Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

Arbeit angefertigt unter der Leitung von Uni.-Prof. Dr. Kurt Pfister

IN VITRO CULTURE STUDIES OF TICK CELL LINES

"Endosymbionts in tick cell culture and evaluation of media conditions"

Inaugural-Dissertation zur Erlangung der tiermedizinischen Doktorwürde
der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

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aus Damaskus in Syrien

München 2012

Gedruckt mit Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Braun
Berichterstatter:	Univ.-Prof. Dr. Pfister
Korreferent:	Priv.-Doz. Dr. Zakhartchenko

Tag der Promotion: 21. Juli 2012

Die vorliegende Arbeit wurde nach § 6 Abs. 2 der Promotionsordnung für die Tierärztliche Fakultät der Ludwig-Maximilians-Universität München
als kumulative Dissertation gestaltet.

Mein Schatz

Roda

ABBREVIATIONS

bp	base pair
°C	Degree Celsius
$CaCl_2$	Calcium chloride
CoCl	Cobalt chloride
$CuSO_4$	Cupper sulfate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ds	double-stranded
EDTA	diaminoethanetetraacetic acid
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
$FeSO_4$	Iron sulphate
FRET	Fluorescent resonance energy transfer
g	gram
gyrB	DNA gyrase subunit B
h	hour
HGA	Human granulasis agent
H_2O	Water
KCl	Potassium chloride
l	liter
MEM	Minimum Essential Medium
mg	miligram
$MgCl_2$	Magnesium chloride
Min	minute
ml	mililiter
mM	miliomolar
$MnSO_4$	Manganese sulfate
mOsm	milliosmole
NaCl	Sodium chloride
Na_2SeO_3	Sodium selenite
$NaMoO_4$	Sodium molybdate

NaH_2PO_4	Sodium phosphate
$NaCO_3$	Sodium carbonate
ng	nanogram
nM	nanomolar
PCR	Polymerase Chain Reaction
PDT	Population Doubling Time
PVP	Polyvinylpyrrolidine
Redox	Reduction-oxidation
rRNA	ribosomal ribonucleic acid
SFG	Spotted Fever Group
ss	single-stranded
TAE	Tris Acetate EDTA
Taq	*Themus aquaticus*
TBEV	Tick-Borne Encephalitis Virus
TBP	Tryptose Phosphate Broth
Tris	Trishydroxymethylaminomethane
Tm	melting temperature
U	Unit
UV	Ultraviolet
$ZnSO_4$	Zinc sulfate
µl	Microliter
µM	Micromolar
µm	micrometer
%	Percent

1. INTRODUCTION AND OBJECTIVES

Ticks harbour, besides being a vectors of many pathogens, endosymbionts which are non-pathogenic for the ticks but in contrary may benefit the ticks by antagonizing super infection of the host tick in closely related species that are pathogenic for ticks themselves (Azad and Beard, 1998). One of these symbionts is *Candidatus* Midochloria mitochondrii, which has been detected in several tick species and especially in the ovarial tissues of females *Ixodes ricinus* (Lewis, 1979; Sassera et al., 2006). The major route of transmission for the bacterium is vertical transmission (Lo *et* al. 2006). This bacterium is the only known bacterium that can invade the mitochondria within the infected cells (Sacchi et al., 2004). However, the way in which the bacteria enter the mitochondria has not been known. In addition, the biology of these bacteria is poorly understood. In order to close these gaps in knowledge, studies of this bacterium at the cellular level are of paramount importance. To bridge the gap in this area of study, tick cell cultures represent a good model to unlock the mechanism of invasion for these bacteria.

The tick cell lines that are available so far have been initiated and maintained in different media. Varma and others (1975) used L-15 medium (Leibovitz, 1963) supplemented with tryptose phosphate broth (TPB) and Serum to establish three cell lines from *Rhipicephalus appendiculatus.* Furthermore, five tick cell lines were established from the tick *Hyalomma anatolicum anatolicum* using the mixture medium L-15/H-Lac supplemented with 20 % fetal calf serum (FCS) (Bell-Saki, 1991). The use of different media among tick cell line is a matter of consuming time needed for preparing and changing medium for each line. Therefore, the use of one medium for different cell lines will save the time needed for preparing and changing several media, as well as reducing the costs required.

Hence, the aims of this thesis were in twofold:

Firstly, an attempt was made to determine if selected tick cell lines derived from *Ixodes ricinus*, *I. scapularis*, *Rhipicephalus (Boophilus) microplus* and *R. (Boophilus) decoloratus* are infected with the symbiont *Candidatus* Midichloria mitochondrii, which has been detected in several tick species, taking into account that all these cell lines were derived from embryonic tissues.

Secondly, investigation was carried out to examine the ability of the mixture L-15/H-Lac medium to support the growth of cells in four different cell lines kept under different media conditions and to study the effect of different concentrations of serum on growth rates of the cells.

2. LITERATURE REVIEW

2.1. Ticks

Ticks are obligate haematophagous Acari parasitizing vertebrates. They are of considerable medical and veterinary importance because of their direct effect as ectoparasites and indirect as vectors of many pathogens (Sonenshine, 1993). Ticks also harbour non pathogenic *Rickettsia*- and *Wolbachia*-like bacteria which are possibly mutualistic endosymbionts (Clay et al., 2008).

2.1.1. Taxonomy of ticks

Ticks are known to belong to the phylum *Arthropoda*, class *Arachnida*, subclass *Acari* and they constitute suborder *Ixodida* (= *Metastigmata*) of the order *Parasitiformes* (Figure 1). The suborder *Ixodida* contains three families namely: (1) the *Ixodidae*, or hard ticks, (2) the *Argasidae* or soft ticks, and (3) the *Nuttalliellidae*, in which the latter contains just one species which is *Nuttalliella namaqua* (Sonenshine, 1991; Eckert et al., 2008). The family *Ixodidae* comprises approximately 80% of the world's tick fauna with 12 genera and 683 species. The most important genera in this family are *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Rhipicephalus* and *Boophilus*. Recently, the genus *Boophilus* has been re-classified as a subgenus under the genus *Rhipicephalus* (Horak et al., 2002). The family *Argasidae* or soft ticks, is divided into four genera: *Argas*, *Carios*, *Ornithodorus* and *Obtobius*, in which there are 183 species (Jongejan and Uilenberg, 2004).

Phylum: Arthropoda
Subphylum: Amandibulata
Class: Arachnida
Subclass: Acari
Order: Parasitiformes
Suborder: Metastigmata (Ixodida)
Ixodidae ..... *Amblyomma, Dermacentor (Anocentor), Haemaphysalis ,Hyalomma, Ixodes, Rhipicephalus and Boophilus*
Argasidae ……... *Argas, Carios, Ornithodorus, Otobius*
Nuttalliellidae…… *Nuttalliella*

Figure 1: Taxonomy of ticks (Sonenshine, 1991; Eckert et al., 2008)

2.1.2. Ixodid ticks

2.1.2.1. Life cycle

All ixodid ticks have remarkably the same life cycle consisting of four stages: the embryonated egg and the active stages: larva, nymph and adult. Females suck enormous amounts of blood and become bigger than 100 times their unfed body weight. Adults of all ixodids need blood as meal to start gonotrophic cycle except for species of the genus *Ixodes*. Following mating, which occurs exclusively on their host in metastriate ticks (i.e. while feeding), females drop from their hosts and start oviposition in some sheltered microenvironment. This could take place in some cracks, crevices, or under stones where the females lay several thousand of eggs in one continuous cycle of ovipositional activity. Egg production increases rapidly to reach the peak within 3-5 days after the beginning of oviposition and then decreases gradually, where the hole oviposition period range from 2 to 4 weeks. For most species each active stage seeks a host, feeds and drops off to develop in the nature (3-host life cycle) (Figure 2). Whereas in a few other species active stages either remain, feed and develop on one host (1-host life cycle) or need another host to complete its life cycle (2-host life cycle) (Sonenshine, 1991).

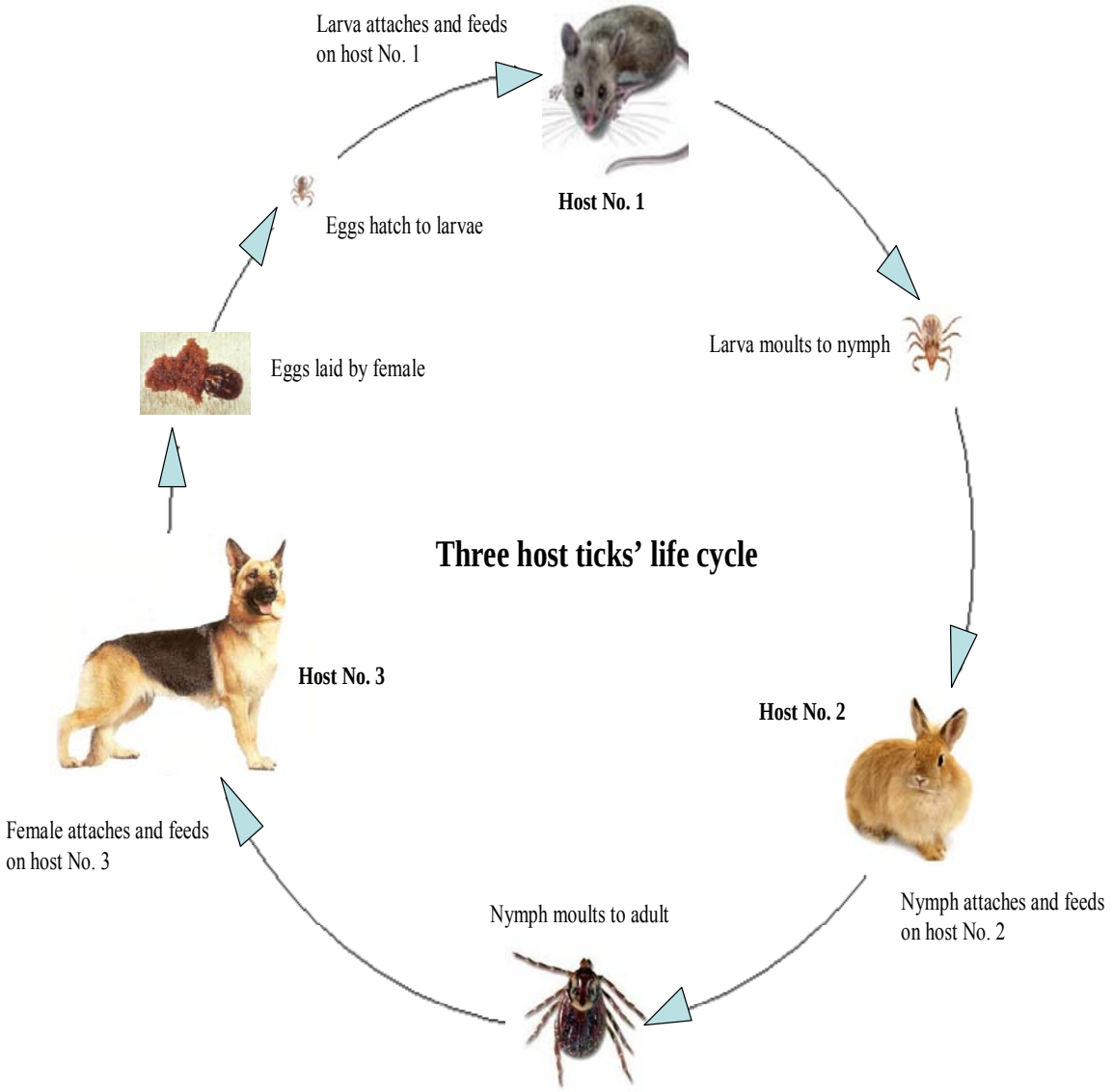

Figure 2: Life cycle of *Dermacentor variabilis* as a three host tick representative.

2.1.2.1.1. Three-host life cycle

After hatching, the emerging larvae seek hosts, attach to them and feed slowly to repletion. In most species, larvae drop after repletion from their hosts and find a sheltered microenvironment to undergo molting. Afterwards the unfed nymphs seek hosts again and the process of finding host, attaching and feeding is repeated. The engorged nymphs drop and also find a place for molting. After nymphal molt, the adult male and female crawl towards the host and attach to it. They mate; feed and the fed females drop off to lay eggs, thereby completing the cycle. This pattern of host-seeking, feeding, dropping off the host and molting in each life stage represents the 3-host life cycle. This cycle from the hatching of the larvae to the hatching of the next generation can be completed in less than one year under favourable environmental conditions. The environmental limitations could extend the duration of the cycle to more than 3 years. This can be reduced to 3 to 4 months in the laboratory, where all conditions can be optimized (Sonenshine, 1991).

2.1.2.1.2. One and two-host life cycle

In some species all stages remain on the host after attaching the larvae. Larvae and nymphs fed and molt in situ. Following molting to the adult stage, the male and the female remain to feed and mate on their host. Only the fed-mated female drops off to lay eggs. This pattern is known as 1-host life cycle.

In some other species, fed larvae remain there, molt on the host and the unfed nymphs re-attach. Following their repletion, they drop off and molt to the adult stage. These ticks are termed as 2-host ticks (Sonenshine, 1991).

2.1.3. Veterinary importance of ticks

Ticks can be harmful to livestock and cause major problems because of their direct effect besides acting as vectors of diseases (Uilenberg, 1992). The direct effect of ticks might appear in several ways. For example, ticks reduce the value of hides because of their damage to the skin. Even ticks with short mouth parts such as *R.* (*Boophilus)* spp. can be important in this direction when they are present in huge numbers on the host. Ticks with long and massive mouth parts such as *Amblyomma* spp. and some *Hyalomma* spp. may cause abscesses due to secondary bacterial infections and might lead to lameness or loss of teats (Jongejan and Uilenberg, 2004). Some tick species contain paralysing toxins in their saliva. The most important paralysis-inducing ticks include *I. holocyclus* (Australia), *D. andersoni*, *D. variabilis* (North America), *R. evertsi evertsi*, *I. rubicundus* (South Africa), *A. cajannense* and *A. ovale* (South America) (Mans et al., 2004). The species *Hy. truncatum* acted in addition as a vector of sweating sickness, a disease associated with an eczema-like skin conditions (Jongejan and Uilenberg, 2004). In most parts of the world, the veterinary importance of tick-borne diseases is much greater than the effect of ticks themselves (Uilenberg, 1992).

2.1.4. Veterinary importance of tick-borne diseases

The most important diseases transmitted by ticks, particularly in domestic ruminants are babesiosis, theileriosis, anaplasmosis and ehrlichiosis. Dogs also can be infected with *Babesia* spp. and *Ehrlichia* spp., where the infection with *E. canis* is often fatal (Jongejan and Uilenberg, 2004). In addition, human tick-borne diseases have gained more importance since the discovery of Lyme borreliosis in 1982 (Burgdorfer et al. 1982). Ticks may transmit many viral infections to man and/or livestock. Some of these tick-borne viruses have a significant threat to the health of humans (tick-borne encephalitis [TBE] transmitted by *Ixodes* spp. and Crimean-Congo haemorrhagic fever transmitted by *Hyalomma* spp.) or livestock (African

swine fever virus transmitted by *Ornithodoros* spp. and Nairobi sheep disease virus transmitted by *R. appendiculatus*) (Labuda and Nuttall, 2004). Man may become infected with other pathogens transmitted by ticks such as *Rickettsia* spp. and *Babesia* spp. (Jongejan and Uilenberg, 2004).

2.2. Tick cell lines

Tick cell lines are cultures derived from ticks embryos (eggs), moulting larvae or nymphs. Establishment can take between 1 and 5 years, and the success rate is generally low (Bell-Sakyi et al., 2007). The term "line" as it was defined by the Tissue Culture Association Terminology Committee (1978) states that a cell line arises from a primary culture at the time of the first subculture (Yunker et al., 1981). Tick cells may grow as monolayer, in suspension, or a mixture of both. Tick cells can survive for long periods – months or even years – without subculture; this characteristic is particularly useful for attempted isolation *in vitro* of slow-growing microorganisms (Bell-Sakyi et al., 2007).

2.2.1. Characteristics

2.2.1.1. Karyology

The existing tick cell lines are derived from tick species having diploid chromosome number (2n) of 21 (male) or 22 (female), autosomes and sex chromosomes which are larger than the autosomes (Chen et al., 1994). Most of tick cell lines are diploid in which either the chromosome number of male or female is predominant (Pundy et al. 1973; Varma et al., 1975; Esteves et al., 2008 and Bhat and Yunker 1977). Examination of three cell lines from the tick *I. scapularis* revealed that 57.7 – 77 % of the cells were diploid and the modal diploid number of all these three lines was 28 chromosomes with 26 autosomes and XX (female) or XY (male) sex chromosomes. Furthermore, it was also found that 0.7 - 35.7 % of the cells

were aneuploid or tetraploid (Chen et al., 1994). Cell lines with aneuploid chromosome number are also present (Mattila et al., 2007). Some changes in chromosome number occur in the early stage of *in vitro* cultivation. The male diploid complements formed 61 % of cells from a *R.* (*Boophilus) microplus* line at the 6th passage and after three passages 80 % of the cells were male and the incubation temperature may play a role in the findings (Holman and Ronald, 1980). Subsequently, continuous cell lines seem to be karyologically stable (Kurtti and Munderloh 1982).

2.2.1.2. Growth rate

Tick cells grow relatively slowly in culture and that can be measured by the frequency of transfers and population doubling time (PDT). Split ratios vary among the lines, where the number of passages provides an estimate of the growth of the line. Young cultures are split mostly 1 to 2 (Varma et al., 1975; Pudney et al., 1979; Holman 1981) or 1 to 3 (Baht and Yunker, 1977). Lallinger et al. (2010) mentioned the use of 1 to 1 split ratio for several tick cell lines. Some tick cell lines can survive for a long time with regular medium changes and occasional subcultures, reflecting the ability of ixodid ticks to exist for long periods in nature between blood meals (Bell-Sakyi et al., 2007). Temperature may have an effect on the multiplication rate, where a rise of incubation temperature resulted in faster cell multiplication (Yunker et al., 1981). Baht and Yunker (1977) reported in details, the growth pattern of the line RML-14 derived from embryonic tissue of a *D. parumapertus.* Cells had a 1-2 days lag phase in which the cell number decreased and a logarithmic phase started 48 h after seeding and continued up to 6 days at which a stationary phase was reached. During the logarithmic phase of growth, the PDT was about three days and could extend to 21 days (Yunker et al., 1981).

2.2.1.3. Cell types

Cells in cultures show shapes and sizes different from those normally recognised in tick tissues. This makes it difficult to assign the cells to their origin (Martin and Vidler, 1962). All tick cell lines comprise two or more cell types (Yunker, 1987, cited by Bell-Sakyi, 2007). Several types of cells have been described such as epithelial-like, fibroblast-like, round, long, bipolar or muscle etc. With subculturing, the diversity of cell types in the line decline and 1 or 2 cell types become dominant (Munderloh, et al., 1994). Holman (1981) was able to establish a new subline with small round cells from previously cultured cell line on the basis of morphology.

Physical and chemical environment (i.e. constituents of the media, pH, temperature, etc.) appears to affect shape and size of the cells. Pudney et al. (1973) reported that cells cultured in H-Lac medium supplemented with lactalbumin hydrolysate and FCS, tend to enlarge and become granular. However, replacement of this medium with L-15 medium supplemented with TPB and FCS produces smaller cells with clearer cytoplasm. Increasing the incubation temperature from 28 °C to 32 °C resulted in domination of epithelial- and fibroblast-like cells over small round cells that exist at 28 °C (Holman and Ronald, 1980). The morphological heterogeneity appears to dwindle with increasing passage numbers and the lines finally tend to have rather homogeneous cell populations (Kurtti and Munderloh, 1982).

2.2.1.4. Developmental patterns

Tick cell lines have a long adaptation period compared to insect cell lines most especially for those from diptera. The line RA-243 isolated from developing adult tissues of *R. appendiculatus* was subcultured only 14 times within its first year in culture (Varma et al., 1975). Whereas line RU-TAE 12 V, isolated from embryos of the mosquito *Toxorhynchites amboinensis* was transferred 50 times within the first year (Munderloh et al., 1982). The time of adjustment to the culture conditions is distinguished by unsettled subculture intervals of

long duration. A given cell line can be considered as "continuous" as soon as a regular subculture rate has been achieved (Kurtti and Munderloh, 1982). This was obvious after nearly 500 days in the development of RA 243 cell line (Varma et al., 1975) compared to 100 days in the case of mosquito line RU-TAE 12 V (Munderloh et al., 1982).

