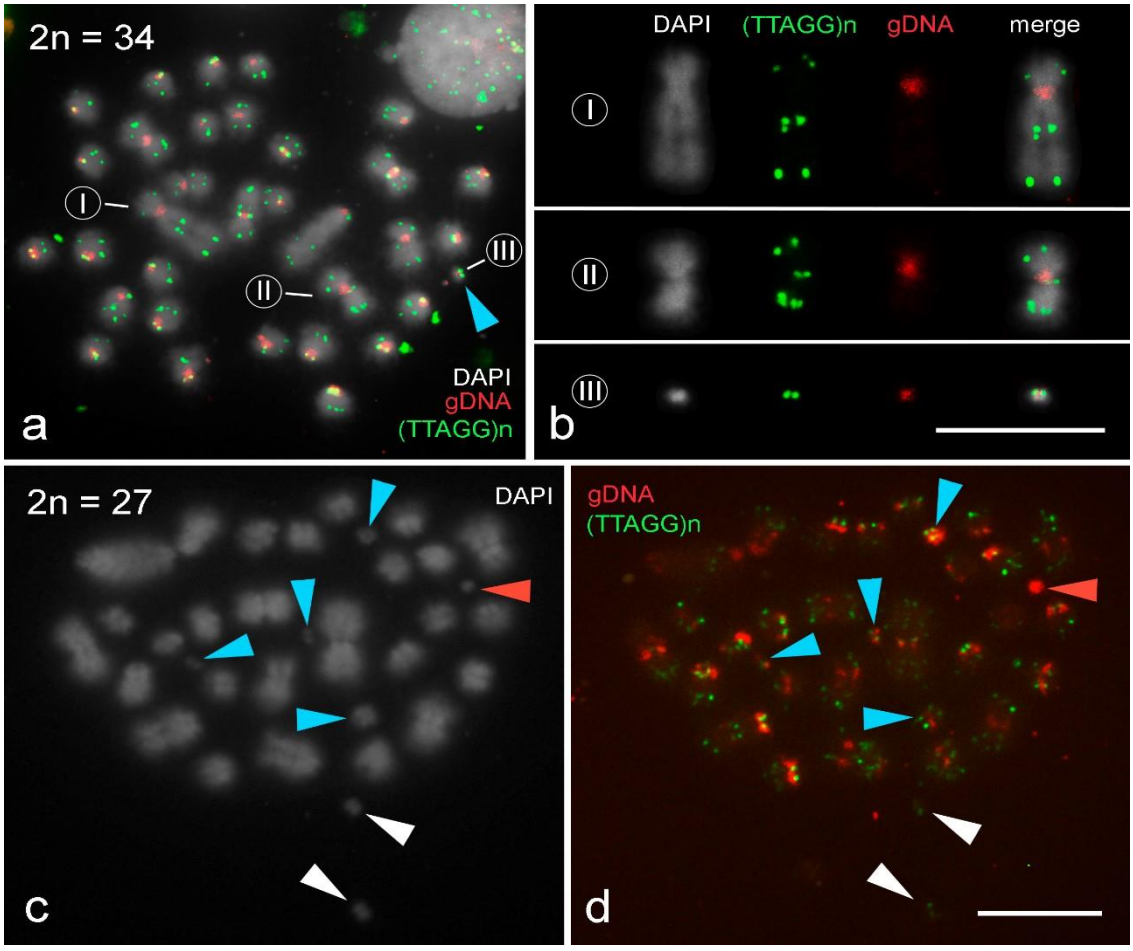

Figure 5. Fluorescence *in situ* hybridization on metaphase mitotic spreads from cell culture of passage 42 of *Amblyomma sculptum* cell line ASE-14 using probes consisting of the telomeric motif $(TTAGG)_n$ in green and gDNA in red. Note that the gDNA probe labels the centromeric region of chromosomes and that the telomeric probe labels the terminal region and also reveals marks in interstitial chromosome areas and pericentromeric regions, corresponding to ITS. (a) A mitotic chromosome spread with 34 chromosomes, which was the modal number of chromosomes in the cell culture, and one

microchromosome or chromosome fragment. (bI) A chromosome with ITS on long arm. (bII) A chromosome with ITS in the pericentromeric area. (bIII) A microchromosome or chromosome fragment containing a telomere and centromeric repeats. (c, d) A metaphase mitotic spread with 27 chromosomes plus seven microchromosome or chromosome fragments, with distinct constitutions, as follows: telomere alone (white arrowheads), centromere alone (red arrowhead) and centromere plus telomere (blue arrowheads). (c) DAPI staining and (d) probe signals. Note the distinct sizes of the microchromosome or chromosome fragments. Scale bars = 5 μm.