2.2.2. Growth requirements

2.2.2.1. Basal media

Several insect and tick culture media were formulated based on analysing the hemolymph. They could only be successfully used after being supplemented with mammalian serum, arthropod hemolymph, tissue and/or egg extracts (Rehacek and Hana, 1961; Grace, 1962; Rehacek and Brzostowski, 1969a).

Most of tick cell cultures have been initiated and maintained in media formulated for mammalian cells. The successful use of these commercially available media precluded the use of media formulated for invertebrate cultures, especially for those based on hemolymph analyses. Leibovitz's L-15 medium (Leibovitz, 1963) supplemented with TPB and FBS or its mixture with Eagle's MEM medium in Hanks' base are most commonly being used to grow several tick cultures (Kurtti and Munderloh, 1982). Eagle (1959) found that provision of 28 essential metabolites supplemented with serum protein (Table 1) supports cultivation of a wide variety of cell cultures and the cells degenerate and die on the omission of a single essential growth factor, be it a vitamin, an amino acid or glucose.

Table 1: Minimum Essential Medium (MEM) for cultivation of mammalian cells according to Eagle (1959)

Concentration		Concentration	
Compound	mg/l	Compound	mg/l
L- Amino acids		**Salts**	
Arginine	105	NaCl	6800
Cystine	24	KCl	400
Glutamine	292	$CaCl_2$	200
Histidine	31	$MgCl_2 . 6H_2O$	200
Isoleucine	52	$NaH_2.PO_4 . 2 H_2O$	150
Leucine	52	$NaHCO_3$	2000
Lysine	58	**Vitamins**	
Methionine	15	Choline	1
Phenylalanine	32	Folic acid	1
Threonine	48	Inositol	2
Tryptophan	10	Nicotinamide	1
Tyrosine	36	Pantothenate	1
Valine	46	Pyridoxal	1
Carbohydrate		Riboflavin	0.1
Glucose	1000	Thiamine	1
		Serum protein 5 to 10%	

Leibovitz (1963) based on Eagle's medium, developed the L-15 medium in an effort to obtain a medium which would enhance both tissue cell and virus growth. He used the maximum amount of amino acids which could be employed without retarding cell growth and replaced bicarbonate with free base amino acid especially L- arginine and glucose with D (+) galactose, sodium pyruvate and DL- alpha alkaline.

Utilization of amino acids and sugars by embryonic *R. sanguineus* primary cell culture showed that amino acids taken up were aspartic acid, threonine, glutamic acid, proline, methionine, leucine and phenylalanine. There was no change in the levels of valine, cystine or isoleucine. There was an increase in the amount of alanine, tyrosine, lysine, histidine, glycine and arginine. The two sugars, glucose and inositol were extensively utilized but not exhausted within 10 days (Rehacek and Brzostoweski, 1969b). L-proline was found to improve the growth of three different cell lines if it was used with suitable concentrations of fetal bovine

serum (FBS) in the medium (Samish et al., 1985). In 1989, L-15 (Leibovitz, 1963) was modified by the addition of vitamins, trace minerals, alpha-ketoglutaric acid, amino acids and glucose, which are not contained in L-15 (Table 2) (Munderloh and Kurtti, 1989). This medium was termed L-15B (Munderloh and Kurtti, 1985).

Table 2: Nutrients added to L-15 in order to form L-15B medium

Concentration		Concentration	
Compound	mg/100ml	Compound	mg/l
Vitamins		**Carbohydrate**	
p-aminobenzoic acid	100	D-glucose	2239
Cyanocobalamine (B12)	50	**Amino acids**	
d-Biotin	10	L-aspartic acid	299
Trace minerals		L-glutamic acid	500
$CoCl.6H_2O$	20	L-glutamine	292
$CuSO_4.5H_2O$	20	L-proline	300
$MnSO_4.H_2O$	160		
$ZnSO_4.7H_2O$	200	Alpha-ketoglutaric acid	299
Na_2SeO_3	20		
$NaMoO_4.2H_2O$	20		
Reduced glutathione	1000		
Ascorbic acid	1000		
$FeSO_4.7H_2O$	50		

It has been found that combination of the amino acids with alpha-ketoglutaric acid and glucose together gave the best results stimulating growth of three different cell lines in medium supplemented with 5 % FBS and 10 µm/ml cholesterol (Munderloh and Kurtti, 1989). The additional trace minerals and vitamins were not shown to be essential in growth of the three cell lines. In general, the optimal culture nutritional requirements of tick cells are not qualitatively or quantitatively defined yet. Nutritional requirements vary among tick species. While L-15 medium supported growth of cells from *R. appendiculatus* and *R. (Boophilus) microplus*, it showed less satisfactory for growth of cells from *Hy. dromedarii* (Varma et al., 1975).

2.2.2.2. Supplements

Serum is the commonest supplement which has been used in almost all the media formulated in order to initiate cultures from vertebrates or invertebrates. It could be supplemented from different sources, e.g., arthropod sera (Rehacek and Hana, 1961), ox serum (Martin and Vidler, 1962), calf serum (Leibovitz, 1963; Varma et al., 1975; Bell-Sakyi, 2004) etc. Kurtti and Munderloh (1982) reported that horse serum could be used in the maintenance of primary culture from the horse tick *D. nitens*, but it did not support the isolation of a continuous cell line. The most widely used supplement in cell culture is FBS. FBS was found among several sera, to be unique in containing a high level of glutathione mixed disulfide which has an effect on controlling the redox potential in the culture (Bump and Reed, 1977). Concentration of FBS needed for growth of cell lines vary among tick species, where 5 % of FBS were found to be optimal for growth of *R. (Boophilus) microplus* line, whereas other two lines from *R. appendiculatus* and *D. variabilis* needed 10 % of FBS (Samish, et al., 1985). This concentration could be reduced to 5 % FBS for growth of *R. appendiculatus* cell line when the medium was supplemented with 0.5 % tick egg extract (Kurtti et al., 1982).

The second popular supplement in culture media is TPB. It has been used at a concentration of 10 %. TPB provides glucose that is not present in L-15 and the disodium phosphate present improves the buffering capacity of the medium. It contains also sodium chloride and peptones from which part of the beneficial effects of TPB is probably accrued (Kurtti and Munderloh, 1982). The yield of cellular protein from a culture was directly correlated to the concentration of TPB (Kurtti et al., 1982). They found that by using cell lines from R. *appendiculatis* and *R. sanguineus,* both FBS and TPB are essential supplements and concentration of 20 % and 10 % respectively seem to be optimal for growth of tick cells. In contrast, Zweygarth et al., (1997, 1998) were able to initiate and propagate *E.* (*Cowdria*) *ruminantium* in bovine endothelial cell culture using serum-free medium and showed that neither serum nor TPB is

essential for its initiation and propagation. Some media have been supplemented with other supplements such as bovine plasma albumin (0.1 %) (Bhat and Yunker, 1977; Yunker et al., 1981) or lactalbumin hydrolysate (0.5 %) (Pudney et al., 1979; Bell-Sakyi, 2004). Antibiotics such as penicillin (100 - 1000 unites/ml) and streptomycin (0.1-1.0 mg/ml) are also often added to the medium.

2.2.2.3. Physical and chemical environment

The optimal conditions for culturing tick cell lines have not been defined, where growth conditions for ticks vary from one tick species to another. However, most of primary cultures and cell lines have been isolated and maintained in a range of temperature (28°C - 34°C) (Table 3) and they vary in their response to these changes in temperature. Some lines may develop better at temperature above 30°C. Indeed, a *R. (Boophilus) microplus* subline incubated at 32 °C was better and continued to grow in contrast to a subline incubated at 28 °C which died off (Pudney et al., 1979). Increasing the incubation temperature leads to faster multiplication and makes the PDT shorter. Four cell lines from the tick *D. variabilis* were adapted to grow at 37°C and they have a PDT of 70.1 h, whereas the PDT was 181.9 h by incubating the cells at 27°C (Yunker et al., 1981). Tolerance of tick cell lines to medium pH has not been tested. But most cell cultures have been isolated and maintained in medium pH between 6.5 and 7.2. Adjusting the pH to 6.9 was found to produce more rapid attachment of the cells to the glass (Varma and Wallers, 1965). Changing the pH to a basic level led to a change in the form and size of the cells from *R. (Boophilus) microplus* and they started to detach from the flasks (Holman and Roland, 1980). This problem could be solved by increasing the volume of medium used, thus decreasing the air to medium ratio. The high oxygen tension resulted from filling the flasks completely with medium may have a beneficial effect in the early development of tick cell monolayer (Holman and Roland, 1980). Some cell lines are propagated at neutral to alkaline pH and this enable the growth of acid-sensitive

pathogens (Munderloh, et al., 1996a). Tick cell cultures are mostly maintained in stationary cultures. In such system the oxygen tension in the immediate circumference of the cell layer is reduced by cellular utilization and its replacement is influenced by the depth of the medium overlaying the cells. Cells are sensitive to oxidation damage which can be controlled by adding reducing agents to the medium such as reduced glutathione which exist in a high level in FBS (Bump and Reed, 1977). Kurtti and Munderloh (1982) mentioned that the commonly used osmotic pressure of media for tick cell culture is about 320 mOsm/liter. An osmotic pressure of 345 mOsm/liter was used in preparing L-15B medium (Munderloh and Kurtti, 1989) whereas Samish et al. (1985) used an osmotic pressure of 310 mOsm/liter.

2.2.3. Storage of tick cell lines

Cell lines have often been banked in liquid nitrogen especially for long-term cryopreservation and the most commonly cryoprotectant used is dimethyl sulfoxide (DMSO) in growth medium. Baht and Yunker (1977) reported storage of cell line RML-14 from the tick *D. parumapertus* in liquid nitrogen using two different cryoprotectants (DMSO and glycerol). They found that cells stored in medium containing DMSO had a recovery and growth rates better than those stored in medium with glycerol. Similar results were obtained from cryopreservation of IDE8 cell line from the tick *I. scapularis* (Bastos et al., 2006).

Cryopreservation process has mostly been done using 10 % DMSO in the medium and gradual freezing procedures which showed better results after resuscitation than rapid immersion into liquid nitrogen (Bastos et al., 2006). Lower concentration of DMSO have been also used in cryopreservation of tick cell lines, where 7.5 % DMSO was used in storage cells from *R. (Boophilus) microplus* in liquid nitrogen and after thirty days the cells were recovered and 70 % of them were viable by trypan blue dye exclusion test (Holman 1981). Furthermore, some tick cell lines were cryopreservated using 6 % DMSO in medium (Bastos et al., 2006) and using of sucrose with 6 % DMSO revealed an improvement in viability of

cells after resuscitation (Lallinger et al., 2010). It was possible to resuscitate cell lines after a storage period of 12 years (Munderloh, et al., 1994). Attempts to cryopreserve cell lines from the soft tick *O. moubata* using DMSO, polyvinylpyrrolidine (PVP) or mixture of both were unsuccessful. In contrast, short-term storage of these lines at 15 °C for up to 19 weeks was reported (Bell-Sakyi et al., 2009). Carrying out cryopreservation in liquid nitrogen was not reliable and has some limitations (Bell-Sakyi, 1991; Munderloh et al., 1994). This motivated some researcher to store tick cell lines under refrigeration conditions. It was possible to store cell lines up to 60 days at 4 °C (Bastos et al., 2006) and 30 days at 6 °C (Lallinger et al., 2010). It was also possible to store the cell line (CCE3) from the soft tick *C. capensis* at room temperature or at 12 °C without feeding for several weeks (Mattila et al., 2007). Yunker and Cory (1965) found that effectiveness of nymphal tick tissues for tissue culture purposes is not significantly less if they were held at -11 °C for many weeks than unrefrigerated tissues. But the viability decreased greatly after 3.5 months under these storage conditions.

2.2.4. Current trend

Although the tick are the most important vectors of protozoan, bacterial and viral diseases of animals and man, *in vitro* culture of tick tissues had not received enough attention until Rehacek (1958) reported for the first time partial success in growing tick tissue of 3 days-old nymph, *D. marginatus*, *in vitro*. In contrast, the first successful attempt to grow insect tissues *in vitro* had been made over 40 years before that (Goldschmidt, 1915). After the pioneer work by Rehacek, there had been several attempts to establish continuous tick cell lines from a variety of tick species. This resulted in several primary cultures which were capable of surviving up to a maximum of 8 months (Pudney et al., 1973). In 1975 Varma and others reported for the first time the establishment of three cell lines derived from developing adults of *R. appendiculatus.* Since then many cell lines have been established from 13 hard (ixodid) tick species of the genera *Amblyomma, R. (Boophilus), Dermacentor, Hyalomma, Ixodes* and

Rhipicephalus. Moreover, cell lines are now available for two soft (argasid) tick species, *C. capensis* (Mattila et al., 2007) and *O. moubata* (Bell-Sakyi, 2009) Table 3.

Table 3: tick cell lines that are currently available according to The Roslin Wellcome Trust Tick Cell Biobank[1]

Tick species	Cell lines	Instar	Incubation temperature	References
		Hard ticks		
A. americanum	AAE2	Embryo	32°C-34°C	Kurtti et al., 2005
A. variegatum	AVL/CTVM13	Moulting larva	28-32°C, 37°C	Bell-Sakyi et al., 2000,
	AVL/CTVM17	Moulting larva	32°C	Bell-Sakyi 2004
R. (Boophilus) decoloratus	BDE/CTVM12, 14	Embryo	32°C, 28°C	Lallinger et al., 2010
	BDE/CTVM16	Embryo	28°C-32°C	Bell-Sakyi 2004
R. (Boophilus) microplus	BME/CTVM2, 6	Embryo	28°C-32°C	Bell-Sakyi 2004
	BME/CTVM4, 5	Embryo	28°C	Bell-Sakyi et al., 2007
	BME/CTVM23, 30	Embryo	32°C, 28°C	Bell-Sakyi, personal communication
	BmVIII-SCC	Embryo	32°C	Holman, 1981
D.(Anocentor) nitens	ANE58	Embryo	32°C-34°C	Kurtti et al., 1983
D. albipictus	DALBE3	Embryo	32°C-34°C	Policastro et al., 1997
D. andersoni	DAE15	Embryo	32°C-34°C	Simser et al., 2001
	DAE100T	Embryo	32°C-34°C	Kurtti et al., 2005
D. variabilis	DVE1	Embryo	32°C-34°C	Kurtti et al., 2005
	RML-15	Embryo	28°C-32°C	Yunker et al., 1981
Hy.anatolicum anatolicum	HAE/CTVM8, 9	Embryo	32°C	Bell-Sakyi, 1991
I. ricinus	IRE/CTVM19, 20	Embryo	28°C	Bell-Sakyi et al., 2007
	IRE11	Embryo	32°C-34°C	Simser et al., 2002
I. scapularis	IDE2, 8	Embryo	32°C-34°C	Munderloh et al., 1994
	ISE6, 18	Embryo	32°C-34°C	
R. appendiculatus	RA243	Moulting nymph	28°C-32°C	Varma et al., 1975
	RAE25	Embryo	28°C-32°C	Kurtti et al., 1982
	RAN/CTVM3	Moulting nymph	28°C	Bekker et al., 2002
	RAE/CTVM1	Embryo	32°C	Bell-Sakyi, 2004
R. evertsi	REE/CTVM28, 29	Embyro	28°C	Bell-Sakyi, personal communication
R. sanguineus	RSE8	Embryo	32°C-34°C	Kurtti et al., 1982
		Soft ticks		
C. capensis	CCE1	Embryo	32°C-34°C	Mattila et al., 2007
O. moubata	OME/CTVM21, 22, 24	Embryo/neonate-larva	28°C	Bell-Sakyi et al., 2009

[1] Additional tick cell lines mentioned in previous papers are, as far as the authors can ascertain, no longer available (Yunker et al., 1981; Bell-Sakyi, personal comunications)

2.2.5. Importance of tick cell cultures

In vitro culture systems, especially continuous cell lines, represent a useful tool for studying many aspects of tick and tick-borne pathogen research (Bell-Sakyi, et al., 2007). So far, tick cell lines have been used for isolation and propagation of pathogens to create a good model to study the biology of such pathogens, e.g., pathogens' metabolism *in vitro*, as well as their interactions with cultured cells, such as the mechanism of cell invasion. Furthermore, their use enhances the development of vaccines and diagnostic tests. Additionally, tick cell lines can be used in studies on the biology of ticks and pathogen genomics and proteomics, as a result of the recent great advances in molecular biological studies.

2.2.5.1. Isolation and propagation of pathogens

2.2.5.1.1. Virology

Tick cell lines have been used for over 35 years in propagation of arboviruses (Varma, et al., 1975; Yunker et al., 1981). Cultivation of a virulent strain of TBE in an *R. appendiculatus*-derived cell line that was persistently infected with an attenuated strain resulted in a partial inhibition of the growth of the virulent strain (Kopecky and Stankova, 1998). Such *in vitro* studies provide a good model for the study of arboviruses virulence. Furthermore, tick cell lines from *R. appendiculatus*, *A. variegatum*, *I. ricinus* and *I. scapularis* were found to be susceptible to infection with the following tick-borne flaviviruses: Tick-borne encephalitis virus (TBEV), *Langat virus* (LGTV), Louping ill virus (LIV), *powassan virus* (POWV), Negishi virus (NGV) and also the mosquito-borne West Nile virus (WNV). Additionally, a *R. appendiculatus* cell line (RAE/CTVM1) was susceptible to infection by the mosquito-borne alphavirus Venezuelan equine encephalitis virus (VEEV) (Lawrie et al., 2004). More recently, Crimean-Congo hemorrhagic fever virus (CCHFV) was propagated in seven different tick cell lines (Bell-Sakyi et al., 2011).

2.2.5.1.2. Parasitology

Tick cell lines have not been widely used in studying tick-borne protozoa, because primary cell culture was thought to be preferred for this purpose (Mosqueda et al., 2008). Attempts to infect tick cell culture derived from *R. (Boophilus) microplus* with *B. bovis* were unsuccessful (Droleskey et al., 1981). Kurtti et al. (1983) reported for the first time the cultivation of tick stages of *B. caballi* in tick cell cultures. Similarly, *B. bigemina* sporokinetes were cultivated up to 8 days in an *I. scapularis* cell line (Ribeiro et al., 2009). Furthermore, *Leishmania* spp. could be propagated in tick cell lines (Nyindo et al., 1987). Apart from that, *Besnoitia besnoiti* was cultivated in four tick cell lines from *R. (Boophilus) microplus*, *R. appendiculatus* and *D. variabilis* and its morphology was studied (Samish et al., 1987, 1988). Moreover, a *R. appendiculatus* cell line was used as a feeder layer for maintaining the bovine nematode *Onchocerca lienalis* (Litchfield et al., 1991). Thus, maintenance and development of nematodes can be done in such a system.

2.2.5.1.3. Bacteriology

Bacteriologists have been the greatest exploiter of tick cell cultures, in which many pathogens have been isolated or/and propagated. The ability to cultivate such pathogens in tick cell cultures aids their study. *Anaplasma* and *Ehrlichia* were the most recently propagated bacterial pathogens in tick cell lines. *I. scapularis* cell lines have proved to be particularly susceptible to infection with a range of ehrlichial pathogens, whether or not this tick species is their natural vector.

A. marginale was propagated in the IDE8 cell line derived from *I. scapularis* and remained infective for cattle after several passages in culture (Munderloh et al., 1996a; Blouin et al., 1998). Subsequently, different strains of *A. marginale* were established in this cell line (Blouin et al., 2000; Bastos et al., 2009). Nonetheless, other cell lines derived from *R. appendiculatus, D. variabilis* and *D. albipictus*, which represent natural vectors for *A.*

marginale, did not support intracellular growth of this *Anaplasma* (Munderloh et al., 1996a). Culture-derived *A. marginale* (CAM) was used as a good antigen in a competitive ELISA (C-ELISA) (Saliki et al., 1998) and in a semi-automated latex agglutination test (LAT) (Rodgers et al., 1998) for the serological diagnosis of anaplasmosis. Moreover, CAM showed promise for use as antigen in development of a new killed vaccine for anaplasmosis. Cattle immunized with this antigen did not display clinical anaplasmosis (Kocan et al., 2001; de la Fuente et al. 2002).

In a similar manner, different strains of *A. phagocytophilum,* previously known as *E. phagocytophila,* were also isolated from different animals and propagated in tick cell cultures. For example, a European ovine stain of *A. phagocytophilum* was isolated and propagated, for the first time, in two tick cell line IDE8 and ISE6 derived from *I. scapularis* (Woldehiwet et al., 2002). Recently, several European strains of *A. phagocytophilum* have been isolated and propagated in the IDE8 cell line (Zweygarth et al., 2011). Similarly, Silaghi and others (2011) isolated and propagated *A. phagocytophilum* from roe deer in the IDE8 cell line.

Other *Anaplasma* spp. were isolated and cultivated in tick cell lines (Munderloh et al., 2003; Zweygarth et al., 2006). Additionally, Massung et al. (2007) reported, for the first time, the direct isolation of *Anaplasma* sp. from ticks *in vitro*.

E. equi was successfully isolated in the IDE8 cell line and retained infectivity and pathogenicity for the equine host after being passaged twice in tick cell culture (Munderloh et al., 1996b). In addition, human granulocytic ehrlichiosis (HGE) agent was isolated directly from human blood (Goodman et al., 1996) and from an infected horse and a dog (Munderloh et at., 1999) in *I. scapularis* cell lines IDE8 and ISE6 respectively. *E. (Cowdria) ruminantum* was first continuously propagated in the IDE8 line (Bell-Sakyi, et al., 2000). Subsequently, it was established in different cell lines from *I. scapularis*, *I. ricinus*, *A. variegatum*, *R. (Boophilus) decoloratus*, *R. (Boophilus) microplus* and *R. appendiculatus*. In contrast, cells from *Hy. anatolicum anatolicum* could not be infected with *E. ruminantum* (Bell-Sakyi,

2004). Later, Zweygarth and colleges (2008) reported isolation and propagation of *E. ruminantum* in IDE8 cells using blood from infected sheep.

It was possible to prolong infectivity of *Borrelia burgdorferi* by co-cultivating spirochetes with *R. appendiculatus* (RAE25) cells, which may provide a useful tool for maintaining infective spirochetes in the laboratory for either vector or reservoir competence studies (Kurtti et al., 1993).

Other bacteria such as *R. rickettsii, R. peacockii* and tick-borne spiroplasms were also cultivated in tick cell lines (Yunker et al., 1984; Policastro et al., 1997; Kurtti et al., 2005 and Yunker et al., 1987).

2.2.5.2. Pathogens genomics and proteomics

Tick cells provide an important environment for studies on stage-specific gene transcription and protein expression. These lead to novel prophylactic and therapeutic targets to prevent transmission and infection, developing from understanding the molecular survival and adaptation strategies within hosts. Infection of *I. scapularis* IDE2 and *D. albipictus* DALBE3 cells with *R. rickettsii* showed temperature-dependant protein expression at both 28°C and 34°C (Policastro et al., 1997); there was no difference in protein expression between tick cells and mammalian cells at 34°C. *E. chaffeensis* was transcriptionally more active in tick cells than in human cells (Kuriakose et al., 2011). High expression levels of genes associated with protein modification, energy, conversion and nutrient transport were shown in tick cells. In contrast, the majority of these genes were in moderate levels in human cells.

2.2.5.3. Biology of ticks and pathogens

Tick cell lines proved to form an effective system to investigate the biology of different pathogens as well as the biology of ticks. Indeed, it was possible to generate three organophosphate resistant *R. (Boophilus) microplus* cell lines by exposing BmVIII-SCC cell

line (Holman, 1981) to incrementally increased toxic concentration of the acaricide Coumaphos (Cossio-Bayugar et al., 2002). The existence of such cell lines constitute a useful model in which the ticks' acaricides resistance issue can be studied. Furthermore, tick cell cultures were used in studying the effect of tetracycline on cultured *A. marginale*, as a case study. It was found that tetracycline killed *A. marginale* in cell culture by interfering with the ability of the organism to complete its replicative cycle in the host cell cytoplasm (Blouin et al., 2002).

Since part of the life cycle of tick-borne agents that occurs within the tick is partially or completely unknown, tick cell cultures represent a good method for solving the problems concerning their developmental cycles. Indeed, the invasion and development of *A. marginale* in cell culture was found to be similar to that in infected ticks (Blouin and Kocan, 1998). Also, intracellular development of human granulocytic ehrlichiosis agent showed that the blood stages of the HGE agent were able to infect tick cells (Munderloh et at., 1999). The mechanism by which spotted fever group (SFG) rickettsiae and *B. burgdorferi* invade and move within tick cells was studied using cell lines from different tick species (Munderloh et al., 1998; Kurtti et al., 1988, 1993).

2.3. Symbionts

Symbiosis is an interaction between two organisms living together in more or less intimate association. When a symbiont lives within a host either intracellular or extracellular, it is referred to as an endosymbiont (Rymaszewska, 2007). Several tick cell lines have been found to be persistently infected with symbionts (Simser et al., 2001; Mattila et al., 2007), which can be isolated in tick cell culture (Kurtti et al., 1996).

2.3.1. Symbionts in ticks

Ticks have considerable medical and veterinary importance because of their feeding action and their role as vectors of many pathogens. In addition, many ticks contain intracellular symbionts which sometimes are closely related to known pathogens (Noda et al., 1997). In addition, tick symbionts were found to have close phylogenetic relationship with human pathogens (Clay et al., 2008). Such bacteria are found primarily in the ovaries or Malpighian tubules of ticks (Beninati et al., 2004; Epis et al., 2008; Noda et al., 1997). The Rocky Mountain wood tick *D. andersoni* was found to be infected with *R. peacockii* which was mainly localized in ovarian tissues (Niebylski et al., 1997). Similarly, *Wolbachia*-like symbionts were isolated from ovarian tissue of *D. andersoni* (Burgdorfer et al., 1973). Bacteria related to *Ca.* Midichloria mitochondrii were recently detected also in *D. andersoni* (Dergousoff and Chilton, 2011). In addition, rickettsial symbionts were found in three different tick species of the genus *Rhipicephalus* in Italy (Satta et al., 2011). These symbionts were regarded as obligatory intracellular organisms (Sassera et al., 2006). They are vertically transmitted (transovarial transmission) and do not appear to be pathogenic for ticks themselves (Noda et al., 1997). Indeed, Rehacek et al. (1976) reported detection of *rickettsia*-like organisms in the tick *D. marginatus* which proved to be non pathogenic for ticks and guinea pigs. Furthermore, *Coxiella* was supposed to be a primary symbiont that is required for the long-term survival of *A. americanum* (Zhong et al., 2007). Such symbionts were also

detected in soft ticks. The soft tick *C. capensis* was found to harbor a rickettsial endosymbiont co-isolated along with the cell line that was derived from this tick species (Mattila et al., 2007).

2.3.2. Symbionts in tick cell cultures

Most of the currently available ixodid and argasid tick cell lines were established from embryonic tissues. This increases the possibilities of being infected with some symbionts, since symbionts of ticks were reported to be vertically transmitted (Noda et al., 1997). For instance, the *I. scapularis* cell line IDE2 was also found to be chronically infected with an arbovirus which presumably had been transmitted transovarially and has no apparent effect on the tick cells (Attoui et al., 2001). The cell line DAE100 which had been isolated from intact ticks of *D. andersoni* was found to be chronically infected with the bacterium *R. peacockii*. Despite high levels of infection, the DAE100 infected cells remained viable (Simser et al., 2001). Similarly, the embryonic cell line CCE3 from soft tick *C. capensis* was found to be infected with a rickettsial endosymbiont that was co-isolated along with this cell line (Mattila et al., 2007). Furthermore, cell cultures from *D. (Anocentor) nitens* were found to be transovarially infected with *B. caballi* (Kurtti et al., 1983). The availability of such cell lines which is persistently infected with endosymbionts makes it possible to explore the interaction between these symbionts and host cells. It also provides the opportunity to evaluate the infectiveness and pathogenicity of such symbionts for various host cells *in vitro*.

2.3.3. *Candidatus* Midichloria mitochondrii

Recent studies mainly based on 16S rRNA gene sequence characterization and analysis show the existence of a new independent clade within the order Rickettsiales *(*Alphaproteobacteria*)*. It is thought that this clade should be considered as a new family of the order Ricketssiales (Beninati et al., 2004). This family contained until recently, only one species namely *Ca.*

Midichloria mitochondrii (Sassera et al., 2006). In 2010, Vannini and others detected two new bacteria belong to the "*Ca.* Midichloria" clade in the ciliate *Euplotes harpa*; they were proposed as new genera and species *Ca*. Anadelfobacter veles and *Ca*. Cytobacter comes. *Ca.* Midichloria mitochondrii is an intracellular bacterium dwelling the perimitochondrial space of the tick *I. ricinus* (Beninati et al., 2004) and of many other hard ticks (Epis et al., 2008)

2.3.3.1. Historical overview

In 1970, it was reported during an electron microscopic study of morphology of the *Oligotrichia* ciliates, the presence of bacteria-like microorganisms within the matrix of mitochondria in most cells of ciliate *Halteria geleiana*. That was the first time in which the occurrence of such microorganisms within the mitochondria had been reported (Yamataka and Hayashi, 1970). Lewis (1979) was able to detect *rickettsia*-like microorganism for the first time within the mitochondria of ticks, particularly of developing oocytes from *I. ricinus* ticks. It was difficult to envisage the successful development of such cells in which mitochondria were heavily infected with rickettsial symbionts. Afterwards, such bacteria were detected in *I. ricinus* and *I. scapularis* ticks and were also restricted to the ovarian tissues (Zhu et al., 1992; Noda et al., 1997). Subsequently, several reports concerning detection of this symbiont in *I. ricinus* were published (Beninati et al., 2004; Sacchi et al., 2004; Lo et al., 2006). According to Murray and Stackebrandt (1995), incompletely described procaryotes should be classified as *Candidatus*. Therefore, the name *Ca.* Midichloria mitochondrii was suggested for the new bacterium based on phylogenetic studies, which confirmed the phylogenetic position of this bacterium (Sassera et al., 2006).

2.3.3.2. *Ca.* Midichloria mitochondrii characterization

Ca. Midichloria mitochondrii belongs to the phylum proteobacteria, to the class Alphaproteobacteria and to the order Rickettsiales (Sassera et al., 2006). It appears microscopically as a gram-negative bacillus-shaped bacterium of approximately 0.45 μm in width and 1.2 μm in length (Beninati et al., 2004; Lewis, 1979). *Ca.* Midichloria mitochondrii is the only characterized bacterium that has the ability to invade the mitochondria within ovarian cells and consume them without any effect on the female tick's fertility (Beninati et al., 2009; Epis et al., 2008; Sacchi et al., 2004; Sassera et al., 2006). In particular, the bacteria are localized between the outer and inner membrane of the mitochondria and not inside the matrix (Sacchi et al., 2004). Furthermore, the bacteria are enclosed singly or in groups within: (1) membrane limited vacuoles; (2) vacuolated mitochondria or (3) vacuoles with their wall consisting partially of an elongated mitochondrion and partially of a plasma membrane of the host cell (Zhu et al., 1992). The steps involved in mitochondrial invasion remain to be explored.

2.3.3.3. Distribution in ticks

Ca. Midichloria mitochondrii was found in females of *I. ricinus* with a prevalence of 100 % (Lo et al., 2006). This indicates that its presence is somehow compatible with the survival and reproduction of the host tick. This bacterium and related bacteria have been detected up to now in many genera of ticks distributed over the world: *Ixodes* (Beninati et al., 2004, 2009; Epis et al., 2008; Lo et al., 2006; Noda et al., 1997; Sacchi et al., 2004; Sassera et al., 2006, 2008; van Overbeek, et al., 2008), *Rhipicephalus* (Epis et al., 2008; Noda et al., 1997), *Dermacentor* (Hornok et al., 2008) and *Amblyomma* (Venzal et al., 2008), and also in bed bugs (Richard et al., 2009). Detection of this bacterium was done microscopically (Lewis, 1979; Zhu et al., 1992) or using molecular assays initiated PCR (Sassera et al., 2006; Epis et al., 2008).

Recently, *Ca*. Midichloria mitochondrii has been detected molecularly in *Hyalomma* and *Rhipicephalus* ticks from the Middle East (Harrus et al., 2011).

2.4. Polymerase Chain Reaction (PCR)

2.4.1. Principle of PCR

The polymerase chain reaction or PCR was invented by Kary Mullis (1983) and serves as a procedure during which the DNA is copied quickly and repeatedly to produce a quantity sufficient to be investigated using conventional laboratory methods. It uses repeated cycles each of which consists of three steps:

1- Denaturing or melting: in this step the two complementary strands of the DNA are separated by heating the reaction solution including the DNA molecules, polymerases (which copy the DNA) and primers (which form a starting DNA) up to 95 °C.

2- Annealing or hybridisation: taking the temperature down to 55 °C stimulates the primers to bind to the single-strand DNA. The bonds between the primers and DNA segment are stable if they are complementary. Then start the polymerases to attach complementary nucleotides, thus expanding the bonding between the primers and the DNA.

3- Extension: the temperature is increased again to 72 °C which represents the ideal working temperature for the polymerases. The polymerases add new nucleotides to the new DNA strand. At the same time, all bonds between the primers and the DNA which are not completely complementary are broken.

That represents the normal conventional PCR, where the PCR products obtained at the end of this process are made visible during a final step of agarose gel electrophoresis.

2.4.2. Real-Time PCR

Real-time PCR is a technique in which the data are collected during the PCR process, thus the amplification and detection are combined into a single step. The PCR products are made visible using different fluorescent chemistries which correlate product concentration to fluorescence intensity (Wong and Medrano, 2005). Real-time PCR consist of four major phases: early ground phase, exponential growth phase, linear growth phase and plateau phase (Tichopad et al., 2003). During the early ground phase, fluorescence emission at each cycle has not yet risen above background. The amplification is first detected at the exponential growth phase when the fluorescence emission is higher than background levels. The cycle at which this occurs is called C_t, which can be used as a quantitative measurement of the starting target number (Heid et al., 1996). The linear growth phase represents the optimal amplification period with the PCR product duplicating after each cycle. At the plateau phase, the reaction components become limited and the fluorescence intensity is not enough any more for calculation of the data (Wong and Medrano, 2005). Real-time PCR assays are very sensitive and can detect a single copy of specific transcript (Palmar et al., 2003). This kind of PCR does not require post-PCR sample handling. Thereby preventing potential contamination and resulting in higher and faster throughput assays.

There are two groups of fluorescent chemistries used in real-time PCR assays:

1- Double-stranded (ds) DNA intercalators: a good example in this group is SYBR® Green I (Applied Biosystems, CA, USA), which fluoresces strongly when bound to dsDNA. It is widely used because of its low cost and applicability in several PCR protocols. The mean limitation of dsDNA intercalators is its non-specific binding ability to any dsDNA as primer-dimers and other non-target amplicons. But the formation of non-specific amplicons can be revealed by a product melting curve which makes an essential feature of SYBR® Green I (Bell and Ranford-Cartwright, 2002).

2- Single-stranded (ss) DNA intercalators: in this group multiple DNA species can be detected and/or quantified by using different reporter probes or beacons.

Hydrolysis probes: Hydrolysis probes known as TaqMan® probes, in which the sequence-specific probe is labelled with a reporter dye at the 5′ end and a quencher at the 3′ end. The quencher reduces the fluorescence of the reporter when the probe is intact. When the probe anneals to the target sequence, it is degraded by the activity of 5′- 3′ exonuclease which allows the reporter to be separated from the quencher and results in fluorescence (Bell and Ranford-Cartwright, 2002; Wong and Medrano, 2005).

Hybridization probes: these probes are labelled with an accepter dye at the 3′ end of the upstream probe and a donor dye at the 5′end of the downstream probe. When the probes are bound, an increase in fluorescence resonance energy transfer (FRET) from the donor to the accepter will happen. The accepter dye then emits the transferred energy as fluorescence. Because of the required condition, the two probes are to be bounded in order to give fluorescence, the method is categorized to be very specific (Bell and Ranford-Cartwright, 2002; Wong and Medrano, 2005).

Molecular beacons: they consist of sequence of specific region (loop) which is held by complementary stem sequences. The reporter at one end is blocked by a quencher at the other end. When the beacon is free in solution, the close proximity of the reporter and the quencher causes a reduction in fluorescence emission. Once the beacon binds to the target, the quenching effect is removed and this results in reporter emission (Bell and Ranford-Cartwright, 2002; Wong and Medrano, 2005).

Scorpion primers: they are an adaptation of molecular beacons. A scorpion consists of stem-loop structure labelled with fluorophore at the 5′end and a quencher at the 3′end. A primer is attached to the 3′of the hairpin loop via a PCR stopper. Once the specific probe sequence incorporates into its complement after extension of the primer during PCR amplifications, the

quencher is separated far enough for the reporter, thereby resulting in increased fluorescence (Thelwell et al., 2000).

Sunrise™ primers: they are similar to scorpion primers in that its detection mechanism and PCR primers are combined in the same molecule. The stem-loop structure is labelled with a fluorophore and quencher at the 5´end with the 3´end acting as PCR primer. When the hairpin is free in solution, the reporter is quenched. After annealing and extension, the reporter is not any more quenched to emit its signal (Wong et al., 2005).

3. MATERIALS AND METHODS

Two experiments were carried out; the first to detect the presence of endosymbionts in tick cell lines and the second to evaluate the effect of distinct medium conditions for maintenance of tick cells.

3.1. General materials and methods

3.1.1. Tick cell lines

Eight cell lines, derived from embryonic ticks of four species from two genera, *Ixodes* and *R. (Boophilus*), were used in this thesis. Table 4 lists the cell lines and the tick species from which they were derived. Some of the cell lines were derived from the same tick species but they differ in cell morphology and composition.

Table 4: Tick cell lines used in the thesis

Tick species	Tick cell line	Years in continuous culture	Reference
I. ricinus	IRE/CTVM18[2]	9-10	Bell-Sakyi (2004)
	IRE/CTVM19	9-10	Bell-Sakyi et al. (2007)
	IRE/CTVM20	9-10	
I. scapularis	IDE8	>15	Mounderloh et al. (1994)
R. (Boophilus) microplus	BME/CTVM2	13-14	Bell-Sakyi (2004)
	BME/CTVM6	13-14	
R. (Boophilus) decoloratus	BDE/CTVM12	2-3	Lallinger et al. (2010)
	BDE/CTVM14	3-4	

[2] IRE/CTVM18 is not currently available for distribution because it was found to be infected with a mycoplasma (Bell-Sakyi, personal communication).

3.1.2. Culture media

The complete culture media routinely used for growth of each cell line (Munderloh et al., 1994; and Bell-Sakyi, 2004) were used (Table 5). Cells were maintained in the following media supplemented with 2mM L-glutamine, penicillin 100 unites/ml and streptomycin 100 µg/ml: L-15 (L-15 Leiboviz medium with tryptose phosphate broth [TPB] 10% and inactivated foetal calf serum [FCS] 20%); H-Lac (Hanks balanced salt solution with lactalbumin hydrolysate 0.5% and FCS 20%); L-15B (L-15 Leiboviz medium supplemented according to Munderloh and Kurtti (1989), with TPB 10%, bovine lipoprotein 0.1% and FCS 5%); L-15/L-15B and L-15/H-Lac (equal parts of each complete media).

Table 5: Culture media for the cell lines used in the thesis

Tick cell line	Original medium
IRE/CTVM18	L-15/H-Lac
IRE/CTVM19	L-15
IRE/CTVM20	L-15/L-15B
IDE8[3]	L-15B
BME/CTVM2	L-15
BME/CTVM6	L-15
BDE/CTVM12	H-Lac
BDE/CTVM14	H-Lac

3.1.3. Cultivation conditions

All cells were cultured in sealed flasks (5 ml) or flat-sided tubes (3 ml) and incubated in dry incubators at temperatures 28°C except the line IDE8 which was incubated at 32°C. Medium changing was done once a week by removal and replacement of 1.5 ml (tubes) and 3 ml (flasks) of the medium. Subcultures were made at a split ratio 1:1 at 2-4 weeks intervals based

[3] IDE8 cell line was tested with two different concentrations of FCS (10 and 20%) in the L-15/H-Lac medium.

on the growth rates in the lines. On a medium change day, an equal volume of fresh medium was added to the parent culture; the cells were resuspended by gentle shaking and half of the cell suspension was transferred into a new vessel (conditioned with medium overnight).

3.2. Experiment 1: Detection of endosymbionts in tick cell lines

All cell lines cited on the table 4 were used in this experiment in order to examine them for the presence of the endosymbiont *Ca*. Midichloria mitochondrii or other endosymbionts related to this bacterium.