3.4 Discussion

One of the important variables in starting a tick cell culture is the stage of the egg embryogenesis used in the primary culture. In the present study, the optimal incubation period for achieving successful cell cultures was determined to be 16 d (corresponding to around half the incubation period for *A. sculptum*, under laboratory conditions). Most tick cell lines have been derived from eggs, which form the most easily handled developmental stage and provide a plentiful source of relatively undifferentiated starting material (Bell-Sakyi et al., 2018). In the present study, primary cultures of *A. sculptum* eggs that were prepared in complete L-15B medium containing 10% FBS allowed cell growth and adhesion.

With regard to morphology and cell types, we observed in our study that the cultures present two types: spindle cells with cytoplasm vesicles; and rounded cells. In a primary culture derived from *A. sculptum* (cited as *A. cajennense*), Rezende et al. (2012) initially observed fibroblastoid and epithelioid globose cells, along with polyhedral and stellate cells, while some cells with eccentric nuclei and vacuolated cytoplasm appeared later. In previous studies, two or more cell types were also observed in tick cell lines from *I. scapularis* and *R. microplus* (Munderloh et al., 1994; Esteves et al., 2008; Lima-Duarte et al., 2021).

The results from the histochemical study using PAS and bromophenol blue allowed detection of polysaccharides and proteins, respectively, in the cytoplasm of ASE-14 cells. In a previous study on the RBME-6 cell lineage (*R. microplus*), a general pattern of protein and polysaccharide distribution in cells was also evidenced (Lima-Duarte et al., 2021). Despite the different morphology of the cells (spindle cells in *A. sculptum* and

rounded cells in *R. microplus*), the same histochemical pattern was observed in both the ASE-14 and the RBME-6 cell lineage.

Esteves et al. (2008) reported the presence of protein in some cytoplasmic vesicles in *R. microplus* cell lines stained with fluorescent metalloporphyrin Pd-mP, thus indicating the existence of a putative cytosolic heme-binding protein. Here, similar vesicles were also observed in the ASE-14 cell linethat could be similar to those reported by Esteves et al. (2008). The evidence of a cytosolic heme-binding protein suggests that tick cell lines may be a model for drug discovery and indicates that vesicles may be involved in autophagy and endocytosis (Esteves et al., 2008). However, studies with palladium meso-porphyrin (Pd-mP)marking and other markers would be necessary to confirm the composition of these vesicles in the ASE-14 cell line.

In the present study, we observed that spindle cells became dominant after subculturing. This morphological characteristic, as well as the cytoplasmic granulation observed in cells, may be associated with the physical and chemical environment (i.e. pH, density, temperature and constituents of the medium).

Determining the cell growth curve is important for identification of certain parameters specific to a population under certain culturing conditions. In addition, the growth curve is also useful for guiding the clinical use of drugs, investigating the functions of genes and understanding the mechanism of action of drugs (Xu et al., 2020). ASE-14 tick cells were found to grow slowly in cultures, as measured by doubling time (Td). In the present study, the population doubling time (Td) of the cells during the logarithmic growth phase was about 67 h at 30 °C and the overall doubling time of the culture ranged from 3 to 4 d.

In previous studies, the doubling time of distinct cell lines of *R. microplus* ranged from 3.8 to 15 d (Holman, 1981; Munderloh and Kurtti, 1989; Cossio-Bayugar et al., 2002; Esteves et al., 2008; Lima-Duarte et al., 2021). The ASE-14 cell line showed good viability after storage in liquid nitrogen for 90 d. This tick cell line was successfully cryopreserved with 5% DMSO. The cryopreservation process has mostly been done using 10% DMSO, because this has shown better results after thawing (Bastos et al., 2006; Lallinger et al., 2010). A lower concentration of DMSO has been also used in cryopreservation of tick cell lines from *R. microplus*: 7.5% in USA (Holman, 1981) and 10% in Brazil (Lima-Duarte et al., 2021). Moreover, IDE8 tick cells were cryopreserved using 6% DMSO medium (Bastos et al., 2006).