3.2.1. DNA extraction

Altogether three DNA samples, from three different time points (December 2008, February 2009 and March 2009), were extracted from each cell line. DNA was extracted with Qiagen DNA DNeasy® tissue kit (Qiagen, Hilden, Germany) using the protocol for cultured animal cells. Appropriate amounts of cell suspension (3 ml from each cell culture containing a maximum of 5 x 10^6 cells) were centrifuged for 5 min at 300 x *g* and the pellet was resuspended in 500 µl phosphate buffered saline (PBS). The lysis was then carried out with 20 µl proteinase K and 200 µl Buffer AL at 70°C for 10 min. Elution was done with 200 µl AE Buffer. A free DNA sample was added to each extraction line as a quality control to guarantee that no contamination had happened during the extraction procedures.

3.2.2. Quality and quantity of extracted DNA

In order to control the quality and quantity of the extracted DNA, all samples were measured by a spectrophotometer (NanoDrop®1000, PeqLab, Erlangen, Germany) according to manufacturer's instructions.

3.2.3. Polymerase Chain Reaction (PCR)

3.2.3.1. Conventional PCR for detection of the *16S rRNA* gene of *Ca.* Midichloria mitochondrii and related bacteria

A conventional PCR using two sets of general primers Midi-F, Midi-R and Midi-F2, Midi-R2 from Epis et al. (2008) was chosen for screening the tick cell cultures for bacteria related to *Ca*. Midichoria mitochondrii (table 6). The cultures were tested with both primer sets in order to double check the results. The primers, targeting the *16SrRNA* gene, amplify fragments of ~1100 bp (Midi-F –R) and ~350 bp (Midi-F2 –R2) and were designed to be conserved between the sequence of *Ca*. Midichloria mitochondrii from *I. ricinus* and the closely related sequences available in the data bases (Epis et al. 2008). Amplifications were performed in a Thermocycler Mastercycler gradient (Eppendorf, Wesseling-Berzdorf, Germany) with the reaction mix and cycling conditions as shown in the tables 7 and 8.

Table 6: Primers for conventional PCR targeting the *16SrRNA* gene of *Candidatus* Midichloria mitochondrii and related bacteria

Primer	Oligonucleotide sequence	Reference
Midi-F	5′-GTACATGGGAATCTACCTTGC-3′	
Midi-R	5′-CAGGTCGCCCTATTGCTTCTTT-3`	Epis et al. 2008
Midi-F2	5′-CAACGAGCGCAACCCTTAT-3′	
Midi-R2	5′-CAGTCGTCAACCTTACCGT-3`	

Positive control (*Ca.* Midichloria mitochondrii DNA) was kindly provided by Prof. Claudio Bandi, Università degli Studi di Milano, Italy. Furthermore, DNA extracted from a female *I. ricinus* was included in the test as an extra positive control.

Table 7: Reaction conditions for conventional PCR detection of *Candidatus* Midichloria mitochondrii and related bacteria

Reagent	Volume
Buffer 10×	5 µl
dNTPs (10mM each)	1 µl
Taq Polymerase (5U/µl)	0,25 µl
Midi-F (100 µM)	0,15 µl
Midi-R (100 µM)	0.15 µl
Midi-F2 (100 µM)	0.15 µl
Midi-R2 (100 µM)	0.15 µl
Deionised PCR clean water	43.15 µl
Template DNA	5 µl
Total volume	50 µl

Table 8: Cycling conditions for conventional PCR detection of *Candidatus* Midichloria mitochondrii and related bacteria

Cycle	Step	Temperature	Duration
Cycle 1: 1×	Initial denaturation	95 °C	15 min
Cycle 2: 40×	Denaturation	94 °C	30 sec
	Annealing	56 °C	30 sec
	Extension	72 °C	1 min
Cycle 3: 1×	Final extension	72 °C	10 min

3.2.3.2. Quantitative Real-Time PCR (qRT-PCR) for detection of the *gyrB* gene *Ca.* Midichloria mitochondrii

Approximately 20 bacterial equivalents per PCR tube could be detected by the gyrB PCR, which is 50 times more than the 16S rRNA PCR (Lo et al. 2006). A SYBR green qRT-PCR according to Sassera et al. (2008) was done in order to detect the *gyrB* gene of "*Ca.*

Midichloria mitochondrii" by using a specific set of primers targeting this gene and amplifying 125 bp (Table 9).

The reactions were done in a 7500 Real Time PCR System (Applied Biosystem, Weiterstadt, Germany). All reactions were performed in 50 µl reactions containing: 25 µl of SYBR-green master mix (Applied Biosystems, Darmstadt, Germany), 3 µl of each primer at a concentration of 5µM, and 5 µl of DNA, then made up to 50 µl with deionized PCR clean water. The cycling conditions used in here are shown in the table 10.

Table 9: Primers for SYBR green RT PCR targeting the *gyrB* gene of *Candidatus* Midichloria mitochondrii

Primer	Oligonucleotide sequence	Reference
Midi-gyrB-f	5´-CTTGAGAG-CAGAACCACCTA-3´	Sassera et al. 2008
Midi-gyrB-r	5´-CAAGCTCTGCCGAAATATCTT-3´	

Table 10: Cycling conditions for SYBR green RT PCR targeting the *gyrB* gene of *Candidatus* Midichloria mitochondrii

Cycle	Step	Temperature	Duration
Cycle 1: 1×	Initial denaturation	95 °C	10 min
Cycle 2: 40×	Denaturation	95 °C	15 sec
	Anneal / Extension	60 °C	1 min
Cycle 3: 1×	Dissociation	95 °C	15 sec
		60 °C	1 min
		95 °C	15 sec

3.2.4. Agarose gel electrophoresis

Conventional PCR products were run through a 2% RedGel-treated gel (2g Agarose /100 ml Tric Acetate EDTA [TAE] buffer) and visualized with UV light. A standardized DNA Ladder was applied to the agarose gel as a reference to estimate the size of the DNA fragments.

3.2.5. DNA purification

Purification of DNA from the conventional PCR products, which had given bands in the gel electrophoresis, was performed using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as described in the manufacturer's instruction.

3.2.6. Sequencing and sequence- phylogenetic analysis

The PCR products were sent, after purification, for sequencing (MWG Biotech, Martinsried, Germany). After that, specificity of the results was evaluated with Chromas©Lite (http://www.technelysium.com.au/chromas_lite.html). The *16S rRNA* gene sequences were then subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/) and aligned with close relatives and other proteobacteria sequences using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2). Phylogenetic analysis was performed using distance methods with the program PHYLIP, version 3.69. Confidence values for individual branches of the resulting tree were determined by a bootstrapping analysis in which 100 bootstrap trees were generated from resampled data.

3.3. Experiment 2: Evaluation of medium conditions for maintenance of tick cell lines

The *I. ricinus* cell line IRE/CTVM19, the *I. scapularis* cell line IDE8, the *R. (Boophilus) microplus* cell line BME/CTVM6 and the *R. (Boophilus) decoloratus* cell line BDE/CTVM14 were included in this experiment to test their susceptibility to the medium L-15/H-Lac.

3.3.1. Experimental conditions

Six aliquots of IRE/CTVM 19, BME/CTVM 6 and BDE/CTVM 14 cell lines and nine aliquots for IDE 8 cell line were taken from their parent flasks and distributed into flat-sided tubes, where every tube had nearly same number of cells for each cell line. Numbers of cells differed among the lines and ranged from 6.75×10^5 to 12.25×10^5 cells/ml. The aliquots obtained from the cells were divided in two groups (Original medium as control and L-15/H-Lac medium), whereas aliquots from IDE 8 cell line were divided in three groups (Original medium as control, L-15/H-Lac 10% FCS and L-15/H-Lac 20% FCS). Each group included three tubes. Medium changes were done weekly by replacement of 1.5 ml of the medium with fresh medium. Viability and morphology of cells were evaluated on the medium change day. Subcultures were carried out as mentioned before (3.1.3. Cultivation conditions).

3.3.2. Monitoring of cultures

Cultures were monitored weekly by examination of cell morphology and viability.

3.3.2.1. Cell morphology

Cell morphology including growth patterns, shape and size of the cells was primary monitored every week before changing medium by looking directly at the cultures under an inverted microscope (DIAVERT-LEITZ, Germany) and by examination of Giemsa-stained cytospin smears.

At the time of changing medium a volume of 50 µl of cell suspension were taken and placed in the prepared centrifuge vessel. The centrifugation was done for 5 minutes at 1000 rpm. The

smears were then fixed in pure Methanol and put into a freshly prepared 5% GIEMSA solution for 40 minutes. After staining the slides were left to dry and examined under the microscope with 630× magnification oil immersion lens. Photographs for the smears were taken using AxioCam MRc and edited with Axio Vision 4.7.1 software (Figure 3).

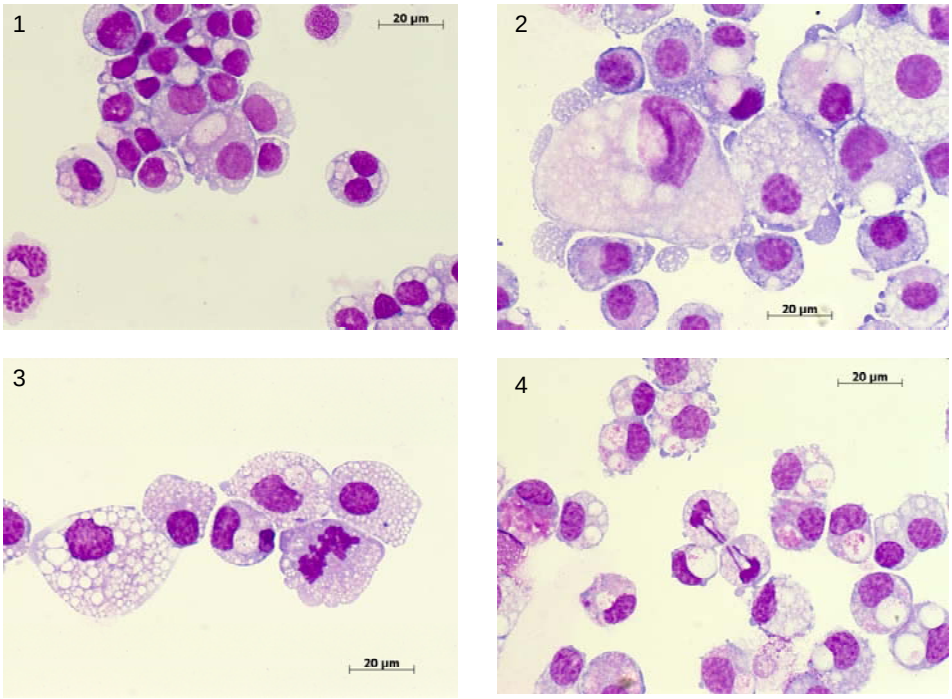

Figure 3: Cytocentrifuge smears for cell lines used in this experiment stained with 5% Giemsa -solution. Magnification 630x. (1) IDE 8; (2) IRE/CTVM 19; (3) BME/CTVM 6; (4) BDE/CTVM 14.

3.3.2.2. Cell viability

Cultures and subcultures were weekly evaluated for viability by the trypan blue exclusion method (Mary et al. 1994; Bastos et al. 2006) depending on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do (Figure 4).

Trypan blue solution was prepared by dissolving 0.5g trypan blue in 100ml PBS to have 0.5% solution. Then the solution was filtered through 2µm filter and stored at -20 °C. To evaluate

cell viability, 20 µl of cell suspension were added to 80 µl of trypan blue and incubated at room temperature at least 5 minutes but not longer than 30 minutes.

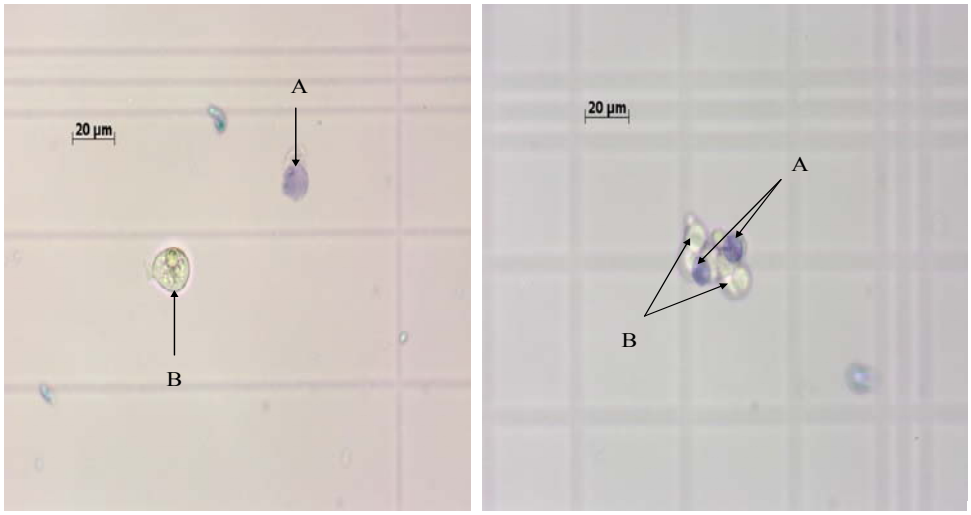

Figure 4: Evaluation of cells' viability by trypan blue exclusion method; A: dead cells, B: live cells

Cell counting took place in a hemocytometer (Improved Neubauer Counting Chamber). The cell viability was calculated by using the following formulae:

Cells per ml = the average count per square × dilution factor × 10^4 (mostly four squares were counted)

Total cells = cells per ml × the original volume of fluid from which cell sample was removed.

Cell viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100

3.3.3. Statistical analysis

Results obtained from each cell line under different treatments were statistically analyzed using the Kruskal-Wallis test, Chi-square test and Duncan test (Sampaio, 2007).

4. RESULTS

4.1. Experiment 1: Detection of endosymbionts in tick cell lines

Results from this experiment have been published in the journal Parasitology Research (Peer reviewed, IF 1.812)

4.1.1. Publication

Detection of bacteria related to *Candidatus* Midichloria mitochondrii in tick cell lines

Nour Addeen-Najm[1]; Cornelia Silaghi[1*]; Lesley Bell-Sakyi[2*]; Kurt Pfister[1*]; Lygia M. Friche Passos[1, 3§]

[1]Lehrstuhl für Vergleichende Tropenmedizin und Parasitologie, Ludwig-Maximilians-Universitaet Muenchen, Leopoldstr. 5, 80802, Munich, Germany

[2]The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, Scotland, UK.

[3]Departamento de Medicina Veterinária Preventiva, Escola de Veterinária- UFMG, Belo Horizonte, Minas Gerais, Brazil

*These authors contributed equally to this work

[§]Corresponding author Tel.: +49-89-21803622; Fax: +49-89-21803623

E-mail: Lygia.Passos@lmu.de

Abstract

Many ticks have been shown to be infected with intracellular bacteria. One of these bacteria is *Candidatus* Midichloria mitochondrii which is the only characterized bacterium that has the ability to invade the mitochondria within ovarian cells and consume them without any effect on the female tick's reproduction. Tick cell lines, which are derived from embryonic ticks, could be infected with such bacteria. In the present study eight cell lines derived from the ticks *Ixodes ricinus*, *I. scapularis*, *Rhipicephalus (Boophilus) microplus* and *R. (B.) decoloratus* were examined for the presence of the bacterium *Ca.* Midichloria mitochondrii. PCR assays for this bacterium were carried out using two sets of primers targeting the eubacterial *16SrRNA* gene and a set of primers specific for the *gyrB* gene of *Ca.* Midichloria mitochondrii. With the *16S rRNA* primers, DNA was amplified from two cell lines (*R.* (*B.*) *decoloratus* line BDE/CTVM14 and *I. ricinus* line IRE/CTVM19) on one out of three occasions each. Sequencing of the PCR products showed that the two cell lines gave sequences with 100% similarity to *Ca*. Midichloria mitochondrii when compared to those available in the GenBank. However, all cell lines, including the two positive cell lines, were negative with the specific primers. Phylogenetic analysis shows that our sequences belong to the subclass α-proteobacteria. They were identical to sequences amplified from the tick *Ixodes ricinus*. The results suggest that two cell lines, IRE/CTVM19 and BDE/CTVM14, may contain bacteria closely related to *Ca*. Midichloria mitochondrii and identical with it in a 350 bp part of the 16S rRNA gene sequence. To our knowledge this constitutes the first report of the presence of DNA similar to DNA of *Ca*. Midichloria mitochondrii in tick cell lines.

Keywords: Ixodes ricinus; Midichloria; Tick cell lines; Endosymbionts; Hard ticks

Introduction

Ticks are known to be vectors of many pathogens (viral, bacterial, and protozoan). In addition, many ticks contain intracellular bacteria that are apparently not harmful to humans, animals or the ticks themselves. These are usually termed symbionts, which in some cases are closely related to known pathogens (Noda *et* al. 1997). These bacteria share the common characteristic that they have never been cultured outside eukaryotic cells and are thus regarded as obligately intracellular (Sassera *et* al. 2006). Such bacteria are found primarily in the ovaries or Malpighian tubules of ticks (Beninati *et* al. 2004; Epis *et* al. 2008; Noda *et* al. 1997). This tissue specificity reduces the chances of these bacteria being transmitted to the tick's host, thereby reducing the probability of horizontal transfer to other blood-sucking arthropods and increasing the probability of vertical transfer (from one generation to the next through the eggs); despite the possibility that horizontal transfer might occur, how it could take place is unknown (Epis *et* al. 2008). One of these symbiotic bacteria is *Ca.* Midichloria mitochondrii which is the only characterized bacterium that has the ability to invade the mitochondria within ovarian cells and consume them without any effect on the female tick's fecundity (Beninati *et* al., 2009; Epis *et* al. 2008; Sacchi *et* al. 2004; Sassera *et* al. 2006). The presence of bacteria in mitochondria was first demonstrated in the 1970s within ciliate species (Yamataka and Hayashi, 1970) and in the tick *I. ricinus* (Lewis, 1979). Sassera and collaborators confirmed in 2006 the phylogenetic position of the intramitochondrial bacterium in *I. ricinus* and proposed the name *Ca.* Midichloria mitochondrii (Sassera *et* al, 2006). It appears microscopically as a gram-negative bacillus-shaped bacterium, of approximately 0.45 μm in width and 1.2 μm in length (Beninati *et* al. 2004; Lewis, 1979). This bacterium and related bacteria have been detected up to now in many genera of ticks: *Ixodes* (Beninati *et* al., 2004, 2009; Epis *et* al. 2008; Lo, 2006; Noda *et* al. 1997; Sacchi *et* al. 2004; Sassera *et al.* 2006, 2008; van Overbeek, *et* al. 2008), *Rhipicephalus* (Epis *et* al. 2008; Noda *et* al. 1997),

Dermacentor (Hornok *et* al. 2008) and *Amblyomma* (Venzal *et* al. 2008), and also in bed bugs (Richard, 2009).

Continuous tick cell lines serve as a useful model to study host–vector–pathogen relationships and to understand the parasites' biology (Bell-Sakyi et al. 2007). Most of the more than 50 currently available ixodid and argasid tick cell lines were set up from embryonic tissues, and some are known to be infected with symbionts. For instance, the *I. scapularis* cell line IDE2 was found to be chronically infected with a virus which has no apparent effect on the tick cells and is presumably transmitted transovarially (Attoui *et* al. 2001). The *Dermacentor andersoni* cell line DAE100 is chronically infected with the bacterium *Rickettsia peacockii* (Simser et al., 2001), which has also been isolated from intact ticks of this species. The aim of the present study was to determine if selected tick cell lines derived from *Ixodes ricinus*, *I. scapularis*, *Rhipicephalus (Boophilus) microplus* and *R. (B.) decoloratus* are infected with *Ca.* Midichloria mitochondrii, taking into account that all were derived from embryonic tissues.

Methods

Tick cell lines

Eight cell lines, derived from embryonic ticks of four species from two genera, *Ixodes* and *Rhipicephalus (Boophilus*), were examined for the presence of *Ca.* Midichloria mitochondrii. Table 1 lists the cell lines and their respective culture media. All media were supplemented with 2mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin The IDE8 cell line was incubated at 32 °C and the remaining 7 cell lines were incubated at 29 °C.

Table 1 - Tick cell lines screened for the presence of the bacterium *Candidatus* Midichloria mitochondrii

Tick species	Tick cell line	Years in continuous culture	Culture medium	Reference
Ixodes ricinus	IRE/CTVM18	9-10	L-15/HLac[a]	Bell-Sakyi (2004)
	IRE/CTVM19	9-10	L-15[b]	Bell-Sakyi et al. (2007)
	IRE/CTVM20	9-10	L-15/L-15B[a]	
Ixodes scapularis	IDE8	>15	L-15B[c]	Mounderloh et al. (1994)
Rhipicephalus (Boophilus) microplus	BME/CTVM2	13-14	L-15	Bell-Sakyi (2004)
	BME/CTVM6	13-14	L-15	
Rhipicephalus (Boophilus) decoloratus	BDE/CTVM12	2-3	H-Lac[d]	Lallinger et al. (2010)
	BDE/CTVM14	3-4	H-Lac	

[a] A 1:1 mixture of the two complete media

[b] L-15 (Leibovitz) medium supplemented with 10% tryptose phosphate broth (TPB), 20% foetal bovine serum (FBS)

[a] L-15B medium (Munderloh & Kurtti, 1989) supplemented with 10% TPB, 5% FBS, 0.1% bovine lipoprotein

[d] Hanks' balanced salt solution supplemented with 0.5% lactalbumin hydrolysate, 10% FBS

DNA extraction

DNA was extracted from the eight tick cell lines with the Qiagen DNA DNeasy® tissue kit (Qiagen, Hilden, Germany) using the protocol for cultured animal cells. Appropriate amounts of cell suspension (3 ml from each cell culture containing a maximum of 5 x 10^6 cells) were centrifuged for 5 min at 300 x *g* and the pellet was resuspended in 500 µl phosphate buffered saline. The lysis was then carried out with 20 µl proteinase K and 200 µl Buffer AL at 70°C for 10 min. Elution was done with 200 µl AE Buffer. The amounts and the quality of DNA

extracted were measured with NanoDrop®1000 (PeqLab, Erlangen, Germany) and then the DNA was stored at -20°C until use. Altogether three DNA samples, from three different time points (December 2008, February 2009 and March 2009), were extracted from each cell line. The positive control (*Ca.* M. mitochondrii DNA) was kindly provided by Prof. Claudio Bandi, Università degli Studi di Milano, Italy. Furthermore, DNA extracted from a female *I. ricinus* collected in Bavaria (Germany) was included in the test as an extra positive control.

Conventional PCR screening

PCR screening of all tick cell cultures for bacteria related to *Ca*. Midichoria mitochondrii was performed using two sets of general primers: Midi-F (5´-GTACATGGGAATCTACCTTGC-3´) and Midi-R (5´-CAGGTCGCCCTATTGC-TTCTTT-3`); Midi-F2 (5´-CAACGAGCGCAACCCTTAT-3´) and Midi-R2 (5´-CAGTCGTCAACCTTACCGT-3`) (4). These primers, targeting the *16SrRNA* gene, amplify fragments of ~1100 bp (Midi-F –R) and ~350 bp (Midi-F2 –R2) and were designed to be conserved between the sequence of *Ca*. Midichloria mitochondrii from *I. ricinus* and the closely related sequences available in the data bases (Epis *et* al. 2008). Amplifications were performed, with both primer sets, in 50 µl reactions containing: 5 µl of buffer (10x concentrated contains Tris.Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7) with 10 mM of each deoxynucleoside triphosphate (dNTP), 1.25 U of Taq polymerase, 300 nM of each primer, 5 µl of DNA sample and made up to 50 µl with deionised PCR clean water. The thermal profile was: 15 min at 95 °C; 40 cycles of 94 °C for 30 sec; 56 °C for 30 sec and 72 °C for one minute; the elongation was completed at 72 °C for 10 min. PCR products were electrophoresed in a 2% agarose gel stained with GelRed@ dye (Biotium, Hayward, USA). The resultant PCR products were purified with the QIAquick PCR product purification kit (Qiagen) and sent for sequencing to Eurofins MWG Operon

(Martinsried, Germany). The *16S rRNA* gene sequences were evaluated using ChromasLite (www.technelysium.com.au/chromas_lite.html).

Phylogenetic analysis

The *16S rRNA* gene sequences obtained from the conventional PCR were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/) and aligned with close relatives and other proteobacteria sequences using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2). Phylogenetic analysis was performed using distance methods with the program PHYLIP, version 3.69. Confidence values for individual branches of the resulting tree were determined by a bootstrapping analysis in which 100 bootstrap trees were generated from resampled data.

SYBR green real-time PCR

One set of primers specifically targeting the *gyrB* gene of "*Ca*. Midichloria mitochondrii" was used for the SYBR green real time PCR: Midi-gyrB-f (5´-CTTGAGAG-CAGAACCACCTA-3´) and Midi-gyrB-R (5´-CAAGCTCTGCCGAAATATCTT-3´) amplifying 125 bp (Sassera *et* al 2008). All reactions were performed in 50 µl reactions containing: 25 µl of SYBR-green master mix (Applied Biosystems, Darmstadt, Germany), each primer at a concentration of 300nM in the final Master Mix, and 5 µl of DNA, then made up to 50 µl with deionized PCR clean water.

Real-time PCR cycling conditions for *gyrB* were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for one minute, followed by a dissociation stage of 95 °C for 15 sec, 60 °C for one minute and 95 °C for 15 sec after the 40 reaction cycles to check the specificity of the PCR.

Results and Discussion

Three DNA extractions from each cell line were examined in duplicate by conventional PCR assays for the presence of bacteria related to *Ca*. Midichloria mitochondrii. No amplifications occurred in any of the three DNA extractions for the primers targeting the 1100 bp fragment (Midi F- Midi R), while for the primers targeting the 350 bp fragment (Midi F2- Midi R2), one amplification occurred in the first extraction and one in the second extraction, but in samples from different cell lines. In the third DNA extraction no amplifications occurred. The cell lines BDE/CTVM14 and IRE/CTVM19 gave bands of the correct nucleotide size in the electrophoresis from the first and second DNA extractions respectively. Sequencing of these PCR products with forward and reverse primers showed that the two cell lines gave sequences (350 bp) with 100% similarity to *Ca*. Midichloria mitochondrii sequences already deposited in the GenBank. The two sequences have been deposited in the GenBank database under accession numbers HQ638198 and HQ638199 respectively.

The positive results with primers for only the small fragment and not the larger one could be explained by taking into account that each fragment represents a different part of the gene. The putative Midichloria-like bacteria present in the tick cell lines could be identical to Midichloria at the section of the *16S rRNA* gene amplified by the small fragment primers, but sufficiently different from it at the section of the gene targeted by the large fragment primers that they were unable to amplify a product. The absence of *16S rRNA* PCR positivity in most cell lines could reflect an undetectably low level of Midichloria-like microorganisms (if present) at the time of DNA extraction.

Phylogenetic analysis revealed that our sequences were identical to sequences of bacteria of the subclass α-proteobacteria amplified from the tick *Ixodes ricinus* (Lo *et* al. 2006). Other bacteria containing DNA closely related to our sequences were *Ca. Nicolleia massiliensis* (from *Ixodes ricinus*), *Ca*. Midichloria mitochondrii sp. (from *Ixodes holocyclus*) and endosymbionts of *Acanthamoeba* spp. (Fig.1).

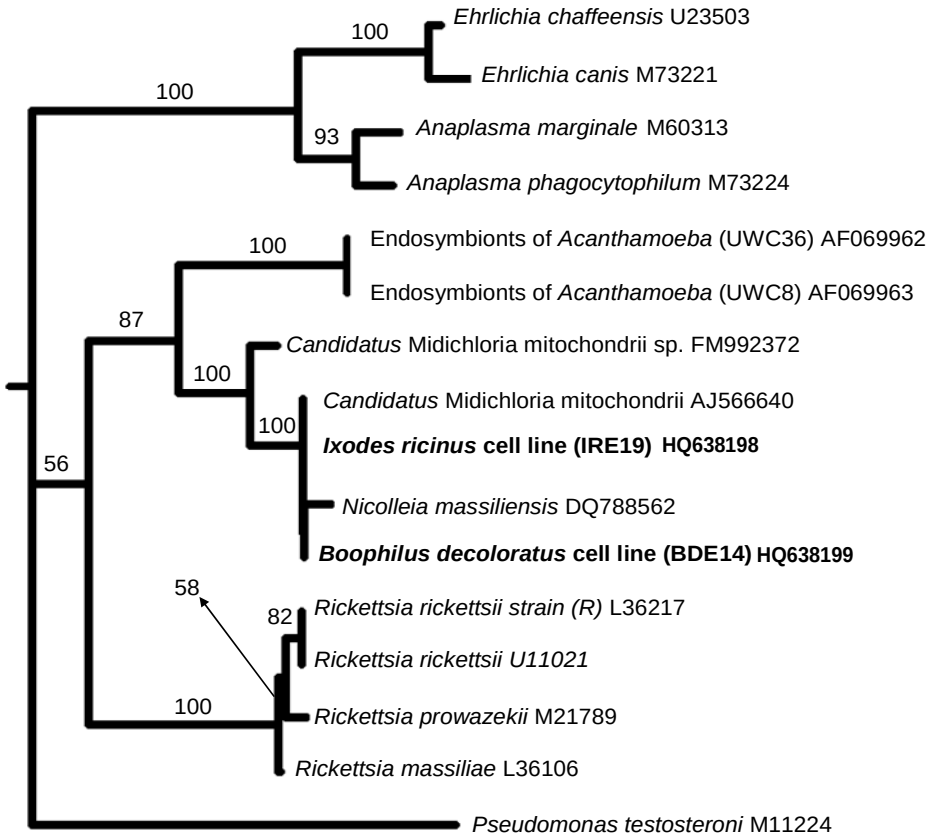

Figure 1 – Phylogenetic relationship of tick cell lines

Phylogenetic relationship of a 350 bp part of the *16S rRNA* gene detected in the BDE/CTVM14 and IRE/CTVM19 cell lines to selected species of proteobacteria. Bold print indicates sequences derived from this study. The bacterium *Pseudomonas testosteroni* was selected as an out-group species. The tree was constructed by a neighbour-joining procedure with the software package PHYLIP, version 3.69. Numbers indicate bootstrap percent confidence values.

In an attempt to prove if the negative results in the conventional PCR reflected real absence of *Ca*, Midichloria mitochondrii in the cell lines, a SYBR green real time PCR assay with *gyrB* primers specific for *Ca*. Midichloria mitochondrii was carried out. Amplifications occurred in the positive control and the *I. ricinus* tick with a melting temperature (T_m) 75.97 °C, while for the tick cell lines, no specific amplifications occurred. Taken all together we can say that the two positive cell lines contain bacterial DNA closely related to *Ca*. Midichloria mitochondrii and the 100% similarity between the sequences reflects the similarity in this part of the DNA

sequence. The inconsistent positive results with the conventional PCR on these two cell lines could indicate real differences in the infection rates of symbionts between the lines at different time points or between different nutritional and developmental stages (Van Overbeek *et* al. 2008).

An alternative hypothesis could be proposed to explain the intermittent positive results obtained with the primers targeting the smaller 16S fragment, and the negative results obtained with the primers targeting the larger 16S fragment and the *gyr b* gene. Incorporation of fragments of DNA originating from the genome of another invertebrate endosymbiont, *Wolbachia*, has been detected in the genomes of host nematodes (McNulty et al., 2010) and woodlice (Martin et al., 2010). It is possible that fragments of DNA, including the 350 bp 16S fragment amplified in the present study in two of the tick cell lines, could have become incorporated within the genomes of a small proportion of the thousands of individual tick embryos from which the lines were derived, and thus be intermittently detectable by PCR (carried out on a small sample of the total number of cells within the culture) in the absence of entire bacteria.

The eight tick cell lines appeared not to contain the bacterium *Ca*. Midichloria mitochondrii, which has been detected in some of the tick species (*Ixodes ricinus, I. scapularis)* from which they were derived (Beninati *et* al., 2004; Epis *et* al. 2008; Lo *et* al 2006; Noda *et* al. 1997; Sacchi *et* al. 2004; Sassera *et al.* 2006, 2008; van Overbeek, *et* al. 2008). The bacterium was detected in 100% of eggs produced by infected female *Ixodes ricinus*, indicating that the major route of transmission for the bacterium is vertical transmission (Lo *et* al. 2006), which theoretically enhances the possibility of the existence of this bacterium in tick cell lines with embryonic ancestry. The prevalence of this bacterium in field-collected *I. ricinus* females was found to be 100%, falling to less than 50% in ticks maintained for a long time in the laboratory (Lo *et* al. 2006). Arthropod symbionts are known to be lost under laboratory conditions, and temperature may play a role in some cases (Weeks *et* al., 2002). That might

explain why the cell lines BME/CTVM 2 and 6, which were derived from ticks maintained in laboratory colonies, were consistently negative. On the other hand these negative results could reflect real absence of Midichloria in the tick *R. (B) microplus*, in which Midichloria has not yet been reported to occur. Two of the cell lines derived from field ticks (IRE/CTVM19 and BDE/CTVM14) were positive. The three *I. ricinus* lines were derived from the same pool of *I. ricinus* egg batches and similarly the two *R.* (*B.*) *decoloratus* lines were derived from the same pool of *R.* (*B.*) *decoloratus* egg batches. A possible reason for only one of the lines of each of these tick species being positive is that the bacteria or bacterial DNA in the tick genome were present in the original pool of egg batches at such a low level that they were only included in one of the primary cultures and not the others.

A previous study found 100% of female larvae and nymphs derived from a single engorged female to be infected with *Rickettsia*-like bacteria (named later Midichloria), while no infection was found within testicular primordia of male progeny (Zhu *et* al. 1992). Taken together, these results suggest that if an infected egg develops into a female, the bacteria continue to survive in ovarian tissues, but if the egg develops into a male, most or all of the bacteria could be lost. Moreover, maintenance of ticks in the laboratory for a long time might lead to a loss of the bacterium in the majority of female ticks, which could be caused by antibiotics given to the mammalian hosts on which the ticks were fed, and/or by exposure of the ticks to constant room temperature conditions (Lo *et* al. 2006). Such conditions (use of antibiotics and constant incubation temperature) are also found in tick cell cultures, which could have an effect on the survival of endosymbionts, depending on the time of exposure to these conditions (passage number and total time in culture).

Conclusions

The results presented here revealed that two tick cell lines included in this study contain DNA from symbionts closely related to *Ca.* Midichloria mitochondrii. The inconsistent results

indicate that additional cell lines might contain endosymbiont DNA at undetectable levels or that the negative results may reflect real absence of DNA from these endosymbionts. Further studies are needed to identify the endosymbionts and to determine whether the actual bacteria are present in the tick cells or if only small segments of their DNA are incorporated in the tick genome. If entire, viable bacteria are present, their location within the tick cells should be confirmed using microscopy, and the influence of culture conditions on their relative abundance should be investigated.

Acknowledgements

The authors would like to thank Prof. U. G. Munderloh (University of Minnesota, USA) for permission to use the cell line IDE8, Ms Claudia Thiel (LMU-Munich, Germany) for technical support in molecular techniques and Prof. Claudio Bandi (Università degli Studi di Milano, Italy) for providing positive control DNA.

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4.2. Experiment 2: Evaluation of medium conditions for maintenance of tick cell lines

All cell lines showed in general high viability rates under different media conditions. The viability rates were calculated as the mean of replicates for each condition (Appendix 12.1). Each passage was treated as an individual line. Evaluation of the means statistically brought out that there was a significant difference ($P = 0.001$) between the cultures growing in their original media and the cultures in L-15/H-Lac medium for each cell line (Appendix 12.2). Growth rates and morphological aspects differed among the cell lines.

4.2.1. IDE8 cell line

Statistical analysis showed that the cultures growing in L-15/H-Lac medium with 20% serum gave the best viability rates along the experiment followed by the cultures in L-15/H-Lac with 10% serum and behind them the control cultures. In general, all cultures showed high viability rates (60-82%) along the whole experimental period (Figures 5 and 6). In addition, the cultures showed different characteristics according to the medium, therein they grew. The cultures growing in L-15/HLac medium with 20% serum grew faster than the others, but they showed different shapes and sizes of cells. In contrast, the cells in the cultures growing in L-15/Lac medium with 10% serum grew slowly and separately, where the fusiform cells were the most common cells in these cultures with more big cells than in the other cultures. Cells in these cultures needed at least four weeks to grow nicely and abreast. However, the growth in the control group represented steady rates and the cells showed more stability and homogeneity.

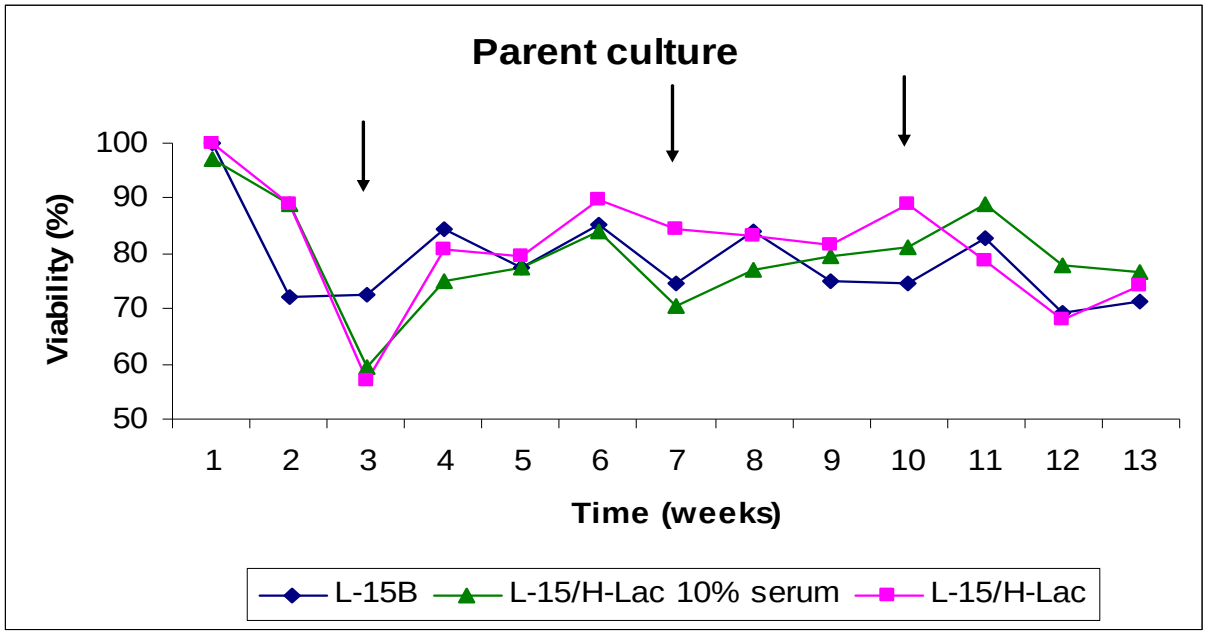

Figure 5: Viability rates of IDE 8 cells (starting cultures) under three different media conditions (Control: L-15B, Tested: L-15/H-Lac with 10% serum and L-15/H-Lac with 20% serum). Arrows indicate subculture points.

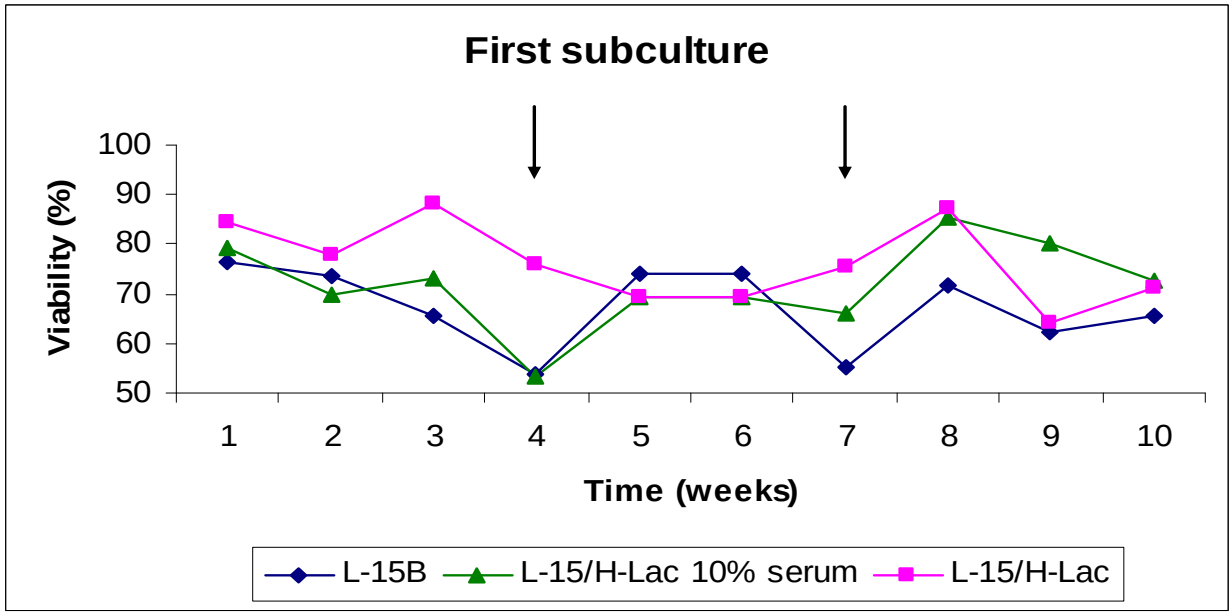

Figure 6: Viability rates of IDE 8 cells (first passage) under three different media conditions (Control: L-15B, Tested: L-15/H-Lac with 10% serum and L-15/H-Lac with 20% serum).