Concerning the numbers of chromosomes, some tick cell lines are diploid and either the number of male or female chromosomes is predominant (Pudney et al. 1973; Varma et al., 1975; Esteves et al., 2008; Bhat and Yunker 1977; Lima-Duarte et al. 2021). Our comparison of the numbers of chromosomes between *A. sculptum* adult males with the ASE-14 cell line on passage 42 clearly suggests the occurrence ofpolyploidization events, thus allowing cells with distinct diploid numbers to be generated. Besides the polyploidization, occurrences of chromosome rearrangements, like fusions and translocations, are also evident through the presence of biarmed chromosomes, that are also involved with the emergence of distinct diploid numbers. These biarmed chromosomes are not observed in adult males of *A. sculptum*. Interestingly, distinct types of fusions were noticed, involving the pericentromeric areas, with occurrence of ITS on centromeres or in tandem fusions, as documented by ITS on chromosomal arms.

In a previous study, high numbers of chromosomes were observed in the IRE/CTVM19 cell line (Bell-Sakyi, 2004) from *Ixodes ricinus* (Linnaeus, 1758) and in the ISE18 cell line (Kurtti et al., 1996) from *I. scapularis*. These were possibly polyploidizations or integral duplications of chromosomes (Kotsarenko et al., 2020), which suggests that this could be the common fate for distinct cell cultures. Even though some cell lines previously studied presented variable numbers of chromosomes, these failed with the occurrence of ITS, as documented here (Kotsarenko et al., 2020; Lima-Duarte et al. 2021). The intense dynamics in the ASE-14 chromosomes was also shown by occurrences of distinct types, concerning size and composition, of microchromosome or chromosome fragments. This type of chromosome fragment was also observed in one cell line from *R. microplus* (RBME-6) (Lima-Duarte et al. 2021).

Although the chromosomes showed much rearrangement, our FISH analysis revealed that the pericentromeric region was maintained in all chromosomes, independently of the diploid number numbers in the chromosome spread, thus indicating that the chromosomes are probably stable in these cells during the chromosome divisions. Moreover, the telomeres at canonical foci (i.e. chromosome termini) assured their stability. Interestingly, this chromosome stability was also shown for microchromosome or chromosome fragments, such that some of them had telomere and pericentromeric DNA.

The consensus sequence obtained for ASE-14 cell line (cells from the 20th subculture) from the showed 100% identity to *A. sculptum* isolated from Brazil, São Paulo (MF353127 and KY172626). In a study by BEATI et al. (2013), in phylogenetic analyses

performed on specimens of *A. cajennense*, it was observed that there was genetic segregation in different clades, the authors concluded that this ixodid constitutes a complex of species, each associated with a particular biogeographic region.

Posteriorly, other studies confirmed that *A. sculptum* Berlese,1888 is a member of the *A. cajennense* complex and has a wide distributed in peri-Amazonian areas in Brazil, encompassing the states of Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Pernambuco, Piauí, Mato Grosso, Mato Grosso do Sul and Goiás (NAVA, SANTIAGO et al., 2014).

In vitro culture systems represent a tool for studying many aspects of tick and tick-borne pathogen research.The Arkansas strain of *Ehrlichia chaffeensis* was grown in the *A. americanum* cell line AAE2 (Singu et al., 2006), *Ehrlichia ruminantium* was grown in two cell lines derived from the vector tick species *A. variegatum* (Bell-Sakyi, 2004). Policastro et al. (1994) reported that *R. rickettsii* was grown in two cell lines derived from *Dermacentor albipictus* Packard, 1869 (DALBE3) and *Ixodes scapularis* Say 1821 (IDE2). Moreover, successful infection of the *A. americanum* cell line (AAE12) by seven *Bartonella* isolates and three of '*Candidatus Bartonella* spp.' has also been reported (Billeter et al., 2009).

Tick cell lines have been used for over 35 years for propagation of arboviruses (Varma, et al., 1975; Yunker et al., 1981). A tick cell line from *A. variegatum* (AVL/CTVM17) was found to be susceptible to infection with the following tick-borne flaviviruses: tick-borne encephalitis virus (TBEV), Langat virus (LGTV), Louping ill virus (LIV) and Powassan virus (POWV), Negishi virus (NGV); and to infection with the mosquito-borne West Nile virus (WNV) (Lawrie et al., 2004).