4.2.2. IRE/CTVM 19 cell line

The cultures and subcultures showed generally high viability rates (79.5-92%). Statistical and graphical analysis of the results obtained from this cell line revealed that the cultures with their original medium (L-15) showed better viability rates than the cultures with L-15/H-Lac medium (Figures 7 and 8).

Generally, the cells in the culture with L-15/H-Lac medium were morphologically bigger than the cells in the culture with control medium. But the cells in both cultures were nearly homogeneous (almost circle with some big cells)

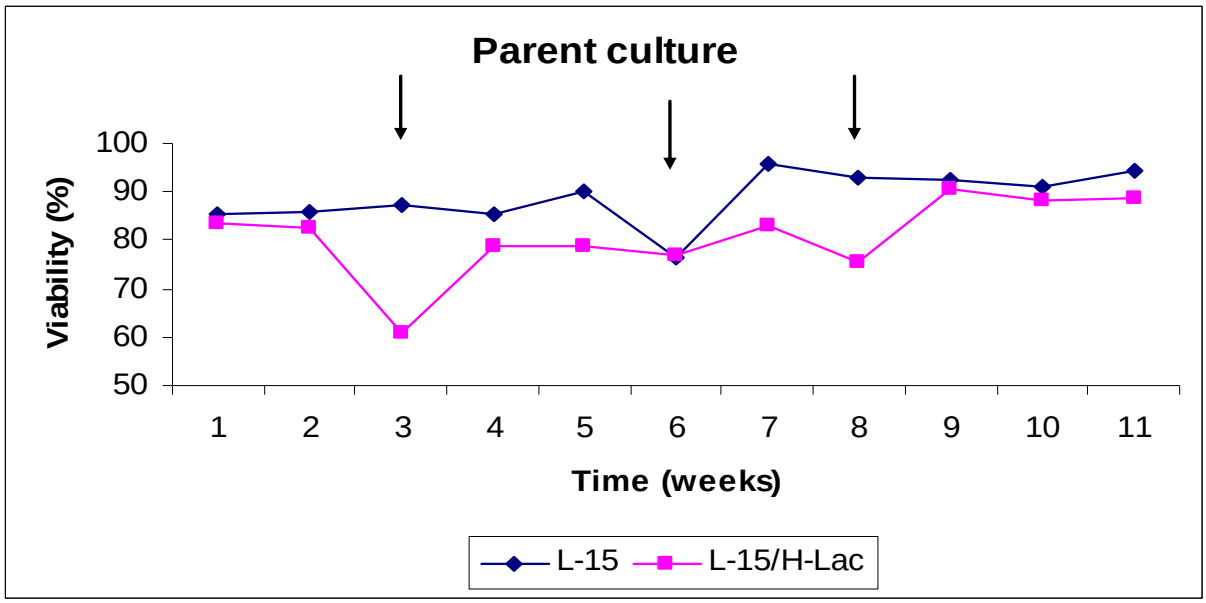

Figure 7: Viability rates of IRE/CTVM 19 cells (started culture) under two different media conditions (Control: L-15 and Tested: L15/HLac). Arrows indicate subculture points.

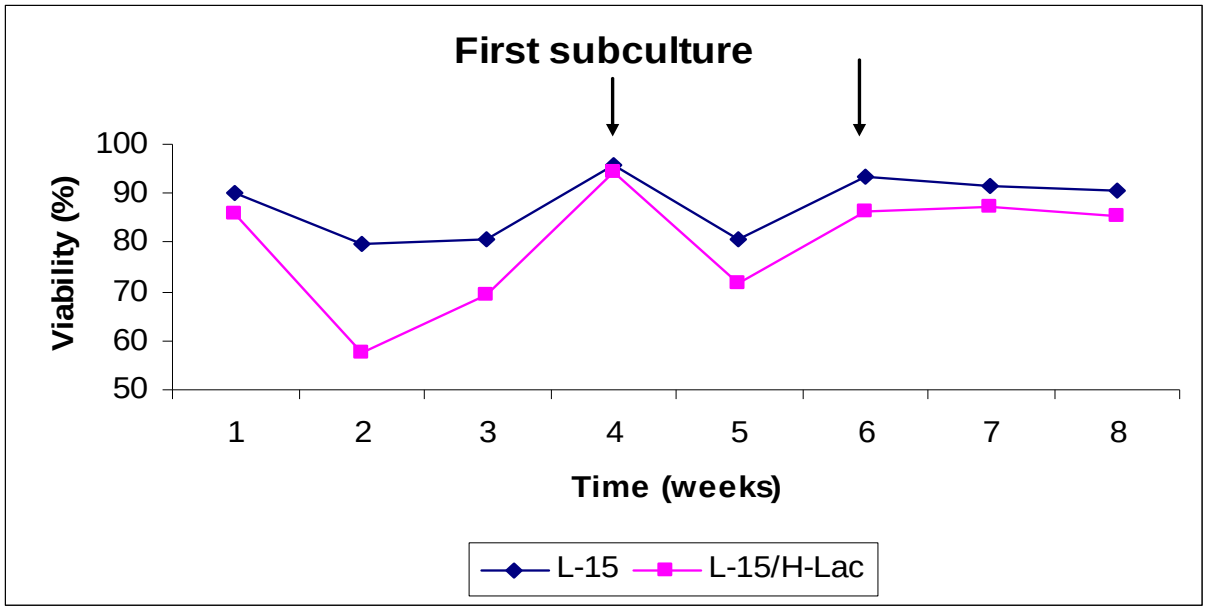

Figure 8: Viability rates of IRE/CTVM 19 cells (first passage) under two different media conditions (Control: L-15 and Tested: L15/HLac).

4.2.3. BME/CTVM 6 cell line

As shown in figures 9 and 10 cultures needed sometimes up to four weeks to be split due to their slowly growing. In spite of that, all cultures showed high viability rates (71-89.5%). Statistically, the cultures in control medium showed relatively better viability rates compared to those obtained from the cultures in L-15/H-Lac medium. Morphologically the cells in both cultures were generally middle-sized, almost circle and grew in multilayer form.

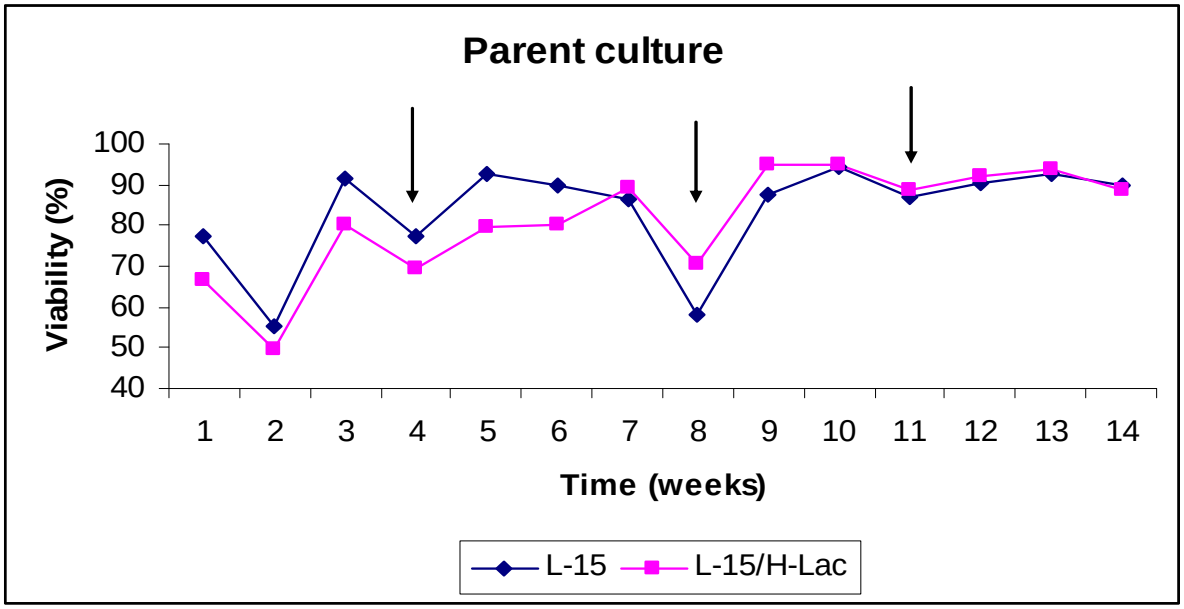

Figure 9: Viability rates of BME/CTVM 6 cells (started culture) under two different media conditions (Control: L-15 and Tested: L15/HLac). Arrows indicate subculture points.

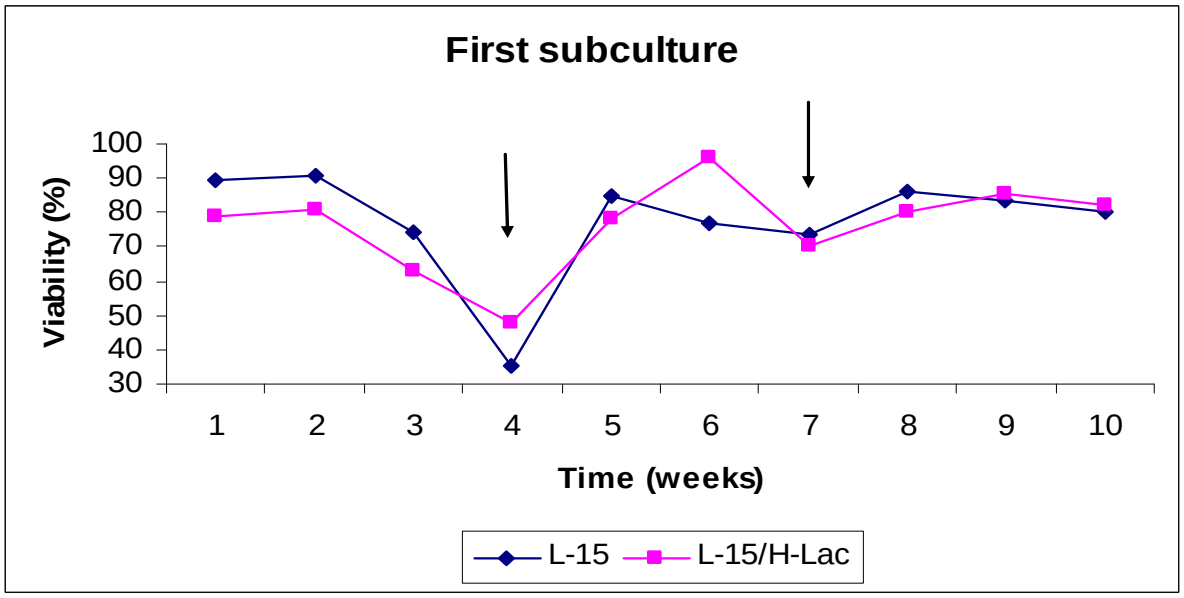

Figure 10: Viability rates of BME/CTVM 6 cells (first passage) under two different media conditions (Control: L-15 and Tested: L15/HLac).

4.2.4. BDE/CTVM 14 cell line

The cultures and subcultures were split at three weeks interval. The cultures showed mostly high viability rates (74-89.5%) with priority of the cultures under control medium conditions over the cultures with L15/H-Lac medium along the whole experiment (Figures 11 and 12). In deed, that was proved statistically. The cells were morphologically almost similar in shape and size (circle and small). The cells grew in some cultures with L15/H-Lac medium faster than those in the control cultures.

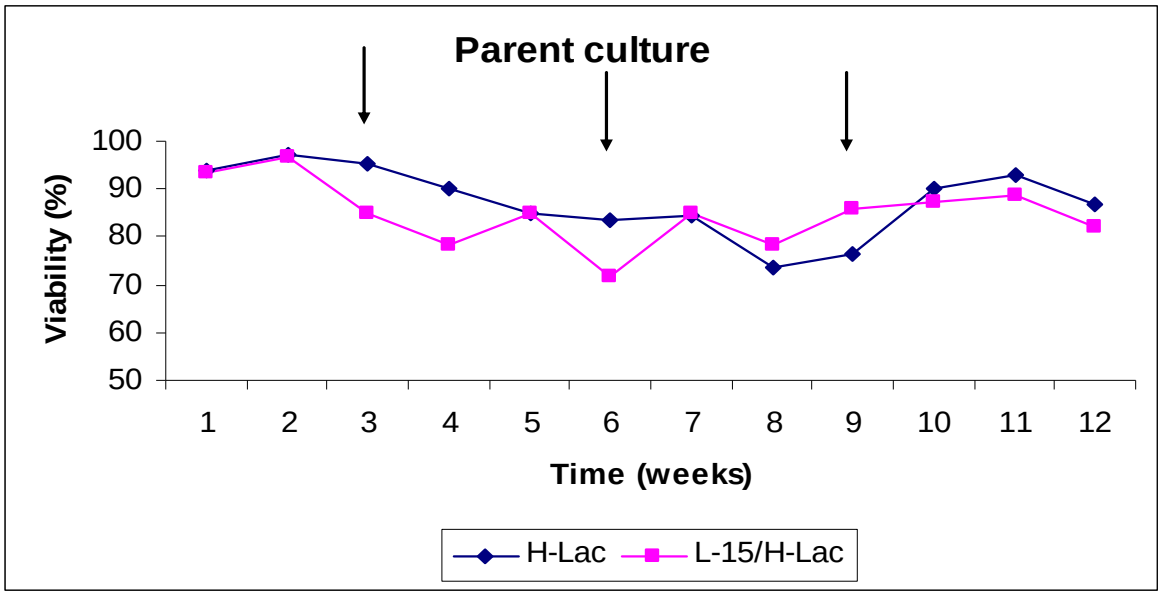

Figure 11: Viability rates of BDE/CTVM 14 cells (started culture) under two different media conditions (Control: H-Lac and Tested: L15/HLac). Arrows indicate subculture points.

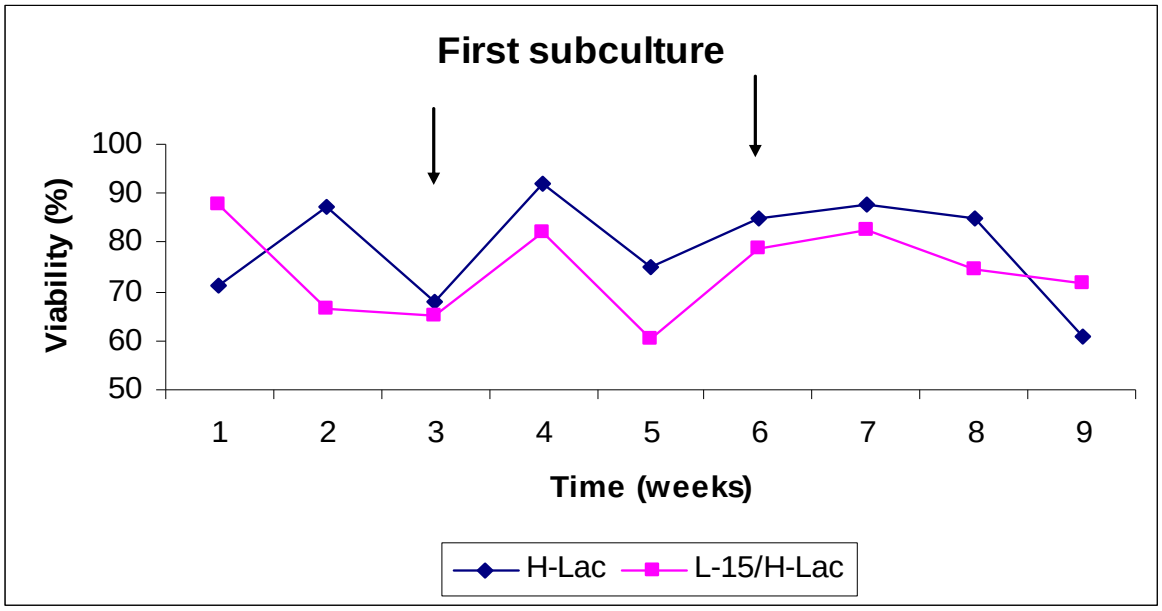

Figure 12: Viability rates of BDE/CTVM 14 cells (first passage) under two different media conditions (Control: H-Lac and Tested: L15/HLac).

5. DISCUSSION

5.1. Detection of *Ca*. Midichloria mitochondrii in tick cells

Three DNA extractions from each tick cell line were examined by conventional PCR assays using two sets of primers targeting the *16SrRNA* gene, amplifying fragments of ~1100 bp (Midi-F –R) and ~350 bp (Midi-F2 –R2). No amplifications occurred in any of the three DNA extractions for the primers Midi F- Midi R; whereas for the primers Midi F2- Midi R2 amplifications occurred in BDE/CTVM14 cell line from the first DNA extraction and in IRE/CTVM19 cell line from the second extraction. In the third DNA extraction no amplifications were observed. Each primer set amplified a fragment which represents a different part of the *16SrRNA* gene; that may explain having positive results with primers for only the small fragment and not the larger one. The two positive cell lines gave bands of the correct nucleotide size in the electrophoresis and sequencing of their PCR products with forward and reverse primers resulted in sequences (350 bp) with 100 % similarity to *Ca*. Midichloria mitochondrii sequences that were already deposited in the GenBank. Phylogenetic analysis revealed that our sequences were identical to sequences of bacteria of the subclass α-proteobacteria amplified from the tick *I. ricinus* (Lo *et* al. 2006) and closely related to other sequences such as *Ca.* Nicolleia massiliensis (from *I. ricinus*), *Ca*. Midichloria mitochondrii sp. (from *I. holocyclus*) and endosymbionts of *Acanthamoeba* spp.

The subjective Midichloria-like bacteria present in the tick cell lines could be identical to Midichloria at the section of the *16S rRNA* gene amplified by the small fragment primers, but sufficiently different from it at the section of the gene targeted by the large fragment primers which were unable to amplify a product. The absence of *16S rRNA* PCR positivity in other cell lines could reflect an undetectably low level of Midichloria-like microorganisms (if present) at the time of DNA extraction or real absence of this bacterium in the tick cell lines.

Subsequently, a SYBR green real time PCR assay with *gyrB* primers specific for *Ca*. Midichloria mitochondrii was carried out to prove if the negative results in the conventional

PCR reflected real absence of *Ca*. Midichloria mitochondrii in the cell lines. Amplifications occurred in the positive control and the *I. ricinus* ticks with a melting temperature (T_m) 75.97 °C. Non-specific amplification was observed in IRE/CTVM18 cell line and therefore it was considered negative. Summarizing all these facts, we can conclude that the two positive cell lines contain bacterial DNA that is closely related to *Ca*. Midichloria mitochondrii and identical with it in this part of the DNA sequence. Incorporation of fragments of DNA originating from the genome of *Wolbachia* (invertebrate endosymbiont), has been detected in the genomes of host nematodes (McNulty et al., 2010) and woodlice (Martin et al., 2010). This could form an alternative hypothesis to explain the remittent positive results obtained with the primers targeting the smaller 16S fragment and also the negative results obtained with the primers targeting the larger 16S fragment and the *gyr b* gene. It is most likely that fragments of DNA, including the 350 bp 16S fragment amplified in the present study in two of the tick cell lines, could have become incorporated within the genomes of a small proportion of the thousands of individual tick embryos from which the cell lines were derived and thus become intermittently detectable by PCR (carried out on a small sample of the total number of cells within the culture) in the absence of the entire bacteria.

The cell lines BME/CTVM 2 and 6 were derived from ticks that were maintained in laboratory colonies. This might elucidate the reason for these two cell lines being consistently negative, taking into consideration that this bacterium seems to get gradually lost from ticks that were maintained for a long time in the laboratory. That could be caused by antibiotics given to the mammalian hosts on which the ticks were fed (Lo et al. 2006). Antibiotics are also used in tick cell cultures, which could have an effect on the survival of endosymbionts, in some lines, taking into account species specificity of the cell lines (Kurtti and Munderloh, 1989). On the other hand, these negative results could reflect real absence of Midichloria in the tick *R. (Boophilus) microplus*, in which Midichloria has not yet been reported to occur. The two positive cell lines (IRE/CTVM19 and BDE/CTVM14) were derived from field ticks.

The three *I. ricinus* lines were derived from the same pool of *I. ricinus* egg batches and similarly, the two *R.* (*Boophilus*) *decoloratus* lines were derived from the same pool of *R.* (*Boophilus*) *decoloratus* egg batches. A possible reason for only one of the lines of each of these tick species being positive is that the bacteria or bacterial DNA in the tick genome were present in the original pool of egg batches at such a low level that they were only included in one of the primary cultures and not in the others.

A previous study found 100 % of female larvae and nymphs derived from a single engorged female to be infected with *Rickettsia*-like bacteria (named later Midichloria), while no infection was found within testicular primordia of male progeny (Zhu et al. 1992). Summing up these evidences, these results suggest that if an infected egg develops into a female, the bacteria continue to survive in ovarian tissues; but if the egg develops into a male, most or all of the bacteria could be lost. The same thing could happen in tick cell lines, since some lines have cells with male origin predominant and some with that of female (Varma et al., 1975; Esteves et al., 2008).

5.2. Evaluation of different medium conditions

In this experiment the medium L-15/H-Lac, which comprise a mixture of equal part of two media (L-15 and H-Lac) was used to test its ability to support growth of four different tick cell lines. In general, the cultures and subcultures showed high viability rates in average (60-90 %) under different medium conditions. Statistically there was a significant difference (P = 0.001) between different media for all cell lines which were tested. Indeed, the cell lines IRE/CTVM19, BME/CTVM6 and BDE/CTVM14 showed better viability rates when they were maintained in their original medium (L-15 for the first two lines and H-Lac for BDE/CTVM14 line). While IDE8 cells cultured in L-15/H-Lac medium gave better viability rates compared to those maintained in their original medium (L-15B).The non-parametric method was necessary to analyse the results statistically where the variable (cell viability) may look normally distributed; but the experimental groups do not present homoscedasticity (as their means decrease, their standard deviations increase). H-Lac comprises a solution of several salts supplemented with 5 % lactalbumin hydrolysate, which provides several amino acids to the medium and 20 % FCS. In contrast, L-15 (Leibovitz, 1963) contains amino acids and vitamins that do not exist in H-Lac in addition to the salts. L-15 was supplemented with 10 % TPB and 20 % FCS. In the mixture of these two media the components that exist in each medium separately such as TPB and lactalbumin hydrolysate concentrations will decrease because of the dilution factor. That may explain the priority of the origin medium over the mixture L-15/H-Lac as a suitable medium for each of the cell lines IRE/CTVM19, BME/CTVM6 and BDE/CTVM14. Furthermore, L-15 medium, supplemented with 10 % TBP and 15 % FCS, produces smaller cells and higher subculture levels of cells compared to cells in H-Lac medium (Pundy and et al., 1973); that can explain why the cells of IRE/CTVM19 and IDE8 lines were smaller in their original medium. Guru et al. (1976) reported that out of several media tried, L-15 supplemented with 10 % TBP and 10 % FCS proved to be the most suitable medium for cells originated from several tick species *(H.*

spinigera, *H. obesa* and *R. sanguineus).* On the other hand, Martin and Vidler (1962) found that the medium contained Hanks' balanced salt solution, amino acids and vitamins of Eagle's basal medium together with 20 % ox serum supported the survival and growth of tissues of the tick *R. appendiculatus* for a long time. In addition, Bell-Sakyi (1991) reported establishment of 5 continuous cell lines from the tick *Hy. anatolicum anatolicum* in L-15/H-Lac medium supplemented with 20 % FCS. In summary, the susceptibility of tick cell lines to different media differs among the cell lines. That could reflect a kind of species specificity among the tick cell lines; for instance L-15 medium supported growth of cells from *R. appendiculatus* and *R. (Boophilus) microplus*, while it showed less satisfaction for the growth of cells from *Hy. dromedarii* (Varma et al., 1975). Additionally, a toxic batch of glutamine contained in L-15B medium did not affect growth of different cell lines from *R. appendiculatus, R. sanguineus, D. nitens* and *D. variabilis* ticks, whereas it was found to be toxic to a cell line from *R. (Boophilus) microplus* (Munderloh and Kurtti, 1989).

Finally, L-15B is a medium modified from L-15 by adding some amino acids and glucose. It had been supplemented with 20 % FBS and 10 % TPB (Munderloh and Kurtti, 1985); these concentrations of both supplements mentioned above were proved to comprise optimal concentrations for these two supplements in the medium (Kurtti et al., 1982). Later on, it was possible to reduce the concentration of the FBS from 20 to 5 % by adding lipoprotein to the medium in order to decrease the undefined components in the medium (Kurtti and Munderloh, 1989). The results obtained from IDE8 cell line confirmed the findings by Kurtti et al. (1982), where the cultures with 20 % FCS present in L-15/H-Lac medium produced the best growth rates. This fact also elucidates the reason behind paucity of cells in the cultures cultivated in 10 % FCS.

Essentiality of serum in cultures indicates that the protein acts in part as a carrier of growth factors which are bound to the protein and slowly released into the medium (Eagle, 1959).

6. CONCLUSIONS

The results presented here revealed that eight tick cell lines (IRE/CTVM18, IRE/CTVM19, IRE/CTVM20, IDE8, BME/CTVM2, BME/CTVM6, BDE/CTVM12 and BDE/CTVM14 were not infected with *Ca.* Midichloria mitochondrii. However, DNA from symbionts closely related to *Ca.* Midichloria mitochondrii was detected in two cell lines (IRE/CTVM19 and BDE/CTVM14). Other cell lines might contain endosymbiont DNA at undetectable levels or the negative results reflect real absence of DNA from these endosymbionts.

In addition, the results obtained from the evaluation of different medium conditions indicate that the cell lines grew better in their self-originated media. In spite of that, L-15/H-Lac medium supported growth of all tested cell lines with high viability rates. In other words, the media used for the cell lines could be replaced by L-15/H-Lac for routine maintenance of cultures. Moreover, the differences in the shape and size of the cells among the lines are normal and may reflect species specificity since none of them had been cloned. Similarly, the response of tick cell lines to different media conditions may also reflect species specificity. Furthermore, serum proved to be essential supplement to the culture media and 20 % were confirmed to be the best concentration for the growth of the cells.

7. RECOMMENDATIONS

Based on the findings in this research work, further studies are imperative to identify the endosymbiont of which DNA was detected in the two lines and to determine whether the actual bacteria are present in the tick cells or if only small segments of their DNA are incorporated in the tick genome. If entire, viable bacteria are present, their location within the tick cells should be confirmed using microscopy, and the influence of culture conditions on their relative abundance should be investigated.

Also, additional studies are needed to determine the influence of other components of the media on the growth of cells.

7. SUMMARY

On the basis of polymerase chain reaction (PCR) assays, this thesis evaluated eight tick cell lines originated from four tick species for the presence of the endosymbiont *Candidatus* Midichloria mitochondrii or bacteria related to it. The cell lines were IRE/CTVM18, 19, 20; IDE8; BME/CTVM2, 6 and BDE/CTVM12, 14 that derived from *Ixodes ricinus*, *I. scapularis*, *Rhipicephalus (Boophilus) microplus* and *R. (Boophilus) decoloratus* respectively. DNA was extracted at three different time points for each cell line and two PCR assays were carried out. Conventional PCR assay included two sets of primers targeting the *16S rRNA* gene of bacteria related to *Ca*. Midichloria mitochondrii and amplifying two different parts of the gene. Amplification occurred in two cell lines from different DNA extractions. Sequencing of the PCR products showed 100 % similarity with *Ca*. Midichloria mitochondrii DNA sequence already deposited in the GenBank. In order to confirm the negative results real-time PCR using a specific primer set for *Ca*. Midichloria mitochondrii was carried out. The results from real-time PCR were all negative, which leads to the conclusion that the two positive results in the conventional PCR represent infection with bacteria related to *Ca*. Midichloria mitochondrii. It was also postulated that the negative results may reflect real absence of the bacteria or was below the detectable DNA levels in the cultures.

Subsequently, four out of the eight cell lines were randomly selected, representing the four tick species in order to study the effect of the mixture medium L-15/H-Lac supplemented with 20 % fetal calf serum (FCS) on their growth rates. Both cell lines IRE/CTVM19 and BME/CTVM6 were originated and maintained in L-15 medium supplemented with 20 % FCS, whereas BDE/CTVM14 and IDE8 were maintained in H-Lac medium supplemented with 20 % FCS and L-15B medium supplemented with 5 % FCS respectively. In general, all cultures and subcultures showed high growth rates under different media conditions, but the first three lines (IRE/CTVM19, BME/CTVM6 and BDE/CTVM14) shows exceptionally better growth rates under their self-originated media conditions. The IDE8 line experienced

the highest growth rates under L-15/H-Lac medium conditions. As already reported, the results obtained from IDE8 cell line confirmed that the concentration of 20 % serum comprise the optimal concentration of this supplement in the medium. In conclusion, L-15/H-Lac medium proved to support growth of cells in different tick cell lines.

8. ZUSAMMENFASSUNG

Acht Zeckenzelllinien, die sich von 4 Zeckenspezies ableiten, wurden auf die Anwesenheit des Endosymbionten *Candidatus* Midichloria mitochondrii oder damit verwandter Bakterien mittels Polymerase-Kettenreaktion (PCR) untersucht. Die Zelllinien waren IRE/CTVM18, 19 und 20; IDE8; BME/CTVM2 und 6 sowie BDE/CTVM12 und 14, die sich entweder von *Ixodes ricinus*, *I. scapularis*, *Rhipicephalus (Boophilus) microplus* oder *R. (Boophilus) decoloratus* ableiten. Von jeder Zelllinie wurden an drei verschiedenen Zeitpunkten DNS extrahiert und jeweils zwei PCR Tests durchgeführt.

Die konventionelle PCR schloss zwei Primersets ein, die zwei verschiedene Abschnitte des 16S rRNS Gens von Bakterien, die verwandt sind mit *Ca.* Midichloria mitochondrii, amplifizieren. Bei zwei verschiedenen DNA Extraktionen gab es eine Amplifikation bei den Zelllinien IRE/CTVM19 und BDE/CTVM14. Die Sequenzierung dieser PCR Produkte zeigte eine 100%-ige Übereinstimmung mit der *Ca*. Midichloria mitochondrii DNS Sequenz, die bereits bei GenBank hinterlegt war. Um die negativen Ergebnisse zu bestätigen, wurde mit einem für *Ca*. Midichloria mitochondrii spezifischen Primer Set eine PCR durchgeführt.

Die Ergebnisse der PCR waren alle negativ, was die Vermutung nahe legt, dass die zwei positiven Ergebnisse bei der konventionellen PCR eine Infektion mit Bakterien darstellt, die mit *Ca.* Midichloria mitochondrii verwandt sind. Die negativen Ergebnisse lassen vermuten, dass keine Bakterien in der Kultur vorhanden waren, oder dass die DNS Konzentrationen unter der Nachweisgrenze lagen.

Vier der acht Zelllinien, jeweils eine Zeckenart repräsentierend, wurden anschließend ausgewählt, um die Wirkung des Kulturmediums L15/H-Lac, das mit 20% fötales Kälberserum (FKS) ergänzt wurde, auf das Zeckenzellwachstum zu untersuchen. Beide Zelllinien, IRE/CTVM19 und BME/CTVM6 wurden ursprünglich in L-15 Medium etabliert und propagiert, das 20% FKS enthielt, wohingegen BDE/CTVM14 und IDE8 in H-Lac Medium propagiert wurden, das mit 20% FKS, beziehungsweise L-15B Medium, das mit 5%

FKS ergänzt wurde. Im Allgemeinen zeigten alle Kulturen und Subkulturen hohe Wachstumsraten mit den verschiedenen Medien, aber die ersten drei Zelllinien (IRE/CTVM19, BME/CTVM6 and BDE/CTVM14) zeigten bessere Wachstumsraten mit den ursprünglichen Medien. Die IDE8-Zelllinie zeigte die besten Wachstumsraten wenn L 15/H LAC benutzt wurde. Die mit der IDE8 Zelllinie erhaltenen Ergebnisse bestätigten, dass eine Serumkonzentration von 20% im Kulturmedium die optimale Konzentration darstellt. Abschließend kann gesagt werden, dass L 15/H LAC Medium das Wachstum der getesteten Zeckenzelllinien unterstützte.

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10. TABLES

11. FIGURES

12. APPENDIX

12.1. Viability rates as the mean of replicates for each cell line and each condition.

1- IDE 8

Week	Passage number	L-15B	L-15/H-Lac 10% serum	L-15/H-Lac
1	105	100.00	96.97	100.00
2	105	72.22	88.89	88.89
3	105	72.54	59.58	56.89
4	105	84.39	75.00	80.72
4	106	76.60	79.18	84.26
5	105	77.43	77.63	79.44
5	106	73.48	69.77	77.96
6	105	85.06	84.07	89.93
6	106	65.76	73.05	88.34
7	105	74.39	70.40	84.33
7	106	53.84	53.11	75.89
8	105	83.85	77.05	83.26
8	106	74.21	69.49	69.30
8	106A	72.83	58.43	78.04
8	107	78.02	75.09	74.01
9	105	75.07	79.52	81.70
9	106	61.05	45.83	82.73
9	106A	67.01	71.38	89.33
9	107	52.78	58.89	78.16
10	106A	78.07	64.60	74.73
10	107	61.36	60.32	77.27
11	105	74.46	81.02	89.03
11	106	55.34	65.91	75.39
12	106A	72.22	69.05	82.90
12	107	69.06	68.63	63.33
12	107B	73.72	62.58	81.17
12	108	59.01	70.51	67.69
13	105	82.96	88.74	78.73
13	106	71.65	85.19	87.21
13	106B	70.45	85.79	68.81
13	107A	70.45	70.05	83.39
14	106A	70.81	71.46	82.85
14	107	51.67	74.99	74.07
14	107B	71.99	84.94	79.01
14	108	66.32	56.12	65.42
15	105	69.25	78.01	68.05
15	106	62.42	80.32	64.07
15	106B	68.19	82.28	85.59
15	107A	73.33	91.11	80.76
16	106A	65.52	55.68	85.22
16	107	47.17	47.53	70.45
16	107B	64.52	81.04	79.73
16	108	57.96	42.63	83.40
17	105	71.36	76.72	74.24
17	106	65.46	72.64	71.45
17	106B	71.33	77.46	86.14
17	107A	72.50	74.44	69.58

2- IRE/CTVM 19

Week	Passage number	L-15	L-15/H-Lac
1	124	85.39	83.59
2	124	85.67	82.53
3	124	87.04	61.01
4	124	85.40	78.61
4	125	90.22	85.87
5	124	89.93	78.68
5	125	79.92	57.32
6	124	76.23	76.95
6	125	80.56	69.17
7	124	95.71	83.10
7	125	95.98	94.16
7	125A	94.54	94.13
7	126	91.56	85.89
8	124	92.94	75.51
8	125	80.69	71.53
8	125A	93.09	85.07
8	126	89.83	75.24
9	124	92.41	90.66
9	125	93.19	86.13
9	125A	94.25	91.30
9	126	90.78	86.39
9	125B	92.12	87.28
9	126A	94.57	84.48
9	126B	89.00	88.37
9	127	94.22	85.39
10	124	91.27	88.21
10	125	91.67	87.10
10	125A	92.85	89.41
10	126	92.15	85.07
10	125B	88.94	76.70
10	126A	90.04	77.24
10	126B	92.85	92.22
10	127	95.08	90.15
11	124	94.34	88.79
11	125	90.77	85.15
11	125A	87.93	83.12
11	126	85.92	80.68
11	125B	83.73	84.21
11	126A	83.16	73.38
11	126B	97.56	89.37
11	127	99.11	92.95

3- BME/CTVM 6

Week	Passage number	L-15	L-15/H-Lac
1	179	77.32	66.43
2	179	55.28	49.50
3	179	91.41	80.23
4	179	77.31	69.17
5	179	92.75	79.79
5	180	89.51	79.17
6	179	89.97	80.20
6	180	90.48	80.95
7	179	86.31	89.40
7	180	74.53	63.34
8	179	57.97	70.69
8	180	35.04	47.92
9	179	87.36	94.84
9	180	84.95	77.96
9	180A	85.76	73.28
9	181	78.31	58.89
10	179	94.59	95.18
10	180	76.66	96.14
10	180A	91.79	87.92
10	181	91.90	86.88
11	179	86.85	88.77
11	180	73.47	70.23
11	180A	96.35	89.54
11	181	82.40	70.97
12	179	90.17	91.92
12	180	86.38	79.96
12	180A	73.57	76.12
12	181	93.14	82.14
12	180B	87.19	72.64
12	181A	80.16	76.77
13	179	92.74	93.77
13	180	83.68	85.72
13	180A	98.04	91.96
13	181	78.19	65.29
13	180B	90.92	74.69
13	181A	73.05	74.22
13	181B	62.94	77.45
14	179	89.70	88.51
14	180	80.03	82.47
14	180A	92.46	82.83
14	181	93.16	61.74
14	180B	92.93	91.05
14	181A	78.42	85.34
14	181B	73.01	71.52
14	182	62.36	53.33
15	181B	87.00	82.62
15	182	82.09	76.87
16	182	86.46	81.55

4- BDE/CTVM 14

Week	Passage number	H-Lac	L-15/H-Lac
1	33	93.92	93.33
2	33	97.02	96.74
3	33	95.30	85.08
4	33	90.06	78.18
4	34	71.11	87.83
5	33	84.85	84.77
5	34	87.30	66.74
6	33	83.69	71.91
6	34	67.96	65.14
7	33	84.25	85.02
7	34	92.02	81.89
7	34A	85.56	86.97
7	35	94.28	85.73
8	33	73.74	78.10
8	34	75.08	60.59
8	34A	89.64	83.78
8	35	78.83	70.30
9	33	76.47	85.69
9	34	84.99	78.65
9	34A	85.98	84.28
9	35	70.26	78.21
10	33	90.13	87.28
10	34	87.92	82.41
10	34A	94.37	95.63
10	35	88.07	87.07
10	34B	87.08	86.70
10	35A	88.00	85.07
10	35B	86.15	88.55
10	36	80.84	75.49
11	33	93.10	88.67
11	34	84.73	74.36
11	34A	94.14	96.84
11	35	86.46	96.37
11	34B	88.04	86.67
11	35A	88.84	83.17
11	35B	87.12	85.84
11	36	87.94	72.16
12	33	86.68	82.05
12	34	60.74	71.74
12	34A	87.58	61.95
12	35	67.22	74.49
12	34B	89.38	86.15
12	35A	87.66	81.71
12	35B	72.57	73.60
12	36	75.11	69.50

12.2. Statistical analysis

Means with different letter showed statistical difference (P = 0.001). [a] presents the best viability rates.

Table 1: IRE/CTVM 19 cell viability (%) means according to culture medium and time.

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
L-15	85.3 a	85.6 a	87 a	85.3 ab	89.9 a	76.2 a	95.7 a	92.9 a	92.4 abcd	91.2 abcd	94.3 abcd
L-15/H-Lac	83.5 a	82.5 a	61 b	78.6 b	78.6 cd	76.9 a	83.1 c	75.5 def	90.6 abcdef	88.2 bcd	88.7 defghijklm
L-15				90.2 a	79.9 bcd	80.5 a	95.9 a	80.6 cdef	93.1 abcd	91.6 abcd	90.7 bcdefghijkl
L-15/H-Lac				85.8 ab	57.3 d	69.1 a	94.1 a	71.5 ef	86.1 def	87 cd	85.1 ghijklm
L-15							94.5 a	93 a	94.2 ab	92.8 abcd	87.9 efghijklm
L-15/H-Lac							94.1 a	85 bcdef	91.3 abcdef	89.4 abcd	83.1 hijklm
L-15							91.5 ab	89.8 ab	90.7 abcdef	92.1 abcd	85.9 fghijklm
L-15/H-Lac							85.8 bc	75.2 f	86.3 cdef	85 def	80.6 jklm
L-15									92.1 abcd	88.9 abcd	83.7 klm
L-15/H-Lac									87.2 abcdef	76.7 ef	84.2 ijklm
L-15									94.5 a	90 abcd	83.1 lm
L-15/H-Lac									84.4 f	77.2 f	73.3 m
L-15									89 abcdef	92.8 abc	97.5 ab
L-15/H-Lac									88.3 bcdef	92.2 abcd	89.3 cdefghijkl
L-15									94.2 ab	95 a	99.1 a
L-15/H-Lac									85.3 ef	90.1 abcd	92.9 abcde

Table2: IDE 8 cell viability (%) means according to culture medium and time.