In conclusion, a new tick cell line derived from embryos of *A. sculptum* was generated and characterized in this study. It can be tested as a substrate for maintenance of pathogens in a future study.

CRediT authorship contribution statement

Leidiane Lima-Duarte: Conceptualization, Investigation, Methodology, Writing - original draft. Ana Carolina Castro-Santiago: Investigation, Writing - review & editing. Jaqueline Valéria Camargo: Investigation, Writing - review & editing. Rosangela Zacarias Machado: Formal analysis, Resources, Writing - review & editing. Marcos Rogério André: Formal analysis, Resources, Writing - review & editing. Diogo Cavalcanti Cabral-de-Mello: Investigation, Methodology, Writing - review & editing.

Ana Beatriz Stein Machado Ferretti: Investigation, Methodology, Writing - review & editing. Maria Izabel Camargo-Mathias: Investigation, Methodology, Writing - review & editing. Gustavo Seron Sanches: Resources, Methodology. Priscila Ikeda: Investigation, Writing - review & editing. Luís Adriano Anholeto: Investigation, Methodology, Writing - review & editing. Melissa Carolina Pereira: Investigation, Methodology, Writing - review & editing. Carlos Perez: Resources. Darci Moraes Barros-Battesti: Conceptualization, Methodology, Supervision, Writing - review & editing.

Acknowledgements

This work was supported by the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq) [through grant nos. 312331/2013-4 and 302420/2017-7] and the Research Support Foundation of the State of São Paulo (Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP) [through grant nos. 2015/26209-9 and 2020/03190–9]. It was also partially supported by the Coordination Office for Improvement of Higher- Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES) under finance code 001. We are grateful to Timothy Kurtti and Urlike G. Munderloh, of the Department of Entomology, University of Minnesota, Minneapolis, United States, for training us in Minneapolis/St Paul in 2007, in relation to preparation of tick cell cultures.

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4. CONCLUSIONS

The obtaining and maintenance of primary culture and cell lines from embryonic eggs of *R. microplus* and *A. sculptum* were described here.

The egg incubation time for successful primary cultures was 12 days for *R. microplus* and 16 days for *A. sculptum*. Furthermore, the ideal FBS (fetal bovine serum) concentration for cells cultures maintenance was 20% for *R. microplus* and 10% for *A. sculptum*.

Cell lines derived from *R. microplus* and *A. sculptum* can be successfully stored in liquid nitrogen using 5% DMSO as a cryoprotectant. Only cultures frozen in the 6th subculture from *R. microplus* and 14th subculture from *A. sculptum* were recovered after thawing.

The results presented here revealed a comprehensive analysis of some aspects of these cell lines (RBME-6 and ASE-14) by morphology analysis, karyotyping, absence of contaminants, cell viability before freezing and after thawing and specie identification.

Cell growth showed that the population doubling time (Td) during the logarithmic growth phase was about 62 hours and 56 minutes for *R. microplus* (6th subculture) and 67 hours for *A. sculptum* (24th subculture).

Cell morphology analysis revealed clusters of small, rounded cells together with cells presenting a fibroblast-like appearance in the RBME-6 cell lines.While in the ASE-14 cell line spindle cells with cytoplasmic vesicles and round cells were observed.

The histochemical study using PAS and bromophenol blue dyes allowed the detection of polysaccharides and proteins in the cytoplasm of RBME-6 and ASE-14 cells.

The karyotyping of RBME-6 cell line revealed modal diploid number in both passages analyzed (7th and 29th subculture) was 2n = 21, with one X chromosome. Karyotyping (43th subculture) revealed the occurrence of chromosome multiplication in ASE-14 cell line, probably by events of polyploidization, allowing generating cells with distinct diploid numbers.All the chromosomes were acro-telocentric. Slight variations were observed between the two passages, with occurrence of aneuploid metaphase chromosome spreads

The origin RBME-6 and ASE-14 cell line was confirmed by conventional PCR and sequencing of a fragment of the mitochondrial 16S rRNA gene. No DNA from *Anaplasma* spp., *Anaplasma marginale*, *Babesia/Theileria* spp., *Coxiella* spp., *Ehrichia canis*, *Mycoplasma* spp. and *Rickettsia* sp. was detected in the cells lines through PCR

assays. Overall, we report the successful primary cultures and establishiment of two new cell lines of two tick species from Brazil.