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14	Week 15	Week 16	Week 17
L-15B	100 a	72.2 A	72.5 a	84.3 a	77.4 a	85 ab	74.3 abc	83.8 a	75 abcdef	78.6 a	74.4 abc	72.2 abc	82.9 abcd	70.8 abcd	69.2 cdef	65.5 abcdefg	71.3 ab
L-15/H-lac 10% serum	96.9 a	88.8 a	59.5 a	75 a	77.6 a	84 ab	70.3 abc	77 ab	79.5 abcd	64.6 a	81 ab	69 abc	88.7 a	71.4 abcd	78 abcdef	55.6 defg	76.7 ab
L-15/H-lac 20% serum	100 a	88.8 a	56.8 a	80.7 a	79.4 a	89.9 a	84.3 a	83.2 a	81.6 abc	74.7 a	89 a	82.8 a	78.7 abcd	82.8 a	68 def	85.2 a	74.2 ab
L-15B				76.6 a	73.4 a	65.7 c	53.8 c	74.2 ab	61 ef	61.3 a	55.3 c	69 abc	71.6 cd	51.6 b	62.4 f	47.1 g	65.4 b
L-15/H-lac 10% serum				79.1 a	69.7 a	73 bc	53.1 bc	69.4 ab	45.8 def	60.3 a	65.9 bc	68.6 abc	85.1 abcd	74.9 abcd	80.3 abcd	47.5 fg	72.6 ab
L-15/H-lac 20% serum				84.9 a	77.9 a	88 a	75.8 ab	69.3 ab	82.7 abc	77.2 a	75.3 abc	63.3 abc	87.2 a	74 abcd	64 ef	70.4 abcdefg	71.4 ab
L-15B								72.8 ab	67 cdef			73.7 abc	70.4 abcd	71.9 abcd	68.1 bcdef	64.5 bcdefg	71.3 ab
L-15/H-lac 10% serum								58.4 b	71.3 abcdef			62.5 bc	85.7 abcd	84.9 a	82.2 abcd	81 ab	77.4 ab
L-15/H-lac 20% serum								78 ab	89.3 a			81.1 a	68.8 d	79 abc	85.5 ab	79.7 ab	86.1 a
L-15B								78 ab	52.7 f			59 c	70.4 bcd	66.3 abcd	73.3 abcdef	57.9 cdefg	72.5 ab
L-15/H-lac 10% serum								75 ab	58.8 bcdef			70.5 abc	70 d	56.1 cd	91.1 a	42.6 efg	74.4 ab
L-15/H-lac 20% serum								74 ab	78.1 abcde			67.6 abc	83.3 abcd	65.4 bcd	80.7 abcd	83.4 a	69.5 ab

Table 3: BME/CTVM 6 cell viability (%) means according to culture medium and time.

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14	Week 15	Week 16
L-15	77.3 a	55.2 a	91.4 a	77.3 a	92.7 a	89.9 a	86.3 ab	59.9 a	87.3 abc	94.5 a	86.8 abcd	90.1 abc	92.7 abcd	89.6 abc	87 a	86.4 a
L-15/H-Lac	66.4 a	49.4 a	80.2 a	69.1 a	79.7 a	80.1 a	89.4 a	70.6 a	94.8 a	95.1 a	88.7 abcd	91.9 abc	93.7 a	88.5 abc	82.6 a	81.5 a
L-15					89.5 a	90.4 a	74.5 ab	35 a	84.9 abc	76.6 b	73.4 bcd	86.3 abc	83.6 abcdef	80 abcdef	82 a	
L-15/H-Lac					79.1 a	80.9 a	63.3 b	47.9 a	77.9 abc	96.1 a	70.2 cd	79.9 abc	85.7 abcdef	82.4 abcdef	76.8 a	
L-15									85.7 abc	91.7 ab	96.3 a	73.5 bc	98 a	92.4 ab		
L-15/H-Lac									73.2 bc	87.9 ab	89.5 abcd	76.1 abc	91.9 abcd	82.8 abcdef		
L-15									78.3 abc	91.8 ab	82.3 abcd	93.1 a	78.1 abcdef	93.1 ab		
L-15/H-Lac									58.8 c	86.8 ab	70.9 d	82.1 abc	65.2 ef	61.7 ef		
L-15												87.1 abc	90.9 abcd	92.9 ab		
L-15/H-Lac												72.6 c	74.6 cdef	91 abc		
L-15												80.1 abc	73 bcdef	78.4 abcdef		
L-15/H-Lac												76.7 abc	74.2 df	85.3 abcdef		
L-15													62.9 f	73 bcdef		
L-15/H-Lac													77.4 abcdef	71.5 cdef		
L-15														62.3 def		
L-15/H-Lac														53.3 f		

Table 4: BDE/CTVM 14 cell viability (%) means according to culture medium and time.

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12
H-Lac	93.9 a	97 a	95.3 a	90 a	84.8 a	83.6 a	84.2 a	73.7 abc	76.4 a	90.1 abcdef	93 abc	86.6 abcde
L-15/H-Lac	93.3 a	96.7 a	85 a	78.1 a	84.7 d	71.9 a	85 a	78 bc	85.6 a	87.2 abcdef	88.6 a	82 bcdefghi
H-Lac				71.1 b	87.3 bcd	67.9 a	92 a	75 c	84.9 a	87.9 abcdef	84.7 abc	60.7 abcdefghi
L-15/H-Lac				78.8 ab	66.7 cd	65.1 a	81.8 a	60.5 abc	78.6 a	82.4 ef	74.3 c	71.7 efghi
H-Lac							85.5 a	89.6 abc	85.9 a	94.3 abc	94.1 a	87.5 abcdefg
L-15/H-Lac							86.9 a	83.7 a	84.2 a	95.6 a	96.8 abc	61.9 fghi
H-Lac							94.2 a	87.8 abc	70.2 a	88 abcdef	86.4 a	67.2 ghi
L-15/H-Lac							85.7 a	70.3 ab	78.2 a	87 abcd	96.3 a	74.4 cdefghi
H-Lac										87 f	88 a	89.3 abc
L-15/H-Lac										86.6 abc	86.6 abc	86.1 abcde
H-Lac										88 abcdef	88.8 a	87.6 abcde
L-15/H-Lac										85 cdef	83.1 abc	81.7 abcdefgh
H-Lac										86.1 abcdef	87.1 abc	72.5 i
L-15/H-Lac										88.5 abcdef	85.8 abc	73.6 hi
H-Lac										80.8 bcdef	87.9 abc	75.1 defghi
L-15/H-Lac										75.4 def	72.1 b	69.5 a

12.3. Sequencing data

12.3.1. *16S rRNA* sequence comparison of *Ca.* Midichloria mitochondrii

AJ566640: *Ca.* Midichloria mitochondrii of the tick *I. ricinus*.

HQ638198: Sequence obtained from *I. ricinus* cell line IRE/CTVM 19 in this thesis

HQ638199: Sequence obtained from *R. (Boophilus) dicoloratus* cell line BDE/CTVM14 in this thesis

```
HQ638198       1   CCTTAGTTGCCAGCGAGTGATGTCGGGAACTTTAAGGAAACTGCCGGTGATAAGCCGGAG
AJ566640       1   CCTTAGTTGCCAGCGAGTGATGTCGGGAACTTTAAGGAAACTGCCGGTGATAAGCCGGAG
HQ638199       1   CCTTAGTTGCCAGCGAGTGATGTCGGGAACTTTAAGGAAACTGCCGGTGATAAGCCGGAG

HQ638198      61   GAAGGTGGGGATGACGTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTAC
AJ566640      61   GAAGGTGGGGATGACGTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTAC
HQ638199      61   GAAGGTGGGGATGACGTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTAC

HQ638198     121   AATGGTGGTGACAAAAAGAAGCAATAGGGCGACCTGGAGCAGATCTTATAAAAGCTATCT
AJ566640     121   AATGGTGGTGACAAAAAGAAGCAATAGGGCGACCTGGAGCAGATCTTATAAAAGCTATCT
HQ638199     121   AATGGTGGTGACAAAAAGAAGCAATAGGGCGACCTGGAGCAGATCTTATAAAAGCTATCT

HQ638198     181   CAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATCGCTAGTAATCGCAGA
AJ566640     181   CAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATCGCTAGTAATCGCAGA
HQ638199     181   CAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATCGCTAGTAATCGCAGA

HQ638198     241   TCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AJ566640     241   TCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
HQ638199     241   TCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACTGCCCGTCACGCCATGGG

HQ638198     301   AGTCGGTTTTGCCTGAAGTAGGTGCGCTAACCGCAAGGAGGCAGCTTACC
AJ566640     301   AGTCGGTTTTGCCTGAAGTAGGTGCGCTAACCGCAAGGAGGCAGCTTACC
HQ638199     301   AGTCGGTTTTGCCTGAAGTAGGTGCGCTAACCGCAAGGAGGCAGCTTACC
```

12.3.2. Phylogenetic analysis sequences

> *R. (Boophilus) dicoloratus* cell line BDE/CTVM14 obtained in this thesis

CCTTAGTTGCCAGCGAGTGATGTCGGGAACTTTAAGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTACAATGGTGGTGACAAAAAGAAGCAATAGGGC
GACCTGGAGCAGATCTTATAAAAGCTATCTCAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATC
GCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AGTCGGTTTTGCCTGAAGTAGGTGCGCTAACCGCAAGGAGGCAGCTTACC

> *I. ricinus* cell line IRE/CTVM 19 obtained in this thesis

CCTTAGTTGCCAGCGAGTGATGTCGGGAACTTTAAGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTACAATGGTGGTGACAAAAAGAAGCAATAGGGC
GACCTGGAGCAGATCTTATAAAAGCTATCTCAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATC
GCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AGTCGGTTTTGCCTGAAGTAGGTGCGCTAACCGCAAGGAGGCAGCTTACC

> Endosymbionts of *Acanthamoeba* (UWC36) AF069962

CCTTAGTTACCAACAGGTTATGCTGGGCACTCTAAGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTACAATGGTGGTGACAATTGGACGCAATAGGGC
GACCTGGAGCAAATCCC-TAAAAGCCACCTCAGTTCGGATTGTACCCTGCAACTCGGGTACATGAAGTCGGAATC
GCTAGTAATCGCAGATCAGCATGCTGTGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCAAGCCATGGG
AGTTGGTCTTACCTTAAGTAGGTGTGCTAACCGTAAGGAGGCAGCCTACC

> *Ca.* Midichloria mitochondrii sp. FM992372

CCTTAGTTGCCAGCGAGTAATGTCGGGAACTTTAAGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTACAATGGTGGTGACAAAAAGAAGCAATAGGGC
GACCTGGAGCAAATCTTATAAAAGCCTCCTCAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATC
GCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AGTTGGTTTTACCTGAAGTAGGTGTGCTAACCGCAAGGAGGCAGCTTGCC

> *R. rickettsii* strain (R) L36217

TCTTATTTGCCAGCGGGTAATGCCGGGAACTATAAGAAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGACGAC
GTCAAGTCATCATGGCCCTTACGGGTTGGGCTACACGCGTGCTACAATGGTGTTTACAGAGGGAAGCAAGACGGC
GACGTGGAGCAAATCCC-TAAAAGACATCTCAGTTCGGATTGTTCTCTGCAACTCGAGAGCATGAAGTTGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCTCGGGCCTTGTACACACTGCCCGTCACGCCATGGG
AGTTAGTTTTACCTGAAGGTGGTGAGCTAAC-GCAAG-AGGCAGCCAACC

> *R. rickettsii* U11021

TCTTATTTGCCAGCGGGTAATGCCGGGAACTATAAGAAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGACGAC
GTCAAGTCATCATGGCCCTTACGGGTTGGGCTACACGCGTGCTACAATGGTGTTTACAGAGGGAAGCAAGACGGC
GACGTGGAGCAAATCCC-TAAAAGACATCTCAGTTCGGATTGTTCTCTGCAACTCGAGAGCATGAAGTTGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCTCGGGCCTTGTACACACTGCCCGTCACGCCATGGG
AGTTAGTTTTACCTGAAGGTGGTGAGCTAAC-GCAAG-AGGCAGCCAACC

>*E. chaffeensis* U23503
CCTTAGTTACCAACAGGTAATGCTGGGCACTCTAAGGAAACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGAT
GTCAAGTCAGCACGGCCCTTATAAGGTGGGCTACACACGTGCTACAATGGCAACTACAATAGGTCGCGAGACCGC
AAGGTTTAGCTAATCC-ATAAAAGTTGTCTCAGTTCGGATTGTTCTCTGCAACTCGAGAGCATGAAGTCGGAATC
GCTAGTAATCGTGGATCATCATGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AATTGGCTTAACTCGAAGCTGGTGTGCTAACCGCAAGGAAGCAGCCATTT

>*A. marginale* M60313
CCTTAGTTACCAGCGGGTAATGCCGGGCACTTTAAGGAAACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGAT
GTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGGCGACTACAATAGGTTGCAACGTCGC
AAGGCTGAGCTAATCC-GTAAAAGTCGTCTCAGTTCGGATTGTCCTCTGTAACTCGAGGGCATGAAGTCGGAATC
GCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AATTGGCTTAACTCGAAGCTGGTGCGCCAACCGTAAGGAGGCAGCCATTT

> Ca. Nicolleia masilliensis DQ788562
CCTTAGTTGCCAGCGAGTGATGTCGGGAACTTTAAGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTACAATGGTGGTGACAAAAAGAAGCAATAGGGC
GACCTGGAGCAGATCTTATAAAAGCTATCTCAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATC
GCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AGTCGGTTTTGCCTGAAGTAGGTGCGCTAACCGCAAGGGTTTCAGCTTACC

> *R. massiliae* L36106
TCTTATTTGCCAGCGGGTAATGCCGGGAACTATAAGAAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCATCATGGCCCTTACGGGTTGGGCTACACGCGTGCTACAATGGTGTTTACAGAGGGAAGCAAGACGGC
GACGTGGAGCAAATCCC-TAAAAGACATCTCAGTTCGGATTGTTCTCTGCAACTCGAGAGCATGAAGTTGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCTCGGGCCTTGTACACACTGCCCGTCACGCCATGGG
AGTTGGTTTTACCTGAAGGTGGTGAGCTAAC-GCAAG-AGGCAGCCAACC

>*A. phagocytophilum* M73224
CCTTAGTTGCCAGCGGGTTAAGCCGGGCACTTTAAGGAAACTGCCAGTGGTAAACTGGAGGAAGGTGGGGATGAT
GTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGGTGACTACAATAGGTTGCAATGTCGC
AAGGCTGAGCTAATCC-GTAAAAGTCATCTCAGTTCGGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATC
GCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AATTGGCTTAACTCGAAGCTGGTGCGCCAACCGAAAGGAGGCAGCCATTT

>*E. canis* M73221
TCTTAGTTACCAACAGGTAATGCTGGGCACTCTAAGGAAACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGAT
GTCAAATCAGCACGGCCCTTATAGGGTGGGCTACACACGTGCTACAATGGCAACTACAATAGGTTGCGAGACCGC
AAGGTTTAGCTAATCC-ATAAAAGTTGTCTCAGTTCGGATTGTTCTCTGAAACTCGAGAGCATGAAGTCGGAATC
GCTAGTAATCGTGGATCATCACGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AATTGGCTTAACTCGAAGCTGGTGTGCTAACCGCAAGGAAGCAGCCATTT

>*R. prowazekii* M21789
TCTTATTTGCCAGTGGGTAATGCCGGGAACTATAAGAAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGACGAC
GTCAAGTCATCATGGCCCTTACGGGTTGGGCTACACGCGTGCTACAATGGTGTTTACAGAGGGAAGCAATACGGT
GACGTGGAGCAAATCCC-TAAAAGACATCTCAGTTCGGATTGTTCTCTGCAACTCGAGAGCATGAAGTTGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCTCGGGCCTTGTACACACTGCCCGTCACGCCATGGG
AGTTGGTTTTACCTGAAGGTGGTGAGCTAAC-GCAAG-AGGCAGCCAACC

>*Ca.* Midichloria mitochondrii AJ566640
CCTTAGTTGCCAGCGAGTGATGTCGGGAACTTTAAGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTACAATGGTGGTGACAAAAAGAAGCAATAGGGC
GACCTGGAGCAGATCTTATAAAAGCTATCTCAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTCGGAATC
GCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACTGCCCGTCACGCCATGGG
AGTCGGTTTTGCCTGAAGTAGGTGCGCTAACCGCAAGGAGGCAGCTTACC

> Endosymbionts of *Acanthamoeba* (UWC8) AF069963
CCTTAGTTACCAACAGGTTATGCTGGGCACTCTAAGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTACAATGGTGGTGACAATTGGACGCAATAGGGC
GACCTGGAGCAAATCCC-TAAAAGCCACCTCAGTTCGGATTGTACCCTGCAACTCGGGTACATGAAGTCGGAATC
GCTAGTAATCGCAGATCAGCATGCTGTGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCAAGCCATGGG
AGTTGGTCTTACCTTAAGTAGGTGTGCTAACCGTAAGGAGGCAGCCTACC

> *Pseudomonas testosteroni* (M11224) was used as an out-group species.

CCTTAGTTGCCAGCGAGTGATGTCGGGAACTTTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCTGGTACAAAGGGTTGCCAACCCGC
GAGGGGGAGCTAATCCCATAAAGCCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATC
GCTAGTAATCGTGGATCAGAATGTCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGG
AGCGGGTCTCGCCAGAAGTAGGTAGCCTAACCGTAAGGAGGGCGCTTACC

13. ACKNOWLEDGEMENTS

All thanks to the God (Allah), the merciful, the beneficent, and the guidance of all my mankind. He is the source of my power and inspiration and makes my life more bountiful. May your name be exalted, honored, and glorified.

It would not have been possible to accomplish this thesis without the help and support of the kind people around me.

Above all, I owe my most sincere gratitude to Professor Dr. Kurt Pfister, the head of Comparative Tropical Medicine and Parasitology department, Ludwig-Maximilians-University, Munich, for giving me the opportunity to work on this thesis under his stewardship.

The good advice and support of my supervisor, Prof. Dr. Lygia Passos, has been invaluable on both academic and personal level, for which I am extremely grateful. I thank her for critically reviewing this thesis and for extensive encouraging discussions.

I am most grateful to Dr. Cornelia Silaghi, who has guided me a great deal about molecular techniques and also helped me most of the time through her advice and great discussions.
During this work I have collaborated with many colleagues for whom I have great regard, and

I wish to extend my warmest thanks most especially to Dr. Erich Zweygarth, Dr. Miriam Scheuerle, Heidrun Schöl and Claudia Thiel for their kindness, friendship and support, together with the other members of Parasitology institute, who created a lovely and enabling environment to work.

My sincere thanks are due to Dr. Lesely Bell-Sakyi and Dr. Ulrike Munderloh for their permission to use the tick cell lines that formed the soul of this thesis.

I also wish to thank Mrs. Rachel Kenneil and Mr. Abdulhakeem Salami for revising the English parts of my manuscript.

I warmly thank PhD. Dr. Sabine Bork-Mimm for her valuable advice and friendly help, when I first started work on my thesis.

I would like to acknowledge the financial support of the Al-Baath University-Syria, which gave me the opportunity to continue my studies abroad and also I would like to express my deep and sincere gratitude to my supervisor in Syria Prof. Dr. Abdulkarim Al-Khaled. As my teacher and mentor, he has taught me more than I could ever give him credit for here.

I owe my loving thanks to my parents, whose love and prayers have been with me all the time. They are the ultimate role models. Most importantly, I would like to thank my wife ***Roda*** for her personal support and great patience at all times and my two wonderful daughters, ***Zainab*** and ***Maria***, who provide unending inspiration.

Last, but not the least, I thank my friends in Syria, Egypt, Lebanon, Tunisia and elsewhere for their support and encouragement throughout.